DIVERSITY OF CANCER STEM CELLS IN ACUTE LYMPHOBLASTIC LEUKAEMIA

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ABSTRACT

For many cancers research led to controversial results regarding frequency and identity of cancer stem cells, cells that self-renew and are able to reconstitute the full phenotype of the original malignancy. In some malignancies such as acute myeloid leukaemia the hierarchical stem cell model that suggests that only rare and immunophenotypically immature blasts exhibit stem cell characteristics, resembling the normal physiological haematopoietic hierarchy, has been well established. In contrast to that, the stochastic model states that all or at least a substantial proportion of malignant cells has stem cell potential whereby this is supported by extrinsic stimuli. Initially, several studies suggested that acute lymphoblastic leukaemia (ALL) also is organised in a hierarchy. However, using more immunocompromised mouse strains and refined transplantation techniques for *in vivo* xenotransplantation models, previous findings regarding frequency and restriction of stem cells to very early B-precursor cell stages have been challenged in more recent studies.

In order to address the questions of identity and frequency of stem cells in ALL, a robust orthotopic mouse model with the most immunocompromised mouse strain currently available (NOD/scid IL2Rγnull; NSG) was established. Primary diagnostic patient ALLs or blasts harvested from engrafted mouse bone marrow were sorted for B-cell lineage differentiation markers CD10, CD19, CD20 or CD34 to purify candidate stem cell populations of different maturity. All transplanted cell populations, from the most immature $CD34^{+}CD19^{low}$ stage to the already more differentiated stage of $CD19^{+}CD20^{high}$ cells were able to reconstitute the original ALL in mouse bone marrow and followed a typical dissemination pattern with infiltration of the spleen. Furthermore, this more mature $CD19^{+}CD20^{high}$ subpopulation proved self-renewal ability in serial transplantation experiments. To investigate, whether stem cells in ALL are a rare entity or more abundant, unsorted bulk leukaemia blasts were transplanted in limiting dilutions

from 1 x 10^4 to 10 cells per mouse. Cell numbers required for engraftment varied between leukaemias but the leukaemia engrafted in mice when only 10 to 1,000 cells were transplanted. The limiting dilution experiments were repeated with sorted blast populations according to the surface antigens CD10, CD20 and CD34. Stem cell frequencies in all sorted populations were comparable and as few as 10 to 100 cells were sufficient to reconstitute the leukaemia in mice.

Stem cells do not seem to be restricted to immature blast populations as in the hierarchical model but a broad spectrum of different blast immunophenotypes display stem cell capabilities. Furthermore, the frequency of stemness among unsorted bulk ALL cells as well as subpopulations of different maturity according to the blast immunophenotype is high and similar. The results from this thesis provide strong evidence for the stochastic cancer stem cell model in B precursor ALL.

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LIST OF ABBREVIATIONS

AGM – Aorta-gonad-mesonephros

ALL – Acute lymphoblastic leukaemia

AML – Acute myeloid leukaemia

APC – Allophycocyanin

BCP – B cell precursor

BCR – B-cell receptor

BM – Bone marrow

BMP – Bone marrow puncture(s)

BSA – Bovine serum albumin

CALLA – Common ALL antigen

CCG – Children´s Cancer Group

CD – Cluster of differentiation

CFU – Colony forming unit

CLP – Common lymphoid progenitor

CML – Chronic myeloid leukaemia

CMP – Common myeloid progenitor

CNA – Copy number alteration

CNS – Central nervous system

CSC – Cancer stem cell

DAPI – 4’,6-diamidino-2-phenylindole

ddH_2O – Distilled, deionised water

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

ds – Double strand

DWH – Died without harvest

EFS – Event Free Survival

FACS – Fluorescence activated cell sorting

FCS – Foetal calf serum

FISH – Fluorescence *in situ* hybridisation

FITC – Fluorescein isothiocyanate

FSC – Forward scatter

g – Gravitational-force

GMP – Granulocyte-macrophage progenitor

HSC – Haematopoietic stem cell

if – Intrafemoral

Ig – Immunoglobulin

iv – Intravenous

IVC – Individually ventilated cage

IVIG – Intravenous immunoglobulin

LAK – lymphokine-activated killer cell

LMPP – Lymphoid-primed multipotent progenitor

LSC – Leukaemic stem cell

LT-HSC – Long-term haematopoietic stem cell

mAb – Monoclonal Antibody

MFI – Median Fluorescence Intensity

MGG – May-Grünwald-Giemsa

MNC – Mononuclear cell

MPP – Multipotent progenitor cell

MRD – Minimal residual disease

NAD – Nicotinamide adenine dinucleotide

NCI – National Cancer Institute

NHEJ – Nonhomologous end joining

NK – Natural killer (cells)

NOD – Non obese diabetic

NOG – NOD/Shi-Scid IL-2Rγ $^{-/-}$ hybrid mouse strain

NSG – NOD/LtSz-Scid IL-2Rγ $^{-/-}$ hybrid mouse strain

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffered saline

PBSC – Peripheral blood stem cell

PBSA – Phosphate buffered saline containing bovine serum albumin

PCR – Polymerase chain reaction

PE – Phycoerythrin

PE-Cy7 – Phycoerythrin with Cyanine 7 conjugate

PerCP-Cy 5.5 – Peridinin chlorophyll protein with Cyanine 5.5 conjugate

Ph – Philadelphia chromosome

PMT – Photomultiplier tube

Pol – positive outside limits

rpm – Revolutions per minute

RQ-PCR – Real-time quantitative polymerase chain reaction

SCF – Stem cell factor

sh-RNA – Short hairpin ribonucleic acid

SL-ICs – Scid leukaemia initiating cells

SRC – Scid repopulating cells

SSC – Side scatter

ST-HSC – Short-term haematopoietic stem cell

UCB – Umbilical cord blood

WCC – White cell count

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1.1 General Introduction

Every year, one in 500 children under the age of 15 years faces the diagnosis of one form of cancer in the United Kingdom, which adds up to around 1,500 new cases per year. This accounts for only 0.5% of all cases of cancer in the whole population and therefore childhood cancer is a very rare disease compared to adulthood. Despite this low incidence it is the second most common cause of death after non-disease related death in the age group from 1 – 14 years, with nearly 300 children dying from cancer in the UK every year. The incidence (ratio of around 1.2:1) as well as the mortality (ratio of around 1.4:1) of malignancies is slightly higher in boys than in girls (CancerResearchUK, 2010).

The most common types of cancer in children are leukaemias, accounting for approximately one third of childhood cancers. In contrast to adults the acute forms of leukaemias are far more common than the chronic forms. Acute forms are further classified according to their cell lineage into lymphoid and myeloid leukaemias. In children around 80% of the leukaemias are acute lymphoblastic leukaemias (ALL). Acute myeloid leukaemias (AML) account for approximately 15% and chronic myeloid leukaemias (CML) make less than 4% of all forms of leukaemia (Cancer Research UK 2010). Today, with current multiagent chemotherapy regimens, overall long-term relapse-free survival of children affected by ALL is around 80% but within this group of leukaemias several cytogenetic subgroups have been described that correlate well with different prognostic outcomes (Möricke et al., 2008; Pui et al., 2004). In developed countries survival rates of affected children are still variable due to the distinct biologies rather than access to and use of modern treatment (Pui et al., 2002). ALL is the subject

of considerable research with more than 1,000 relevant papers released in 2010 (PubMed search: Acute lymphoblastic leukemia). However, continued research is necessary both to increase survival rates and reduce short and long-term toxic side effects.

1.2 Acute Lymphoblastic Leukaemia

1.2.1 Historical Background

The first descriptions of alterations in the blood that retrospectively can be interpreted as leukaemias date back to the early nineteenth century. However, these observations published by Cullen in 1811, Alfred Velpeau in 1827, Alfred Donné in 1844 and David Craigie in 1845 were interpreted as inflammation with purulent cells in the blood by the authors. When David Craigie admitted another patient to the Royal Infirmary Edinburgh with similar symptoms as the patient he had described in 1845, a post mortem was carried out by the Scottish pathologist John Hughes Bennett and he was the first who concluded that the alterations in the blood and the spleen were independent of inflammation and were entities of their own. Only six weeks after Bennet´s publication in the Edinburgh Medical and Surgical Journal the German physician and pathologist Rudolf Virchow at the Charité in Berlin reported a similar patient in "Weisses Blut" with a history of weakness and bleeding and autopsy findings of an enlarged spleen and white blood corpuscles. In 1847 he eventually gave this disease the name Leukhemia, from the Greek words for white (leukos) and hemia (blood) (Piller, 2001).

1.2.2 Classification of Leukaemias

Leukaemias are characterised by the replacement of normal bone marrow by malignant blasts. The term blast refers to primitive precursor cells that show no or minimal signs of

differentiation. Leukaemias are principally categorised according to the lineage and stage of differentiation of the leukaemic cells that form the malignancy. Leukaemias can either arise from the lymphatic or the myeloid lineage of haematopoiesis and both can present as an acute form when immature haematopoietic or lymphoid precursors are involved or a chronic form when the clone consists of more mature cells. Today, morphology and cytochemistry have lost importance and immunophenotypic classification is leading the way to diagnosis of subtypes (Silverman, 2009). The World Health Organisation (WHO) regularly updates and publishes a comprehensive classification of tumours of haematopoietic and lymphoid origin according to their morphology, cytogenetics, immunology, cytochemistry and cytogenetics (Swerdlow et al., 2008).

The following subchapters focus mainly on childhood B cell precursor (BCP) ALL that arise within the B lineage of lymphatic development.

1.2.3 Epidemiology

Leukaemias are the most common of all cancers among children under the age of 15, accounting for around one third (31%) of the approximately 1,500 yearly cases. Between 1991 and 2000 on average 470 children per year were given the diagnosis Leukaemia in the UK of which 372 (79%) were of the acute lymphoblastic form. This results in an annual age-specific incidence of 36 childhood ALL cases in 1,000,000 in the UK. The peak incidence of the disease occurs in children aged 2 and 3 years and decreases after that. Comparable with childhood cancer in general, ALL more often affects boys than girls with a ratio of 1.3:1. Each year approximately 300 children under the age of 15 die from cancer in the UK. The 5 year event free survival (EFS) rate for children is now 80% and the 5 year overall survival rate is close to 90%. However, because of the

relatively high incidence, leukaemia still is responsible for 30% of all deaths from cancer in children, being second only to deaths from tumours of the central nervous system (CNS, brain and spinal cord) (32%) (CancerResearchUK, 2010; Pui et al., 2010).

In developed countries there are racial and ethnic differences in EFS of children with ALL. Treated using the same Children´s Cancer Group (CCG) therapeutic protocols in the USA, children of Asian origin had a significant better outcome compared to white children, while children from a black family background and of Hispanic origin did worse. So far the reasons for this remain unclear (Bhatia et al., 2002), even allowing for differences in socioeconomic status. It has been established that in developing countries there is a lower peak incidence of ALL in early childhood and this was thought to be due to lower socioeconomic status, although under-diagnosis cannot be excluded (Greaves et al., 1993; Stiller and Parkin, 1996). An interesting retrospective analysis from the Czech Republic was able to add more evidence to support the hypothesis that poor socioeconomic status is related to a reduced incidence of ALL. Hrusák and colleagues found a marked increase in the incidence of ALL among children 1 to 4 years old in the last two decades of the last century. This increase paralleled the improved socioeconomic status of the post communist countries in these years and was especially marked in girls (Hrusák et al., 2002).

1.2.4 Clinical Presentation

In general the presenting symptoms of patients with ALL are rather non-specific and are mainly caused by the rapid proliferation of the leukaemic blasts and the resulting effect on the different organs of the body. The clonal expansion of blasts in the bone marrow eventually leads to the suppression of normal physiologic haematopoiesis. As a result progressive anaemia causes fatigue and lethargy and patients may appear pale.

Neutropenia makes patients more susceptible to infections with concomitant fever, one of the most common presenting features. Children may show signs of bleeding and bruising as a consequence of thrombocytopenia. Other features are caused by dissemination of leukaemic blasts. Infiltration of the spleen and liver cause splenomegaly and hepatomegaly respectively and accumulation of blasts in lymph nodes leads to enlargement of mainly cervical and nuchal glands. Pain in the legs and a resulting refusal to walk or limping, mainly in younger children, is presumably caused by infiltration of bones and the periostium although this is not entirely proven. Involvement of the thymus and mediastinal lymph nodes is more common in T-cell ALL and can result in narrowing of the trachea or bronchi with dyspnoea whereas infiltration of mesenteric lymph nodes can potentially lead to obstruction of the bowels due to invagination and present with abdominal pain. Involvement of the CNS can be found in up to 20% of patients but only rarely causes clinical symptoms. If present, they include signs of raised intracranial pressure or cranial nerve palsies. Occasionally indolent swelling of the testes can be observed (Schrappe, 2005; Silverman, 2009).

1.2.5 Prognosis

1.2.5.1 Significance of prognostic factors

Without therapy the outcome of all leukaemias in children is dire but treatment according to the current intensive multidrug chemotherapy regimens has led to an overall cure rate of ALL in children of over 80%. This stands in contrast to adult patients where only 40% to 50% of patients can be cured (Onciu, 2009; Pui et al., 2004). However, treatment outcome in ALL patients varies extensively and a number of significant clinical and biological prognostic factors have been established to separate leukaemias that fare better from those with a less favourable outcome. It is important to

note that any prognostic factor is established in the context of the specific treatment modalities applied at the time of observation and its significance regarding 5 year EFS may not be relevant in all regimes. An increasing number of prognostic factors are taken into account by current treatment protocols and are used to allocate patients to different treatment arms in clinical trials. With the intensified treatment regimens now in use the actual chance of survival for patients carrying adverse prognostic factors can be improved and be comparable to that of patients without these prognostic features in some cases (Möricke et al., 2008).

1.2.5.2 Clinical prognostic factors

Of clinical factors, the age at diagnosis and the presenting peripheral blast count are the strongest prognostic indicators. Children between 1 and 9 years of age have a better outcome than infants and children aged 10 years and above. Regarding the peripheral blast count it has been established that patients with counts of less than 50,000/µl have a more favourable outcome than patients with higher counts at presentation due to a smaller risk of relapse. Additionally, the detection of lymphoblasts in the cerebrospinal fluid at diagnosis is an adverse prognostic factor (Smith et al., 1996). The current UKALL 2003 trial and others use these National Cancer Institute (NCI) criteria for risk stratification.

1.2.5.3 Immunophenotype as a prognostic factor

Determining the immunophenotype still is very important in the process of establishing the diagnosis and classifies ALL into pro-B ALL, common ALL, pre-B ALL and T-cell ALL with significant differences in treatment outcome (Schrappe et al., 2000b). However, with advances made in cytogenetics immunophenotyping has lost some of its

independent prognostic value. Differences in outcome that were previously attributed to specific immunophenotypes now can more precisely be assigned to specific cytogenetic changes within the subgroups. In pre-B ALL the generally poorer outcome observed in earlier treatment studies was found to be caused by the cytogenetic subgroup of patients that carry the t(1;19) translocation (Crist et al., 1990). However, treatment intensification now results in an overall outcome for this subgroup comparable to that of the standard risk group (Kager et al., 2007; Schrappe et al., 2000a; Uckun et al., 1998). Conflicting reports were published regarding the prognosis of ALL with coexpression of myeloid cell surface antigens on the lymphoblasts. However, myeloid coexpression has been found more often in association with chromosomal translocations like the Philadelphia chromosome, *MLL* rearrangements and the *ETV6/RUNX1* (*TEL/AML1)* fusion gene and is not considered to be of independent prognostic value outside the context of these cytgogenetic features anymore (Carbonell et al., 1996; Pui et al., 1998; Putti et al., 1998). Cure rates in childhood T-cell acute lymphoblastic leukaemia historically were inferior to average rates but with intensified treatment EFS now is similar to patients with B-precursor ALL (Goldberg et al., 2003).

1.2.5.4 Cytogenetic prognostic factors

Chromosomal abnormalities have an important impact on the prognosis of ALL and are considered in risk stratification. They include gains or losses of parts or whole chromosomes or reciprocal translocations between two chromosomes and can be associated with either a good or a poor prognosis.

ALL with high hyperdiploidy (blasts carrying 51 to 65 chromosomes) usually have a lower risk of relapse due to the high sensitivity of leukaemic blasts to chemotherapy,

especially Methotrexate, and a high tendency to undergo apoptosis. They comprise approximately 25% of childhood cases (Ito et al., 1999; Pui et al., 2004).

The ETV6/RUNX1 (formerly known as TEL/AML1) fusion results from a balanced reciprocal translocation of the chromosomes 12 and 21 (t(12;21)) and is associated with an excellent treatment outcome of the affected 22% of paediatric ALL patients. ETV6 as well as RUNX1 code for genes of transcription factors which are involved in haematopoiesis and the fusion protein affects the self-renewal and differentiation ability of haematopoietic stem cells (HSC) (Loh et al., 2006; Pui et al., 2004). Interestingly, this translocation was found to arise prenatally as it could be detected in DNA isolated from blood spots of Guthrie cards but is not sufficient to cause malignant transformation on its own (Wiemels et al., 1999b).

The Philadelphia chromosome (Ph) was the first specific genetic lesion identified in human cancers by Nowell and Hungerford and the first report of it being involved in ALL was published by Propp and Lizzi in 1970 (Nowell, 2007; Propp and Lizzi, 1970). This translocation between the chromosomes 9 and 22 (t(9;22)) consists of a fusion of the human homologue of the Abelson Murine leukaemia virus ABL1 (a tyrosine kinase) on 9q34 with the breakpoint cluster region BCR (a signalling protein) on 22q11 and an in-frame BCR/ABL1 fusion transcript leads to an upregulation of ABL activity. Three different breakpoints in the BCR gene result in three main BCR/ABL1 chimeric transcripts and the molecular masses of the protein products are 190, 210 and 230 kDa respectively. Three to 5% of children with ALL carry the Philadelphia chromosome with the majority expressing the p190 BCR/ABL1 protein, the constitutively activated tyrosine kinase (Jones and Saha, 2005). For leukaemic transformation, secondary oncogenic events in addition to the expression of the BCR/ABL1 fusion protein are necessary. The deletion of the *IKZF1* gene with consecutive loss of function of the encoded Ikaros transcription factor, required in lymphoid development, has been found

in most B-precursor ALL as well as in CML during the phase of transition to lymphoid blast crisis and is assumed to be a cooperating oncogenic lesion. Less frequent deletions have been found of *CDKN2A* and *PAX5* (Mullighan et al., 2008). On the basis of genome wide gene expression arrays, more recently, one subgroup of patients has been identified that clusters with BCR/ABL1 positive patients and also has an unfavourable prognosis. This subtype was called BCR/ABL-like ALL (Den Boer et al., 2009).

Patients with Ph^+ ALL are always generally allocated to the high risk treatment group but still have a poor survival of less than 50% (Pui et al., 2002; Schrappe et al., 1998a).

A subgroup of ALL patients (2%) carrying an intrachromosomal amplification of chromosome 21 (iAMP21) resulting in additional copies of the RUNX1 gene has been recently identified and found to have a significantly higher risk of relapse. These patients are now stratified to the high risk treatment arm in the current UKALL 2003 study and monitored if improved survival can be achieved with treatment intensification (Moorman et al., 2007).

Another chromosomal abnormality associated with an unfavourable outcome in ALL consists of rearrangements of the *MLL* gene on chromosome 11, the most commonly found being t(4;11), t(11;19) and t(9;11) (Pui et al., 2004; Szczepański et al., 2010). MLL rearrangements occur in around 80% of infant leukaemias and only 30% to 40% of cases with this abnormality can be cured. Physiologically, MLL acts as a transcriptional repressor but in MLL rearranged cells, overexpression of *HOXA5*, *HOXA9* and *MEIS1* can be detected by gene expression profiling and this is believed to play an important role in leukaemogenesis (Harper and Aplan, 2008). In contrast to the BCR/ABL1 (t(9;22)) and the ETV6/RUNX1 (t(12;21)) fusions, rearrangements of the *MLL* gene seem to be sufficient for leukaemic transformation of haematopoietic cells without a

second leukaemogenic event (Clarkson et al., 2003; Krivtsov et al., 2006; Mori et al., 2002; Szczepański et al., 2010; Wiemels et al., 1999a).

1.2.5.5 Response to treatment

The prognosis of a patient under current treatment protocols is highly dependent on the initial response to induction chemotherapy treatment. The morphologically identifiable persistence of blasts in peripheral blood one week into treatment has been shown to be an independent poor prognostic factor after administration of prednisone (and one dose of intrathecal Methotrexate) alone, as well as after one week of multidrug chemotherapy (Gajjar et al., 1995; Möricke et al., 2010).

A more recently established highly predictive and independent prognostic marker for the risk of ALL relapse is the detection of sub-microscopic minimal residual disease (MRD) early in the course of treatment, at the end of induction treatment. Patients with a high MRD load have an unfavourable prognosis independent of other risk factors classically used for risk stratification. Several methods to detect very low numbers of residual blasts in the bone marrow have been developed. Multicolour flow cytometry detects blasts utilising the leukaemia specific immunophenotype while real-time quantitative polymerase chain reaction (RQ-PCR) detects leukaemia associated fusion gene transcripts or leukaemia specific immunoglobulin gene or T-cell receptor gene rearrangements. With these methods a sensitivity of up to one cell in 10^{-4} (3 to 4 colour flow cytometry) or 10^{-6} (RQ-PCR based methods) can be achieved. The ALL-BFM 2000 protocol measured MRD levels at 33 and 78 days after start of treatment and used them for risk stratification for the first time. MRD of 10^{-3} or more at the later time point was classified as MRD high risk, negative MRD levels (10^{-4} or less) at both time points as MRD standard risk and levels in between as MRD intermediate risk. Applying this post-

induction risk stratification had an excellent correlation with treatment outcome in the ALL BFM 2000 study and interestingly, MRD was able to discriminate the risk of relapse in subgroups defined by conventional stratification criteria and could additionally identify patients with favourable outcomes in patients with BCR/ABL1 positive ALL, traditionally regarded as being at high risk for relapse (Conter et al., 2010; Szczepański, 2007).

1.2.5.6 Clinical and treatment-induced complications and their impact on prognosis

There are factors other than relapse or resistance to therapy that have an influence on survival. Massive accumulation of blasts in the vascular system and the effects of induction therapy are mainly responsible for deaths occurring early after diagnosis of ALL. Hyperleukocytosis with only poorly deformable blasts and counts higher than 100 x 10^9 per litre can lead to increased viscosity of the blood and intravascular clumping. Resulting local hypoxaemia may lead to endothelial damage with the consequence of haemorrhage and infarction particularly involving the lungs and central nervous system (Nicolin, 2002). The tumour lysis syndrome is mainly treatment related but can occur spontaneously and is caused by the release of metabolites and electrolytes from dead blasts. Renal failure can result from crystallisation of uric acid and precipitation of calcium phosphate in renal tubules and life threatening arrhythmias can follow high serum potassium concentrations (Halfdanarson et al., 2006). During intensive chemotherapy treatment bacterial infections during phases of neutropenia pose the major threat of therapy related death.

1.2.5.7 Secondary malignancies

Survivors of cancer face a higher risk of developing an unrelated form of malignancy than would be expected by chance alone. Chemotherapy, radiotherapy and genetic predisposition are thought to be the most important factors contributing to the increased risk. In children treated for ALL a significantly higher risk of developing a secondary AML has been recognised for over 20 years and is linked to the administration of the epipodophyllotoxins; Etoposide and Teniposide (Pui et al., 1991). Therapy related AML was also the most common type of secondary malignancies after treatment according to the ALL-BFM protocols, followed in frequency by neoplasms of the CNS, lymphomas and thyroid cancer. Overall, 15 years after initial therapy, children treated for ALL had a 14-fold increased risk of developing cancer as compared to children not being treated for cancer before. Among those, patients treated with cranial radiotherapy had a higher risk for secondary cancers than non-irradiated patients (Löning et al., 2000).

1.2.6 Therapy of acute lymphoblastic leukaemia

Until the middle of the last century ALL was an inevitably lethal disease. The first complete, albeit only temporary, remission in a child with ALL was achieved by Farber and colleagues in 1947 by intramuscular administration of the folic acid antagonist aminopterin (Farber and Diamond, 1948). Following this several single agent therapies in the 1950s and early combination chemotherapies in the 1960s led to complete remissions but all failed to cure the disease and resulted in fatal relapse. With recognition of the CNS as a blast sanctuary site and the introduction of specific CNS-directed treatment first cure rates of around 50% were eventually achieved in the 1970s (Silverman, 2009). Studies on cycling times of leukaemic cells in relation to normal haematopoietic cells and the finding that non-cycling blasts go into cycle as a response to chemotherapy led to elaborated time courses of combined antiproliferative

chemotherapy to increase the chance to hit all blasts in the vulnerable phase of cell division (Cheung et al., 1972; Lampkin et al., 1971; Rubinow and Lebowitz, 1976). All the main drugs that are still used in the treatment of ALL were already known to be effective in the 1950s (corticosteroids, 6-Mercaptopurine and Methotrexate) and 1960s (anthracyclines, Asparaginase and epipodophyllotoxins) but it was the introduction of consecutive large-scale multi-centre clinical trials that led to the great improvements in patient outcome. The main contributing factors to the increasing success rate of several treatment protocols in the next decades were the sophisticated design of complex multidrug chemotherapy regimens, the introduction and adaption of risk-stratified treatment arms, development of specifically CNS-directed compartment treatment and major improvements in supportive care. Risk-adapted regimens were continuously developed further, when new prognostic factors could be established that separated patients with successful treatment from those that relapsed. The ultimate goal of risk-adapted regimens is to give patients with a low risk of relapse as little toxic chemotherapy as possible and high risk patients as intensive therapy as necessary to achieve the best possible 5 year EFS for all (Möricke et al., 2008; Silverman, 2009).

Most contemporary paediatric protocols use the prognostic characteristics mentioned above for risk stratification. Once allocated to the different risk groups, treatment is usually given in four phases of remission induction, CNS-directed therapy and intensification or consolidation followed by less intensive and toxic, but longer, continuation or maintenance therapy.

Remission induction therapy is designed to diminish the presenting tumour burden ideally to a morphologically and MRD undetectable level. Complete remission is achieved if the bone marrow consists of less than 5% blasts, no blasts can be detected in peripheral blood and CNS and if no localised disease can be detected. Drugs usually given in induction include a corticosteroid (prednisone or dexamethasone), Vincristine,

L-Asparaginase and an anthracycline (Möricke et al., 2008). Most children (>95%) achieve complete remission after induction therapy and induction failure mainly occurs in the high risk treatment groups (Oudot et al., 2008). CNS treatment is also started early in induction with intrathecal application of chemotherapy given at the first diagnostic lumbar puncture in order to prevent seeding of lymphoblasts from peripheral blood to the CNS (Gajjar et al., 2000). It was soon discovered that after successful remission relapse was often initiated from residual blasts in the CNS occupying a "pharmacologic sanctuary" where the blood-brain barrier prevents penetration of systemically administered chemotherapeutic drugs (Evans et al., 1970). The consecutive introduction of cranial radiation therapy and later combinational intrathecal compartment and high dose systemic chemotherapy greatly reduced treatment failure due to CNS relapse (Rivera et al., 1993; Schrappe et al., 2000b). Because of the serious side effects on IQ, growth, neuroendocrinology and the higher risk of radiation induced secondary malignancies, modern treatment regimens for B precursor ALL mainly use intensified systemic treatment including Methotrexate and Dexamethasone (with a better CNS penetration than Prednisone) in combination with frequent intrathecal therapy as an effective substitute for radiotherapy (Hill et al., 2004; Pui et al., 2003; Pui et al., 2010; Schrappe et al., 1998b). This CNS compartment treatment is mainly contained in intensification or consolidation phases of ALL treatment protocols. They are risk adapted and also aim to reduce the remaining leukaemic burden and to reach other sanctuary places like the testes and use drugs mainly identical to those used in the phase of remission induction. The intensive phases of treatment are followed by two to three years of lower dose continuation or maintenance therapy to further reduce the risk of relapse initiated by remaining blasts that possibly evaded the previous phases of chemotherapy (Eden et al., 1991; Möricke et al., 2010; Pui et al., 2010; Silverman et al., 2010).

1.3 Haematopoiesis

Leukaemias are non-epithelial cancers that arise from the haematopoietic system and cancer research has made huge progress by applying and investigating the concepts of physiological haematopoiesis to the malignant disease. Haematopoiesis describes the process by which all mature cell components (red cells, granulocytes, monocytes and lymphocytes) as well as platelets are generated. For learning more about the biology of any type of leukaemia it is essential to understand the process of physiological haematopoiesis first. It harbours a great potential to improve our knowledge about disease initiation and progression, cellular interactions, but also about possible relapse. Finally and most importantly the gained insights can potentially be exploited to develop better therapies in the future. Much about haematopoiesis has been learned from animal models as this process is highly conserved in vertebrate evolution (Orkin and Zon, 2008).

1.3.1 Introduction to the haematopoietic system

The corpuscular part of the blood system consists of three main components, red cells (erythrocytes), white cells (leukocytes) and platelets (thrombocytes). While the main function of red cells is the transport of oxygen to the different organs, the white cells are mainly involved in protecting the body from infections and the platelets have an invaluable impact in maintaining the integrity of the vascular system. Throughout life the homeostasis of blood formation needs to be maintained and every day an adult person approximately produces about 10^{12} blood cells of the different lineages (Dick, 2003; Ogawa, 1993). As the terminally differentiated blood cells are relatively short lived, they constantly need to be replenished by stem cells. Therefore haematopoiesis is organised in a hierarchy. Rare haematopoietic stem cells (HSC) with long term blood

reconstituting capability (LT-HSC) reside at the top of this hierarchy and give rise to all blood cell lineages (Osawa et al., 1996). They produce haematopoietic stem cells with short term blood reconstituting capability (ST-HSC), also called multipotent progenitors (MPPs), which can still produce all lineages but already have limited self-renewal potential. They consecutively give rise to the lineage committed common lymphoid progenitor (CLP) cells and common myeloid progenitor (CMP) cells. At this point, myeloid and lymphoid development is thought to become separated and mutually exclusive (Akashi et al., 2000; Bryder et al., 2006; Kondo et al., 1997). However, one group has suggested the existence of an intermediate progenitor unable to undergo erythroid and megakaryocyte lineage differentiation, but still harbouring a combined lympho-myeloid differentiation potential and have named it the lymphoid-primed multipotent progenitor (LMPP) (Adolfsson et al., 2005). Extensive proliferation takes place during the progression of the progenitor cells through the hierarchy and while increasingly losing self-replicative capacity, they continue to differentiate to finally become effector cells. Terminally differentiated functional cells arising from the myeloid lineage comprise erythrocytes, neutrophilic, eosinophilic and basophilic granulocytes, monocytes and macrophages, mast cells and thrombocytes (cell fragments of perisinusoidally located megakaryocytes that are sequestered into the vasculature). The lymphoid lineage results in B- and T-lymphocytes and natural killer (NK) cells (Schrappe, 2005).

Figure 1 depicts a schematic model of the process of sequential differentiation and increasing commitment of the progenitor cells starting with the HSC and eventually leading to terminally differentiated mature blood effector cells.

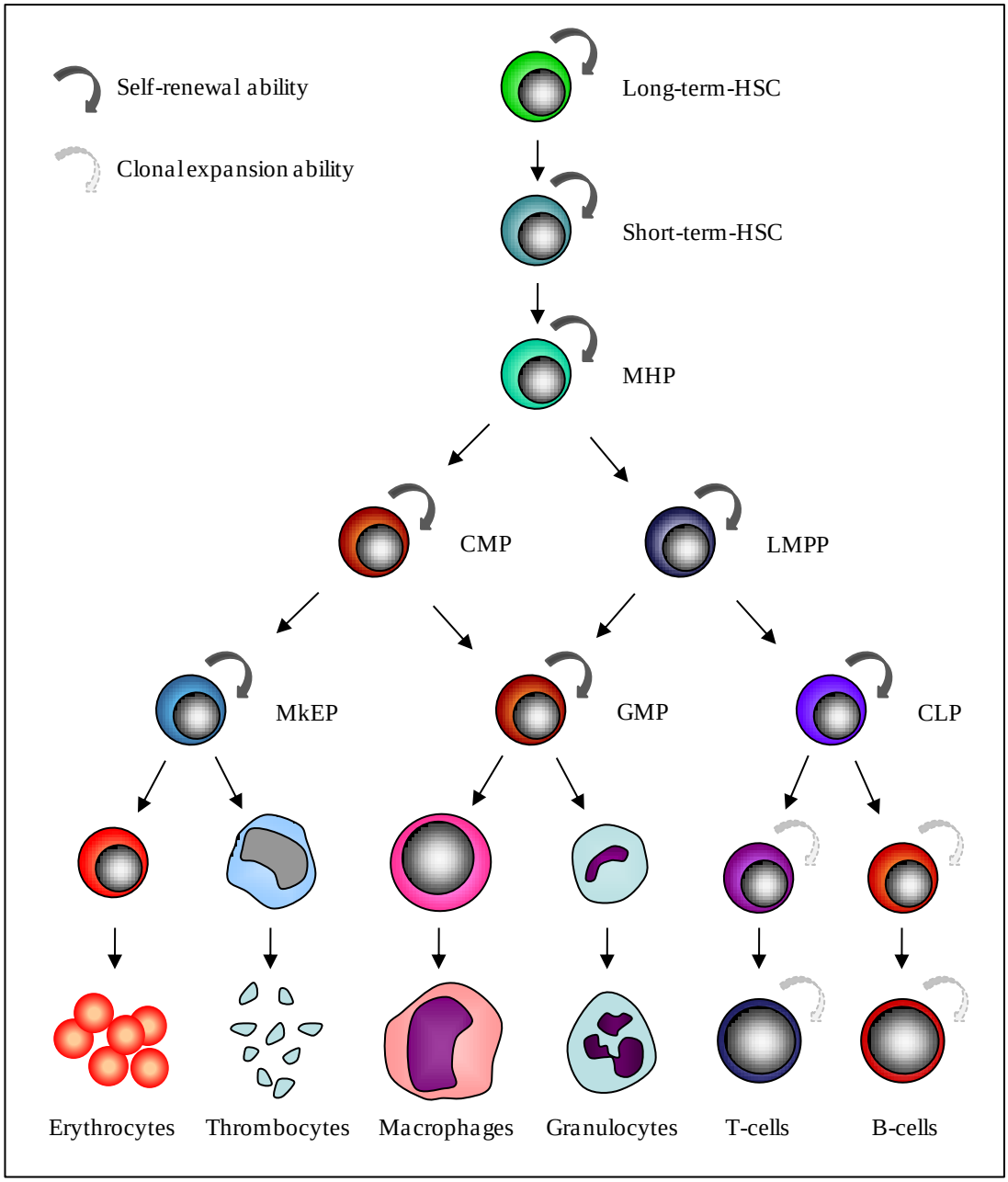


Figure 1: Simplified model of haematopoietic development. Long-term HSCs stand at the apex of the haematopoietic hierarchy. In a stepwise differentiation process, developing cells become more lineage committed and progressively lose the ability to self-renew to finally become postmitotic effector cells. Only lymphocyte lineages retain the ability for clonal expansion.

HSC, haematopoietic stem cells; MHP, multipotent haematopoietic progenitor; LMPP, lymphoid-primed multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MkEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor.

1.3.2 Haematopoietic stem and progenitor cells

1.3.2.1 Origin of haematopoietic stem cells

Haematopoietic stem cells belong to the group of pluripotent adult stem cells and are, due to relatively easy accessibility, probably the best characterized in the field of stem cell biology. Much of the origin of haematopoietic stem cells has been learned from animal models. The first haematopoietic stem cells are descendents of the haemangioblast which itself originates from mesodermal precursor cells and migrate to the yolk sac where they initially form "blood islands" of primitive erythroid cells followed by precursors of the other blood cell lineages. Whether the progenitor cells of the extraembryonic yolk sac contribute to definitive adult haematopoiesis via seeding into the embryo is yet unclear. Early intraembryonic haematopoiesis occurs in the aorta-gonad-mesonephros (AGM) and progenitor cells from this region migrate to the fetal liver where they expand and from there finally to the fetal bone marrow, the physiological site of the adult HSC pool and definitive haematopoiesis (Huang et al., 2007; Orkin and Zon, 2008).

1.3.2.2 Defining properties of haematopoietic stem cells

In a recent study, the frequency of haematopoietic stem cells, defined as $CD34^{+}CD90^{+}CD38^{-}CD45RA^{-}CD10^{-}$ was determined as lying between 0.001 and 0.01% (Kuranda et al., 2011), which actually is very similar to findings of the first attempts to quantify HSC more than 50 years ago (Till and McCulloch, 1961). This very small number of multipotent HSC needs to replace all terminally differentiated and postmitotic cells of the blood system during the entire life without being depleted themselves (Dick, 2003). Therefore these stem cells must combine two apparently conflicting properties which had already been recognised by Till *et al*. in the early 1960s. They must be able to

give rise to all lineages of the committed progenitors and finally mature effector cells and at the same time they must be able to maintain or even expand their own pool of multipotent and undifferentiated adult stem cells (Till and McCulloch, 1961; Till et al., 1964). To meet both criteria of differentiation and self-maintenance, stem cells have one outstanding characteristic; they are able to undergo asymmetric as well as symmetric cell division. In a steady state haematopoietic situation with constant replenishment of terminally differentiated blood cells, HSC usually perform asymmetric cell divisions with one daughter cell adequately replacing the stem cell that underwent division and the other daughter cell pursuing the path of exponential proliferation and differentiation. A symmetric division on the other hand can drive in two directions. It can either result in two daughter cells identical to their ancestor which leads to expansion of the stem cell pool or the generation of two multipotent progenitor cells (MPP) which inevitably will lose stem cell characteristics and become committed to differentiation (Ho, 2005; Warren and Rossi, 2008). The latter decision of a stem cell will lead to a diminished total number of stem cells but growth in early life, haematologic stress and regeneration make it necessary to shift the balance towards symmetric cell division. The process of asymmetric cell division was elegantly demonstrated in human haematopoietic progenitor cells by Leary et al. (Leary et al., 1984; Leary et al., 1985). The different options of cell divisions in stem cells are illustrated in Figure 2.

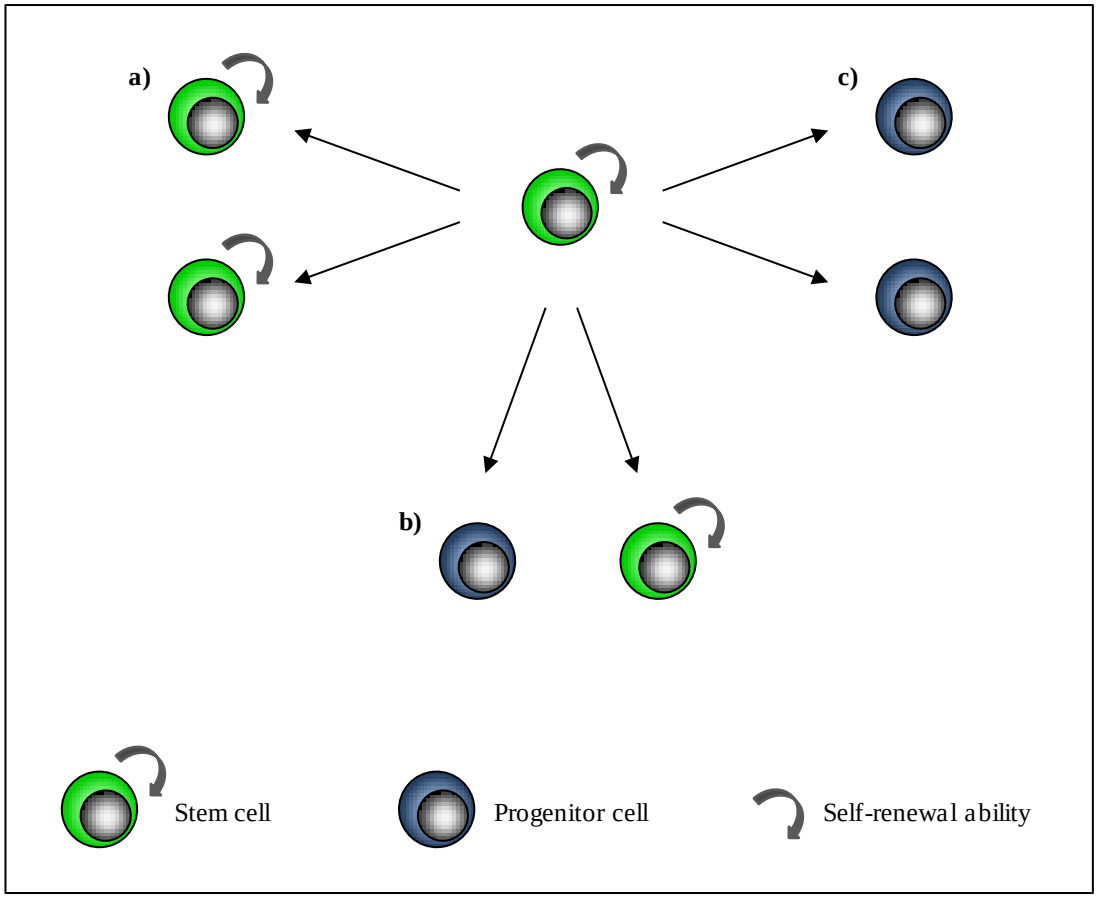


Figure 2: Haematopoietic stem cell division options. **a)** Symmetric stem cell division with both daughter cells retaining stem cell properties resulting in expansion of the stem cell pool. **b)** Asymmetric stem cell division with one daughter cell remaining a stem cell and the other becoming a committed progenitor cell resulting in tissue homeostasis. **c)** Symmetric stem cell division into two committed progenitor cells resulting in stem cell depletion and extinction of the clone.

Although the ability of HSC to self-renew is often described as unlimited, this may not be true. As differentiated mature blood cells still need to be replenished throughout life, stem cells must be available at all times to give rise to these progeny effector cells (Warren and Rossi, 2008). Therefore it was proposed by Kay that most HSC within the bone marrow lie dormant in a non-cycling state and only enter the cell cycle in the manner of a clonal succession, once previously cycling stem cells were depleted (Kay, 1965). However, a recent study using clonal tracking of presumed HSC in a murine

model could not support this hypothesis but suggested that individual HSC may contribute to haematopoiesis throughout life (McKenzie et al., 2006).

In summary, the two main properties of HSC, self-renewal and differentiation, enables them to maintain the entire haematopoietic system throughout life.

1.3.2.3 Characteristics of haematopoietic stem and progenitor cells

The low cycling frequency allows the stem cell to maintain its genomic integrity to a great extent as replication of DNA during the process of cell division and therefore the risk of mitosis associated mutation, are reduced. The G_0 phase of the cell cycle is also associated with a low rate of metabolism and therefore a reduction of exposure to metabolic products or reactive oxygen species. Lineage committed precursor cells have a great proliferation potential but progressively lose the ability to self-renew and the main expansion of blood cells takes place in this compartment (Bryder et al., 2006). Committed precursor cells, however, eventually result in non-proliferating and differentiated cells and for this reason are thought to have a lower chance of accumulating cancer initiating mutations (Dick, 2003). In contrast, despite the low proliferative pressure with infrequent cell divisions, the longevity of stem cells and their capability to self-renew makes them an ideal collector of the multiple genetic hits that usually are involved in the process of oncogenesis. The rising incidence of haematological cancers with age supports this assumption (Warren and Rossi, 2008).

1.3.2.4 The haematopoietic stem cell niche

Definitive adult haematopoiesis is a dynamic process and mainly takes place in the bone marrow. Only at times of haematopoietic stress, the liver and the spleen can serve as

alternative sites of blood production (Kiel and Morrison, 2008). The homeostasis of the defining properties of self-renewal and differentiation needs to be tightly regulated and this is the function of the stem cell niche (Orkin and Zon, 2008). The niche is a specialised supportive and regulatory microenvironment, which, through cell to cell interaction regulates the balance of self-renewal, differentiation and quiescence. The concept of a niche composed of differentiated somatic cells, extracellular matrix and the stem cells themselves as well as part of their progeny was first proposed by Schofield in 1978 (Eckfeldt et al., 2005; Schofield, 1978). Under normal physiological conditions the niche provides an environment that predominantly inhibits both proliferation and differentiation although it provides proliferating signals intermittently to support tissue regeneration (Li and Neaves, 2006). The inner surface of the bones, the bone marrow endosteum, with its osteoblasts, osteoclasts and dense vascularisation has long been thought to play a major part in providing the HSC niche (Gong, 1978). Osteoblasts secrete the soluble and diffusible factors angiopoietin and thromopoietin that trigger quiescence of the stem cells and also the stromal-cell derived factor (SDF)-1 (often referred to as chemokine ligand CXCL12) which is involved in migration and homing of stem cells to the bone marrow (Kiel and Morrison, 2008; Kollet et al., 2002). However, osteoblasts are not the only source of these factors and they could as well diffuse from other sites of production to the bone marrow (Kiel and Morrison, 2008). Furthermore, whether or not N-cadherin is necessary for anchoring stem cells to the osteoblasts remains unclear. Even specific signalling pathways in HSC like the notch pathway that is activated by Jagged1 produced by osteoblasts and together with WNT-signalling supports self-renewal and inhibits differentiation, do not seem to be crucial for stem cell maintenance. Osteoclasts seem to play a role as well. Their proteases can release growth factors like stem cell factor (SCF) from membranes and extracellular matrix and also remodel bone to provide space for the niche (Duncan et al., 2005; Kiel and Morrison,

2008). The observation that extramedullary tissues like the liver and the spleen can take over or contribute to haematopoiesis, suggests that osteoblasts and osteoclasts are not the only cell types that can provide a suitable niche. SDF-1/CXCL12-secreting perivascular reticular cells and mesenchymal progenitors that additionally produce angiopoietin, together with endothelial cells of sinusoids and megakaryocytes are believed to provide such an environment outside the bone marrow. However, they also can be found within it and adult HSC have been localised in their immediate vicinity. As the endosteum is very well vascularised, the endosteal and the vascular niche may well not be independent or clearly separated entities and it is entirely possible that they indeed form a common niche with a contribution of all cells to stem cell maintenance, differentiation and localisation (Kiel and Morrison, 2008; Lo Celso et al., 2009).

1.3.3 CD surface antigens and haematopoietic hierarchy

During the development from the haematopoietic stem cell to the various effector cells, the progenitor and precursor cells pass through several maturation stages in a multistep differentiation process. It was only possible to unravel this differentiation pathway by finding ways to precisely separate cells of one defined developmental step from the ones further upstream and downstream. Furthermore, if biological or pathobiological properties and functional behaviour is to be addressed within distinct cells of this hierarchical haematopoietic system or within blasts of the supposedly related leukaemias, means to differentiate and physically separate discrete populations from the whole is indispensable. Morphology only very roughly allows categorisation into earlier and more mature cells but every cell displays an abundance of cell surface antigens, which now can be exploited for deeper characterisation. The cluster of differentiation (CD) antigens are a heterogeneous group of molecules (proteins, glycoproteins and glycolipids) first described on leukocytes and often involved as receptors or ligands in

cell signalling or in cell adhesion. Each subset of leukocytes exhibits a unique combination of these epitopes. Specific monoclonal antibodies (mAb) have been developed to bind these antigens. Binding of these antibodies can be used to estimate the number of cells in a mixed population bearing a specific CD antigen and also to measure the amount of it on individual cells (Mason et al., 2002; Zola, 2006).

Methods also have been developed to separate cell populations according to their CD surface antigen pattern and fluorescence activated cell sorting (FACS) uses specific antibodies conjugated with a fluorescent dye to purify cells with the same expression level of these markers. Usually combinations of several markers are necessary to identify distinct cell populations and this is possible by using different fluorescent dyes bound to different mAbs.

Within the haematopoietic hierarchy, expression profiles for CD antigens are well defined for different subpopulations and progressively change during maturation. By analogy, the same marker combinations are used in malignancies of haematopoietic origin to identify subpopulations and to position them in a hypothetical corresponding malignant hierarchy.

1.3.3.1 B-cell differentiation

Like all other cells of the haematopoietic system, B lymphocytes are developed from the haematopoietic stem cell through multiple steps of differentiation. Mature B cells have to recognise an enormous number of antigens not innate of its own organism to ultimately protect the body from infective agents. For this purpose a broad repertoire of immunoglobulin antigen receptors is created by gene rearrangements in the immunoglobulin (Ig) heavy (H)- and light (L)-chain during the process of B cell differentiation. Every step of this complex process can be separated in a time response or

developmental manner and the distinct subpopulation of B lineage committed cells identified by the state of IgH and IgL rearrangements can then be correlated with the expression profile of CD surface antigens (Ghia et al., 1998; Noordzij et al., 2002b).

B cell development is initiated when multipotent $CD34^{+}/lin^{-}$ ($CD3/13/19/33/56^{-}$) cells commit themselves to become pro-B cells. Only at that stage in the hierarchical development of B cells the immunoglobulin recombination machinery is first activated and creates double strand (ds) DNA breaks between segments of the *IGH* loci, which it then re-ligates in the following steps of nonhomologous end joining (NHEJ). Pro-B cells are the earliest cells in the B cell lineage and can be characterised by the surface expression of CD34 and CD22 but not yet CD19. These pro-B cells initiate gene rearrangements between the genes for the diversity (D) and joining (J) segments of the immunoglobulin heavy chain but they do not start rearranging the variable (V) segment to the DJ rearranged segment. In the next step of differentiation pro-B cells progress to pre-B-I cells with expression of the specific marker profile $CD34^{+}CD19^{+}CD10^{+}CD20^{-}$. At this level of maturation rearrangements, still only within the *IGH* locus, between the already existent DJ_H segment and the V_H segment are first observed and the D_H to J_H rearrangements decline in percentage. When pre-B-I cells move forward in the B-cell hierarchy they emerge as pre-B-II large cells which undergo some cycles of proliferation mediated by a pre-B-cell receptor (BCR) formed by the already completed heavy chain together with a surrogate light chain. These large cycling pre-B-II cells are now devoid of CD34 and are characterised by the distinct immunophenotype $CD34^{-}CD19^{+}CD10^{+}CD20^{dim}$. When the pre-B-II large cells give rise to the pre-B-II small cells, they lose the only low expression of CD20 and become $CD34^{-}CD19^{+}CD10^{+}CD20^{-}$. At this phase of B-cell development, pre-B-II small cells begin with IgL gene rearrangements between the V and J segments of the *IGK* and the *IGL* loci respectively. In the case of successful expression of a functional Ig light chain a surface membrane

immunoglobulin, the complete B cell receptor, is formed together with the IgH chain and displayed on immature B cells. The CD20 antigen is now highly up-regulated and the immature B cell defining surface marker pattern is $CD34^{-}CD19^{+}CD10^{+}CD20^{+}$. Only non-autoreactive immature B cells finally leave the bone marrow and home to peripheral lymphatic organs as naive mature B cells that lose CD10 expression (Galy et al., 1995; Hystad et al., 2007; LeBien, 2000; Loken et al., 1987; van Zelm et al., 2005).

This B cell development is described as being linear in the cited publications. However, recent work by Sanz and colleagues suggests parallel but distinct developmental pathways of first pre/pro $CD34^{+}CD10^{-}CD19^{+}$ and then CLP/early-B $CD34^{+}CD10^{+}CD19^{-}$ cells from $CD34^{+}CD10^{-}CD19^{-}$ stem cells, at least in cord blood (Sanz et al., 2010).

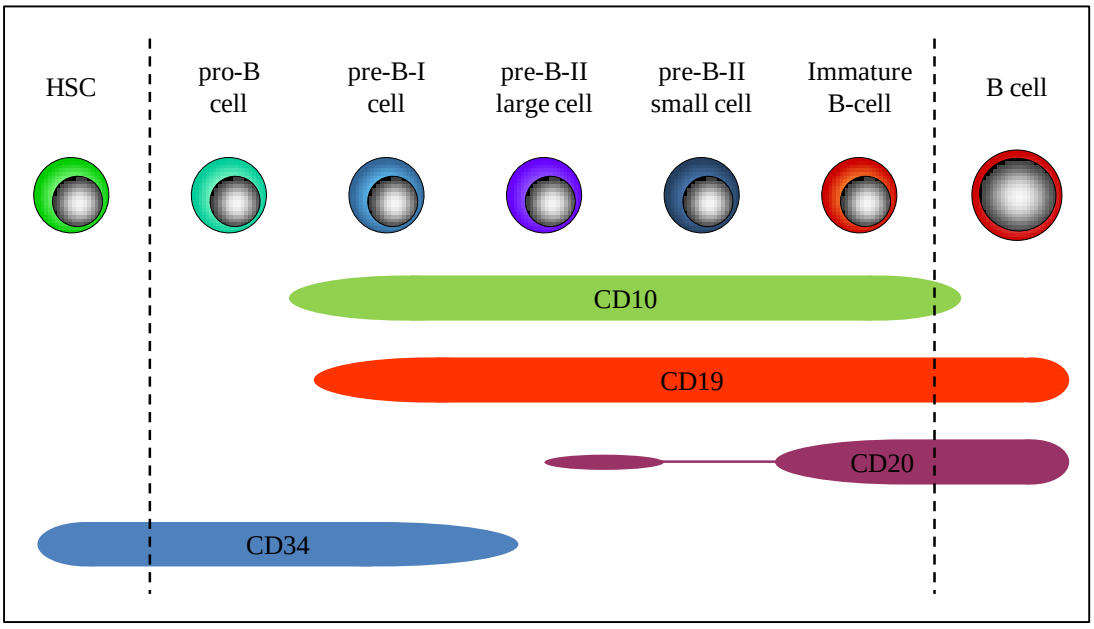


Figure 3: CD surface antigen expression during B cell development. The stem cell marker CD34 is expressed on HSC´s but also retained in B lineage committed cells up to the pre-B-I developmental stadium. Of surface markers restricted to B cell development in the haematopoietic hierarchy, CD10 expression only slightly precedes first expression of CD19 but disappears on mature B cells while CD19 remains positive. CD20 shows the latest appearance of these markers with expression initiated only at the level of pre-B-II large cells. CD20 as well is found on mature B cells.

1.3.3.2 CD10

The CD10 surface antigen was first described by Greaves and co-workers in 1975 in an attempt to produce antisera in rabbits that would be able to reliably differentiate between ALL and other types of leukaemia or normal cells respectively. Their efforts resulted in antisera that were reactive to a large subgroup of ALL but only very few other types of leukaemia and it was assumed that it probably could define an antigen relatively specific to ALL. However, binding to a small number of cells in adult bone marrow and spleen as well as in fetal liver was observed as well (Greaves et al., 1975). Later, when monoclonal antibodies against CD10 could be produced, the expression of CD10 on B-lineage ALL was confirmed and evidence was provided that the original rabbit antiserum, despite being polyclonal and containing antibodies to other cell surface antigens, indeed recognized the CD10 molecule (Greaves et al., 1983; LeBien and McCormack, 1989).

Because of the widespread reactivity with ALL, this antigen was first named common ALL antigen (CALLA) It was subsequently given the cluster of differentiation number 10 during the first international workshop on human leukocyte differentiation antigens in Paris 1982 (Bernard and Boumsell, 1984). Clinically, the subgroup of ALL expressing CALLA and therefore termed common ALL (cALL), soon was associated with a better prognosis in comparison to other types of ALL (Greaves, 1981).

The initial observation that the anti-CD10 rabbit antiserum reacted to very few normal lymphoid bone marrow cells strongly suggested the origin of ALL in normal lymphoid progenitor cells (Hokland et al., 1983; Janossy et al., 1979). As a consequence, using this new marker, CD10 expressing lymphoid cells could be further analysed. It was found that $CD10^+$ lymphoid cells represent early stages of B cell development and they only represented a very small part of the B lineage population (<1% of bone marrow cells). Under further scrutiny they always co-expressed CD19 and subpopulations also

CD34, but mostly not CD20, which could be detected on more mature cells that already presented IgM on the cell surface (Loken et al., 1987; Ryan et al., 1986). Mature peripheral blood B cells were found to be devoid of CD10 surface antigen (Stashenko et al., 1981). Today, CD10 is a well established marker in lymphoid development used in many studies. Recently it was established by Ichii and co-workers that differentiation potential is increasingly lost with up-regulation of CD10 expression in B lymphoid development but that strict B lineage commitment occurs only in $CD34^{+}CD10^{high}$ cells, whereas $CD34^{+}CD10^{low}$ cells still harbour some myeloid differentiation potential (Ichii et al., 2010).

Expression of CD10 is not restricted to the haematopoietic system and the related leukaemias and lymphomas but has also been detected in several other tissues and cancers (LeBien and McCormack, 1989). Regarding its function, CD10 was found to be identical to neprilysin, a zinc metalloendopeptidase on the cell surface. It hydrolyses regulatory oligopeptides and thereby terminates their function of initiating intracellular signalling (Turner et al., 2001).

1.3.3.3 CD19

Early in the 1980s CD19, then known as B4 or Leu-12, was characterised as a glycoprotein being expressed uniformly and specifically throughout B-cell development from the earliest stages to mature B cells. Resting as well as activated B lymphocytes are $CD19^{+}$ and the expression is not lost when B cells terminally differentiate and become plasma cells. It was also found on B lymphoid derived malignancies but neither on normal and activated T lymphoid cells and their malignant counterparts nor on other haematopoietic cells (Harada et al., 1993; Hokland et al., 1983; Meeker et al., 1984; Stamenkovic and Seed, 1988). Indeed, lymphoid progenitor cells with the

immunophenotype $CD34^{+}CD10^{+}CD19^{-}$ are still not completely B lineage restricted and are able to additionally generate T cell progeny, dendritic cells and natural killer cells (Galy et al., 1995; Hystad et al., 2007; LeBien, 2000).

CD19 plays an important role in the generation of germinal centres and in antibody production after contact to antigens as demonstrated in CD19 deficient mice that failed to do so (Carter and Myers, 2008). Hypogammaglobulinaemia was also found in patients carrying homozygous mutations in the *CD19* gene that resulted in lack of CD19 antigen display on the cell surface (van Zelm et al., 2006). On mature B lymphocytes CD19 usually is present in a complex with CD21, CD81 and CD225 which is involved in the activation of B cells. When it co-ligates with the B cell receptor it significantly decreases the threshold for B cell activation upon antigen contact. This enables B lymphocytes to respond to very low concentrations of antigens and through that the highly specific BCR gains a high sensitivity (Carter and Fearon, 1992; van Zelm et al., 2010).

1.3.3.4 CD20

CD20 or formerly named B1 and Leu-16, is a marker recognised to be expressed only on later stages in B-cell development and is retained on mature peripheral blood B lymphocytes. Pre B II cells are the first that initiate display of CD20 on their cell membrane and expression reaches the highest levels on immature B cells. However, when peripheral B lymphocytes are activated and develop into terminally differentiated and Ig-secreting plasma cells, CD20 expression usually disappears (Harada et al., 1993; Noordzij et al., 2002a; Stashenko et al., 1981). CD20 is a phosphoprotein and like CD19, it is exclusively found on members of the B cell lineage. Studies with blocking antibodies to CD20 provided some evidence that it is involved in B cell activation and differentiation (Tedder et al., 1985). The natural ligand of CD20 is not known but

employing monoclonal antibodies it was suggested that it can serve as a calcium channel and probably is involved in intracellular signalling and also has effects on the cell cycle (Bubien et al., 1993; Kanzaki et al., 1995; Perosa et al., 2010). However, in a murine model, lack of CD20 surface expression in $CD20^{-/-}$ mice did not have a major effect on B cell function (Uchida et al., 2004).

With its specificity to the B lineage and expression on mature B lymphocytes, the CD20 surface antigen constitutes an effective target for mAbs and these are now widely applied in immunotherapy of autoimmune diseases as well as in oncology (Oflazoglu and Audoly, 2010; Perosa et al., 2010).

1.3.3.5 CD34

The surface glycophosphoprotein CD34 (in earlier publications termed My10) is one of the most important marker proteins in haematopoietic stem cell research. $CD34^{+}$ populations clearly contain rare haematopoietic stem and progenitor cells with long-term repopulating ability but lineage committed progenitors progressed further down in the haematopoietic hierarchy and mature blood cells are CD34 negative. In normal bone marrow, only 1.5% of all low density mononuclear cells (MNCs) are CD34 positive (Krause et al., 1996). However, expression is not restricted to early haematopoietic cells. CD34 is also expressed in the vascular endothelium, not only in adults, but already in endothelial cells of the yolk sac and the aorta-gonad-mesonephros region of the developing embryo. Furthermore, the presence of CD34 antigen on some subsets of stromal progenitor cells of the bone marrow, the muscle and the placenta suggests that at least some mesenchymal stem cells are part of the $CD34^{+}$ population (Furness and McNagny, 2006).

The function of CD34 still remains largely unknown or controversial. Its expression in non-quiescent activated haematopoietic precursor cells may suggest that it plays a role in supporting proliferation and impeding differentiation. It also has been implicated in leukocyte migration and homing of progenitors to the niche and this characteristic was connected to the pro-adhesive properties of the molecule (Furness and McNagny, 2006; Krause et al., 1996).

As the CD34 antigen is expressed on early haematopoietic stem and progenitor cells, it was not too surprising to discover that the $CD34^+$ cell fraction was capable of reconstituting and maintaining the haematopoietic system, first in animal models and then in humans. This knowledge led to important and now routinely used clinical applications. CD34 usually is not expressed in solid tumours and sorted $CD34^+$ haematopoietic cells that are purged of tumour cells can be used for autologous bone marrow transplantation in patients that have undergone myeloablative high dose chemotherapy (Berenson et al., 1991). In diseases of the haematopoietic system itself, haematopoietic cells enriched in $CD34^+$ progenitors are used for allogeneic transplantation (Krause et al., 1996).

However, haematopoietic stem cell characteristics do not seem to be exclusively confined to $CD34^+$ cell populations, although this was first thought to be the case. Initiation of long-term lympho-myeloid reconstitution has also been demonstrated in very rare $CD34^-c\text{-}kit^-Sca\text{-}1^-Lin^-$ cells in mouse to mouse transplantation experiments (Osawa et al., 1996) and following this also in xenotransplant mouse models for human cells with the immunophenotype $Lin^-CD34^-CD38^-$ (Bhatia et al., 1998; Wang et al., 2003). Interestingly, the surface expression of CD34 on reconstituting human HSC seems to be reversible to some extent. This has been demonstrated by Dao *et al* by showing that in a first round of xenotransplantation primary human bone marrow $CD34^+$ cells can convert to $CD34^-$ cells that do have long-term repopulating capability and

themselves give rise to $CD34^+$ progeny in a secondary round of transplantation (Dao et al., 2003). However, as proved by years of successful clinical SCT with longterm haematopoietic reconstitution from purified $CD34^+$ cells, HSCs certainly are enriched in the $CD34^+$ subfraction and $CD34^-$ stem cells are only detected in Lin^- and $CD38^-$ $CD34^-$ cells but not in $CD34^-$ cells that are further down of $CD34^+$ cells in the haematopoietic hierarchy which make up for the vast majority of $CD34^-$ cells. Furthermore, as determined by limiting dilution experiments, SCs seem to be a very rare entity (one in 125,000) within the $Lin^-CD34^-CD38^-$ fraction (Bhatia et al., 1998).

1.3.3.6 CD38

CD38 is a surface glycoprotein that, unlike CD19 and CD20, is not restricted to the B lineage and can also be found on other blood cells including T lymphocytes, NK cells, monocytes, myelocytes, red cells and platelets as well as on cells of various solid tissues. CD38 is multifunctional; it can function as an ectoenzyme and is involved in the degradation of NAD^+ by hydrolysis and can act as a receptor and in cell-to-cell adhesion. Because of the two latter properties and the finding that its ligand, CD31, is mainly expressed on vascular endothelial cells, it has been connected with lymphocyte transendothelial trafficking and migration. CD38 seems to have contrasting impacts on cells of the B lineage. While ligand activation of the receptor initiates proliferation in mature splenic B lymphocytes, it signals towards apoptosis in immature bone marrow B cells (Deaglio et al., 2001).

As CD38 is displayed on cells of the blood system, the differential degree of its expression has also contributed to elucidation of the haematopoietic hierarchy. However, the expression pattern in physiological haematopoiesis is somewhat different to other markers; CD38 is not displayed over a continuous time span of development but has its

first appearance at the level of normal multipotent progenitors before it disappears until the level of mature lymphocytes, where it is expressed again. The highest surface density in the B cell lineage is found after differentiation to plasma cells (Malavasi et al., 2008; Ramos et al., 2010).

It was first shown by Dick´s group in Toronto that the $CD34^{+}CD38^{-}$ bm cell fraction is able to reconstitute the haematopoietic system (Bhatia et al., 1997; Larochelle et al., 1996) and later, using CD38 as a marker for sub classification of human $CD34^{+}$ haematopoietic cells it was possible to functionally separate different stages of maturation. Hogan and colleagues demonstrated that $CD34^{+}CD38^{-}$ cells were able to repopulate NOD/SCID mice and maintain haematopoiesis of human origin for more than 20 weeks, while haematopoietic reconstitution from $CD34^{+}CD38^{+}$ cells was short lived (Hogan et al., 2002).

1.4 Cancer stem cells

1.4.1 Definition

With a growing body of evidence emerging that, at least in some forms of cancer, only a small number of cells within the tumour harbour the ability to sustain the cancer, this subset of cells has gained a lot of attention. It was self-evident that these cells needed to be the target of any efficient cancer therapy that aimed at eliminating the possibility of relapse. These cancer stem cells would be functionally analogous to physiological stem cells. To compare the work published on cancer stem cells made it necessary to find a consensus definition regarding the exact properties of this subset of cells. For this purpose a group of specialists in the field formed a workshop on cancer stem cells at the meeting of the American Association for Cancer Research in Lansdowne, Virginia, USA in 2006. Cancer stem cells were defined as the cells within a tumour that have a) self-

renewal capacity and b) were able to generate the heterogeneous lineages of cancer cells present in tumours Although it was noticed that normal stem cells with their inherent capacity to self-renew were the ideal object of malignant transformation, it was stated that cancer stem cells could also evolve from progenitor cells that re-acquired self-renewal properties. It was also clarified that cancer stem cells must contain not only the ability to proliferate but in fact have the capacity of symmetrical or asymmetrical self-renewing cell divisions like their normal counterparts in non-cancerous tissues (see Figure 2) (Clarke et al., 2006).

1.4.2 The history of cancer stem cell research

Already with the relatively rough means of staining techniques and light microscopy alone it has been noticed, that most malignancies, haematological and solid tumours alike, are not a homogenous assembly of completely identical cells but rather consist of cell types with significantly differing morphology. In epithelial cancers, blasts even seemed to resemble various differentiation stages of the normal corresponding tissue. This was thought to be caused by the influence of the microenvironment and the cancer inherent genomic instability on the actual phenotype of different cells within the tumour (Clarke et al., 2006).

Malignancies were for a long time thought to be caused by transmissible agents, namely viruses and therefore any cell transduced by such a vector would be expected to be imbued with the same ability regarding initiation and maintenance of abnormal and uncontrolled proliferative growth, the main characteristic of cancer. With the development of the first murine *in vivo* assays, huge and rapid progress regarding research into the functional biology behind the morphologic heterogeneity of normal and malignant tissues was initiated. Remarkable work in this field was conducted by Furth

and Kahn and published in 1937. They used inbred and therefore largely syngenic mice for their experiments, thereby elegantly overcoming immunologic barriers that inevitably negatively effect any functional *in vivo* assay. From murine leukaemic tumours that were initiated by subcutaneous inoculation of leukaemic blasts they produced single cell suspensions and transplanted those subcutaneously, intraperitoneally or intravenously into inbred sister mice. Using two different mouse leukaemias they were able to successfully initiate the original disease in 5 out of 97 mice transplanted intravenously with a single cell only. Aliquots with crushed cells from their cell suspensions, in contrary, were not able to reconstitute the leukaemia in mice and they concluded that live cells rather than cell free agents were essential to transmit the leukaemia (Furth and Kahn, 1937).

With their experiments they not only showed that it is blasts from which the leukaemia is recapitulated in the host but they made another important observation; not every cell seemed to be capable of transferring the disease but only a low percentage has this ability, an important contribution towards the hypothesis of functional heterogeneity within malignancies. Following this seminal publication, further evidence of functional heterogeneity accompanying the morphologic heterogeneity arose from several publications. Hewitt *et al* performed experiments with a mouse lymphocytic leukaemia that they transplanted intraperitoneally on inbred mice and confirmed that acellular suspensions could not re-establish a leukaemia and that leukaemia growth could not be initiated by every cell (Hewitt, 1958).

Similar experiments were also performed using isolated rat sarcoma cells. Single cells from these solid cancers could grow tumours following intraperitoneal inoculation but the success rate varied considerably. Furthermore, serial transplantations were conducted and chromosomal characteristics of the tumours were compared between the different generations and the original tumour. While the initial tumour contained cells with

various chromosome constitutions, the tumours grown from single cells exclusively showed one distinct set of chromosomes that was maintained over several transplant generations (Makino, 1956; Makino and Kano, 1955). These experiments proved self-renewal abilities of the transplanted cells and also suggested that different subsets of tumour cells were able to reconstitute the sarcoma.

Other groups followed another *in vivo* approach to assess the biology of normal and tumour growth. After Till and McCulloch developed their quantitative spleen colony forming assay in sub-lethally irradiated mice and first determined the frequency of HSC, this assay was soon adapted and applied to malignancies as well (Till and McCulloch, 1961). Bruce and van der Gaag transplanted lymphoma cells from several mice with spontaneous lymphomas into irradiated and non-irradiated host mice and found a range of 100 to 100,000 cells being necessary to result in one colony per spleen (Bruce and Van Der Gaag, 1963).

The single cell transplantation assays and the spleen colony forming assays, both of normal and malignant cells, provided strong evidence that only a small minority of cells had the capability to transfer tumour growth on host animals. The serial transplantations additionally demonstrated the self-renewal capacity of the transplanted cells. It was the work of these groups that founded the first body of evidence for the concept of cancer stem cells.

In addition, in the 1960´s Pierce and colleagues observed that in teratocarcinomas, embryonal carcinoma cells were able to differentiate into mature postmitotic and therefore benign tissues. They understood that this type of carcinoma was organised in a hierarchy with the embryonal cancer cells at the apex and proposed the concept that cancers mimic physiological tissue proliferation and differentiation, in which regulation is deregulated (Pierce and Speers, 1988; Pierce and Verney, 1960).

Further progress in CSC research was made with another approach. In 1946 Belanger and Leblond developed a method for the direct experimental visualization of a hierarchical organization of normal and malignant tissues. They exposed a photographic gelatine emulsion to histological sections of radiolabelled (P^{32} or I^{131}) tissues and developed it. Additionally they stained the slide with Methylene blue or Haematoxylin and were then able to locate the cells within the tissue that had incorporated the radioactive molecules (Belanger and Leblond, 1946). Applying this autoradiographic method on blood smears, several studies were performed to investigate the cellular proliferation kinetics of normal haematopoietic and acute myeloid and lymphoblastic leukaemic cells using H^3-Thymidine (Cheung et al., 1972; Clarkson et al., 1967; Clarkson et al., 1975; Todo et al., 1971). Interestingly it was observed that during continuous infusions of H^3-Thymidine the kinetics of uptake was different according to the blast size. Large leukaemic cells were radiolabelled much faster than small cells and it was concluded that these small cells lay dormant for most of the time, have self-renewal capacity and replenish the large blasts (Clarkson et al., 1970).

However, while these studies were able to show the heterogeneity in blast populations of leukaemias and led to the suggestion of a minor, slow-cycling stem cell fraction within the leukaemia that replenishes rapidly proliferating cells, prospective isolation of these cells could not be performed with the methods available, making functional enquiries impossible.

1.4.3 Models of tumour heterogeneity

As described above, cancer cells, in addition to their morphologic heterogeneity, do apparently exhibit pronounced functional heterogeneity regarding their proliferative and cancer initiating potential. Two mutually exclusive models in terms of the nature of

cancer stem cells giving rise to the heterogeneous cancer populations exist (see Figure 4).

In the stochastic model, CSC are not biologically distinct but form the bulk tumour population. In this model any random cell within the tumour has the rare capability to acquire CSC properties in response to endogenous and/or exogenous factors. Endogenous factors could, for example, comprise variable expression of transcription factors, differential induction of signalling pathways and several cell cycle control mechanisms while extrinsic factors include the combination of cytokines a particular cancer cell is exposed to, cell to cell communication and especially the location in respect to the microenvironment of the stem cell niche (Dick, 2008b). Given the appropriate stimuli, any cell within a tumour can act as a CSC and initiate and maintain the complete tumour phenotype but any cell can as well revert to a tumour cell lacking CSC characteristics as the stimuli do not confer stable changes onto exposed cells. It is therefore the ability of any cell within a tumour to acquire CSC status that makes it a potential CSC but not any cell necessarily needs to actually be in that functional state at any given time point. In fact, there may even be an equilibrium between the CSC and non-CSC state within a tumour that can shift according to differing signals from the microenvironment and this plasticity of cells has been called bidirectional interconvertibility (Gupta et al., 2009).

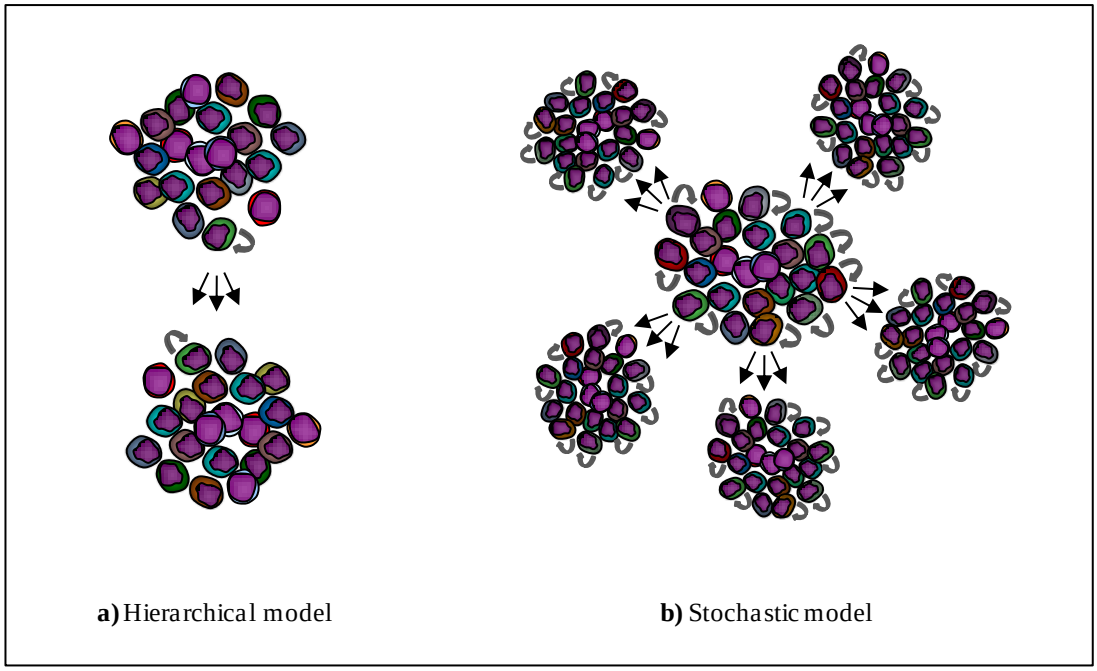


Figure 4: The hierarchical and stochastic cancer stem cell model. a) Only rare cancer stem cells (depicted in green) within the malignancy are able to self-renew and reconstitute the tumour heterogeneity. The bulk population of tumour cells is progressed in a developmental hierarchy (represented by cells of different colours) and lost CSC properties. **b)** Cancer cells of different phenotypes have the same CSC capabilities given the appropriate stimulus. A hierarchical order of cell development as existent in the physiological tissue counterparts, in which the malignancies originated can not be established.

Opposed to the stochastic model stands the hierarchy model. In this model the organisation of a cancer mimics the tightly regulated normal tissue development. Biologically distinct stem cells stand at the apex of a hierarchy in which the ability to self-renew is increasingly lost with differentiation. These cells maintain themselves via symmetric or asymmetric cell divisions to produce identical daughter cells and therefore maintain the malignant clone. Their progeny differentiates into the heterogeneous cells comprising the tumour but despite potentially still having pronounced proliferative capacities lack self-renewal characteristics. It needs to be borne in mind that, although it provides a likely target, the cell of origin of a cancer is not necessarily a normal stem cell.

The concept of a hierarchy of cells existing in tumours led to research to identify the cells that stand at the apex. To prove the hierarchy model, it must be possible to prospectively isolate distinct cell fractions that exclusively have the ability to reconstitute a certain tumour. On the other hand, if the stochastic model is correct, cell populations sorted in regards to any chosen characteristic should have the same potential to initiate tumour formation (Dick, 2008b). While the stochastic model describes the older view of cancer, the hierarchy model is the one that was more recently developed and favoured. However, there is emerging evidence coming from research on different cancer entities, that while the hierarchy model might be applicable to some tumour entities, other tumours may support the stochastic model.

In addition to the two described models of tumour heterogeneity, more lately the concept of clonal evolution of tumour cell populations has begun to influence CSC research and adds considerable complexity to it. In a review of the then available literature, Nowell in 1976 proposed the concept of clonal evolution for cancers. Mirroring Darwin´s natural selection theory, malignant cells are exposed to external pressures on survival and have to pass evolutionary bottlenecks that modulate or dictate their fates. An initial event in a single cell imposes a growth advantage on it that leads to clonal expansion. The unlimited proliferation potential of that clone, combined with increased genetic instability of transformed cells eventually leads to genetic alterations in some progeny resulting in subclonal genetic diversity. Most genetic changes in the cells will lead to cell death but by chance one mutation will confer the cell with additional selective advantage and a new dominant clone emerges. This stepwise process goes along with the biological features of tumour progression (Nowell, 1976). Although it was acknowledged that this may confer chemoresistance, clonal evolution was regarded as a process of sequential selection leading to increasingly more malignant characteristics of a tumour.

Malignant clonal evolution was recently demonstrated by the group led by Greaves in *ETV6-RUNX1* positive ALL. They found up to eight genetic abnormalities in single leukaemic cells using multiplexing FISH probes and were able to infer the existence of up to ten subclones with different genetic profiles. Of note, these subclones showed sequential as well as concurrent, branching occurrence. From these data they gained insight into the subclonal architecture and deduced the likely ancestral relationships of the clonal evolution. Most interestingly, and deviating from sequential clonal evolution, they demonstrated the leukaemia propagating potential of cells from different subclones and therefore the existence of CSC within these subclones (Anderson et al., 2011). John Dick and his group in Toronto also contributed to this evolving field of subclonal diversity in CSC in Philadelphia chromosome positive ALL. In diagnostic patient samples they detected several genetically distinct subclones with leukaemia propagating capability by using DNA copy number alteration (CNA) assessment and the NSG xenotransplant model. In agreement with the Mel Greaves group they reconstituted a branching rather than a sequential ancestral tree of clonal evolution by CNA profiling (Notta et al., 2011).

However, the branching clonal evolution theory can be integrated into both the stochastic and the hierarchical model of cancer heterogeneity. It seems obvious that it will have an impact on the kinetics of disease progression, invasion and metastasis, potential relapse after treatment and inevitably successful treatment strategies as different genetic subclones might well have to be targeted in a different way.

1.4.4 Identification of cancer stem cells in leukaemias

Technical improvements paved the way for identifying the first stem cells in human cancers. Two major problems had to be overcome in order to do so. First, if candidate

stem cells are to be investigated, it is essential to discriminate them reliably from the rest of the malignant population and also to separate them so that they can be further used in functional assays.

The ability to sort bulk populations of cells into distinct fractions according to their expression of surface antigens by the development of monoclonal antibodies and fluorescence activated cell sorting (FACS) opened the field for functional analysis. With the identification of distinguishable patterns of surface antigen expression in different fractions of the bulk cancer population these immunophenotypically defined fractions could be purified and following this their properties could be investigated in functional assays.

The second problem lies in the functional assay itself. The appropriate assay would need to prove the essential characteristics of stem cells: long term self-renewal, high proliferation potential and differentiation into different daughter cells. Human cells are generally not able to survive for a long time *in vitro* and cell lines derived from physiologic or malignant tissues are not suitable for identifying a possible hierarchy as they consist of rather homogenous populations of cells that have been immortalised. An important achievement to circumvent this problem was the development of suitable xenotransplant animal models that can host human cells. In a healthy condition the mammalian body mainly defends its integrity against foreign organisms or agents with the help of the innate and acquired immune system and the highly complex and coordinated interplay between them. Transplanting foreign cells into immunocompetent animals would soon activate the hosts' immune system and lead to the rejection of the foreign material. To create a hospitable xenoenvironment, increasingly immunocompromised mice were bred taking advantage of naturally occurring accidental immune defects and by actively manipulating genes involved in immune defence. For studies on lymphoid cells an additional advantage is that animals that lack lymphoid

tissues readily provide empty niches to accommodate transplanted lymphocytes (Dick, 2008a).

1.4.4.1 AML stem cells

Seminal work in the process of identifying haematopoietic and consecutively cancer stem cells was carried out by researchers in the laboratory led by Dick in Toronto, Canada. He developed powerful assays and his work inspired the search for cancer stem cells in many other malignancies.

Already in *in vitro* experiments rather short term reconstituting AML-colony forming units (CFU) with variable replating abilities could be established suggesting a hierarchical order of AML blasts (McCulloch, 1983). Building on their insights in HSC John Dick and colleagues then tried to characterise long term repopulating AML stem cells by transplanting flow sorted candidate stem cells into their murine xenotransplant model. As distinguishing markers they used the surface antigens CD34 and CD38. For normal haematopoiesis it was already known that early CD34 positive cells increasingly express CD38 during maturation. Flow cytometric studies on AML discovered differential expression of these developmental markers in fractions of the tumour population as well. Correlating the patterns of CD34 and CD38 expression with risk of relapse, an event initiated by LSC surviving chemotherapy, again the existence of a hierarchical organization comparable to that in normal haematopoiesis was suggested but LSC were not formally proved to be present (Terstappen et al., 1992).

Using severe combined immune-deficient (SCID) mice in their assay, the search for AML LSC was finally successful. First, they transplanted unsorted patient AML samples of the FAB subtypes M1, M2 and M4 into the tail veins of sublethally irradiated mice and samples of all subtypes led to disease in the mice with a morphology and

dissemination patterns similar to the patients from which the cells were taken. Furthermore, not only the number of human cells but also the number of AML-CFU initiating cells increased over time in the bone marrow of mice, indicating that these arose from more immature AML stem cells in the process of proliferation and differentiation. Then they went on and transplanted cell fractions of one M1 AML purified according to CD34 and CD38 surface marker expression. Engraftment of the leukaemia could only be observed in mice transplanted with the $CD34^+CD38^-$ blast population but not with $CD34^+CD38^+$ cells. As both fractions contained AML-CFU initiating cells it was concluded, that more mature $CD34^+CD38^+$ cells developed from $CD34^+CD38^-$ cells that constitute AML stem cells. These cells were then termed SCID leukaemia-initiating cells (SL-IC) (Lapidot et al., 1994). However, the SCID transplantation assay only allowed the group to successfully show a hierarchy for a primitive AML M1 sample and other types of AML could potentially not follow this model.

With the development of the more immunocompromised nonobese diabetic SCID (NOD/SCID) mice the hypothesis eventually could be extended to other types of AML. This more robust murine model was already successfully used to characterise normal haematopoietic stem cells to be of the $CD34^+CD38^-$ immunophenotype (Bhatia et al., 1997; Larochelle et al., 1996). In limiting dilution xenotransplantation experiments also the frequency of LSC in unsorted populations of different types of AML (M1, M4 and M5) was examined. The frequency was found to be between 0.2 and 200 SL-IC in one million unseparated AML mononuclear cells in this quantitative assay and was found to have no correlation with factors such as FAB subtype, patient age and sex or $CD34^+$ cell proportion in the sample.

To identify the immunophenotype of the SL-IC the surface markers CD34 and CD38 were used for purification, as in the preceding experiments on the AML M1 and studies

of the normal haematopoietic stem cells. In all transplanted AML samples, the undifferentiated M1 phenotype as well as the myelomonocytic lineage designated M4 and M5 AML, the $CD34^{+}CD38^{-}$ cell subfraction exclusively harboured the SL-IC. Furthermore, the leukaemias initiated by these cells clinically resembled the original AML subtypes, were able to give rise to more differentiated blasts that acquired lineage markers and the cells displaying the immunophenotype $CD34^{+}CD38^{-}$ were maintained in serial transplantations into secondary recipients, indicating self-renewal potential. Taken together, these studies strongly suggested a hierarchical organisation for human AML clones similar to that in the physiological developmental pathway. As SL-IC for the different AML subtypes were all found in the cells with the $CD34^{+}CD38^{-}$ immunophenotype, it was concluded that leukaemic transformation took place in early haematopoietic stem cells and not in committed progenitor cells regardless of the AML subtype (Bonnet and Dick, 1997).

Further studies elucidated an even more complex hierarchical composition in AML. In normal HSC individual cells with short-term and long-term repopulating abilities could be detected where increased differentiation goes along with reduced self-renewal capacity and a transition from long-term to short term repopulating capacity (Guenechea et al., 2001). Similar experiments were then performed on AML by lentiviral clonal tracking of individual clones in the xenotransplant model. In serial transplantations clones were identified that largely contributed to the bulk leukaemia in several rounds of transplantation indicating extensive self-renewal potential. Other clones only reconstituted the AML in the primary recipient mice but disappeared in secondary transplantations therefore representing short-term SL-IC. A third type of clone emerged in secondary mice after apparently having remained dormant in the primary transplant. These cells were called quiescent long-term SL-IC. Furthermore, it was possible to

demonstrate that short-term SL-IC were generated by long-term SL-IC and that quiescent long-term SL-IC gave rise to both of these cell types (Hope et al., 2004).

Support that haematopoietic stem cells are the target for malignant transformation came from a study of Kroon and colleagues. They showed that a combined over expression of *Hoxa9* and *Meis1a* in primary bone marrow cells resulted in the development of a hierarchically organised AML and that malignant transformation took part in primitive haematopoietic cells (Kroon et al., 1998). These striking similarities in the organisation between haematopoiesis and AML in the succession of clones highly suggest that the cell of origin for AML is a HSC.

The hypothesis that AML stem cells are a rare entity did not remain unchallenged. Syngenic mouse models that are devoid of the caveats of xenotransplantation models provided opposing results. When single blasts from a mouse AML, caused by PU.1 deficiency, were transplanted into syngenic recipients, positive engraftment could be detected, at least in some mice. Certainly, human and murine leukaemias do not match each other in many respects, but the much higher proportion of AML initiating cells in the syngenic model may well point out, that a xenotransplant model underestimates the real frequency of stem cells (Kelly et al., 2007). However, to argue against a hierarchical model in human AML, the existence of LSC would need to be proved in cells of other immunophenotypes than the rare $CD34^{+}CD38^{-}$ cells but mostly, even in more recent studies using the most immunocompromised NSG mice available, the earlier findings that SL-IC in human AML are exclusive to the $CD34^{+}CD38^{-}$ compartment were confirmed (Ishikawa et al., 2007). However, there are first studies now, which identified LICs in immunophenotypically more mature $CD34^{+}CD38^{+}$ or $CD34^{-}$ blast subfractions in cases of AML carrying a nucleophosmin mutation (Taussig et al., 2008; Taussig et al., 2010). Furthermore, another study provided convincing evidence that LICs are present in AML blast populations resembling normal granulocyte-macrophage progenitors

(GMP) and lymphoid-primed multipotential progenitors (LMPPs) (Goardon et al., 2011).

1.4.4.2 ALL stem cells

Unlike in AML where the model of hierarchical organisation of the tumour population is widely accepted, the situation for ALL is quite different. Investigations focussing on LSC in lymphoid malignancies were long held back as in contrast to AML suitable clonogenic *in vitro* assays were limited. However, the extensive body of work done on AML paved the way for the search of CSC in other malignancies. In particular, in the case of ALL, the SCID mouse model was shown to successfully engraft primary patient samples, providing a powerful tool to investigate candidate stem cell populations (Kamel-Reid et al., 1989). Applying this method, investigations could be performed to establish whether ALL follows the hierarchical model or whether many cell types, resembling the stem cell as well as the progenitor compartment of normal haematopoiesis could sustain the disease, thereby supporting the stochastic model of CSC.

The discovery that in AML, regardless of the actual immunophenotype of the bulk blast population, LSC only can be detected in the multipotent $CD34^+CD38^-$ compartment that had not undergone maturation towards lineage commitment led to the assumption that this could also be the case for ALL. Undeniably, earlier studies on ALL pointed in this direction. In a murine xenotransplant model Cobaleda et al indeed determined only a very low frequency of SL-IC between 0.2 and 41 in one million cells of Ph^+ ALL samples and transplantation of $CD34^+CD38^-$ and $CD34^+CD38^+$ subfractions only resulted in mouse engraftment from the less mature $CD34^+CD38^-$ cells. Furthermore, engrafted blasts partly expressed CD19, a clear sign of differentiation along the B

lineage and $CD34^+CD38^-$ cells were able to serially repopulate mice proving self-renewal capacity of these immature cells (Cobaleda et al., 2000). These results strongly argued in favour of a hierarchical organisation of ALL.

Following this, Cox and colleagues investigated 16 mainly standard risk pre-B ALL and c-ALL samples. To achieve engraftment between 5.0×10^5 and 1.0×10^6 blasts had to be transplanted. In further experiments with blast populations sorted according to CD34, CD10 and CD19 expression they found that only $CD34^+CD10^-$ and $CD34^+CD19^-$ subfractions but not cells of the $CD34^-$, $CD34^+CD10^+$ or $CD34^+CD19^+$ immunophenotype could reconstitute the ALL in mice. Again self-renewal capability of engrafting populations was proved in serial transplantations (Cox et al., 2004). These results of a second study confirmed the findings of Cobaleda et al and supported the assumption that stem cells in ALL were only present in the pluripotent immature cell fraction which gives rise to more differentiated blasts without stem cell characteristics.

Cox *et al* subsequently confirmed their hypothesis of a hierarchical order in ALL with other surface markers 5 years later. CD133 was earlier found to characterise very early multipotent HSC (Gallacher et al., 2000; Kuçi et al., 2003) and was chosen as it was shown to be a useful marker in identifying CSC of a variety of solid cancers, including those of the colon and the CNS but also to be differentially expressed on leukaemic cells (Bühring et al., 1999; O'Brien et al., 2007; Singh et al., 2004). Blasts with co-expression of CD133 and CD19 or CD38 were sorted as candidate stem cell populations and transplanted onto mice. Again, only immature immunophenotypes with $CD133^+CD19^-$ or $CD133^+CD38^-$ expression were able to transfer the original ALL onto mice. Additionally they demonstrated a relative high resistance of these subgroups towards Dexamethasone and Vincristine. Drug resistance is thought to be especially pronounced in stem cells (Cox et al., 2009).

Other groups did not confirm these results and a dispute was ignited whether or not only rather immature and not lineage restricted blasts harboured SL-IC in ALL. In 2005 Castor *et al* published their findings in ETV6/RUNX1 and BCR/ABL1 positive ALL. Firstly they confirmed earlier findings that the ETV6/RUNX1 fusion gene can be detected in the $CD34^{+}CD38^{-}$ subfraction of leukaemic cells but when they subjected these cells to further scrutiny they found that the translocation was only found in an aberrant, but largely predominant, population with the immunophenotype $CD34^{+}CD38^{-}CD19^{+}$ and not in the very small $CD34^{+}CD38^{-}CD19^{-}$ population which constitutes an immature developmental stage of the normal haematopoietic hierarchy. CD19 expression only occurs in already B-lineage committed lymphoid precursor cells and, in combination with the finding that none of additionally isolated $CD34^{+}CD33^{+}CD19^{-}$ myeloid cells carried the ETV6/RUNX1 fusion gene, they concluded that leukaemic transformation takes place after definite separation of myeloid and lymphoid development with the multipotential immature $CD34^{+}CD38^{-}CD19^{-}$ compartment being unaffected. In Ph^{+} ALL they had different findings. While in ALL with the p210 BCR/ABL1 fusion the $CD34^{+}CD38^{-}CD19^{-}$ compartment was involved and lymphoid as well as myeloid cell lineages continued to express the fusion gene, in ALL harbouring p190 BCR/ABL1 the fusion gene could only be detected in the lymphoid restricted lineage. Leukaemia reconstitution ability was then assayed in irradiated NOD/SCID mice by intravenous cell transplantation. As expected for ETV6/RUNX1 and p190 BCR/ABL1 positive ALL, the original leukaemia only was reconstituted and serially transplantable from $CD19^{+}$ and not $CD19^{-}$ blasts but surprisingly this was also the case for p210 BCR/ABL1 positive ALL. In conclusion, LSC in all investigated types of ALL were only part of the lymphoid committed precursor cell compartment (Castor et al., 2005).

These data contradicted the studies of Cobaleda *et al* and Cox *et al* and reopened the debate regarding the nature of LSC in ALL. In support of the findings of Castor *et al*, Hong *et al* demonstrated tumour propagating abilities in B-lineage restricted lymphocytic blasts. They purified the same abnormal $CD34^{+}CD38^{low/-}CD19^{+}$ cells from an ETV6/RUNX1 positive c-ALL, an immunophenotype that is not part of normal haematopoiesis. This population, containing the t(12;21) translocation, from four patient samples engrafted and reproduced itself in primary and secondary generations of NOD/SCID mice, providing evidence for self-renewal. The group interestingly also showed that this specific subpopulation must form part of an ancestral pre-leukaemic clone as they detected it, including the t(12;21) translocation, in the blood of the still healthy identical twin sister of one patient, whereas her other progenitor B cells or non lineage committed HSC did not express the fusion transcript. The leukaemic blasts of the patient had an additional loss of the not translocated ETV6 allele (Hong et al., 2008). This is a known "second hit" in this subtype of ALL (Zuna et al., 2004).

More studies continued to provide support for the hypothesis that ALL blast populations with an already committed B-precursor immunophenotype have leukaemia propagating abilities. Kong *et al* intravenously injected purified populations, not only of the aberrant $CD34^{+}CD38^{-}CD19^{+}$ but also of the $CD34^{+}CD38^{+}CD19^{+}$ immunophenotype which resembles a physiological B-cell precursor, into the more recently developed and even more immunocompromised NOD/SCID/IL2rγ^{null} (NSG) mice. Remarkably, both subfractions were able to transfer the patient ALL onto the mice with a typical ALL dissemination pattern and to show self-renewal by serial transplantation. The samples used were from higher risk paediatric patients, one defined by a high presenting blast count and two by the presence of the MLL-rearrangement. However, again opposing to the studies performed earlier, no leukaemic but only normal haematopoietic engraftment was achieved from the immature $CD34^{+}CD38^{+}CD19^{+}CD10^{-}$ compartment (Kong et al.,

2008). The decisive contribution of this work regarding the dispute over the differentiation level of LIC in ALL is that it not only determined LIC in the abnormal $CD34^{+}CD38^{-}CD19^{+}$ population but also in the $CD34^{+}CD38^{+}CD19^{+}$ fraction, that truly resembles a committed stage in the physiological lymphoid hierarchy. Additionally supporting the hypothesis that immunophenotypically B-lineage committed blasts harbour LIC le Viseur *et al* published the observation that not only $CD34^{+}CD19^{-}$ and $CD34^{+}CD19^{+}$ but interestingly as well developmentally further progressed $CD34^{-}CD19^{+}$ and $CD19^{+}CD20^{low/-}$ or $CD19^{+}CD20^{+}$ blasts serially reconstituted the patient leukaemia. This study used mice and transplantation techniques most favourable of xenoengraftment known to date. In contrast to earlier studies, additionally candidate LSC populations were orthotopically injected into the bone marrow of the mice femora. The hosts consisted of either NOD/SCID mice in which residual natural killer cells (NK-cells) were depleted by anti-CD122 treatment or NSG mice that naturally lack any NK-cells (le Viseur et al., 2008).

Another recent study did not address the question of the immunophenotypic identity of a potential ALL stem cell but aimed to establish the frequency of LIC within the unsorted bulk ALL population. The high frequency that was found in their NSG mouse model, with 1 to 100 blasts being sufficient to transfer the leukaemia, provided additional support that LIC could not be restricted to the very small compartment of immature blast immunophenotypes (Morisot et al., 2010).

To compare these *in vivo* studies can be challenging as they all were carried out using xenotransplant models with marked experimental differences. These lie in the use of markers and antibodies but more importantly are affected by the development of increasingly immunocompromised mouse strains that were available in the time course of the studies. Refined transplantation techniques (orthotopic transplantations) are as well likely to have contributed to the conflicting findings.

In summary, these studies leave the identity or the distribution of LSC in subpopulations of ALL open to question. However, the aim to develop specifically designed and targeted therapies that are able to improve clinical patient outcome can only be addressed and potentially realised once the identity of the target LSC is convincingly established.

1.4.4.3 Cancer stem cells in solid tumours

The attractive prospect of developing targeted therapies for leukaemias has initiated a huge interest in identifying CSC for solid cancers as well as they comprise the majority of malignancies in adulthood with frequently very low success rates using current treatment strategies. Despite significantly more difficulties in investigating stem cells in solid tumours, an increasing number of publications now address a variety of tumour entities. Besides problems with accessibility, one major obstacle lies in the lack of clearly established and tissue specific developmental hierarchies defined by differential surface marker expressions that can be used for the discrimination of cell subsets within cancers (Clevers, 2011). Studies on solid cancers performed so far therefore often used markers that are known to be differentially expressed in certain cancers rather than being known as specific stem cell markers in the referring normal tissue.

One of the first studies successful in identifying a definable subset of cells with CSC characteristics was performed on breast cancer using the surface markers CD24 and CD44 in combination. CD24 and CD44 both belong to the group of adhesion molecules. Al-Hajj and colleagues transplanted sorted cell subsets of human primary breast cancers or metastasic pleural effusions orthotopically into the mammary fat pads of NOD/SCID mice and evaluated serial transplantation potential. They found that, of the tumour cells, those with the $CD44^{+}CD24^{-/low}$ immunophenotype were highly enriched in tumourigenic

cells with self-renewal and differentiation capabilities (Al-Hajj et al., 2003). However, malignant cells of the $CD44^{+}CD24^{-/low}$ immunophenotype can only be detected in about one third of breast carcinomas, particularly on the BRCA1 hereditary basal-like subgroup (Honeth et al., 2008). Pancreatic cancer stem cells, in contrast, have been suggested in populations being positive for surface marker expression of CD44 and CD24 (Li et al., 2007). $CD44^{+}$ subfractions of cells have also been associated with stem cells of gastric cancer and ovarian cancer (Alvero et al., 2009; Bapat et al., 2005; Takaishi et al., 2009). Another surface marker widely used to identify CSC of solid tumours in xenotransplant mouse models is CD133. In brain tumours (medulloblastomas and glioblastomas), Singh *et al* could demonstrate that while as few as 100 cells of the purified minor $CD133^{+}$ subfraction were able to give rise to a brain tumour in mice that could be recapitulated over several generations, none developed after intracranial injection of 10^{5} cells of the larger supplementary $CD133^{-}$ subpopulation, thereby suggesting a functional hierarchy (Singh et al., 2004). Advanced aggressive stages of gliomas were also found to have a higher proportion of $CD133^{+}$ cells and these are also more radioresistant than the bulk tumour mass (Bao et al., 2006; Thon et al., 2010). Similar results were obtained from transplant experiments on colon cancer. Despite CD133 not being expressed on the majority of tumour cells, colon cancer initiating cells were highly enriched in the minor $CD133^{+}$ cell fraction compared to the unfractionated cell population in the studies of O´Brien *et al* and Ricci-Vitiani *et al* (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). However, as in ALL, the existence of a functional hierarchy for colon cancer has recently been challenged by another group, which showed tumour engraftment from $CD133^{+}$ as well as $CD133^{-}$ subpopulations. Additionally they showed that CD133 is universally expressed in the differentiated normal colon epithelium and linked CD133 expression to the expression of CD24 and CD44. Interestingly, $CD133^{+}$ cells were $CD44^{low}CD24^{+}$ but $CD44^{+}CD24^{-}$ cells, previously

described to identify CSC of other tumour entities, could be found among the $CD133^-$ population (Shmelkov et al., 2008). CD44 in combination with CD133 expression has also been found in a small fraction of prostate cancer cells that exhibit stem cell characteristics *in vitro* (Collins et al., 2005). CD133 additionally has been linked to hepatic, pancreatic and lung cancer propagating cells (Eramo et al., 2008; Hermann et al., 2007; Ma et al., 2007).

Other groups used the so-called side population in the search for CSC, cells identified by flow cytometry with the ability to exclude Hoechst dye due to ATP-binding cassette transporter family expression and therefore regarded to be enriched for normal and malignant stem cells. Side population cells with enriched tumour initiating capability and other stem cell characteristics like self-renewal, differentiation ability and cell cycle quiescence were found in lung cancer, gliomas and gastric cancers (Fukuda et al., 2009; Harris et al., 2008; Ho et al., 2007). For oral squamous cell cancer, CSC within this side population were additionally found to be associated with CD133 surface marker expression (Chiou et al., 2008).

Interesting conflicting findings have been reported from the field of melanoma stem cell research. Melanoma has been suggested to be organised in a hierarchical order following the cancer stem cell model by *in vitro* and *in vivo* experiments (Fang et al., 2005). Schatton et al found malignant melanoma initiating cells with self-renewal and differentiation capacity enriched in a subpopulation of cells expressing the chemoresistance mediator ABCB5 in a non-irradiated NOD/SCID xenotransplantation assay after subcutaneous tumour cell injection. In unsorted cell populations approximately 1 in 10^6 cells had stem cell activity and that rose to 1 in around 1.5×10^5 cells in the $ABCB5^+$ subfraction (Schatton et al., 2008). Notably, the percentage of tumour initiating cells in unfractionated melanoma cells shortly after was heavily adjusted upwards to approximately 25% by the group of Morrison. Interestingly, that

was achieved by using the more immunodeficient NSG mouse model and by improving the tumour cell environment by coinjection of Matrigel, an extracellular protein mixture. Furthermore, they tested melanomas for heterogeneous expression of a variety of surface markers, among them CD133 and CD166 which correlate with the expression of ABCB5 and CD44. When purified according to these surface markers, none of the subgroups was found to be enriched in CSC´s. Additionally, $CD133^+$ as well as $CD133^-$ subfractions both reconstituted the opposing immunophenotype in NSG host mice. In conclusion, this study puts the hypothesis of a hierarchical organisation in malignant melanomas into question and also challenged studies on other tumour entities that used less immunocompromised NOD/SCID mice (Quintana et al., 2008). In contrast, the Weissman group at Stanford, California, suggested in a recent very sophisticated study, that a hierarchy in melanoma does exist and can be found in early stages but that a self-renewing CSC population outgrows in later stages, being the only population detectable. The CSC subfraction in early stages of malignant melanoma was characterised by the expression of the neural crest stem cell marker CD271 (Boiko et al., 2010). However, this study was challenged by the Morrison group, who reported that in a comparable patient group $CD271^+$ as well as $CD271^-$ single primary malignant melanoma cells had CSC frequencies of around 25% (Quintana et al., 2010).

These examples of solid cancers illustrate how complex and controversial the field of CSC research is and that many, if not all, questions regarding the definite identity and proportion of CSC within various cancers still remain unanswered.

1.4.5 The cell of origin in cancers

As a clonal disease, cancer is initiated in a single cell of origin in the process of malignant transformation and the whole tumour mass subsequently arises from it. The

observation that the hierarchical organisation of AML stem cells closely mimics the normal HSC compartment with reduced self-renewal capacity in cells which are further progressed in the differentiation pathway strongly suggests that the cell that undergoes malignant transformation is part of the HSC compartment. This is also supported by the fact that, despite different maturation stages of blasts in different AML samples, their LSC all had an undifferentiated immunophenotype resembling that of a normal HSC (Bonnet and Dick, 1997; Hope et al., 2004). However, an alternative model could also be true. The initiating step in cancer development could also take place in an already committed progenitor cell that had already lost self-renewal capacity but retains proliferation potential. It would then be essential that this cell reacquires the ability to self-renew in order to establish a hierarchy (Passegué et al., 2003). The first scenario appears to be more attractive for a cancer organised in a hierarchy. If the normal stem cell inherent hallmark of self-renewal is simply taken over and maintained by CSC, fewer mutagenic events need to accumulate in the process of malignant transformation than if a progenitor cell would need to gain this property to drive tumour progression and survival. The fact that stem cells persist over a very long time in the organism, gives them a much higher chance to collect transforming mutations in comparison to rather short lived, more differentiated, cells of a tissue hierarchy (Reya et al., 2001). On the other hand, the leukaemic transformation of committed progenitor cells was successfully demonstrated in a murine model and it could be shown that this involved reactivation of some genes normally highly expressed in haematopoietic stem cells and associated with self-renewal. Krivtsov and colleagues expressed the MLL/AF9 fusion protein in mouse granulocyte macrophage progenitors and these led to the development of an AML in their syngenic siblings (Krivtsov et al., 2006). However, MLL-rearrangements are thought to have leukaemogenic transformation potential as a single hit and extended

survival of progenitor cells therefore does not seem to be necessary to acquire the otherwise required second hit (Szczepański et al., 2010).

For chronic myeloid leukaemia (CML) it was shown more that 30 years ago that the cell of origin lies within the HSC compartment. The finding that non-leukaemic lymphocytes arose from the same precursor as the malignant clone indicated that a multipotential HSC must have been the cell of origin of the CML (Fialkow et al., 1978).

The situation in ALL could be different as lymphocytes maintain self-renewal capacity even in advanced stages of differentiation and only loose it when they progress to become immunoglobulin secreting plasma cells.

1.4.7 Implication of cancer stem cells on therapeutic strategies

Current therapeutic strategies for curing cancer generally consist of chemotherapy, radiotherapy, surgery or different combinations of these. All these strategies have one aim in common; they are designed to eliminate all cells comprising the malignancy. In other terms, they are highly unspecific. This approach pays tribute to the classical stochastic theory that all or at least the majority of malignant cells within a tumour contribute to its growth by having the equal and unlimited ability to proliferate. As chemotherapy and radiotherapy both mainly target rapidly dividing cells, they not only kill cancer cells but also have cytotoxic effects on normal cells, especially of highly regenerative tissues like the gastrointestinal mucosa or the bone marrow. As a simple general rule, successful treatment of cancers will need to effectively eradicate those cells which sustain the disease and lead to its progression. With emerging evidence for the more recent cancer stem cell model for at least some cancer entities new challenges but also new therapeutic chances arise. If cancers are organised in a similar way to physiologic non-malignant tissues with a stem cell at the apex of a cell hierarchy, then

only these cancer stem cells are responsible for maintaining the tumour and give rise to progenitor or transit amplifying cells with no, or limited, self-renewal potential. From a therapeutic aspect that would mean that the cells that form the bulk cancer mass do not need to be eliminated but that the CSC are the prime target of any therapy as they would initiate possible relapse. Learning more about the biology of these CSC offers a great opportunity to develop specific therapies with higher effectiveness and potentially fewer side effects. The challenges, however, are partly imposed by the biological features of stem cells themselves. Stem cells usually lie dormant in their niches and only very occasionally enter a cycling status in response to physiological demand. This imposes a natural resistance to current therapies that target cycling cells (Clarke et al., 2006).

Like their normal counterparts, LSC are also believed to lie quiescent for most of the time and therefore are difficult to eradicate with treatment modalities that are cell-cycle dependent (Holyoake et al., 1999; Ravandi and Estrov, 2006). Even if malignant cells with stem cell properties could be targeted selectively it still might be necessary to eradicate non-CSC as well. The stem cell phenotype may not be a stable trait but subject to bidirectional interconvertibility between the CSC and the non-CSC compartment as proposed by Gupta *et al*. In this case non-CSC could replenish CSC after those have been eliminated successfully with targeted therapy (Gupta et al., 2009). Similarly, the more recent findings of subclonal diversity of leukaemic blasts with tumour propagating potential impose another level of complexity to cancer therapy. If mutant molecules are specifically targeted, they may only be present in one distinct subclone of tumour propagating cells that was dominant at presentation. However, relapse could then derive from other subclones that lack this mutant molecule and become dominant because of the selective evolutional pressure as a consequence of the targeted therapy (Anderson et al., 2011; Notta et al., 2011; Nowell, 1976). Difficulties of a similar type can arise in CML. It is established that the disease is initiated in HSC that naturally are endowed

with self-renewal capacity and would need to be eradicated if therapy is to be successful. However, in the case of CML progression to blast crisis malignant cells of the downstream progenitor population have been shown to reacquire self-renewal capability resulting in LSC status of these cells (Jamieson, 2008).

Selectively targeting CSC with all its therapeutic advantages is a very attractive prospect but will be very difficult to achieve considering how challenging it is to identify them. An alternative approach is to force stem cells out of their quiescent stage and to make them vulnerable to classic antiproliferative cancer therapy. First evidence for the success of this strategy was provided in an AML mouse model. Entry into the cell cycle could be induced in quiescent AML $CD34^{+}CD38^{-}$ LIC by administration of granulocyte colony-stimulating factor (G-CSF) and they then became susceptible to conventional chemotherapy (Saito et al., 2010).

Relapse can emerge not only from residual LSC of the dominant or minor malignant subclones but also from a persistent preleukaemic clone that, as a result of an independent second hit, initiates overt leukaemia. This possibility has been demonstrated for ETV6/RUNX1 positive ALL (Zuna et al., 2004). To include a preleukaemic clone with self-renewal capacity in treatment strategies would add another level of complexity.

Additionally, the inherently cytoprotective abilities of stem cells such as enhanced drug exclusion due to up-regulation of ABC/MDR transporter genes may partially explain why cancers initially often respond well to chemotherapy but that treatment fails to completely eradicate the disease, finally resulting in relapse (Rossi et al., 2005; Zhou et al., 2001). Furthermore, upregulation of anti-apoptotic genes like Bcl-X(L) and Bcl-2 in leukaemic $CD34^{+}$ cells impose a resistance to apoptotic stimuli in the blasts and increase chance of survival of LSC. In AML blasts, expression of BCL-2 has also been found to

be inversely correlated with the ability to enter the cell cycle, thereby additionally protecting blasts from chemotherapy (Konopleva et al., 2002; Tacke et al., 2004).

The aim of CSC directed treatment is not only to specifically target them but also to preserve their normal equivalents within the non-malignant tissue. As malignant stem cells supposedly only accumulated the smallest number of mutagenic events within the bulk tumour and are still most similar to normal stem cells, this aim remains a big challenge in cancer research (Ravandi and Estrov, 2006).

However, it has been shown, that at least cells of some cancers are still able to differentiate to acquire a postmitotic cell status. In *in vitro* and *in vivo* studies on teratocarcinomas, Pierce *et al* convincingly demonstrated differentiation abilities of malignant cells (Pierce and Verney, 1960) and suggested that this could potentially be exploited for cancer therapy. A differentiation therapy would force malignant cells to terminally differentiate to benign progeny in response to administered chemicals or naturally occurring factors (Pierce and Speers, 1988). Induction of differentiation by using all-trans-retinoic acid (ATRA) is successfully applied in one form of leukaemia, the acute promyelocytic leukaemia (Sanz and Lo-Coco, 2011).

The described potential consequences of CSC characteristics for cancer treatment strategies have already been investigated in some exemplary cancers and were found to be a hindrance for conventional antiproliferative chemotherapy. In glioblastomas, for instance, it has been shown that $CD133^{+}$ cells represent CSC that are able to initiate and maintain the disease in a xenotransplant assay and that these cells are especially resistant to chemotherapy as well as to radiation therapy (Bao et al., 2006; Liu et al., 2006; Singh et al., 2004). Similarly, in breast cancers, the subset of $CD44^{+}CD24^{-/low}$ cells was found to be more chemotherapy resistant than the bulk tumour population (Li et al., 2008).

1.5 Significance and summary

Improvement in treatment outcomes of childhood ALL from the first achieved remissions over 60 years ago to a cure rate of now over 80% is an outstanding story of success. Additionally, allocation of patients to treatment arms of different intensity by applying increasingly refined prognostic markers at diagnosis and during chemotherapy has led to a significant reduction of adverse events and long-term sequelae resulting in a higher quality of life of former ALL patients, almost comparable to the general population. Despite this, due to the relative high incidence, ALL still remains one of the major threats for the life of children and adolescents and new therapeutic strategies to successfully treat those who fail to be cured by current chemotherapy approaches are yet to be developed. Much hope is invested in the field of cancer stem cell research, with the ultimate prospect of specifically targeting the cells that are responsible for tumour maintenance and growth. However, drug development essentially relies on the definitive identification and characterisation of these stem cells but cancer researchers, especially those interested in ALL, have obtained inconsistent and partly contradictory insights regarding the frequency and identity of these candidate target cells. Several recent studies provide possible explanations for these differences by demonstrating the critical impact of the experiment set up, especially in the *in vivo* murine model but also of the preparative steps and transplantation techniques. Using the most sensitive assays available to date and taking experimental pitfalls into account as well as standardising experiments between research groups holds the biggest promise in finally succeeding to decisively settle the dispute over the nature of the leukaemic stem cell in ALL.

1.6 Hypothesis and aims of study

On the basis of findings for AML, in which LSC represent a rare immature entity at the apex of a developmental hierarchy, closely related to normal physiological haematopoiesis, it traditionally is assumed that ALL follows an analogous pattern. More recent findings, however, suggest the possibility that opposed to AML and several solid cancers, ALL may not follow the classical hierarchical model of stem cells.

This study addresses the question whether the immunophenotypic heterogeneity of ALL reflects a functional heterogeneity of blasts regarding stem cell characteristics and whether or not LIC are a rare occurrence within ALL.

Conflicting results in the field of ALL stem cell research produced by experimental models that altered significantly in the last two decades highlight the need for an assay that reliably and consistently generates reproducible results. Considering this, the purpose of this research is to:

- Establish a robust *in vivo* murine transplant model for ALL, using the most appropriate mouse strain available and applying the most promising transplantation techniques to address the defining leukaemic stem cell properties of self-renewal and leukaemia reconstitution.
- Develop a flow cytometry based method of reliably and quantitatively detecting leukaemic blasts of human origin in a murine xenoenvironment.

Studies published prior to the start and during the time course of this project (Castor et al., 2005; Hong et al., 2008; Kong et al., 2008; le Viseur et al., 2008; Morisot et al., 2010) suggest that not only blasts with an immature immunophenotype similar to HSC´s

are capable to transfer ALL onto severely immunocompromised mice but also leukaemic cells of the $CD19^+$ population and that LIC may have a higher frequency than demonstrated by earlier studies. Therefore, this study addresses following questions:

- Do blast populations of various maturity, defined by the differentially expressed surface marker antigens CD10, CD19, CD20 and CD34, have the capability to initiate ALL in an orthotopic severely immunodeficient murine xenotransplant model?
- Are blast populations of different maturity able to serially engraft several generations of mice, providing evidence of self-renewal?
- Do identified LSC immunophenotypes reconstitute the original and complete patient immunophenotype?
- What is the frequency of LIC in unsorted blast populations of different ALLs?
- If leukaemic subfractions of different maturity are able to transfer the leukaemia, is there a difference in LSC frequency between these subfractions?

CHAPTER 2: MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 General equipment and reagents

2.1.1 General equipment

IKA MS3 basic vortex mixer (Fisher Scientific, Leicestershire, UK)

Allegra X-12R centrifuge (Beckman Coulter, Buckinghamshire, UK)

Allegra X-22R centrifuge (Beckman Coulter)

5415 R microfuge (Eppendorf, Cambridgeshire, UK)

Olympus CK30 transmitted light microscope (Olympus, Japan)

2.1.2 General reagents

Phosphate buffered saline (PBS) was prepared by dissolving PBS tablets (Invitrogen, Life Technologies, Paisley, UK) in deionised water. For sterilisation, the solution was autoclaved at >120°C.

Phosphate buffered saline 0.2% w/v bovine serum albumin (0.2% w/v PBSA) solution was prepared by adding 1 g bovine serum albumin (Sigma-Aldrich, Gillingham, Dorset, UK) to 500 ml sterile PBS followed by sterile filtration.

Ammonium chloride red cell lysis buffer was prepared by dissolving 8.3 g NH_4Cl, 1 g $KHCO_3$ and 0.036 g EDTA (all Sigma-Aldrich) in 800 ml ddH_2O, adjusting the pH to 7.35 with 0.1 M NaOH and then adding ddH_2O to the total volume of 1000 ml.

2.2 Flow cytometry

2.2.1 General equipment, materials and solutions

BD FACSCanto II flow cytometer equipped with 488 nm and 633 nm wavelength lasers, fitted with photomultiplier tube (PMT) detectors to allow up to 6 colour multiparameter flow cytometry (including FITC, PE, PerCP-Cy5.5, PE-Cy7 and APC) (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).

FACSDiva analysis software, v6.1.2 (BD Biosciences).

FACS tubes (BD Biosciences).

0.2% w/v PBSA

2.2.2 Directly fluorochrome labelled surface antigen antibodies for flow cytometry

Commercially available monoclonal antibodies to specific human and mouse surface antigen markers of interest directly labelled to fluorescent dyes were used for multicolour flow cytometry and fluorescence activated cell sorting (FACS). All antibodies were obtained from BD Biosciences. Details of the antibodies used in this project are listed in Table 1. Differences between flow cytometry and FACS existed only in the choice of the conjugated fluorochrome.

Target surface antigen	Target species	Clone	Fluorochrome	Volume per 10^6 cells [µl]	Application
CD10	Human	HI10a	FITC	20	Flow cytometry / FACS
CD19	Human	SJ25C1	PE	20	Flow cytometry / FACS
CD20	Human	L27	PerCP-Cy5.5	20	Flow cytometry
			FITC	20	FACS
CD34	Human	8G12	APC	5	Flow cytometry
			FITC	20	FACS
CD38	Human	HB-7	PE-Cy7	5	Flow cytometry
CD45	Mouse	30-F11	PE-Cy7	5	Flow cytometry
			APC	5	FACS
TER-119	Mouse	TER119	PE-Cy7	5	Flow cytometry
			APC	5	FACS

Table 1: Monoclonal antibodies directly conjugated to fluorochromes used for flow cytometry and FACS.

2.2.3 Reference normal human peripheral blood mononuclear cells (PBMC)

Human PBMCs were used for compensation experiments and as reference cells in the setup of multicolour flow cytometry. Healthy volunteers donated approximately 10 ml of peripheral venous blood after informed consent. An aliquot of the samples was diluted with 10 volumes of 1x ammonium chloride red cell lysis buffer and incubated for 10 minutes at room temperature. The red cell lysed cell suspension was centrifuged at 650 g for 5 minutes at room temperature and after decanting the supernatant, the resulting cell pellet was resuspended in 10 ml PBS and centrifuged with the same centrifuge settings for a first washing step. A second washing step was carried out identically and the cells finally resuspended in 0.2% w/v PBSA for counting and further use in flow cytometry.

2.2.4 Principles of flow cytometry

Flow cytometry is a method that became very popular in both biomedical research and clinical medicine over the last 30 to 40 years. The basic principle of flow cytometry is

that it can rapidly identify a variety of surface or intracellular markers of an individual cell or particle by measuring emitted optical signals, mostly fluorescence. The optical signals from several variables then can be analysed electronically in detail to identify and characterise different populations within a single cell suspension (Robinson, 2004). In haematology and oncology flow cytometry now is an invaluable tool of modern diagnostics (Brown and Wittwer, 2000; Kroft, 2004).

In order to analyse single cells, it is necessary to generate a stream of individual cells separated by fluid. This physical cell separation is carried out in the flow cell of the fluidic system of the flow cytometer. Through an injection tube, cells from a single cell suspension are delivered into the centre of a stream of sheath fluid, resulting in a coaxial flow. A laminar flow of this coaxial fluid stream is then generated when the fluid is forced under pressure from a larger diameter through a smaller orifice. This creates a parabolic velocity profile with the highest velocity at the centre of the fluid stream. The acceleration of fluid speed in the centre spreads the cells out, resulting in a hydrodynamically focused stream of single, spatially separated cells in a file. The individual cells now can be analysed according to their light emission profile if specific antibodies conjugated with fluorescent molecules (fluorochromes) are bound to the cell. When they pass through the interrogation point the various fluorochromes are usually excited by gas-ion or solid state lasers with distinct specific excitation wavelengths matching the absorption spectra of the fluorochromes. On excitation, electrons of atoms of the fluorescent conjugate to the antibody are elevated to an orbital of higher energy and when they leave the laser beam, the electrons return to their initial state, emitting a specific wavelength of less energy (longer wavelength). The more antibodies with a specific fluorochrome bind to a single cell, the more intense the emission signal becomes, allowing a quantitative in addition to a qualitative analysis. Each emission spectrum is then registered and amplified by a spectral wavelength specific detector, the

photo multiplier tube (PMT). A complex optical system with dichroic mirrors directing the different emission spectra and narrow bandpass filters in front of the PMTs allows recording various wavelengths simultaneously. Figure 5 illustrates these principles of flow cytometry.

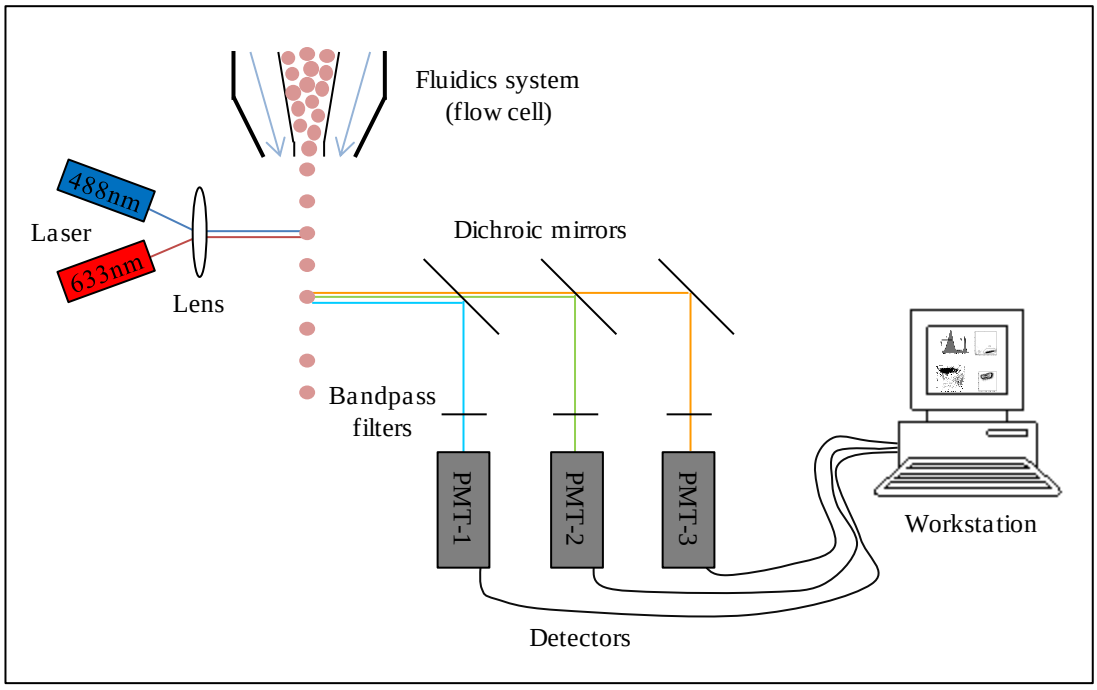


Figure 5: The principles of flow cytometry. The flow cell of the fluidic system generates a stream of spatially separated single cells. The various fluorochromes of the directly labelled surface antigen antibodies bound to the cells are excited by laser beams at the interrogation point and consecutively emit light of a specific longer wave length. The emitted light is diverted by various dichroic mirrors before it passes narrow bandpass filters and finally is detected and amplified by photo multiplier tubes. The analogue signals are converted to a digital output and analysed with specialised software at the work station.

PMT, photo multiplier tube; nm, nanometers

Laser light is also scattered by the cells that pass the laser beam. Unlike photons emitted by the fluorochromes that have a longer wavelength (lower energy) than the laser used for excitation, the scattered light of the laser is not altered in wavelength. Laser light is

scattered in a small angle to the direction of the laser beam (forward scatter) in proportion to the size or volume of the cell. It is also scattered orthogonal to the laser beam (side scatter) and this is proportional to the complexity of the cell content (granularity). Measuring the forward and side scatter profile of cells can provide important information about cell morphology.

The analogue signals generated by the photons in the PMT are converted to a digital output which is then fed into a computer and analysed with specialised flow cytometry software.

Contemporary research often requires the analysis of several surface markers on a single cell and this can be achieved by labelling the cells with distinct mAbs conjugated with fluorochromes of different emission spectra. However, for example both a PMT with a band pass filter for FITC (525 nm fluorescent emission wavelength) and a PMT with a band pass filter for PE (550 nm fluorescent emission wavelength) will register photons emitted by FITC and PE as the emission bands are very close to each other and partly overlap (fluorescence spillover). To determine which of the two fluorochromes actually generated the photons, spectral compensation is necessary. In this process, a signal detected in one PMT must be subtracted from the signal in the other PMT as a percentage. The more fluorochromes with different overlapping spectral bands are used, the more complex this system gets as a percentage of each signal must be subtracted from all other PMTs (Kumar et al., 2007; Robinson, 2004).

2.2.5 Multicolour staining for flow cytometry

PBMCs of healthy donors, cells of primary patient samples or cells retrieved from transplanted mice upon bone marrow punctures (bmp), bm harvests or single cell suspensions of spleens, livers or kidneys were resuspended in 200 µl of 0.2% w/v PBSA

in a FACS tube. The bovine serum albumin reduces unspecific antibody binding during incubation with the antibody panel. Saturating amounts of antibodies according to Table 1 (section 2.2.2) were added to each FACS tube. The content of the FACS tubes was briefly spun down and tubes then vortexed to assure that cells were evenly mixed with all antibodies. Thereafter, the samples were incubated for 20 minutes, protected from light to avoid fluorochrome bleaching. Occasionally the samples were flick-mixed to prevent irregular staining of cells due to sedimentation. Subsequently, the samples were washed twice to remove unbound excess antibodies. For both washing steps 2.5 ml of 0.2% w/v PBSA were added to each sample, the FACS tubes were centrifuged at 650 g for 5 minutes at ambient temperature and the supernatant was then carefully discarded. After the second wash, stained cells were resuspended in 500 µl 0.2% w/v PBSA and the samples were kept in the dark until the flow cytometer was ready for use.

Before the first xenotransplantation experiment, surface antigen expression of CD10, CD19, CD20, CD34 and occasionally CD38 was determined on a small cell aliquot of each diagnostic patient ALL sample in order to characterise the original ALL immunophenotype. Cells from mouse bmps, bm harvests and single cell suspensions of mouse spleens, livers or kidneys were routinely stained with anti-human CD10, CD19, CD20 and CD34 and anti-mouse CD45 and TER119 to identify human cells in a xenotransplant background and to characterise their CD surface antigen expression pattern.

2.2.6 FACS analysis of cell samples

FACS analysis was performed on a FACSCanto II flow cytometer. A maximum flow rate of 10,000 events/second was set in order to avoid cell blockage of the flow cytometer nozzle and also to prevent automatic interruption of data acquisition due to

limitations of the computing system. Routinely, 100,000 events were recorded and then analysed using FACSDiva analysis software, v6.1.2. Details of data analysis are described and discussed in chapter 3.

2.3 Fluorescence activated cell sorting (FACS)

2.3.1 General equipment, materials and solutions

BD FACS Vantage SE cell sorter (BD Biosciences)

0.2 µm Minisart syringe filter (Sartorius Stedim Biotech, Germany)

50 µm Cup Filcons syringe to tube filters (BD Biosciences)

0.2% w/v PBSA, sterilised by filtration with 0.2 µm syringe filters

2.3.2 Directly fluorochrome labelled surface antigen antibodies for FACS

All directly fluorochrome labelled monoclonal antibodies used for FACS were of the same clones as those used for flow cytometry and purchased from the same manufacturer (BD Biosciences). In some cases the mAbs were conjugated to different fluorochromes (see Table 1, section 2.2.2).

Where primary diagnostic patient ALL samples were sorted, cells were only labelled with antibodies to human surface antigens of interest. In case of sorting material from bm harvests of engrafted mice for serial transplantation experiments, anti-mouse CD45 and anti-mouse TER-119 mAbs conjugated to APC were added in the staining process. This helped to clearly discriminate between cells of human and mouse origin and to set appropriate sorting gates.

2.3.3 Principles of fluorescence activated cell sorting

Flow cytometry was a major advance in cell characterisation and could be performed on living cells. There soon was the desire not only to identify apparently distinct populations within a heterogeneous cell suspension but also to separate them physically to a high purity and at high speed to address further biomedical questions by assaying these subpopulations. Several developments in the 1960s and 1970s eventually led to the principal components of FACS as they are still used today. The initial process of identification of the cells of interest is identical to flow cytometry but after that, distinct cells have to be first detached from the fluid stream and in a second step collected in separate locations (for example FACS tubes or microscopic slides). To achieve this, the flow cell nozzle and thereby the coaxial fluid stream emerging from it is vibrated at high frequencies (10,000 to 300,000 Hz) by a piezoelectric crystal oscillator, thereby breaking it apart into small droplets, each containing one single and already identified cell only. If the droplet breaking off the stream contains a cell of interest, it is charged either positive or negative by charging electrodes before it passes electrostatically charged deflection plates. Opposite charged plates will attract the droplets and divert them into the direction of a collection system such as FACS tubes, pre-filled with collecting medium to maintain cell viability. The cell is not harmed by imposing a charge onto the droplet as the sheath fluid acts like a Faraday´s cage, protecting the cell. It is also possible to perform cell sorting under sterile conditions- if that is desirable for further biological assays. High speed cell sorting is a very complex process in which variables of nozzle diameter, generation of a stable laminar flow, sheath pressure, droplet generation frequency and analysis computing time play an important role. Higher droplet frequencies can only be achieved by decreasing the size of the nozzle and increasing the stream pressure. This increasing pressure and the smaller volume of the droplet, which eventually is limited by the size of the cells to be sorted, pose threats to

cell viability and therefore limit the speed of cell sorting. However, taking pressure limitations to cell viability into account, droplet and sorting frequencies currently can be increased to around 100,000/s with the latest generations of sorters and high speed computing technologies (Kumar et al., 2007; Robinson, 2004).

2.3.4 Multicolour staining for FACS

Usually 5 x 10^6 to 1 x 10^7 cells of primary patient samples or bm recovered from mice engrafted with human ALL in previous experiments were resuspended in 200 µl of sterilised 0.2% w/v PBSA in a FACS tube. Appropriate amounts of antibodies according to manufacturer's instructions were added, the FACS tube content spun down and briefly vortexed to mix cells and antibodies sufficiently. The samples were incubated and washed in the same way as described in the staining process for flow cytometry (see section 2.2.5). Following the second wash and centrifugation step, the cell pellet was resuspended in 3 ml 0.2% w/v PBSA and the samples were kept in the dark until the BD FACS Vantage SE cell sorter was ready for cell sorting.

2.3.5 Cell sorting

In preparation for fluorescence activated cell sorting, the fluid channels of the BD FACS Vantage SE cell sorter were thoroughly sterilised by running 70% v/v ethanol through the system. After rinsing the system with ddH_2O and cell medium for approximately one minute each, around 1,000 cells from the stained sample were acquired and visualised in dot plots in a first step. Sorting gates were set around target populations with a low and high expression of the CD surface marker of interest (CD10, CD19, CD20 or CD34). The cell sorting rate was set to 1 to 1.5 x 10^3 cells/second and the sorted cells were collected in fresh sterile cell medium.

2.3.6 Purity analysis of sorted cell populations

After desired numbers of cells with low and high expression of a specific surface marker were sorted and collected, purity analysis was performed. For this purpose, 500 to 1,000 cells of each population were acquired on the FACS Vantage SE cell sorter. Ideally, cells sorted according to a high or low expression level of a specific surface marker would form tight clusters with the desired level of antigen expression. However, contamination of sorted populations with cells of the opposing antigen expression profile is difficult to avoid. To determine the percentage of this contamination, one gate was drawn around the desired cluster and one around all cells with a lower expression of the antigen in case of a population sorted for high levels of surface markers or around all cells with a higher expression of the antigen in case of a population sorted for low levels of the specific surface marker. The number of cells in the gate with the contaminating cells was then divided by the combined cell numbers of both gates and the calculated percentage deducted from 100%. The percentage gained from this calculation represented the sorting purity.

2.3.7 Preparation of sorted cell samples for xenotransplantation

Preparation of sorted cell samples for xenotransplantation followed the same protocol as described for samples with unsorted cells (see section 2.6.6). Briefly, the content of viable sorted cells in FACS tubes was counted (see section 2.6.5) and after a centrifugation step (1,750 rmp (713 g), 5 minutes, room temperature) and removal of supernatant, the cell pellet was resuspended in an appropriate volume of cell medium to gain the desired cell concentration. Cell numbers were again counted to confirm accuracy of the cell concentration or to adjust the cell concentration if necessary. Transplantations were then carried out with as little delay as possible.

2.4 Fluorescence *in situ* hybridization (FISH)

2.4.1 General equipment and materials

Hybrite, thermocontrolled programmable hotplate (Abbott diagnostics, Illinois, USA)

Menzel-Gläser superfrost microscope slides, 10 mm circular and 22x50 mm coverglasses (Fisher scientific)

Diamond pen (Fisher scientific)

Olympus BX61 fluorescent microscope fitted with a 100x/1.30 oil objective and CCD camera (Olympus) fitted with digital imaging software by Metasystems (v5.1.9 Metasystems) and Applied Imaging (cytovision v4.5)

2.4.2 General reagents

0.75 M potassium chloride solution, prepared from potassium chloride salt (Sigma-Aldrich) dissolved in distilled deionised water.

Carnoy's fixative solution, comprising of 3 parts methanol and 1 part glacial acetic acid (both chemicals purchased from Fisher Scientific).

Hybridisation buffer (Abbott Diagnostics), containing formamide, dextran sulphate, blocking DNA, salt solution (SSC) and detergent.

2x SSC solution, prepared from 20x SSC solution (Fisher Scientific) and distilled deionised water.

Wash buffer 1, prepared from 20x SSC (17.5% w/v NaCl, 8.8% w/v Sodium Citrate Dihydrate) solution diluted 1 to 50 with distilled deionised water and addition of 3 ml of the detergent NP40 (Sigma-Aldrich) (0.4x SSC/0.3% w/v NP40).

Wash buffer 2, prepared from 2x SSC working solution with addition of 1 ml Igepal-CA-630 detergent (Sigma-Aldrich) per litre (2x SSC/0,1% w/v Igepal-CA-630).

4',6-diamidino-2-phenylindole (DAPI) cell nucleus counterstaining solution (1.5 µg/ml) (Sigma-Aldrich).

BCR/ABL1 fusion fluorescent FISH probe (Spectrum Green/Spectrum Orange) (Abbott Molecular Diagnostics, formerly Vysis).

2.4.3 Principles of FISH

Fluorescence *in situ* hybridisation (FISH) is an invaluable method in molecular biology to determine the cytogenetics of an individual cell nucleus. Chromosome numbers (ploidy) as well as structural chromosome abnormalities can be detected with this technique. FISH is widely used in the clinical diagnostic setting, including ALL initial diagnostics and cytogenetic findings contribute to risk stratification when established as a prognostic factor (Szczepański et al., 2010). Fluorescently labelled probes complementary to specific genomic DNA regions are hybridised to the chromosomes of immobilised cells and chromosome copy numbers and chromosomal alterations can then be determined with fluorescence microscopy. First, the FISH probes are added to cells on a glass slide and the directly labelled probe and the target DNA are both denaturated at 72°C. In the following hybridisation step at 37°C, the probe anneals to the specific target DNA sequence on the chromosome over several hours. After hybridisation, washing steps with solutions of low salt concentrations ensure that only probes that bind precisely to the complementary DNA sequence without mismatches remain on the chromosomes. Following that, DAPI counter stain is added which colours nuclei blue. The fluorescent signals from the FISH probes can now be allocated to one individual cell nucleus (O´Connor, 2008). The principles of FISH are illustrated in Figure 6.

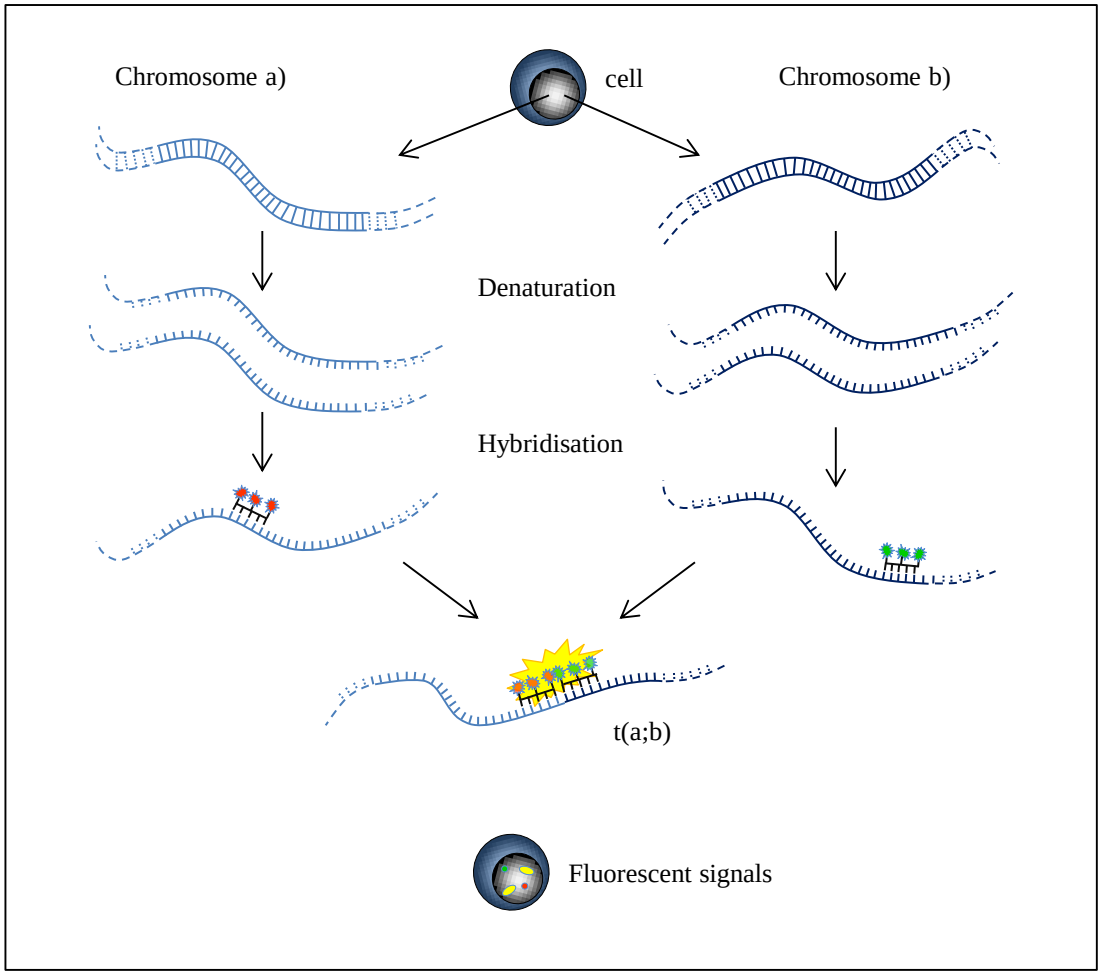


Figure 6: Principles of fluorescence *in situ* hybridisation (FISH). The DNA of the chromosomes of cells fixed to a microscopic glass slide is denaturated over 2 minutes at 72°C in order to generate single DNA strands. Fluorescently labelled FISH-probes (spectrum orange and spectrum green) complementary to specific genomic DNA regions are hybridised to single DNA strands at 37°C over night. A fluorescence microscope is used to visualise the fluorescent signals. Intact DNA regions are either labelled with an orange (chromosome a)) or a green (chromosome b)) signal, while a translocation between chromosome a) and chromosome b) (t(a;b)) results in a yellow fusion signal.

2.4.4 Cell fixation and slide preparation

1-5 x 10^6 fresh or thawed cells were pelleted by centrifugation at 2,000 rpm (932 g) for 5 minutes at ambient temperature and resuspended in 5 ml 0.75 M potassium chloride solution prewarmed to 37°C in a waterbath. During incubation in hypotonic potassium chloride solution over 10 minutes the cells swell due to osmotic water influx. The cell suspension was again pelleted under the same conditions, the supernatant was carefully

and completely discarded and 5 ml of Carnoy's fixative solution was added drop by drop during vortexing. After another centrifugation step, fresh Carnoy´s solution was added until the cell suspension appeared clear and the cell suspension was stored at -20°C if the FISH procedure was not carried out immediately afterwards.

3-5 µl of the fixed cell suspension were dropped onto a pre-labelled microscope glass slide from approximately 5 cm height, allowed to air dry and then aged at 60°C for 15 minutes on a hotplate. Dropping the suspension with the swollen cells causes the cell membrane to burst and allows the nucleus to spread out on the slide as the Carnoy's fixative evaporates. This procedure improves access of the FISH probe to the target DNA and brings the chromosomes closer to one focal layer.

2.4.5 Hybridisation

In the hybridisation step, the FISH probe is annealed to the chromosome DNA. The commercially available BCR/ABL1 fusion fluorescent FISH probe (Spectrum Green/Spectrum Orange) from Abbott Molecular Diagnostics was chosen as it is well established in research and clinical diagnostics. For each slide 0.2 µl of BCR/ABL1 FISH probe were combined with 1.8 µl of the provided hybridisation buffer to prepare a probe mixture of which 2 µl were applied to a round coverslip. The area with the fixed cells was marked with a diamond pen on the back of the slide and the slide was inverted over the coverslip to which it adhered due to surface tension. The edges of the coverslip were sealed with rubber cement to prevent drying of the probe during denaturation and hybridisation. The slides were transferred to a Hybrite chamber, incubated at 72°C for 2 minutes to denature probe and chromosome DNA and then incubated at 37°C overnight for hybridisation.

2.4.6 Post hybridisation washes

Washing steps after hybridisation are performed to remove unbound or only loosely bound FISH probes. The rubber cement was carefully removed from the coverslips with the help of tweezers and the slides were placed in ambient temperature 2x SSC solution to allow the coverslip to soak off without washing off the cells. The slide were then placed in preheated wash buffer 1 and incubated for 2 minutes at 72°C. This step removes background fluorescence from non-specific binding of the probe. The slides thereafter underwent a second wash at room temperature for 2 minutes in wash buffer 2 to remove any remaining unbound probe.

2.4.7 Nuclear counterstaining

The counterstaining of the nucleus allows mapping of the FISH probes to one single cell nucleus. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) directly after the hybridisation washes. 5 µl of DAPI were added to a coverslip and the slides inverted onto the coverslip leading to adhesion. The slides were then stored in the dark at 4°C to prevent bleaching of the fluorochromes.

2.4.8 Fluorescence microscopy

The Olympus BX61 fluorescence microscope was used to visualise the fluorescent FISH probes and the slides were assessed for cytogenetic abnormalities. The x10 objective with a filter for DAPI was initially used to focus on cells. Following this the x100 objective oil immersion lens were used in combination with a red, a green or a combined filter to visualise fluorescent signals of the FISH probe within the blue DAPI stained nuclei. Example images of representative cells were captured using the triple filter that allowed the red, green and DAPI signals to be imaged simultaneously.

2.5 May-Grünwald-Giemsa (MGG) staining

2.5.1 General materials

Menzel-Gläser superfrost microscope slides (Fisher scientific)

Menzel-Gläser 22x50mm coverglasses (Fisher Scientific)

2.5.2 General reagents

May-Grünwald stain (contains eosin, methylene blue, methanol, water) (Sigma-Aldrich)

Giemsa Stain (contains azure B, methylene blue, methanol, glycerine) (Sigma-Aldrich)

DPX Mountant (contains xylenes, polystyrene, dibutyl phthalate) (Sigma-Aldrich)

2.5.3 Principles of MGG staining

The MGG staining procedure is commonly applied in a clinical diagnostic as well as research setting for morphological examination of blood and bone marrow smears. May-Grünwald stain mainly is applied to stain granulas. While eosin has a high affinity to acidic granulas, methylene blue enriches in basophilic granulas. The Giemsa solution stains the DNA of the nucleus purple and the cytoplasm blueish. The staining process is largely dependent on the pH of the solutions. ALL blasts have immature cell morphology as a differentiation arrest occurs at B-cell precursor developmental stages. Applying MGG-staining, lymphoblasts typically appear relatively small and show a high nuclear-to-cytoplasmic ratio. The cytoplasm is usually pale blue without granules and constitutes only a small rim around the relatively round nucleus which is characterised by homogeneous material and indistinct nucleoli. Blast forms are a normal part of haematopoiesis but should only represent up to 5% of bone marrow cells. More than 5% blast forms in the bone marrow or any blasts detected in peripheral blood are highly suspicious of leukaemia. After staining with May-Grünwald and Giemsa solutions,

microscopic glass slides are visualised in normal brightfield microscopy (Schrappe, 2005; Silverman, 2009).

2.5.4 Microscopic slide preparation and staining

Fresh or thawed lymphoblastic cell suspensions with 1-5 x 10^6 cells were centrifuged at 2,000 rpm (932 g) for 5 minutes at ambient temperature, the supernatant was discarded and the cell pellet was resuspended in a small volume of PBS (usually 100 µl) to yield a cloudy suspension. 5-10 µl of cell suspension were added on a labelled microscope slide and left to air dry. In a first step, 1.5 ml of May-Grünwald solution was added onto the slide for an incubation time of 3 minutes. Following that, 1.5 ml of deionised water were pipetted onto the slide and mixed with the May-Grünwald solution. After incubation of 5 minutes, the mixture was removed from the slides by inversion and in the next step 1.5 ml Giemsa solution, 1 to 10 diluted with deionised water were pipetted onto each slide for another 15 minutes of staining. The slides were again drained by inversion, rinsed with deionised water to remove remaining staining solutions and then air dried before finally mounting in DPX mountant.

2.5.5 Brightfield microscopy

MGG stained slides were visualised under the x10 and the x40 objective lens of conventional brightfield microscopy. Samples were analysed regarding content of cells with the typical immature lymphoblast morphology of ALL.

2.6 Transplant ALL Samples

2.6.1 General equipment and materials

BIOMAT^2 Class II microbiological safety cabinet (Medical Air Technology Ltd, Lancashire, UK)

Improved Neubauer Counting Chamber (Hawksley, Sussex, UK)

All plasticware including sterilin universal (20 ml) and bijou tubes (5 ml), Greiner bio-one falcon tubes (50 ml) and pipettes (2 ml, 5 ml, 10 ml and 25 ml) were purchased from Fisher Scientific. As the only exception, cryovials (1.5 ml) were purchased from Invitrogen Life Technologies.

2.6.2 General reagents

RPMI 1640 medium, foetal calf serum (FCS) and trypan blue 0.4% w/v solution were purchased from Sigma-Aldrich.

Unless otherwise stated, RPMI 1640 medium supplemented with 10% v/v foetal calf serum was used as a cell suspension medium for all sample processing steps with primary human ALL samples or engrafted and non-engrafted bone marrow harvested from mice including thawing, sorting, transplanting and for bone marrow punctures. Hereafter this medium is simply referred to as ‘cell medium’.

2.6.3 Patient ALL samples

Unsorted or sorted original patient diagnostic bone marrow or peripheral blood ALL samples were used for primary xenotransplantation experiments. Excess bone marrow from initial diagnostic bone marrow punctures or peripheral blood samples from venopunctures was processed to remove erythrocytes applying density gradient centrifugation or red cell lysis and cryopreserved in the Newcastle Biobank after written

informed consent was obtained from patients or their legal guardians (Ethical approval was obtained, reference numbers – 2002/111 and 07 H0906). ALL samples from the Netherlands and Germany were kindly provided by the Dutch Childhood Oncology Group (DCOG)-ALL8/9 and the ALL-Berlin-Frankfurt-Münster (BFM) 2000 studies, both approved by institutional review boards and written informed consent was obtained from parents or legal guardians. Suitable ALL patient samples for intrafemoral transplantation were chosen by availability of viable material, cytogenetics, expression array profiling and immunophenotype. Cell viability was determined by counting cell suspensions in a haemocytometer after mixing 1:1 with 0.4% w/v trypan blue. Living cells are able to exclude the dye and can be distinguished from dead cells which appear dark blue in normal light microscopy (see section 2.6.5).

2.6.4 Cell thawing

Cryopreserved human primary patient samples were removed from the -80°C freezer or liquid nitrogen storage tank and thawed rapidly in a waterbath at 37°C. The thawed cell suspension (usually 1-2 ml) was slowly diluted up to a volume of 10 ml by drop by drop addition of cell medium, a small aliquot from the diluted cell suspensions (usually 5 µl) was taken for immediate cell counting (see section 2.6.5) and the cell suspension was then centrifuged at 1,750 rpm (713 g) for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in a small volume of fresh cell medium (usually 200 µl to 2 ml) to achieve a high cell concentration for further procedures.

2.6.5 Cell counting and determination of cell viability

In order to determine total cell numbers and cell viability, small aliquots from the thawed and diluted cell suspensions (usually 5 µl) were mixed with 0.4% w/v trypan blue solution (ratio 1:1). This mixture was placed in an improved Neubauer haemocytometer and cells were counted under the light microscope using the x20 objective lens. Living cells exclude the trypan blue solution and appear bright while dead cells lose the efflux ability, retain the dye and therefore look blue. Numbers of dead and alive cells were counted and the percentage of viable cells was calculated. Cell viability of both primary human ALL samples and harvested bone marrow from mice was usually high, typically in the range of 80-95%.

2.6.6 Sample preparation for xenotransplantation

After cell counting, centrifugation and resuspension of cells in fresh cell medium to a high cell concentration, cell suspensions were directly used for transplantation if relatively high but non-defined cell numbers were to be transplanted. In the case that exact numbers of cells were to be injected into mice, the cell concentrations were adapted by addition of fresh cell medium in order to yield the desired number of cells per mouse in an aliquot volume of 30 µl. The total cell suspension volume prepared for each transplantation group was in excess of number of mice transplanted times 30 µl, to have some spare transplant cell suspension. Intrafemoral (if) bone marrow transplantations were subsequently carried out with as little delay as possible to avoid sample degradation and cell death. The cell suspensions were kept in FACS tubes and only just before injection drawn into an insulin syringe after careful flip-mixing the tube to resuspend the cells homogenously.

2.7 *In vivo* xenotransplantation experiments

2.7.1 General equipment and materials

Faster Ultrasafe Class II Microbiological safety cabinet (Wolf laboratories Ltd, Yorkshire, UK)

MIE Cavendish 500 anaesthesia machine, fitted with an Ohmeda Isotec 400 volatile anaesthetic vaporiser and Fluovac scavenge system (Key Health Solutions Ltd, Wiltshire, UK)

Curved blade dissection scissors (Fisher Scientific)

Curved pointed dissection forceps (Fisher Scientific)

BD Biosciences 40 μm cell strainers (VWR, Leicestershire, UK)

2.7.2 General reagents

Isoflurane (IsoFlo, Abbott laboratories, Maidenhead, UK)

Carprofen (Rimadyl, Pfizer, Kent, UK)

Chlorohexidine disinfectant spray (Hydrex Pink, Ecolab, Yorkshire, UK)

2.7.3 Mice for xenotransplantation

The immunocompromised mouse strain used for this project was the NOD/LtSz-Scid IL-2Rγ $^{-/-}$ (NSG) hybrid strain. This strain is currently the most immunocompromised mouse strain available, with no functional B, T or NK cells. NSG mice in addition have functionally impaired macrophages and a compromised complement system. The highly suppressed immune system profoundly prevents rejection of transplanted non-syngenic material by the host. The lack of host lymphocytes potentially also facilitates human

lymphocyte or lymphoblast engraftment as transplanted cells do not have to compete for the niche microenvironment with cells of murine origin. Additionally, NSG mice have a comparably long life span of around 16 months and a low tendency to become “leaky” (regaining some degree of immune function), attributes that make them especially suitable for xenotransplant experiments that potentially require an extended period of time. NSG mice have recently been shown to be probably the most ideal hosts for human haematopoietic engraftment (Agliano et al., 2008; Ishikawa et al., 2005; McDermott et al., 2010; Shultz et al., 2007; Shultz et al., 2005).

Due to the severe immunosuppression, great care was taken to prevent any contact with potential pathogens. All procedures involving mice were carried out carefully under maximally aseptic conditions. The breeding colony was maintained in the animal facility of the Newcastle University. Usually 3 to 6 mice of the same sex were held in individually ventilated cages (IVCs) and provided with irradiation sterilised food and sterile water from individual 500 ml water bags fitted with an autoclaved steel nozzle. Mice were only taken out of the IVCs in the protected environment of a Class 2 safety cabinet or changing station with laminar air flow. All staff involved in mouse care or research had to wear disposable, individually packed lab coats or clean surgical gowns as well as disposable nitrile gloves. Glove covered hands were disinfected regularly between handling of different IVCs. Animal care, xenotransplantation and bone marrow punctures were performed in conformance to personal or group project license regulations. In the case of suspected infection or other problems with the mice, advice was sought and potential treatment was discussed with the on-site veterinary staff. Whenever mice seemed to become ill from overt leukaemia or appeared to suffer for other reasons, experiments were terminated and mice were killed by high cervical dislocation.

2.7.4 General anaesthesia of NSG mice

General anaesthesia of mice was induced in an anaesthesia chamber connected to an Isoflurane vaporiser and oxygen. For safety reasons, excess anaesthetic was scavenged. For induction of anaesthesia, 5% v/v Isoflurane in a constant flow of 1,500-2,000 ml/min oxygen was lead into the anaesthesia chamber until animals became unconscious. Mice were then transferred to a mouse face mask that as well supplied Isoflurane in oxygen (usually 3-5% v/v Isoflurane) to maintain a deep anaesthesia. To determine if an appropriate depth of general anaesthesia was achieved, a pain stimulus was applied to one foot of a mouse and in case of foot withdrawal, the level of anaesthesia was adjusted prior to carrying out any procedure. The non-steroidal anti-inflammatory drug Carprofen was given to mice following intrafemoral bone marrow transplantation or bone marrow punctures to provide post-interventional analgesia and to prevent inflammatory reactions at the site of the invasive procedure. Carprofen solution was diluted to 5 µg/µl with sterile water and 10 µl of the solution per 10 g mouse body weight was usually injected subcutaneously in the abdominal area. After procedures, mice were transferred back to their IVC and observed for full recovery. Femoral puncture sites were carefully inspected for signs of prolonged bleeding. All steps involved in anaesthetising mice were carried out under maximally aseptic conditions.

2.7.5 Intrafemoral (if) transplantation procedure

ALL originates from haematopoietic cells in the bone marrow and blasts home to their appropriate niche within it. Transplanting human lymphoblasts directly into the bm in an orthotopic mouse model carries several advantages. The necessity of homing of intravenously injected blasts to the bm is made redundant and clearance of blasts due to a residual host immune system can possibly be avoided and superior results of orthotopic transplant models compared to conventional intravenous transplantation

techniques have been published (Kushida et al., 2001; Taussig et al., 2008; van Hennik et al., 1999). The rationale for orthotopic xenotransplantation will be discussed in more detail in 3.1.2. In order to endow ALL blasts with the highest chance of engraftment, the orthotopic intrafemoral xenotransplantation technique was the method of choice in this study.

As soon as mice were adequately anaesthetised, they were set in supine position and the right knee was soaked thoroughly with Chlorohexidine, a disinfectant solution commonly used for surgical procedures. In addition to providing an aseptic skin area, wetting the fur made it stick to the skin, making it easier to locate the correct site for the transplantation procedure. The knee joint was bent to around 90 degree and fixed between the index finger, the middle finger and the thumb of the left hand. First a hole was drilled through the intercondylar fossa of the distal femur into the cavity of the femur diaphysis using an insulin syringe (30 g). The correct position of the needle was checked by bending and straightening the leg and palpating for an incorrectly located needle. The leg then was firmly retained in the bent position and the drilling needle carefully withdrawn. The insulin syringe pre-filled with the cells for transplantation was inserted through the same hole and the position of the needle carefully checked again by moving it up and down slightly with minor changes of direction. The needle was correctly positioned in the bone cavity if the rough inner surface of the bone corticalis could be felt. Following this, a 30 µl aliquot was slowly injected into the bone marrow cavity. The injection needle was withdrawn and the leg immediately straightened in the knee joint in order to close the site of the injection hole, preventing outflow of the transplant suspension as much as possible. Transplanted mice were then referred back to the IVC for recovery from anaesthesia.

2.7.6 Mouse bone marrow punctures

Bone marrow punctures of NSG mice were always performed under general anaesthesia (see 2.7.4). Mice were positioned and the right leg manually fixed as described for if transplantation (2.7.5). After disinfection, a hole for access was drilled through the intercondylar fossa of the femur using a 25 g ½" needle, attached to a 2.5 ml syringe. After ensuring the correct position of the needle in the femur cavity it was withdrawn and a new 25 g ½" needle attached to a 2.5 ml syringe containing 200 µl of 0.2% w/v PBSA was inserted. Negative pressure was applied by pulling back the plunger of the syringe and slowly released as soon as a little amount of bm flowing into the syringe could be detected. The aspiration needle was pulled out and the leg straightened in the knee joint to maximally prevent bleeding from the puncture site. Punctured mice were referred back to the IVC for recovery from anaesthesia. The bm collected in the volume of 200 µl 0.2% w/v PBSA was transferred into a labelled FACS tube for direct staining with a desired combination of antibodies for flow cytometry.

2.7.7 Harvest of mouse bone marrow and tissue samples

Generally individual mouse experiments were terminated when a mouse appeared to become ill either from developing overt ALL or for any other non transplant related reason. Occasionally, a previous bmp showing a very high percentage of human engraftment indicated that deterioration of the mouse condition was imminent. Additionally, paleness or a progressively distended abdomen due to spleen enlargement were reliable signs that a mouse had to be killed soon even if it still was active and not obviously affected by disease. Mice were also killed when they approached their natural life expectancy.

The final bm harvest from mice served two main purposes. One was to determine a terminal engraftment level and the other was to collect and preserve bone marrow for following serial xenotransplantation experiments or other analyses. Routinely, bm was collected from both femora and tibiae. Both legs were disarticulated in the hip joints and the skin and muscles stripped of the bones. Femur and tibia were separated by overextending the knee joint and cutting the ligaments and remaining connective tissue with a pair of scissors. The epiphysis of both bone ends was snipped off with the help of tweezers to reveal the bone cavity and bm was flushed out from both ends with cell medium. A 25 g ½" needle attached to a 2.5 ml syringe containing the cell medium was used for this purpose and the bm was usually collected in a total volume of approximately 5 ml.

On the majority of mice that had bm engraftment in a previous bmp a *post mortem* splenectomy was performed to analyse blast infiltration into the organ. A single cell suspension of cells contained in the spleen was prepared by grinding the spleen through a 40 µm BD cell strainer with the help of a syringe plunger and collecting the disaggregated cells in cell medium for further procedures. Spleen single cell suspensions were routinely cryopreserved and stored in liquid nitrogen in addition to bm samples.

From selected mice with obvious hepatomegaly or infiltrated kidneys (indicated by enlargement and subcapsular petechiae) these organs were taken out as well and single cell suspension prepared as described for spleen. Liver and kidney samples were only analysed by flow cytometry and not cryopreserved.

2.7.8 Cryopreservation, long time storage and thawing of mouse bone marrow and spleen single cell suspensions

In order to use mouse bone marrow or spleen single cell suspensions from engrafted mice for further analysis or future xenotransplantation experiments, both cell materials were frozen down and stored in a liquid nitrogen tank. Viable cell numbers for bm but not for spleen samples were counted in an improved Neubauer chamber using trypan blue exclusion as described in 2.6.5 and cells pelleted by centrifugation at 1,750 rpm (713 g) for 5 minutes at room temperature. After decanting the supernatant, the cell pellet was resuspended in 1 ml of foetal calf serum containing 10% v/v DMSO (freeze mixture) per aliquot of approximately 5 x 10^6 viable bm cells. 1 ml each of the freeze mixture cell suspension was then transferred into a pre-labelled polypropylene cryovial tube. The tubes were then wrapped into paper tissue to enable slow freezing in a -80°C freezer. The cryovials were kept for a minimum of 12 hours at -80°C and then transferred to the liquid nitrogen tank for long term storage.

When cryopreserved cell samples stored in liquid nitrogen were needed for further analysis or serial xenotransplantation experiments, individual cryovials were removed from the liquid nitrogen tank and quickly thawed at 37°C in a waterbath. Immediately after thawing the cell suspension was pipetted into a 20 ml universal tube and cell medium, prewarmed to 37°C was added drop by drop to a total volume of 10 ml to enable slow diffusion of DMSO out of the cells. A small cell suspension aliquot (usually 5 µl) was taken and the viable cell content of the sample was determined as described above. The cells were centrifuged at 1,750 rpm (713 g) for 5 minutes at ambient temperature and after removing the fluid supernatant, the resulting cell pellet was resuspended in a volume of cell medium adequate for the planned further procedures.

CHAPTER 3: DELIVERY, ENGRAFTMENT AND DETECTION OF HUMAN ALL IN NSG MICE

Chapter 3: Delivery, engraftment and detection of human ALL in NSG mice

3.1 Introduction

Research on cancer stem cells has yielded contradictory results regarding their identity and frequency within the malignant population for many cancers and in particular for ALL. Cancer stem cells are the cells within a cancer that maintain the disease and therefore must combine two defining characteristics. Being capable of self-renewal ensures that the malignant clone does not die out and the ability to differentiate into diverse subpopulations generates and maintains the heterogeneity of blast populations within a tumour. These two properties must be proved for distinct cell populations within a specific cancer, if they are to be identified as cancer stem cells (Clarke et al., 2006). Consequently, to address cancer stem cells an experimental model has to be able to:

- identify distinct cell populations within a tumour and to purify these in order to assay their stem cell properties
- demonstrate self-renewal and the ability to reconstitute tumour heterogeneity of candidate stem cell populations

Leukaemic cells generally express a panel of surface markers that constitute their immunophenotype and within a heterogeneous tumour population these blast surface markers are differentially expressed regarding their type and number to characterise

distinct tumour subpopulations. Leukaemias are believed to originate in the normal hierarchically organised haematopoietic system, where different developmental stages are well characterised and can be determined by a specific surface antigen expression profile. Assessment of the cell specific surface antigen expression pattern on individual cells can be achieved by flow cytometry. Modern multicolour flow cytometry allows to simultaneously interrogate the expression of several different surface markers on a single cell with a high sensitivity, making this method suitable to identify subpopulations within a tumour and to determine their contribution to the bulk tumour mass. Through establishing the cell specific surface marker expression, the immunophenotypes of leukaemic cells can be compared to those of normal haematopoietic maturational stages. The development of FACS made it possible to accurately purify viable cells of a heterogeneous population into distinct subpopulations. These subpopulations subsequently can be further scrutinised in clonogenic assays regarding their potential stem cell characteristics.

Currently, the defining stem cell characteristics of self-renewal and the capability to reconstitute the heterogeneous lineages of a certain tumour can only be evaluated in *in vivo* animal xenotransplantation assays (Clevers, 2011). Methylcellulose or semisolid agar based clonogenic *in vitro* experiments played an invaluable role in identifying several progenitor cells of normal haematopoiesis, especially myeloid development. Colony-forming cell assays usually only detect already lineage restricted progenitor cells and the most immature developmental stage that can be identified is the multipotent myeloid progenitor colony forming unit granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM). The lymphoid lineage is far more difficult to assess and even progenitor cells require complex co-culture methods. However, self-renewal of stem cells can not be addressed with these short-term assays and the gold standard to measure stem cells in normal and malignant haematopoietic cells are long-term

repopulating assays (Purton and Scadden, 2007; Spangrude, 2002). These *in vivo* xenotransplant models usually involve highly immunocompromised mice. The trait of self-renewal can be addressed by determining the ability of candidate stem cell populations to serially repopulate several generations of host mice without dying out. The ability to reconstitute the full tumour or normal haematopoietic cell heterogeneity can be determined by comparing the immunophenotypes of the engrafted populations with the original sample by the means of flow cytometry.

Murine xenograft assays were essential in elucidating the normal haematopoietic hierarchy and now are commonly used in the field of cancer stem cell research.

3.1.1 Selecting a murine xenotransplant model

Despite FACS and flow cytometry being essential in purifying candidate stem cells for further investigations and in studying engrafted cell populations in mouse bone marrow and other tissues, the crucial part of the experiments, assaying CSC properties of leukaemic cell subpopulations, relies on the xenotransplant model. It is essential that the assayed cells meet the best possible conditions for engraftment. The means of delivery of cells into the mouse xenoenvironment (transplantation technique) and the ability of the host mice to provide a maximally supporting microenvironment for the transplanted material are critical for success.

The crucial determinant of mice for successfully hosting human xenotransplant material is the immune system. The immune system is designed to recognise and eliminate xenogenic material in order to protect the integrity of a body system. As a consequence and under normal circumstances, foreign material only has a reasonable chance to survive and thrive in a host environment if the immune defence of the recipient is compromised. Obviously, the more pronounced this immunosuppression is, the higher is

the sensitivity of the assay in detecting xenogenic cell populations with stem cell characteristics.

Combining the most promising way of delivery of ALL cells into the appropriate niche and the use of the most suitable host for accommodating the ALL graft holds the prospect of the highest sensitivity for demonstrating stem cell properties of various blast subpopulations. Several immunocompromised mouse strains have been used in stem cell research over the last decades and their efficacy to engraft human cells proved to be substantially variable and closely linked to the immune status of the mice.

3.1.1.1 Nude mouse strains

Nude mice developed following a spontaneous single gene mutation in BALB/c mice. Flanagan published the first detailed description of these mice in 1966 but it was Pantelouris two year later who described the lack of a functional thymus in homozygote mice (Pantelouris, 1968). The underlying defect in nude mice is a mutation in the *Foxn1* gene (a transcription factor) which results in a failure of T-cell development. Nude mice were the first described to have a certain degree of immunosuppression and are still used in pharmacological and imaging studies on human tumour xenografts. However, these animals still have a partly intact humoral immunity with an ability to produce IgM but not IgG, an increased NK-cell activity and in mice of advanced age, some cells with a T-cell immunophenotype develop. Haematopoietic cells of human origin fail to engraft this mouse strain and therefore they cannot be used in haematopoietic or leukaemic stem cell research (Belizário, 2009; Shultz et al., 2007).

3.1.1.2 Scid mouse strains

Severe combined immunodeficiency (Scid) in CB17 mice also developed after a spontaneous mutation and these mice were discovered and described by Bosma et al (Bosma et al., 1983). The autosomal recessive mutation in the *Prkdc*^{Scid} gene that codes for a DNA-dependent protein kinase, results in a defect of non-homologous end joining of double-stranded DNA, a crucial process in the development of functional B- and T-cells, ultimately leading to agammaglobulinaemia. (Shultz et al., 2007). These mice lack mature B- and T-lymphocytes and due to the defect in non-homologous end joining of double-stranded DNA they are very sensitive to radiation (Fulop and Phillips, 1990). The mice have pronounced innate immunity and NK-cell activity and are prone to develop "leakiness" with the generation of B- and T-cells when they get older (Bosma et al., 1988; Greiner et al., 1998). However, besides their initial main use in human immunodeficiency virus research, these mice were the first described to successfully engraft human haematopoietic cells, at least to a low level. Intrestingly, transplanting $CD34^+$ human cord blood cells, it could be elegantly demonstrated that Scid mice reconstitute the whole physiologic hierarchy of normal human haematopoietic cells from Scid repopulating cells over long term culture initiating cells to colony forming units and mature effector cells (Pflumio et al., 1996). This haematopoietic engraftment ability led to the employment of these mice in many haematological studies providing important insights into normal and malignant haematopoiesis but due to the development of mouse strains with better acceptance of human haematopoietic cells this mouse strain is now mainly of use for tissues and cancers of non-haematologic origin (Lapidot et al., 1992; Mosier et al., 1988; Vormoor et al., 1994).

3.1.1.3 bg/nu/xid (BNX) mouse strains

The innate immunity, enhanced NK-cell and lymphokine-activated killer cell (LAK) activity of the nude and Scid mouse strains still has a pronounced negative impact on human cell growth in mice receiving xenotransplants, especially cells of the myeloid lineage. In order to overcome these problems of graft rejection, a more immunocompromised mouse strain was needed. In the bg/nu/xid (BNX) mouse strain the advantages of reduced NK cell numbers of mice with the beige (*bg*) mutation and reduced number of LAKs from mice with the *xid* mutation, which both have an important role in the reaction of the host against the xenograft were transferred on athymic mice with the nude (*nu*) mutation by cross-breeding (Andriole et al., 1985; Fodstad et al., 1984). Dick and colleagues used this more immunocompromised mouse strain that finally made it possible to not only engraft normal lymphoid but also normal myeloid cells from human bone marrow. However, high numbers of cells need to be transplanted and the additional substituion of human growth factors and irradiation of the mice is necessary for successful engraftment of human cells (Kamel-Reid and Dick, 1988). Little later, an ALL cell line (A-1) was as well successfully transplanted into this mouse strain by the same group (Kamel-Reid et al., 1989).

3.1.1.4 NOD/Scid hybrid mouse strains

Backcrossing the *scid* mutation onto the non-obese diabetic (NOD/Lt) mouse strain resulted in the NOD/Scid hybrid strain. NOD mice were originally used to model autoimmune type 1 diabetes mellitus but were also found to harbour a profound functional deficit in NK-cells and antigen presenting cells and to lack serum complement factors, all important contributors to the innate immune system (Shultz et al., 1995). The combination of the multiple functional deficiencies in the innate immunity of the NOD strain with the impact of the *scid* mutation on B- and T-lymphatic

development highly improved the long term engraftment of human haematopoietic cells in the NOD/Scid hybrid strain (Cashman et al., 1997; Lowry et al., 1996; Shultz et al., 2007). Like in Scid mice the whole physiologic hierarchy of haematopoietic cells is similarly reconstituted in the NOD/Scid mouse strain (Pflumio et al., 1996). Furthermore, with the use of NOD/Scid mice it was possible for the first time to prove that human cord blood contains transplantable totipotent progenitor cells with the ability to differentiate into B and T lymphocytes, natural killer cells and granulocytes (Robin et al., 1999). As a consequence, NOD/Scid mice have also been used in leukaemia stem cell research and were shown to have superior engraftment abilities. To gain the same levels of engraftment of AML blasts, 10 to 20 times fewer leukaemic cells were required for transplantation in NOD/Scid hybrid mice than in mice with the *scid* mutation alone (Bonnet and Dick, 1997). Of note, NOD/Scid hybrid mice do not develop diabetes but there are limitations in their sensitivity as they retain low levels of innate immunity and at least some NK-cell activity. In order to overcome the engraftment barriers related to residual NK-cell activity, an antibody directed against the IL-2Rβ chain (anti-CD122) can be administered to NOD/Scid mice to deplete remaining NK-cells and further members of the innate immune system like macrophages and other myeloid cells (Kerre et al., 2002). This treatment helped to identify members of the haematopoietic hierarchy with short-term repopulating activity (McKenzie et al., 2005; Shultz et al., 2003). A major disadvantage is the relatively short life span of NOD/Scid hybrid mice, limited to around 8 to 9 months at which age they commonly develop thymic lymphomas. This impedes their employment in long-term engraftment studies or limiting dilution experiments with very low numbers of cells transplanted which usually need an extended time of observation (Shultz et al., 1995).

3.1.1.5 β2 microglobulin-deficient (B2m$^{-/-}$) NOD/Scid mouse strains

A mouse strain that lacks NK-cell activity was developed by crossing NOD/Scid mice with mice deficient of β2 microglobulin expression. This additional defect leads to the absence of major histocompatibility complex (MHC) class 1 molecules essential for NK-cell development (Christianson et al., 1997). Using these mice in human Scid repopulating cell assays showed a superior engraftment ability of this strain. The SRC frequency of normal haematopoiesis read out more than 11-fold higher than in conventional NOD/Scid mice and previously unknown members of the haematopoietic hierarchy, one with short-term myeloid and one with short-term myeloid and lymphoid repopulating ability could be identified that completely failed to be detected in NOD/Scid mice (Glimm et al., 2001; Kollet et al., 2000). However, this mouse strain also has a relatively short life expectancy of around 6 months due to the development of thymic lymphomas and the mice were also found to accumulate iron in the liver leading to the development of haemochromatosis (Christianson et al., 1997; Shultz et al., 2007). As in NOD/Scid mice this short lifespan imposes a disadvantage on these mice when the experiments require a long observation period. Furthermore, NOD/Scid mice additionally treated with anti CD122 and transplanted intrafemorally proved to be better hosts for human haematopoietic cell engraftment than NOD/Scid B2m$^{-/-}$ mice in one study of John Dick´s group (McKenzie et al., 2005).

3.1.1.6 NOD/Scid IL-2Rγ $^{-/-}$ (NSG) mouse strains

The finding that even a very low NK-cell activity in NOD/Scid or NOD/Scid B2m$^{-/-}$ mice has a significant impact on human haematopoietic engraftment and lymphomyeloid differentiation prompted efforts to develop immunodeficient mice that lack NK-cell function completely. From X-chromosome linked Scid in humans it is known that the IL-2 common γ-chain, a signalling component of the IL-2, IL-4, IL-7, IL-9, IL-15 and

IL-21, is essential for lymphoid development and mice created with a homozygous disruption in the gene for the IL-2 common γ-chain completely lack NK cells and have severely reduced B- and T-lymphocytes (Asao et al., 2001; Cao et al., 1995). Crossing IL-2Rγ$^{-/-}$ mice with mice of the NOD/LtSz-Scid hybrid strain resulted in the new, highly immunocompromised NOD/LtSz-Scid IL-2Rγ $^{-/-}$ hybrid strain NSG that combined the immunodeficiency characteristics of both maternal strains. These mice readily engrafted human HSCs to high levels and supported multilineage differentiation (Shultz et al., 2005). When compared to conventional NOD/Scid and NOD/Scid B2m$^{-/-}$ mice, NSG mice as well were found to engraft patient samples that failed to reconstitute the leukaemia in both other strains as well as to engraft AML and ALL cell lines or other primary patient samples faster and to a higher level (Agliano et al., 2008). Another big advantage that makes NSG mice highly suitable for long-term engraftment studies and for limiting dilution experiments which potentially need extended time for follow up when small cell doses are transplanted, is the comparably old age of more than 16 months that these animals can achieve. This is partly owed to the fact that NSG mice are highly unlikely to get lymphomas at advanced age or even when sublethal irradiation is applied. Furthermore, NSG mice additionally only have a small tendency to become "leaky" (Ishikawa et al., 2005; Shultz et al., 2005).

Another group used mice with a truncated IL-2 chain for backcrossing on NOD/Scid mice and generated the NOD/Shi-Scid IL-2Rγ$^{-/-}$ strain (NOG). They also demonstrated superior engraftment and multilineage differentiation abilities of these mice compared to anti-asialo GM1 antibody (to deplete residual NK-cells) treated NOD/Scid and NOD/Scid B2m$^{-/-}$ mice (Hiramatsu et al., 2003; Ito et al., 2002).

A potential disadvantage of the NOG to the NSG strain is that in the first the IL-2Rγ-chain is not completely absent but only truncated and still can bind cytokines (Shultz et al., 2007). Indeed, in a recent HSC engraftment study, conducted by McDermott et al,

both the NOG and the NSG strain showed much higher engraftment sensitivity and multilineage engraftment than NOD/Scid mice treated with anti CD122 antibody but NSG mice were found to have slightly higher bone marrow engraftment of human haematopoietic cells than all other transplanted mouse strains (McDermott et al., 2010).

The superior ability of NSG mice to engraft human haematopoietic cells compared to other mouse strains with higher residual levels of innate and adaptive immunity proved in several studies, combined with the longevity of these mice made the NSG strain a robust mouse model of choice for this project.

3.1.2 Intrafemoral transplantation technique

In addition to the applied mouse model, the transplantation technique is another critical step in an *in vivo* xenotransplantation setup. Ideally, every human cell delivered into the mouse should meet the same and most supportive conditions to allow it to prove, if existent, its self-renewal and differentiation capability. Insights regarding the identity and frequency of stem cells closest resembling those in their natural habitat can only be gained if artificial impediments imposed by the experimental setup are alleviated to the smallest possible degree. Stem cells are dependent on the microenvironment of their niche and in a first step after transplantation any stem cell has to find its way into this niche in order to have the chance to engraft. For the conventional approach of intravenous injection of candidate stem cells this means that transplanted cells first circulate through the blood system and have to adhere to and migrate through the endothelial wall of blood capillaries to home in the appropriate bone marrow stroma. The seeding efficiency of normal HSCs (the percentage of HSCs that reaches the murine bone marrow after intravenous injection) has been investigated in irradiated NOD/Scid mice by van Hennik et al and found to be only around 5% (van Hennik et al., 1999).

As homing ability is not a defining characteristic of stem cells it and therefore should be, if possible, excluded from the assay. In order to circumvent this step, several groups have tried to establish an orthotopic transplantation approach in which assayed cells are directly delivered into the most accessible bone marrow in the mouse femur or tibia. Although the orthotopic transplantation technique is more elaborate and time consuming than intravenous injection, as mice have to be anaesthetised in order to inject the cells through a pre-drilled hole into the mouse femur or tibia, the increase of sensitivity in revealing stem cell activity is convincing. The method was developed by Kushida et al in a syngenic mouse model in 2001 and Yahata et al were the first to apply this approach in the human to mouse xenotransplant setting and to prove its superiority to the conventional intravenous transplantation technique (Kushida et al., 2001; Yahata et al., 2003). The frequency of HSCs in $CD34^{+}CD38^{-}$ umbilical cord blood cells, as determined by intratibial injection into NOD/Scid mice, was found to be 15-fold higher than after intravenous injection (Yahata et al., 2003). By transplanting subfractions of human umbilical cord blood cells intrafemorally into NOD/Scid mice, John Dick´s group even managed to identify a new class of rapid myeloerythroid repopulating stem cells that evaded detection by the conventional intravenous transplantation procedure (Mazurier et al., 2003). The advantage of orthotopic transplantation was subsequently demonstrated for several mouse models (NOD/Scid mice, NOD/Scid mice treated with anti CD122 antibody and NOD/Scid $B2m^{-/-}$ mice) by the same group (McKenzie et al., 2005).

In addition to the advantage of delivering the transplanted cells as close as possible to their ideal microenvironment, the intrafemoral transplantation approach potentially also diminishes the problem of antibody mediated clearance of intravenously injected cells by residual peripheral immune cells as suggested by Taussig et al (Taussig et al., 2008).

3.2 Aims

The primary aims of the initial phase of the study were:

- To establish multicolour flow cytometry to detect, characterise and quantify human ALL cells in the NSG murine background with a high sensitivity and specificity.

- To establish the intrafemoral orthotopic transplantation technique for ALL in NSG mice in order to assess the existence and frequency of blasts with LSC characteristics in purified subfractions defined by the immunophenotype.

3.3 Materials and methods

Flow cytometry has played an invaluable role in elucidating B-cell development and the haematopoietic hierarchy in general through the identification of distinct patterns of surface antigens in a time dependant manner (Loken et al., 1987). For xenotransplantation experiments it is also essential to clearly separate cells of mouse origin from human cells. This can also be achieved by taking differential surface antigen expression into account.

The interrogation of a single cell to assess its expression of several surface antigens can be realized by multiparameter flow cytometry that in addition to forward and side light scatter profiles uses multi-colour staining and detection (multi-colour flow cytometry). With a higher number of defining antibodies and therefore colours used in one single assay, the resolving power of flow cytometry increases. Several single fluorochromes (also named fluorophores) or tandem dyes with distinct fluorescence emission spectra are available which can be conjugated to specific mAbs. The detection and intensity of fluorescent signals from individual cells then proportionally indicates the expression profile of certain marker antigens on the cell of interest.

The more fluorescent colours are used in one experiment, the more spectral overlap occurs between different emission spectra of fluorochromes and this creates a problem in unambiguously allocating signals received by a PMT to a specific fluorochrome. Spectral overlap between two fluorochromes is schematically depicted in Figure 7. To prevent false results due to spillover of fluorescent signals into PMTs assigned to other colours the process of compensation is mandatory as bandpass filters are not able to completely filter out light from other fluorochromes. Compensation is designed to mathematically subtract signals generated by several fluorochromes not assigned to a specific PMT detector from the total signal received by it (Roederer, 2001).

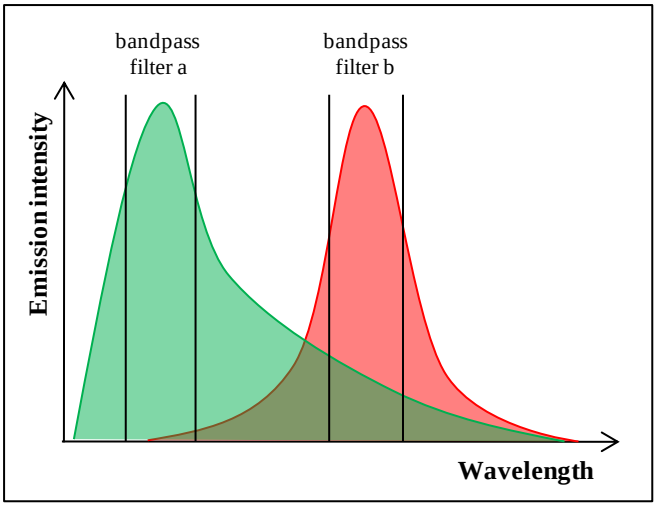


Figure 7: Spectral overlap between two fluorochromes. Emitted light from fluorochromes covers a broad spectrum of wavelengths, although of variable intensity. The emission spectra and overlap between two fluorochromes is shown in this schematic figure. Bandpass filters (a and b, indicated by vertical lines) are designed to allow only light within a narrow bandwidth around the emission maximum of a specific fluorochrome to pass to the PMT. In case of spectral overlap falling into the bandwidth of a bandpass filter, light of a fluorochrome not assigned to the PMT is recorded and has to be compensated for in order to prevent false positive signalling.

3.3.1 Preparation of human and NSG mouse cells for flow cytometry

Human peripheral blood lymphocytes were regarded as being appropriate for the setup of flow cytometry as normal human bone marrow was not available for this procedure. After informed consent, approximately 10 ml of peripheral blood was taken from healthy volunteers via venesection. Red cell lysis was carried out with 1x ammonium chloride buffer and the remaining mononuclear cells were washed twice with PBS (see section 2.2.3). Compensation experiments were carried out with fluorochromes conjugated to the anti-CD3 surface marker antibody (see section 2.2.2). CD3 is commonly expressed on T-lymphocytes as part of the T-cell receptor complex (Clevers et al., 1988). Lymphocytes usually represent 20 to 45% of all peripheral white blood cells and T-lymphocytes make up for 40 to 75% of total lymphocytes in a differential full blood count (Blumenreich, 1990). PBMCs were counted in an improved Neubauer

counting chamber (see section 2.6.5), cell aliquots distributed in FACS tubes and cells were labelled with saturating amounts of one or several monoclonal antibodies to CD3 directly conjugated to FITC, PE, PerCP-Cy5.5, PE-Cy7 or APC. PBMCs were also labelled with anti-CD10 FITC, anti-CD19 PE, anti-CD20 PerCP-Cy5.5, anti-CD38 PE-Cy7 and anti-CD34 APC (see section 2.2.5). All stained and unstained samples were acquired on the BD FACSCanto II flow cytometer equipped with FACSDiva analysis software, v6.1.2 (see section 2.2.6).

In order to detect engraftment of human cells in peripheral blood of transplanted mice, a different method of lysis for mouse red cell was performed. After incubating peripheral blood from mice (usually 30 – 50 µl diluted to 200 µl with 0.2% w/v PBSA) for 20 minutes with antibodies of interest, the samples were incubated for an additional 7 minutes with fixative and lysis buffer, commercially available from BD Biosciences (1x BD FACS Lysis buffer) at room temperature in the dark. Samples were centrifuged at 1,750 rpm (713 g) for 5 minutes at room temperature, the supernatant discarded and then washed twice with 3 ml PBS using the same parameters for centrifugation. Cells were then resuspended in 500 µl of 0.2% w/v PBSA solution for flow cytometry.

3.3.2 Setup of multi-colour flow cytometry

To set up for calculation of a compensation matrix, an unstained cell sample as well as one sample for each individual fluorochrome that is due to be part of multi-colour staining in later experiments, is needed. Data from all samples are acquired into the compensation mask, provided by the FACSDiva analysis software, v6.1.2. Acquisition of the unstained cell sample is needed first in order to set the PMT voltages. Unstained cells have some degree of autofluorescence overlapping the fluorescence spectra of individual fluorochromes. This autofluorescence has to be excluded from being regarded

as a true signal in the PMT. Therefore the autofluorescent signal from unstained cells has to be set into the first quadrant of a signal intensity histogram for each colour recorded by a PMT by adjusting the signal amplifying voltage across the PMT. Signals in the first quadrant are counted as being negative. After adjusting PMT voltages for autofluorescence, the individually stained samples are acquired to record a positive signal for each fluorochrome and to check if clearly defined and separable positive and negative signals are present from populations of cells that did, or did not, bind the fluorochrome conjugated antibody. In the case of acquisition of well defined negative and positive signals for each fluorochrome, the FACSDiva software is able to calculate a compensation matrix. This compensation matrix then adjusts for spectral overlap between the individual fluorochromes, enabling to record fluorescent signals only from cells that actually did bind the specific antibody-fluorochrome conjugate.

3.4 Results

3.4.1 Compensation matrix for 5-colour flow cytometry

Compensation experiments were performed on the BD FACSCanto II to set up a compensation matrix for multi-colour flow cytometry by using the FACSDiva analysis software, v6.1.2. It was possible to compensate for a combination of 5 different fluorochromes. The different steps involved in the process of generating the compensation matrix are shown in Figure 8. This allows the discrimination of expression of 5 different human surface antigens in the case of labelling primary patient samples that are not contaminated with mouse cells with antibodies directed at human surface markers only. The combination of anti-CD10 FITC, anti-CD19 PE, anti-CD20 PerCP-Cy5.5, anti-CD38 PE-Cy7 and anti-CD34 APC, comprising stem cell markers and surface antigens expressed in B-cell development was generally used for this purpose. To analyse cells from transplanted mice, one colour was dedicated to antibodies against mouse cells, anti-mouse CD45 and anti-mouse TER-119. Both antibodies were conjugated to the same fluorochrome (PE-Cy7) as the purpose of identifying mouse cells within mouse human chimerae only was to exclude the cells of mouse origin from analysis and not to measure individual expression levels of these mouse antigens. Determining the expression of 4 human antigens (CD10, CD19, CD20 and CD34) on cells of human origin in the mouse background was considered as being sufficient to characterise the immunophenotype of engrafted blasts.

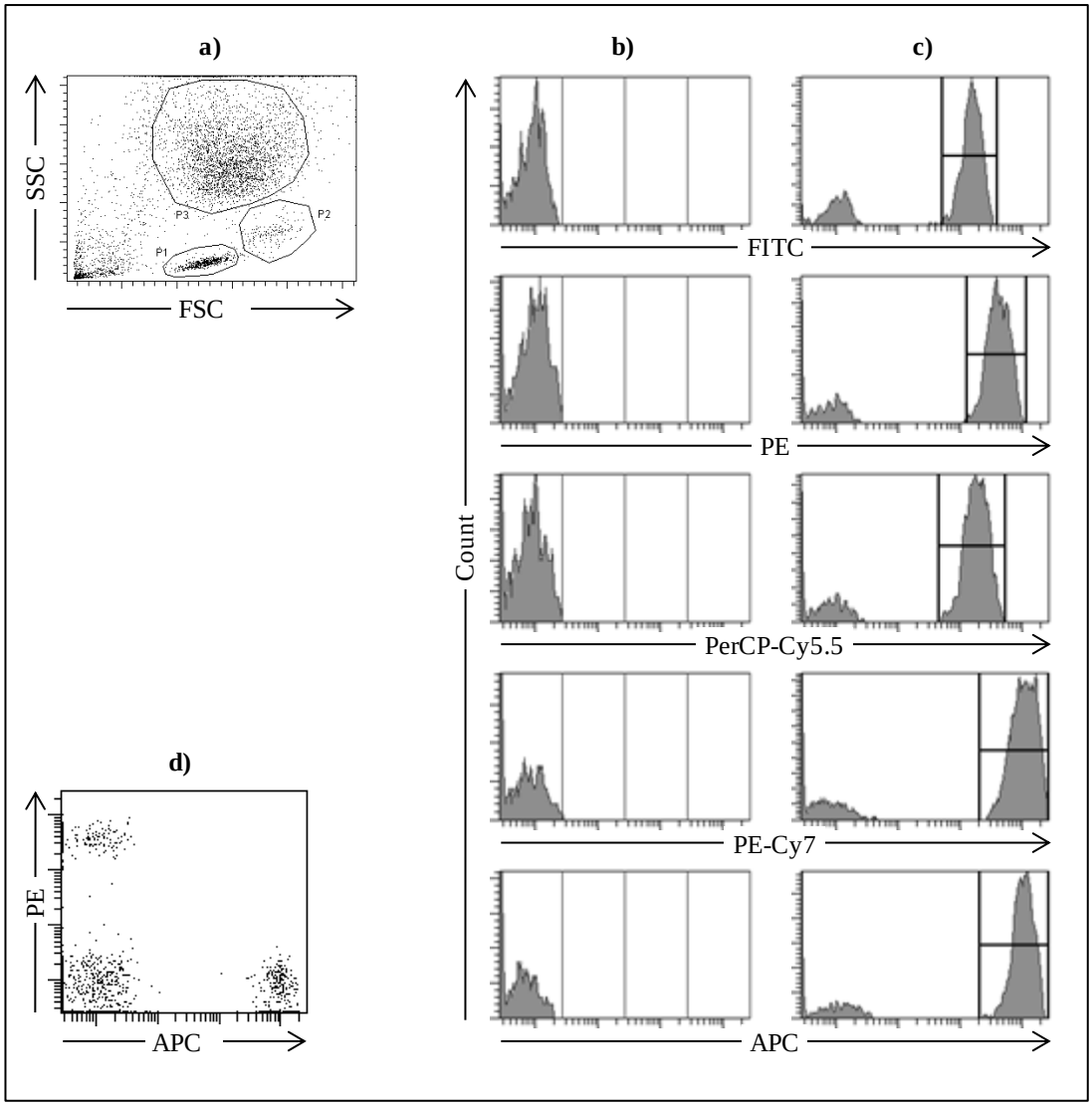


Figure 8: The process of creating a compensation matrix with FACSDiva analysis software, v6.1.2. a) The normal light scatter profile of human PBMCs is depicted after light scatter voltages have been adjusted to clearly separate the three distinct PBMC populations of lymphocytes (gated in P1), monocytes (P2) and granulocytes (P3). The relatively small lymphocytes with cytoplasm of low complexity show a typical low SSC and FSC profile. **b)** The voltages across PMTs for different fluorochromes were adjusted to place autofluorescence signals of lymphocytes (P1) in the first quadrant of the histograms, defined as being negative. **c)** Each of 5 lymphocyte samples was labelled with one individual fluorochrome directly conjugated to an anti-CD3 antibody and the positive signals were recorded and gated. Spectral overlap between the fluorochromes was identified and automatically compensated for. **d)** To test the result, lymphocyte samples individually stained with anti-CD3 PE and anti-CD3 APC were mixed, recorded on the FACSCanto II and depicted as a dot plot. Cell populations with no bound antibody or labelled with one of the two fluorochromes are distinct and clearly separable.

3.4.2 Excluding murine cells from analysis of human blast engraftment by using antibodies to murine CD45 and TER-119

Flow cytometry analysis of all transplantation experiments had to meet two essential criteria for this project, high specificity and high sensitivity of detecting blasts of human origin in the xenoenvironment of the NSG host mice. The purpose of that was to find out whether or not the leukaemia was in general able to engraft into the NSG mice and in the case of successful engraftment to determine the percentage of chimerism between cells of human and mouse origin as reliably and exactly as possible. At later stages of the project it was also necessary to dependably detect very low engraftment levels of human blasts within the mouse bone marrow compartment, comparable to MRD levels in human leukaemia follow up diagnostics, as it could be expected that mice transplanted with only 10 cells in the limiting dilution experiments would need a rather long time to engraft the NSG mice and that the life span of around 16 months of these mice would probably still not be long enough to engraft the blasts to a higher percentage. Detecting first minimal signs of bone marrow engraftment was as well necessary to address the question of differences in engraftment kinetics between sorted blast populations and also in limiting dilution experiments of unsorted and sorted blasts. The rational for this was that in ALL usually an exponential growth pattern is expected and that, with the limited allowed number of bmps and minimum intervals of two weeks between consecutive bmps it would have been difficult to see differences in time to engraftment once the log phase of leukaemic growth with higher engraftment levels was reached. A third reason for the need to undoubtedly document a very low chimerism of human cells was inherent of the animal model. Working with highly immune compromised mice, it was unavoidable that some animals would die during the course of the experiments for other reasons than developing the full clinical picture of an ALL or approaching their expected life expectancy. As this could happen at any time point during the course of

experiments, it was possible that engraftment was only marginal at the last bone marrow puncture before the loss of the individual mouse.

To match these criteria, first of all it was crucial to reliably differentiate between human and mouse cells in the flow cytometry analysis of murine chimeric bone marrow. The light scatter profiles of mouse cells and human lymphocytes were not suitable for discriminating these cells as there is significant overlay between cells of mouse origin and human lymphocytes. Additionally, staining native mouse bone marrow with a panel of human surface antigen antibodies that would be used for characterisation of engrafted leukaemic blasts (antibodies to human CD10, CD19, CD20 and CD34) according to the staining protocol described in section 2.2.5 showed a substantial degree of non specific binding to mouse bone marrow cells (see Figure 9). This unspecific binding would make it difficult to detect and exclusively depict human leukaemic blasts in the murine bone marrow background, especially if only a low number of blasts were engrafted.

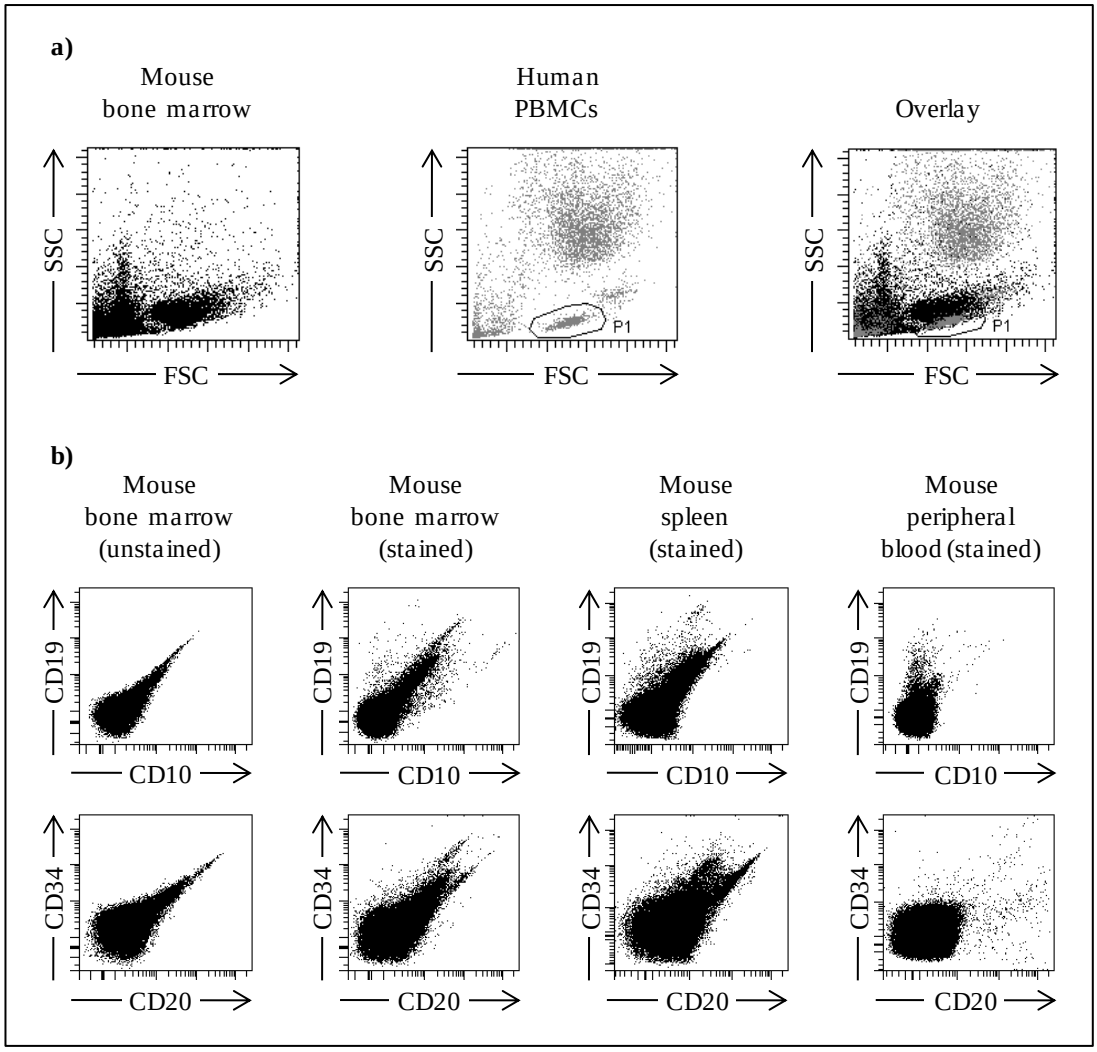


Figure 9: Similar light scatter profiles of human lymphocytes and murine cells and unspecific binding of anti-human surface marker antibodies to murine cells hamper detection of human cells in a murine bm background. a) Human lymphocytes depicted in a light scatter dot plot are overlaid by murine cells with a similar light scatter profile. **b)** Bone marrow, spleen cells and peripheral blood from native, not transplanted NSG mice were stained with anti-human CD10 FITC, CD19 PE, CD20 PerCP-Cy5.5 and CD34 APC according to standard protocol and dot plots compared to unstained samples (only shown for bm). Mouse cells of all three sources show significant unspecific binding of antibodies to human surface antigens.

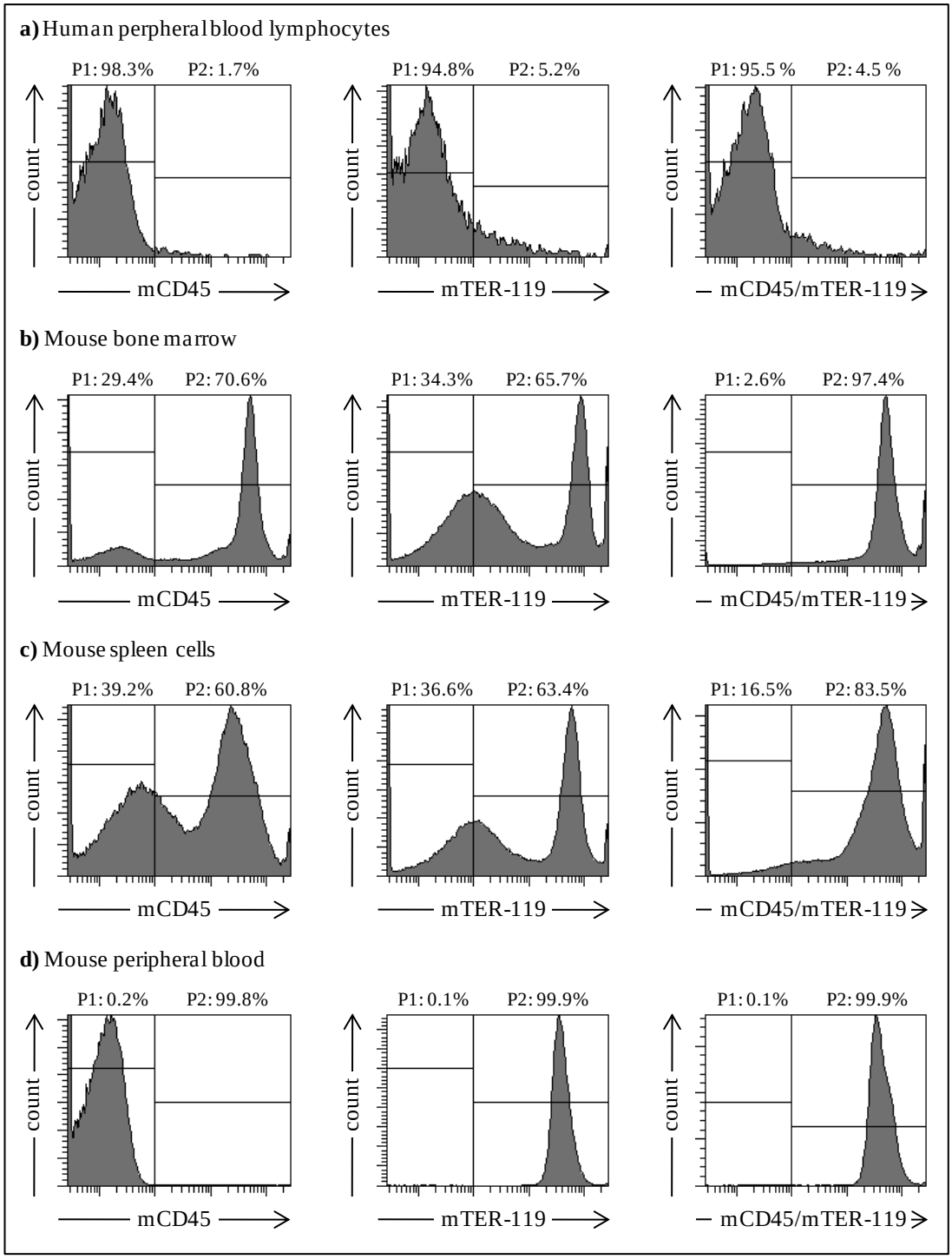
To circumvent the problem of nonspecific binding antibodies against mouse white and red cell lineages were used to gate out the majority of cells of mouse origin in the flow cytometry and exclusively leave engrafted cells of human origin for analysis using the antibody panel directed against human surface antigens (CD10, CD19, CD20 and

CD34). Anti mouse CD45 (alternate names Leukocyte Common Antigen (LCA), Ly-5, T200) which binds to all cells of haematopoietic origin except erythroid cells and anti mouse TER-119 (alternate name Ly-76) which binds to erythroid cells from pro-erythroblasts through to mature erythrocyte stages were chosen for this purpose and evaluated regarding their ability to gate out murine bone marrow cells from engraftment analysis. Initially it was planned to serially analyse bone marrow samples only but from post mortem examinations of mice it soon became obvious after the first sets of successful transplantations that these leukaemias were spreading in the NSG mice and apparently were infiltrating and thereby enlarging the spleens of the animals. This observation suggested analysing harvested mouse spleens regarding their content of human leukaemic blasts as well.

To validate whether a gating strategy to exclude mouse cells from analysis would be feasible, human peripheral blood mononuclear cells, mouse bone marrow, mouse peripheral blood and later a single cell suspension of mouse spleen cells were stained with anti-mouse CD45 (mCD45) or anti-mouse TER-119 (mTER-119) individually and also in combination and analysed by flow cytometry. First the extent of unspecific binding of mouse antibodies to cells of human origin was assessed. As the best substitute for normal human bone marrow, a sample of human leukocytes isolated from peripheral blood of healthy volunteers after red cell lysis first was incubated only with anti mouse CD45 and the fluorescence profile was depicted in a flow histogram. Over 98% of human lymphocytes in a normal distribution could be included into a gate P1 that reached from zero to 10^3 of mCD45 mean fluorescence intensity (MFI). Applying the same strategy, mTER-119 was evaluated. By placing the same gate P1 from zero to 10^3 of mTER-119 MFI, nearly 95% of the human cells were captured within P1. Staining human lymphocytes with both murine antibodies simultaneously placed over 95% of the analysed cells into the same gate P1 in an additional experiment. These data are shown

in Figure 10 and proved that the mouse antibodies CD45 and TER-119 exhibit only low levels of nonspecific binding to blood lymphocytes of human origin and that therefore only a low number of human blood cells would be lost for analysis, if cells with an MFI of higher than 10^3 of mCD45 and mTER-119 in combination were excluded.

Figure 10 (page 118): Separating cells of human and mouse origin with anti-mouse CD45 and TER-119. The histogram gates P1 and P2 separate cells of MFIs lower and higher than 10^3 respectively. **a)** Human lymphocytes show only little binding of anti-mouse CD45 and TER-119 mAbs. **b)** More than 97% of NSG mouse bm cells stained with mCD45 and mTER-119 in combination lie in P2. **c)** More than 83% of mouse spleen cells are included in P2 with the mCD45/mTER-119 combination and mouse blood cells almost completely fall in P2 when labelled with both mouse mAbs (**d)**). Analysing only events in P1 for human CD marker expression excludes the vast majority of mouse cells and diminishes the possibility of false positive results from unspecific human mAb binding to murine cells.



In a second step it was assessed if cells of mouse origin could be readily excluded from analysis with this staining and gating strategy, thereby preventing possible false positive results for human engraftment as much as possible. For this purpose full bone marrow, a single cell suspension generated from a spleen and peripheral blood from a healthy NSG

mouse were stained with the same antibodies individually and in combination. Using mCD45 on murine bone marrow cells alone left less than 30% of the analysed cells in the established gate P1 from zero to 10^3 of mCD45 MFI. Staining another sample of the bone marrow cells with mTER-119 showed acquisition of only less than 35% of murine cells within P1. When both antibodies were used in combination on a third bone marrow sample the exclusion of mouse cells from the gate P1 increased to 97.4% and only less than 3% of mouse cells remained in the gate P1 in which cells were supposed to be analysed for human surface marker expression in the xenotransplant experiments. Accordingly, exclusion experiments were conducted on the mouse spleen cells. Staining the single cell suspension with mCD45 led to less than 40% remaining in the gate P1 and staining the spleen cells with mTER-119 showed that only less than 37% of the mouse cells were included into gate P1. Again, the combination of both anti-mouse antibodies, mCD45 and mTER-119, was able to greatly increase the percentage of mouse cells that successfully could be excluded from being analysed regarding the expression of human surface antigens. The same antibody labelling and histogram gating strategy was applied on peripheral blood from mice. The mCD45 antibody only labelled a very small amount of mouse cells, most likely due to a very low peripheral white cell count, whereas mTER-119 binding approached 100%. The combination of both antibodies therefore could not increase the fraction of mouse cells excluded from P1. Figure 10 illustrates and summarizes the experiments that lead to the labelling and gating strategy applied to mouse bone marrow, spleen and peripheral blood samples in initial xenotransplantation experiments.

3.4.3 A histogram based gating algorithm for flow cytometry analysis on human mouse chimeric cell samples

The experiments with the anti-mouse antibodies directed at mouse white lineage and red lineage cells, CD45 and TER-119 respectively, showed that the majority of cells from mouse bone marrow and peripheral blood could be labelled with a combination of these two antibodies. On the other hand, it could be established that human peripheral lymphocytes were only stained with the two anti-mouse antibodies to a very little extent. The gate placed in the histograms from zero to 10^3 MFI promised to readily isolate cells of human origin out of a chimeric mouse bone marrow and peripheral blood. These binding characteristics and the gate placement founded the basis for a gating algorithm (mainly suggested by PhD fellow student Kerrie Wilson).

Whenever possible, 100,000 events were recorded from a bmp or peripheral blood. A histogram was drawn and a gate P1 set to cover the MFI of PE-Cy7 (the fluorochrome conjugated to both mCD45 and mTER-119) from zero to 10^3. A second gate was set to cover the MFI of PE-Cy7 from 10^3 to maximum. Events restricted to P1 were then depicted in a light scatter dot plot in a second step. Cells that showed a scatter profile typical of lymphocytes or blasts of lymphocyte origin were again gated (P3) to exclude cell debris from analysis and the number of cells within this gate could be used to calculate the percentage of engraftment. In a third step the P3 gated cells from the previous dot plot were consecutively portrayed in several dot plots of the various combinations of fluorochromes on the x- and y-axis representing the human surface antigens CD10, CD19, CD20 and CD34, as well as the mouse surface antigen CD45 in combination with TER-119. These dot plots could then be used for assessment of the immunophenotype of engrafted human cells. This gating strategy is illustrated in Figure 11.

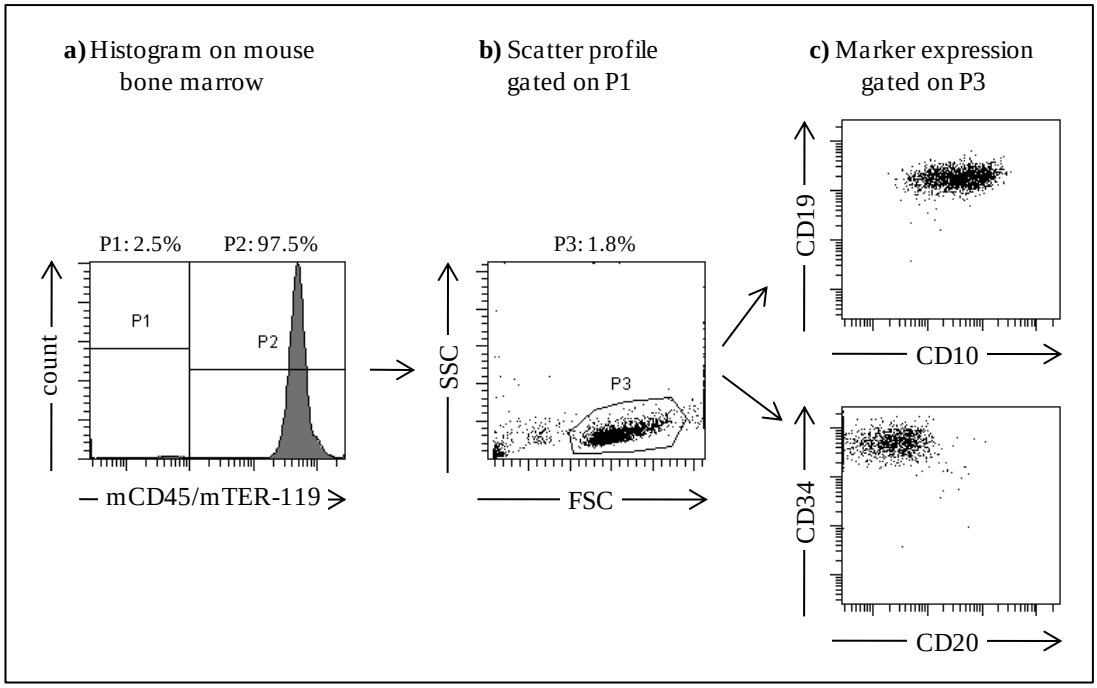


Figure 11: Gating algorithm based on flow histogram (example on bmp1, mouse M19, patient sample A67). a) The majority of NSG mouse cells are excluded from further analysis by gating them out with P2 set between 10^3 and maximum MFI for mCD45 and mTER-119. **b)** Only cells falling into P1 are then visualised in a light scatter dot plot and another gate P3 set to enclose cells with a light scatter profile resembling lymphoblasts. **c)** Cells from P3 are then depicted in dot plots for surface CD antigen expression.

3.4.4 First bone marrow xenotransplantations and engraftment analysis

After developing a method to detect and characterize potential engraftment of human leukaemic blasts in a NSG mouse xenograft background by applying the established gating strategy on a 5 colour flow cytometry, the next crucial step was to carry out first bone marrow transplantations onto NSG mice. These transplantations were performed to assess the technical success of the procedure and to gain a first insight into the behaviour of a human ALL in the xenoenvironment. On the technical side it was essential to see whether all the procedures involved in the xenotransplantation assay would lead to successful engraftment of human ALL blasts into NSG mice and whether engrafted cells could indeed be reliably detected in the mouse background. This involved the steps of

thawing the primary patient material, transplanting it intrafemorally into several NSG mice and following this, performing a series of bone marrow punctures and finally a full bone marrow harvest with application of the flow cytometry analysis on the acquired cells. In the case of positive results for engraftment, the second issue was to determine whether it was possible to amplify blast cell numbers in the mouse for further sorting experiments. An important pathophysiological question was to compare the original diagnostic ALL immunophenotype with the immunophenotype of human blasts grown in the xenotransplant assay and to determine whether or not the xenoenvironment would change the immunophenotypic character of the ALL.

For these reasons, and to achieve the highest possible chance of positive results, the first experiments were kept as simple as possible with relative high cell numbers of freshly thawed and unmanipulated, unsorted diagnostic material used for transplantation. Initially the investigations additionally concentrated on the high risk treatment group of BCR/ABL1 positive ALL patients as high risk leukaemias have been considered to have increased engraftment capabilities compared to ALL samples from lower risk groups.

Two diagnostic ALL patient bone marrow samples with different characteristic were chosen. Sample 8849 (BCR/ABL1 positive, adult patient, sample frozen for 11 years) was transplanted intrafemorally with 1×10^6 cells for each of four mice. Sample A67 (BCR/ABL1 positive, paediatric patient, sample frozen for just over one year) was transplanted into four mice but with a lower cell number of 3×10^5 each.

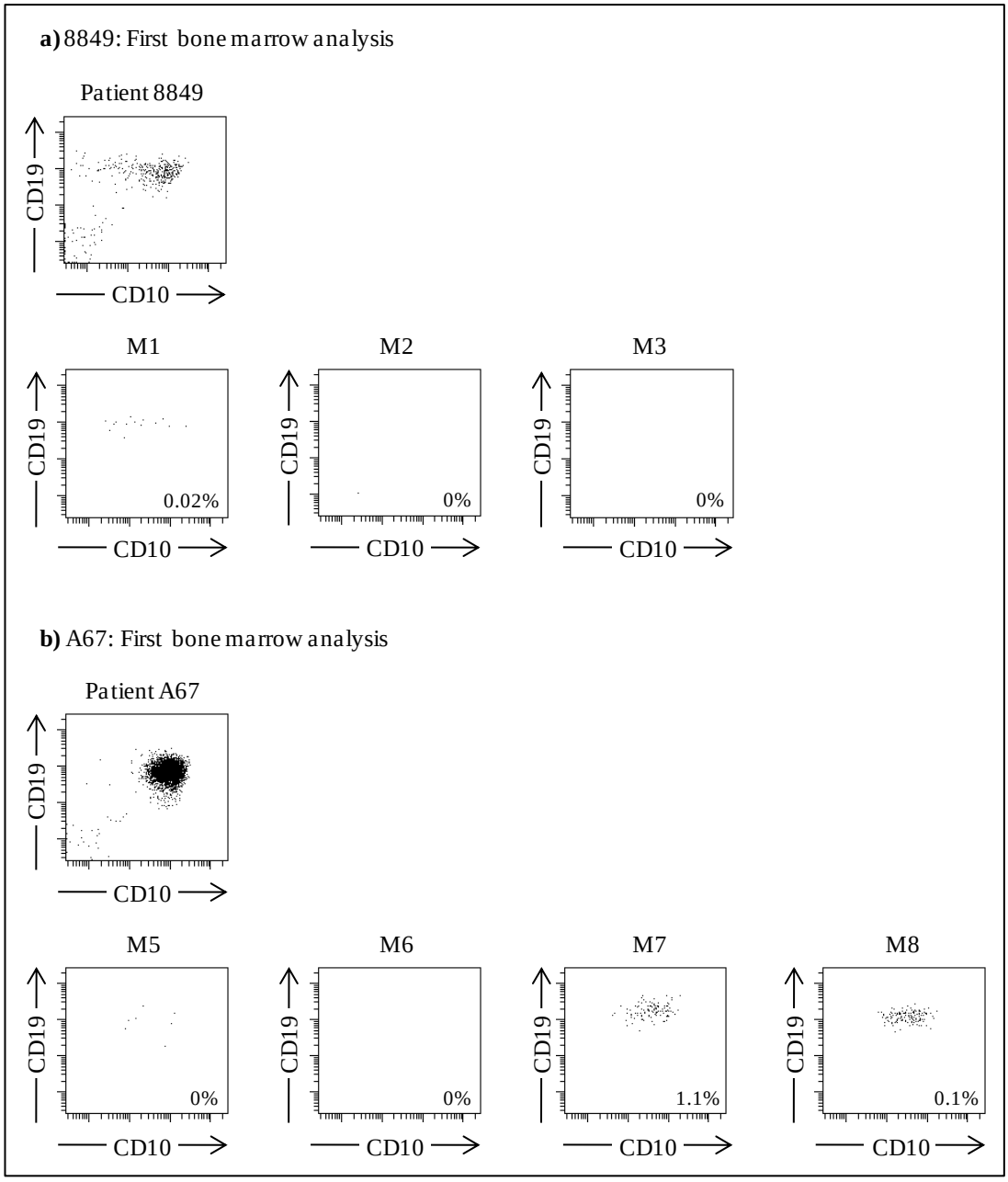
3.4.4.1 Engraftment analysis from mouse bone marrow punctures and bone marrow harvests

Bone marrow punctures form an essential part of patient initial and follow up diagnostics as ALL cells can be present in bm while peripheral blood appears to be

normal. Analysis of cells from bmps was therefore used to detect human engraftment in NSG mice. The first bone marrow punctures of the mice transplanted with the two different patient samples were carried out after 56 days (8 weeks) for sample 8849 and after 65 days (approximately 9 weeks) for sample A67 respectively. The individual samples were stained with the established antibody panel of anti-human CD10 FITC, CD19 PE, CD20 PerCP-Cy5.5 and CD34 APC according to the staining protocol described in 2.2.5 to characterize the potentially engrafted human blast population and with anti-mouse CD45 PE-Cy7 and TER-119 PE-Cy7 in order to exclude cells of mouse origin from flow analysis as described in section 3.4.2. One mouse transplanted with sample 8849 died before the first bmp was carried out. Of the remaining 3 mice the first bmp was performed on day 56 post intrafemoral transplantation and after staining the samples with the established antibody panel, 100,000 events from each sample were acquired on the BD FACSCanto II. Only one mouse (M1) showed engraftment, albeit to a low level of only 0.02%, as determined using the gating strategy described in 3.4.3. This is illustrated in Figure 12 a). The distribution of CD10 and CD19 expression among the engrafted lymphoblasts of mouse M1, despite the low cell number detected, shows close similarities to the original patient blast immunophenotype. All 4 mice transplanted with patient sample A67 were available for the first bmp on day 65 post if transplant. The bmp aspirates were stained with the same anti-human and anti-mouse antibodies and 100,000 events were recorded for mice M5 and M8, while only 10,000 events could be acquired for mice M6 and M7. Flow cytometry data of mice M7 and M8 show human chimerism of 1.1% and 0.1% respectively. As already seen for patient 8849 (Figure 12 a)), levels of expression for CD10 and CD19 in the bmp samples mimic the expression observed in the original patient sample. In the dot plot of CD19 against CD10 expression of mouse M5, only 7 events were detectable with the applied gating strategy. In addition to the very low number, the events do not form a coherent cluster but rather form two

groups and as a consequence they can not confidently be regarded as positive evidence of human lymphoblast engraftment. Dot plots of the original patient sample (A67) and of the bmps from transplanted mice (M5, M6, M7 and M8) with CD19 expression on the y-axis and CD10 expression on the x-axis are shown in Figure 12 b).

Figure 12 (page 125): Analysis of first bone marrow punctures on 8849 and A67. a) Dot plots with analysis of human CD10 and CD19 expression on the original patient sample 8849 and below on cells from the bmp 56 days post if transplant are shown of the 3 mice (M1, M2 and M3) transplanted each with 1 x 10^6 cells. 100,000 events were recorded from each bmp sample. While mouse M1 shows very little engraftment of 0.02%, no cells expressing human CD10 or CD19 were detected in mice M2 and M3. **b)** Dot plots analysis of human CD10 and CD19 expression on the original patient sample A67 and below on cells from the bmp 65 days post if transplant are shown of the 4 mice (M5, M6, M7 and M8) transplanted each with 3 x 10^5 cells. 100,000 events were acquired from mice M5 and M8 and 10,000 events from mice M6 and M7. Mice M7 and M8 exhibit clear clusters of cells representing 1.1% and 0.1% of the total number of events acquired respectively. The only 7 events recorded from mouse M5 do not appear as a clear cluster and were therefore not counted as engraftment.



In order to follow up engraftment levels of human cells, up to 5 consecutive bmps could be performed on mice with a minimum interval of 14 days between two punctures. However, if a mouse appeared to be ill from leukaemia or due to other reasons before completion of 5 bmps, the mouse was killed and a bone marrow harvest from the two femora and tibiae was carried out at that time point. All mice from the first two xenotransplantation experiments with patient samples 8849 and A67 underwent further

bmps for engraftment monitoring. Mouse M1 (patient 8849) showed generally increasing levels of human chimerism over the observed time course. At the time of bone marrow harvest, the overall engraftment level was determined to be 19.3%. The immunophenotype of the harvested bone marrow cells has a generally high expression of CD19 and a spread from low to high CD10 expression, comparable to the expression profile of the original patient blasts. While only two mice (M7 and M8) transplanted with A67 patient material showed engraftment at the time of the first bmp, all 4 mice were positive for cells of human origin, when the samples of the second bmp were analysed. The levels of human chimerism generally increased over the next bmps. This is illustrated in Table 2 (additionally engraftment analyses from bmps of all mice are listed in the table with raw date of Appendix 1).

Mouse ID	Patient / Leukemia	BMP1 Result Wk 8	BMP2 Result Wk 10	BMP3 Result Wk 13	BMP4 Result Wk 16	BMP5 Result Wk 21	Wk	Final Engraftment
M1	8849 prim	0.039%	0.134%	0.09%	4.0%	7.8%	25	75.7%
M2		0	0	0	0	0	49	0
M3		0	0	0	0		>16	
M4							<8	
Mouse ID	**Patient / Leukemia**	**BMP1 Result (Wk 9)**	**BMP2 Result (Wk 12)**	**BMP3 Result (Wk 15)**	**BMP4 Result (Wk 16)**	**BMP5 Result (Wk 19)**	**Wk**	**Final Engraftment**
M5	A67 prim	0.008*%	1.40%	5.2%	4.2%		16	40.6%
M6		0	0.057%	0.041%	0.32%		18	14.8%
M7		2.1%	3.7%				14	74.8%
M8		0.51%	0.064%	8.6%	3.2%	22.5%	19	75.8%

Table 2: Human chimerism of all bone marrow punctures from the first two xenotransplantation experiments on patient samples 8849 and A67.
Wk, week; *, positive outside limits

Analysis of the consecutive bmps usually also showed high consistency in the expression levels of human surface CD antigens. The majority of human lymphoblasts aspirated from mouse M5 at the fourth bmp, however, showed only low expression of CD10. Nevertheless, this expression profile was inconsistent to the one determined at

bmp3 and also for the final bone marrow harvest. A major downregulation of CD10 on most cells therefore seemed to be unlikely and was probably caused by a problem during the process of antibody labelling leading to a false negative result. All 4 mice transplanted with patient material A67 were killed and bone marrow was harvested when the ALL became clinically overt. Final bone marrow engraftment levels were determined and the immunophenotype of the harvested cells compared to the original diagnostic patient blasts. CD19 expression was uniformly high at presentation as well as on cells grown in mice. CD10 expression also was generally high in both original patient blasts and bone marrow obtained from engrafted mice. Only a minor proportion of engrafted human cells showed downregulation of CD10 to some degree in the bm harvests. Figure 13 shows dot plots of the original material from patient A67, 4 consecutive bmps and the final bm harvest.

The first bmp on mouse M5 resulted in only very few cells exhibiting a CD surface marker profile comparable to that of the A67 patient sample and they were not regarded as engraftment due to low number and widespread distribution of events in the dot plot. Considering that this mouse proved to be successfully engrafted with the ALL in the second and following bmps, retrospectively the recorded events in bmp1 were indeed likely to be caused by cells of human origin as the mouse must have had already some albeit very low human chimerism at this stage of the experiment.

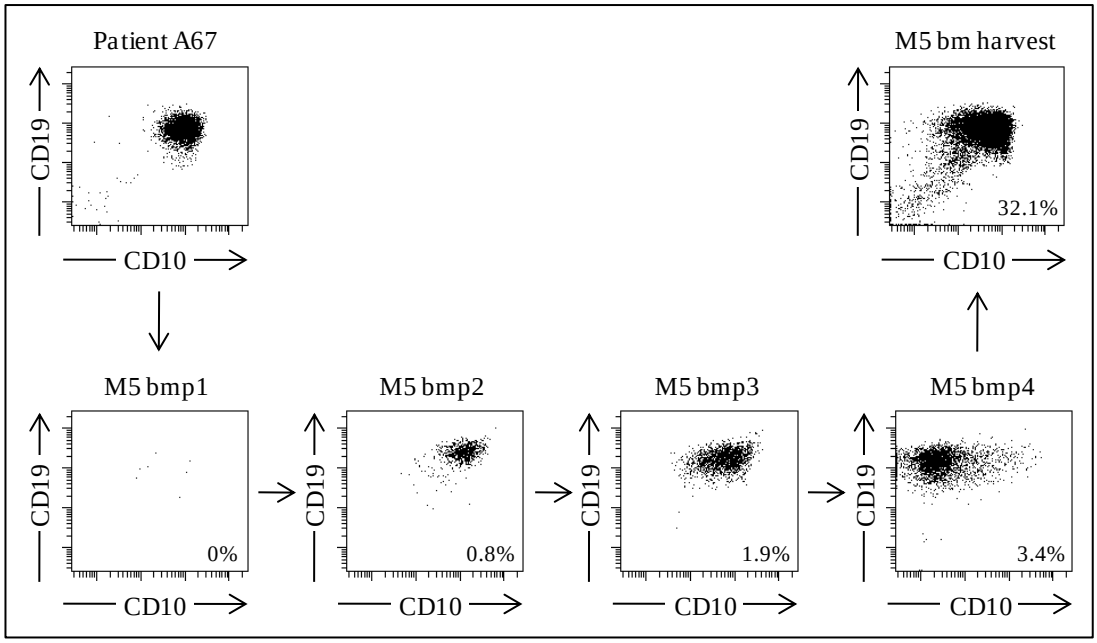


Figure 13: Serial bone marrow punctures and final bone marrow harvest of mouse M5 (Patient A67). Dot plots with analysis of human CD10 and CD19 expression on the original patient sample A67 as well as 4 consecutive bmps and the final bone marrow harvest (both femora and tibiae) of mouse M5 transplanted with 3 x 10^5 cells are shown. 100,000 events from each bmp and 150,000 events from the final bm harvest were acquired and analysed. The serial bmps show an increase in human chimerism over the time course observed. Final engraftment in harvested bm was determined to be 32.1%. Bmps 2 and 3 as well as the final harvest show an immunophenotype closely resembling that of the original patient sample. Cells from bmp 4 appear to express less CD10, most likely due to technical staining issues. Retrospectively, the events recorded from bmp1 probably can be considered as first but very low human engraftment.

Harvested bm of all engrafted mice transplanted with diagnostic material from patients 8849 and A67 was frozen down and stored in liquid nitrogen. A total of 8 x 10^7 cells was retrieved from the only mouse (M1) engrafted from sample 8849. Bone marrow harvests from mice M5-M8, transplanted with patient sample A67, yielded between 2.7 and 3.3 x 10^7 cells. These high numbers of recovered cells were then available for use in further xenotransplantation experiments.

3.4.4.2 Engraftment analysis on mouse peripheral blood samples

Although analysis of bm is the best way of detecting engraftment only 5 bmps could be performed because of considerations of animal welfare. Analysis was therefore also performed using peripheral blood. The time point of first signs of engraftment and engraftment kinetics towards a fully developed picture of leukaemia could be variable between mice even among groups transplanted with the same cell number from the same sample, making it necessary to monitor individual mice for presenting with ALL even when all 5 bmps had already been performed. Additionally, information would be gained whether the xenotransplant experiment would also lead to elevated blast numbers in peripheral blood of the mice, resembling the clinical picture of ALL in humans.

Blood samples of around 50 µl were taken from the tail vein of mice, diluted to a volume of 200 µl with 0.2% PBSA, then incubated with the usual antibody panel and finally the red cell content of the sample was lysed with BD FACS Lysis buffer as described in 3.1.1. Mice transplanted with patient sample 8849 were the first analysed for the presence of peripheral human blood lymphoblasts around week 20 after the start of the experiment. At this time human engraftment of mouse M1 was already well established whereas in the bone marrow of mice M2 and M3 no human cells could be detected via flow cytometry. In conformance to these findings, mouse peripheral blood as well only exhibited human chimerism in mouse M1 and not in mice M2 and M3 (Figure 14).

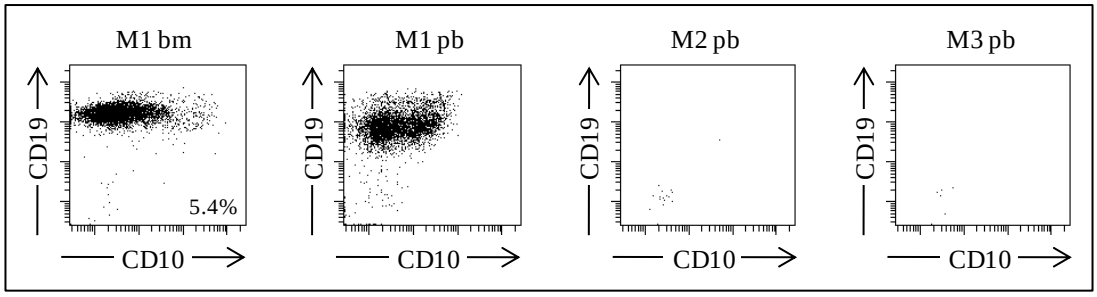


Figure 14: Presence of human lymphoblasts in mouse peripheral blood. Solid human ALL engraftment of 5.4% is detected in the bm of mouse M1 at week 21 post xenotransplantation. Peripheral blood taken from the same mouse 6 days earlier also contains cells of human origin as depicted in the second dot plot from the left. Mice M2 and M3 were not bm engrafted at week 21 and also do not show peripheral human blasts.

Peripheral blood analysis for human engraftment was performed on 22 additional mice from early xenotransplantation experiments performed on cells harvested from mice M5, M6 and M7 (patient A67, secondary material). In 18 of these mice, the leukaemia could be detected in the mouse blood samples with flow cytometry and in all cases it closely resembled the original patient immunophenotype. However, of the remaining 4 mice with no apparent peripheral human lymphoblasts, 3 already showed bm engraftment of 1.7%, 6.2% and 15.7% prior to analysis of the corresponding blood samples. These findings indicated that reconstitution of human cells in the mouse would not be detected in at least some mice if only peripheral blood is analysed.

3.4.4.3 Engraftment analysis from mouse spleen single cell suspension

Enlargement of the spleen due to blast infiltration is a commonly found at clinical presentation of ALL in humans. The spleens of mice transplanted with human ALL cells were also found to be increasingly enlarged concordant with higher human chimerism in mouse bm. Following this observation from the first xenotransplantation experiments and the desire to yield as many as possible human ALL blasts from every individual

mouse, the mouse spleens also were regarded as a possible source for a high number of human cells that could be used in following experiments. Confirmation of blast infiltration into the spleens of transplanted mice additionally would show even closer resemblance of the original clinical disease pattern in the experiment animals.

In order to analyse the spleen for human cell content with flow cytometry, a single cell suspension had to be prepared first. Spleens were passed through a 40 µm cell strainer to disaggregate the tissue and part of the cells were resuspended in 0.2% PBSA for surface marker labelling with the antibody panel already used for mouse bmps, bm harvests and peripheral blood samples. Routinely, 100,000 events were recorded and analysed for expression of human surface antigens. The first mice that were assessed *post mortem* for infiltration of the spleens were mice transplanted with secondary material from patient A67. Spleens of 3 mice (M13, M14 and M17) from that experiment were available for analysis. All 3 mice showed positive human engraftment (M13: 4.0%, M14: 21.5% and M17: 37.7%) in cells from the bm harvest and consecutively were as well found to have lymphoblast infiltrated spleens. Figure 15 shows dot plots of CD19 versus CD10 expression of the bone marrow and spleen from mouse M13 as well as from the spleens of mice M14 and M17. In comparison, the immunophenotypes of human blasts from bm or spleen do not differ to a great extent in the expression profiles of surface antigens.

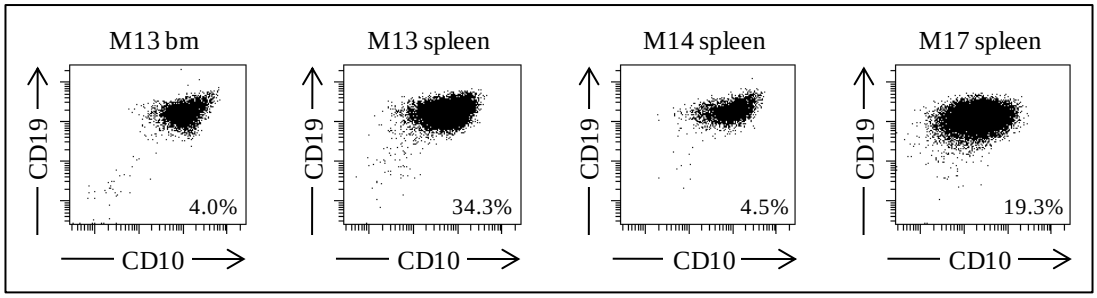


Figure 15: Infiltration of host mice spleens with human lymphoblasts. Enlarged spleens of euthanized mice were assessed for infiltration with cells of the transplanted ALL (secondary material of patient A67) in addition to analysis of harvested bm. Flow cytometry analysis data are shown as dot plots for bm and a single cell suspension of the spleen of mouse M13 and additionally of two further spleen single cell suspensions from mice M14 and M17. Cells of human origin can be identified in all 3 spleens depicted. Comparison of cells from spleens and bm from the 3 mice reveals similarities of immunophenotypes. This is depicted for mouse M13.

After these first analyses of spleens detected substantial fractions of human lymphoblasts within the spleen tissue, every individual mouse with positive bm engraftment at the time of bm harvest from all further experiments was assessed for splenic involvement. Single cell suspensions of all harvested spleens containing human ALL blasts were routinely frozen down and stored in liquid nitrogen for further use. With the large numbers of cells retrieved from a single spleen in addition to bm, a large supply of material could be made available for future experiments.

Furthermore, with bm and spleen chimerism determined by flow cytometry, the level of bm engraftment associated with infiltration of the spleen with lymphoblasts could also be assessed. In mice that had a human chimerism of higher than 10% in bm at the time of harvest, cells of human origin with an immunophenotype resembling that of the bm ALL cells could be detected in the spleen. Therefore, only mice with an engraftment level of less than 10% at the time of experiment termination were assessed for splenic involvement. Mice without overt leukaemia (less than 25% blasts in bm) usually are not clinically unwell and therefore the number of mice that were killed when they still had

relatively low levels of human engraftment was limited. However, of altogether 14 mice, transplanted with cells of 11 different ALLs, bm showed an engraftment of less than 10% at the time of experiment termination. Spleens taken at that time were investigated for the content of leukaemic cells.

Exp. No	Mouse ID	Patient	Cells transplanted	Cell No	Weeks to harvest	Engraftment [%]	
						bm	spleen
KR4	M13	A67	$CD34^{+}Cd19^{low}$	2×10^{3}	20	7.0	61.7
KR24	M126	2510	$CD19^{+}CD10^{low}$	6.9×10^{3}	30	1.2	0
KR35	M228	L858	Unsorted	1×10^{3}	36	1.9	0
KR39	M276	WB51	Unsorted	1×10^{2}	20	0.25	0
KR44	M334	737c	Unsorted	1×10^{2}	19	4.7	25.5
KR47	M377	M120	$CD19^{+}CD10^{high}$	2×10^{3}	58	9.1	8.6
KR49	M394	758b	Unsorted	1×10^{4}	48	0.4	0
KR52	M427	A67	Unsorted	1×10^{4}	15	1.8	3.2
KR53	M448	L876	unsorted	1×10^{2}	31	3.1	0.03
KR56	M512	737c	$CD19^{+}CD10^{low}$	1×10^{2}	46	7.7	43.4
KR59	M568	WB51	$CD19^{+}CD34^{high}$	1×10^{1}	19	0.47	0
	M572				22	1.5	1.3
KR61	M616	4540a	$CD19^{+}CD34^{high}$	1×10^{2}	29	8.9	15.7
KR62	M645	L784	$CD19^{+}CD34^{high}$	1×10^{3}	36	2.7	20.3

Table 3: Infiltration of the spleen with lymphoblasts takes part early in the xenotransplantation experiment. 14 mice transplanted from 11 different patients had bm engraftment of less than 10% at the time of experiment termination. 5 of these mice, all with bm engraftment of less than 2%, had no signs of ALL within the spleens. In contrast, another 2 mice (transplanted from A67 and WB51), with engraftment of less than 2% in bm, had 1.3% and 3.2% spleen involvement respectively. Migration and settling of lymphoblasts to the spleen seems to take place at around 1% to 2% bm engraftment.

The data shown in Table 3, together with the finding that all mice with a bm engraftment of higher than 10% already had spread of cells to the spleen without exception (see table with raw data of all experiments in the Appendix) show that dissemination of ALL takes place at a relatively early stage of the disease. Negative spleen involvement was seen in

only 5 mice and they all had bm engraftment of less than 2%. However, in 2 cases of only 1.5% and 1.8% human chimerism in bm respectively, the spleen already harboured human lymphoblasts. The threshold for human lymphoblasts to migrate to the spleen and colonize it appears to have reached when bm engraftment exceeds 1% to 2%.

3.4.5 Development of a dot plot based gating algorithm for more accurate quantification of human engraftment

The gating algorithm applied to bone marrow cells from punctures and harvests as described in section 3.4.3 was highly specific in detecting human engraftment but it soon became obvious from the initial xenotransplantation experiments of different patient samples, that blasts of human origin did bind mouse specific antibodies (mCD45 and mTER-119) to a greater extent than normal PBMCs from healthy volunteers. This possibility was not considered when the initial gating algorithm was developed. When the gating strategy was performed on xenotransplantation experiments with the ALL samples, however, the method led to the exclusion of a significant proportion of human lymphoblasts contributing to the engraftment level, finally resulting in an underestimation of the real human chimerism. Furthermore, the cells excluded by the initial gate P1 were lost for immunophenotype analysis in the subsequent steps. Figure 16 illustrates how cells of apparent human origin are excluded from further analysis by the boundaries of P1 and P2 respectively.

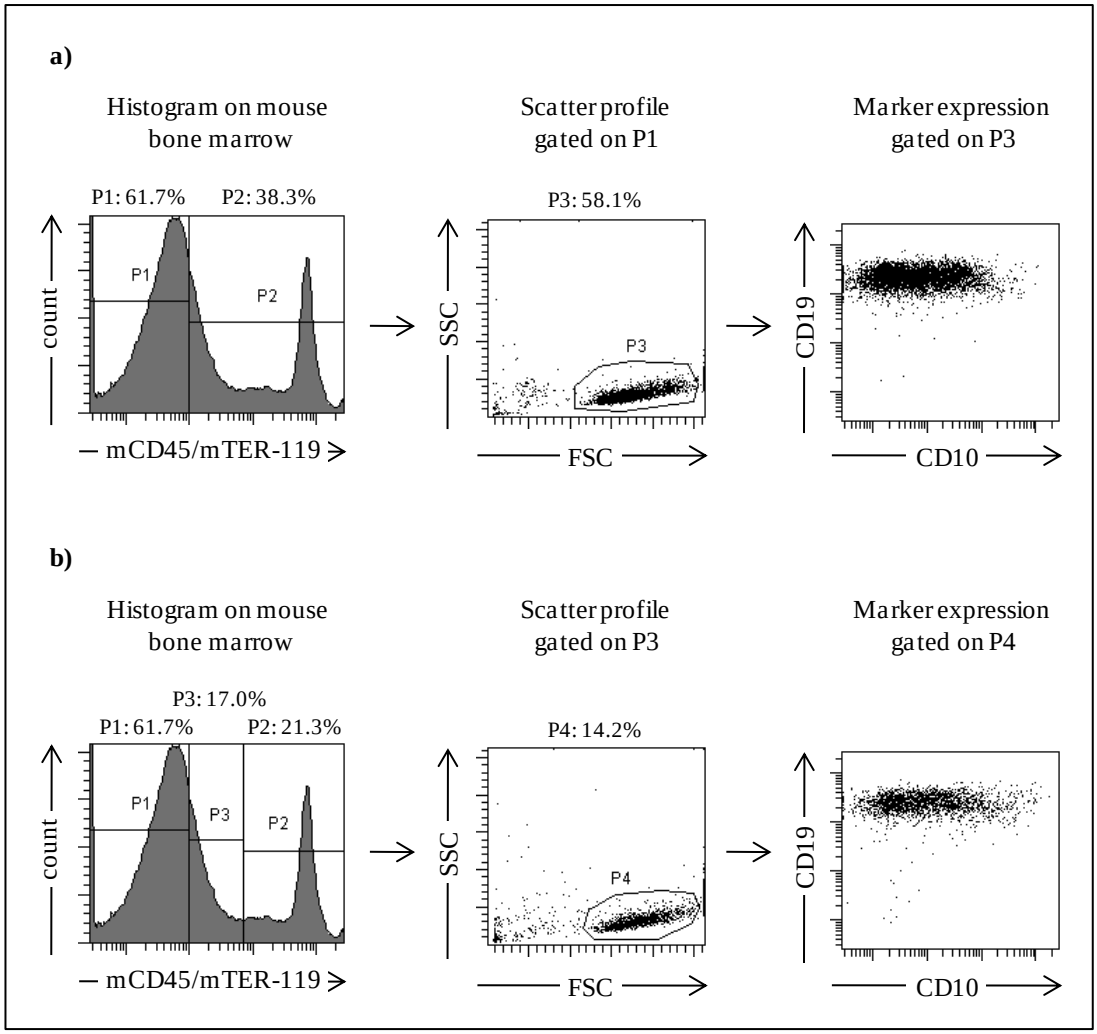


Figure 16: The histogram based gating algorithm excludes a significant proportion of human lymphoblasts from analysis (example on bm harvest, mouse M1, patient sample 8849). a) Gating is based on the algorithm described in subchapter 3.4.3. Gate P3, surrounding cells with a light scatter profile of human lymphoblasts on the dot plot in the middle image is a subgate of gate P1 (histogram on the left). Human ALL engraftment calculated with the cell number enclosed in P3 is 58.1%. The engrafted human cells are characterised by a high expression of CD19 and low to high expression of CD10 (dot plot on the right). **b)** A gate P3 is set in the histogram adjacent to the original gate P1, enclosing cells of assumed human origin. The lower boundary of original gate P2 is moved to a higher MFI for mCD45/mTER-119 in continuation of P3. 14.2% of the total acquired cell number fall into P4, a subgate dependent on P3 only and exhibit an immunophenotype pattern highly resembling the one of human cells analysed in a). Adding this percentage of 14.2% to the 58.1% gained in a) results in a significantly higher total human chimerism of 72.3%.

Eliminating significant numbers of human cells from analysis by the fixed position of gate P1 in a first gating step could also have the consequence that low numbers of

human cells in the mouse bm background would be missed completely and a mouse would be considered as not being engrafted. Especially later in the project, when limiting dilution experiments were introduced and proved to be very important for the conclusions of this work, a higher sensitivity in detecting human cells in the murine cell background became necessary.

In order to more accurately quantify human chimerism in the xenotransplantation assay, a possible gating strategy needed to fulfil two essential criteria. Most importantly it was necessary to unambiguously separate cells of mouse origin visually from human cells. The initial histogram based gating strategy was not designed to differentiate between mouse and human cells but rather to reliably exclude the vast majority of mouse cells. This was done by negative selection. Only cells that had no affinity to mouse directed antibodies (mCD45 and mTER-119) were included in further analysis. The method did not allow for unspecific binding of mouse antibodies to human cells as seen in human lymphoblasts but not normal human lymphocytes. The second essential criterion of including human cells as completely as possible in the following analysis steps of establishing a realistic engraftment level could therefore not be met. The problem was resolved by including a positive selection parameter for human cells in addition to the negative mouse specific selection markers into the process of differentiating between cells of human or mouse origin. The obvious choice from the panel of routinely labelled human surface antigens CD10, CD19, CD20 and CD34 was the human marker CD19. This surface marker is most consistently expressed to a high level on human lymphoblasts of B cell precursor ALL unlike the other markers CD10, CD20 and CD34 which can either be completely negative on some forms of ALL (CD10 on infant ALL) or more frequently present with a biphenotypic expression profile. On a dot plot with CD19 expression on the y-axis versus expression of the mCD45/mTER-119 combination on the x-axis populations of human and mouse origin were separated into

distinct cell clusters. The population of high CD19 expression was surrounded by a gate and visualised in a gate dependent light scatter dot plot. In the next step cells with a characteristic appearance of lymphoblast according to the light scatter profile were again gated and the cells enclosed in this gate were scrutinised for their expression pattern of human surface antigens. This gating hierarchy is shown in Figure 17 using the example of flow cytometry data for the bm harvest of mouse M1, patient sample 8849. For comparison, the same data were used as for the illustration how cells of human origin are lost for analysis using the strict histogram based gating algorithm (see Figure 16 and text above). Engraftment levels determined by the histogram method and the improved dot plot method are 58.1% and 73.6% respectively. This accounts for a considerable difference of 15.5% and demonstrates the superiority of the dot plot based gating algorithm.

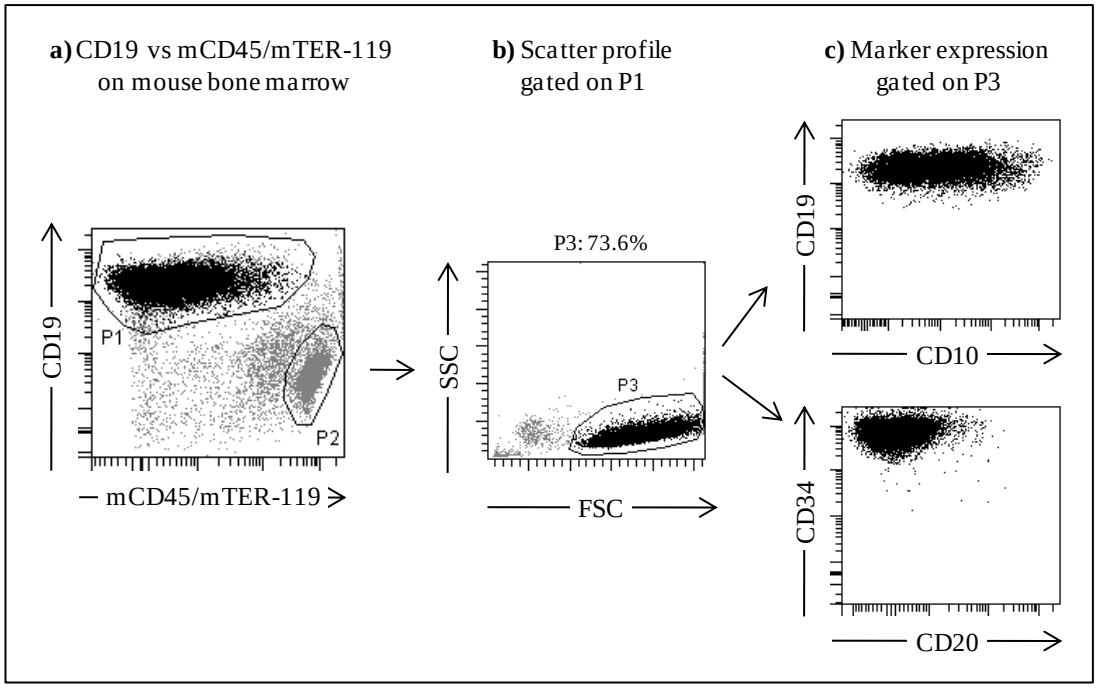


Figure 17: A dot plot based gating algorithm more accurately measures human cells in the NSG bm background (example on bm harvest, mouse M1, patient sample 8849). a) A gate P1 is drawn around the definable cell cluster of presumably human origin with relatively high expression of CD19. The enclosed population includes cells with increasing levels of bound mouse specific antibodies (mCD45/mTER-119). A second distinct population, representing mouse bm cells, is characterised by high expression of mCD45/mTER-119 and is encircled by gate P2 (this gate is drawn for illustration only and does not form a part of the gating hierarchy). b) Events contained in gate P1 are depicted as a light scatter dot plot and a gate P3 is set to surround cells with a typical light scatter profile of human lymphoblasts. Human ALL engraftment calculated with the cell number enclosed in P3 is 73.6%. This engraftment level is even higher than the one determined with the already extended gates of the histogram gating algorithm of the same flow cytometry analysis (see Figure 16 and text). c) The engrafted human cells are characterised by a high expression of CD19 and a low to high expression of CD10 (upper dot plot) as well as a generally high expression of CD34 and a low to high expression of CD20 (lower dot plot).

3.4.6 Definition of thresholds for minimal positive engraftment

The gating method proved to be able to filter out very low percentages of human engraftment in a NSG mouse bm background. Very low numbers of cells were found to form clusters of a specific human leukaemic immunophenotype in flow cytometry analysis and therefore were indeed assumed to be of human origin. This created the

requirement to set levels for the minimal number of clustered events from which a minimal percentage of human chimerism could be calculated that was counted as a reliable positive human engraftment of a mouse in this project.

The high sensitivity of flow cytometric analysis is well established and multi-colour flow cytometry has already been tested by several studies for use in monitoring MRD in ALL to complement or even substitute PCR based methods. These analyses need to reliably detect a minimal number of positive events. Additionally, these measurements cannot use antibodies to gate out most cells of host origin in a xenotransplant setting, but rather have to identify lymphoblasts with a specific leukaemia-associated immunophenotype (LAIP) in a syngenic human bm background. Nevertheless, very low levels of leukaemic cells were shown to be measurable in syngenic human bm by identifying blasts with a LAIP. The UK flow MRD group established a highly sensitive 4 colour flow MRD method that reliably detected malignant cells in a healthy bm background down to a level of 0.01% and lower when a minimum of 50 events forming a cell cluster for one specific LAIP were recorded. Furthermore, the data closely correlated to the gold standard of molecular MRD measurement (Irving et al., 2009). A Swedish multi-centre study also comparing PCR and flow cytometry based MRD measurement even considered a sample MRD positive when a minimum of only 10 or more LAIP clustered cells could be detected by multicolour flow cytometry. They also found a high concordance with the PCR based method (Thörn et al., 2011). The same threshold number of 10 clustered events, associated with a light scatter profile typical of lymphoid cells, was applied by another multi-centre study, in context of the AIOP-BFM-ALL 2000 trial (Dworzak et al., 2008; Ratei et al., 2009). Taking the criteria from these studies as guidance, thresholds were set to consider a mouse as being positively engrafted with human cells in this project. In order to be counted as engraftment, at least 20 clustered events that showed a lymphoid light scatter profile had to be identified and

represent 0.01% or more of all recorded events including those from obvious cell debris. However, in most cases, cell material of consecutive bmps or bm harvests was available and all of those showed eventually increasing human chimerism, indicating that, applying the defined criteria, the risk of getting false positive results was very low. If one of the two criteria was not met, the mouse was regarded as not being engrafted with human cells. In some cases a theoretical engraftment level of less than 0.01% was determined or only less than 20 clustered events of a leukaemic immunophenotype could be recorded. Results of these bmps were counted as negative but labelled as positive outside limits (pol).

3.4.7 Comparison of engraftment levels between the two gating algorithms

The levels of unspecific binding of the anti mouse specific antibodies mCD45 and mTER-119 to engrafted human lymphoblasts were found to be different between transplanted patient samples and also between mice transplanted with the same material. This would not result in a systematic error with equal underestimation of bm engraftment among different mice but rather to different percentages of human cells included for further analyses between individual mice. For example, mouse M1 transplanted with patient sample 8894, used to demonstrate the difference of human engraftment levels determined with the two gating methods in section 3.4.5, did not bind the mCD45/TER-119 antibody combination to a particularly high extent. Other samples were found to exhibit much higher mean levels of unspecific mouse antibody binding according to mCD45/mTER-119 expression histograms. This would result in a much more pronounced underestimation of engraftment. In order to evaluate the range of this underestimation, the two gating algorithms were compared on bmps and bm harvests for an extended set of samples from different experiments. Human engraftment percentages were measured on 29 individual mice transplanted with 7 different ALL samples.

Altogether 101 bmps and 27 final bm harvests were analysed according to both methods and difference in the determined engraftment level was calculated. In general, the dot plot based method resulted in higher human chimerism than the histogram based method in both bmps and final bm harvests. At the time of bmp1, 14 out of 29 mice showed the beginning of bm engraftment. The mean engraftment was 3.45% higher when determined with the dot plot based method than with the histogram based method. Differences for individual samples ranged from 0.02% to 13%. At bmp2, 23 engrafted out of 28 alive and punctured mice had a mean difference of engraftment of 3.58% (range 0.01% - 26%), at bmp3 17 engrafted out of 21 alive and punctured mice had a mean difference of engraftment of 5.1% (range 0.01% - 26.1%), at bmp4 11 engrafted out of 14 alive and punctured mice had a mean difference of engraftment of 2.94% (range 0.03% - 12.2%) and at the last bmp5, 7 engrafted out of 9 alive and punctured mice had a mean difference of engraftment of 2.76% (range 0.04% - 8.4%). Bm of 27 mice was harvested and of those, 25 had engrafted the ALL. The mean increase of engraftment levels determined with the dot plot gating algorithm as compared to the histogram gating algorithm was 16.26% and differences ranged from 0.02% to a maximum of 66.7%. Furthermore, low levels of engraftment were found in 8 bmps with the dot plot method but could not be identified with the histogram method highlighting the higher sensitivity of the dot plot method. The results of the comparison between the two gating strategies are summarised in Table 4.

Table 4 (pages 142 and 143): Comparison of human engraftment levels in NSG mice determined by the histogram or dot plot gating method. The table contains the bm engraftment levels of all bmps and the final bm harvest from 7 different xenotransplantation experiments. Engraftmen levels are given for the histogram gating method and the dot plot gating method and the difference of measured human engraftment between the two methods is calculated. * indicates results that are positive outside limits (pol).

Exp. No.	Mouse ID	Patient / Leukaemia	Injected Cells	Injected Cell No.	bmp1				bmp2				bmp3			
					Weeks post bmt	Engraftment [%]			Weeks post bmt	Engraftment [%]			Weeks post bmt	Engraftment [%]		
						Histogram	Dot plot	Difference		Histogram	Dot plot	Difference		Histogram	Dot plot	Difference
KR2	M5	A67 prim	unsorted	3.0×10^5	9	0.00%	0.008%*	0.008%*	12	0.97%	1.40%	0.43%	15	4.20%	5.20%	1.00%
	M6					0.00%	0.00%	0.00%		0.02%	0.06%	0.04%		0.02%	0.04%	0.02%
	M7					1.50%	2.10%	0.60%		2.90%	3.70%	0.80%				
	M8					0.30%	0.51%	0.21%		0.05%	0.06%	0.02%		4.60%	8.60%	4.00%
KR7	M29	2510 prim	unsorted	1.3×10^6	15	10.80%	14.00%	3.20%	21	14.70%	16.80%	2.10%	25	20.50%	25.30%	4.80%
	M30					1.60%	3.40%	1.80%		9.20%	15.30%	6.10%		47.70%	55.80%	8.10%
	M31					1.10%	1.30%	0.20%		11.50%	12.40%	0.90%		20.40%	24.10%	3.70%
KR16	M64	WB51 prim	unsorted	9.8×10^4	7	7.80%	11.40%	3.60%	11							
	M65					8.30%	19.40%	11.10%		1.90%	8.40%	6.50%				
	M66					32.60%	43.30%	10.70%		5.10%	12.80%	7.70%				
	M67					14.80%	27.80%	13.00%		3.80%	7.70%	3.90%				
KR44	M333	737c prim	unsorted	1.0×10^2	12	0.07%	0.18%	0.11%	15	1.00%	3.90%	2.90%	19			
	M334					0.70%	3.30%	2.60%		29.40%	55.40%	26.00%				
	M335					0.00%	0.016%*	0.016%*		0.03%	0.21%	0.18%		5.70%	11.90%	6.20%
	M336					0.00%	0.00%	0.00%		0.00%	0.00%	0.00%		0.00%	0.00%	0.00%
	M337					0.20%	1.30%	1.10%		15.40%	33.40%	18.00%				
KR46	M357	M120 prim	unsorted	1.0×10^4	9	0.00%	0.00%	0.00%	15	0.00%	0.004%*	0.004%*	19	0.07%	0.15%	0.08%
	M358					0.00%	0.00%	0.00%		0.00%	0.03%	0.03%		0.09%	0.60%	0.51%
	M359					0.00%	0.00%	0.00%		0.05%	0.10%	0.05%		0.00%	0% d	0.00%
	M360					0.00%	0.00%	0.00%		0.00%	0.07%	0.07%		0.00%	0.24%	0.24%
KR53	M443	L876 prim	unsorted	1.0×10^3	9	0.00%	0.02%	0.02%	13	0.00%	0.03%	0.03%	20	0.02%	0.09%	0.07%
	M444					0.00%	0.00%	0.00%		0.02%	0.13%	0.11%		54.90%	74.40%	19.50%
	M445					0.00%	0.00%	0.00%		0.00%	0.03%	0.03%		0.00%	0.63%	0.63%
	M446					0.00%	0.00%	0.00%		0.17%	0.35%	0.18%		29.80%	36.30%	6.50%
KR54	M466	HV101 sec (of M187)	unsorted	1.0×10^1	9	0.00%	0.003%*	0.003%*	13	0.00%	0.00%	0.00%	19	0.17%	0.32%	0.15%
	M467					0.00%	0.03%	0.03%		2.40%	8.60%	6.20%		18.40%	23.50%	5.10%
	M468					0.00%	0.00%	0.00%		0.00%	0.00%	0.00%		0.00%	0.00%	0.00%
	M469					0.00%	0.00%	0.00%		0.00%	0.00%	0.00%		0.00%	0.00%	0.00%
	M470					0.00%	0.00%	0.00%		0.00%	0.01%	0.01%		21.00%	47.10%	26.10%

Exp. No.	Mouse ID	Patient / Leukaemia	bmp4				bmp5				Final bm harvest			
			Weeks post bmt	Engraftment [%]			Weeks post bmt	Engraftment [%]			Weeks post bmt	Engraftment [%]		
				Histogram	Dot plot	Difference		Histogram	Dot plot	Difference		Histogram	Dot plot	Difference
KR2	M5	A67 prim	16	3.50%	4.20%	0.70%	19				16	30.50%	40.60%	10.10%
	M6			0.21%	0.32%	0.11%					18	6.60%	14.80%	8.20%
	M7										14	39.00%	74.80%	35.80%
	M8			2.30%	3.20%	0.90%		13.50%	22.50%	9.00%	19	75.30%	75.80%	0.50%
KR7	M29	2510 prim									25	29.60%	30.90%	1.30%
	M30										25	48.30%	56.50%	8.20%
	M31										25	28.40%	30.60%	2.20%
KR16	M64	WB51 prim									11	29.00%	58.60%	29.60%
	M65										16	14.20%	80.90%	66.70%
	M66										14	7.80%	51.70%	43.90%
	M67										14	26.40%	55.50%	29.10%
KR44	M333	737c prim	24				31				0			
	M334										19	3.10%	4.70%	1.60%
	M335			1.10%	1.70%	0.60%		0.31%	0.32%	0.01%	33	55.30%	65.10%	9.80%
	M336			0.00%	0.00%	0.00%		0.00%	0.00%	0.00%	47	0.03%	0.10%	0.07%
	M337										19	37.70%	69.50%	31.80%
KR46	M357	M120 prim	26	0.90%	4.90%	4.00%	40	32.10%	43.10%	11.00%	43	24.20%	43.60%	19.40%
	M358			1.40%	2.00%	0.60%		7.20%	8.50%	1.30%	43	18.00%	32.50%	14.50%
	M359			4.10%	8.90%	4.80%		17.10%	18.60%	1.50%	40	17.40%	33.10%	15.70%
	M360			0.27%	0.30%	0.03%					27			
KR53	M443	L876 prim	27				39				25	0.04%	0.06%	0.02%
	M444										22	1.80%	3.40%	1.60%
	M445			0.00%	0.00%	0.00%					35	0.00%	0.00%	0.00%
	M446										25	20.10%	26.40%	6.30%
KR54	M466	HV101 sec (of M187)	27	19.60%	28.00%	8.40%	39	28.70%	29.70%	1.00%	40	17.40%	25.40%	8.00%
	M467										25	26.60%	51.20%	24.60%
	M468			0.00%	0.00%	0.00%		0.01%	0.01%	0.00%	40	0.00%	0.002%*	0.002%*
	M469			0.16%	0.20%	0.04%		3.60%	8.00%	4.40%	40	22.90%	51.50%	28.60%
	M470			60.00%	72.20%	12.20%					28	23.70%	32.50%	8.80%

3.4.8 FISH analysis and morphology of engrafted ALL samples

3.4.8.1 Fluorescence *in situ* hybridization (FISH)

The first transplanted patient samples successfully engrafted mice and human cells recovered from NSG mice proved to have an immunophenotype closely resembling that of the original ALL sample. In order to provide additional validation of the concordance of diagnostic ALL lymphoblasts and human cells engrafted in the murine xenotransplant setting, cytogenetic characteristics were analysed on a selection of mouse bm samples. FISH analysis for the presence of the BCR/ABL1 fusion gene (t(9;22)) was an obvious choice as transplantation experiments with Philadelphia chromosome positive ALL formed a substantial part of this project. Altogether 22 diagnostic and engrafted bm samples from 9 different Philadelphia chromosome positive patients were analysed. Additionally, single cell suspensions of the spleen and kidney were analysed in one case (patient sample M120, mouse M376). Hybridisation with the BCR/ABL1 fusion fluorescent FISH probe (Spectrum Green/Spectrum Orange) was performed as described in section 2.4 of the materials and methods chapter.

Mouse M1 was the first mouse engrafted with a Philadelphia chromosome positive ALL from patient 8849. Mice were transplanted with relatively high numbers (1×10^6 cells/mouse) of unsorted patient material. Cells from the final bm harvest were found to be positive for the t(9;22) reciprocal translocation (see Figure 18 a).

High cell numbers (3×10^5 cells/mouse) of blasts from patient A67 were also transplanted without prior cell sorting. Harvested bm of mouse M6 contained human lymphoblasts that presented with 3 Philadelphia chromosomes (Figure 18 b). Engrafted bm from the first transplant experiments were consecutively used for secondary transplants. Harvested bm of mouse M6 was sorted according to expression of surface antigen CD20 and M43, injected with 2×10^4 $CD19^+CD20^{high}$ cells, as well as M45,

injected with 2 x 10^4 $CD19^+CD20^{low}$ cells, showed 3 Philadelphia chromosomes in human cells of the bm from the final bm harvest. Mice M14 and M17 were transplanted with 2 x 10^3 bm cells from mouse M7 (primary unsorted transplant experiment) sorted into $CD34^+CD19^{low}$ and $CD34^+CD19^{high}$ fractions respectively. Bm blasts from both mice also proved to have 3 Philadelphia chromosomes (Figure 18 b).

Diagnostic patient sample WB51 was transplanted unsorted into M64 (9.8 x 10^4 cells) and after two passages through mice, again after sorting into $CD19^+CD34^{high}$ (M551, injected with 1 x 10^3 cells) and $CD19^+CD34^{low}$ (M553, injected with 1 x 10^3 cells). As already seen in transplanted mice from ALL A67, all engrafted material that underwent FISH analysis presented blasts with 3 BCR/ABL1 fusions.

A primary sort of patient sample HV101 into a $CD34^+CD20^{high}$ population (transplanted with 3 x 10^3 cells into M183) and a $CD34^+CD20^{low}$ population (transplanted with 3 x 10^3 cells into M187) resulted in engraftment of both fractions. FISH analysis of cells from the bm harvests of both engrafted mice showed two Philadelphia chromosomes, as did blasts from the primary patient sample.

Patient 2003 primary material and cells from the bm harvest of M525, transplanted with 1 x 10^3 unsorted primary ALL cells, were both found to have two Philadelphia chromosomes. ALL sample 4917 was also transplanted without sorting onto mice with a cell dose of 1 x 10^3/mouse and analysis of cells from M539 confirmed the presence of two BCR/ABL1 translocated chromosomes as found in the transplanted original sample.

ALL patient sample 4540a was directly sorted into fractions of $CD19^+CD34^{high}$ and $CD19^+CD34^{low}$ expressing cells. M609 and M611 represent one mouse each of two groups of 5, which were transplanted with 1 x 10^3 cells per mouse of the opposing cell populations. In engrafted bm cells of both mice ALL blasts with two copies of the Philadelphia chromosome were identified.

M122 (patient 2510) was transplanted with 4.5 x 10^3 $CD19^+CD10^{high}$ sorted cells of secondary material from M31, which engrafted the unsorted original patient ALL. This bm was repopulated with lymphoblasts that carried 3 Philadelphia chromosomes.

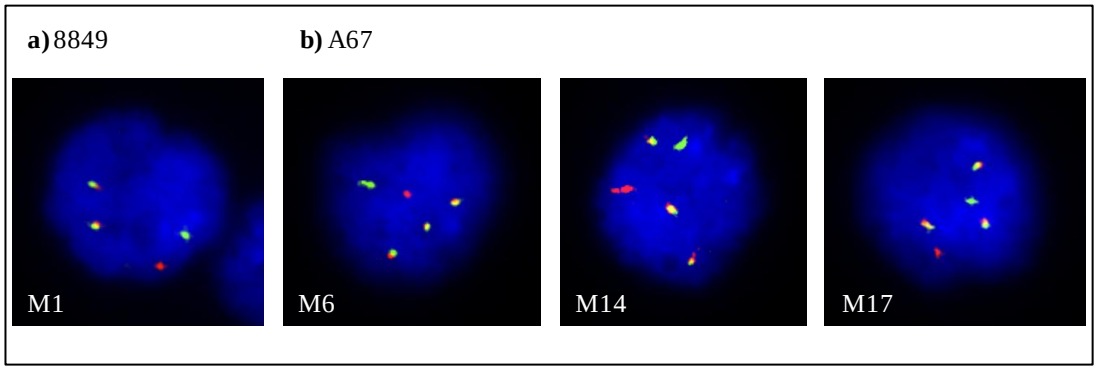


Figure 18: Presence of Philadelphia chromosomes in engrafted mouse bm cells. a) Mouse M1 was transplanted with 1 x 10^6 unsorted cells of patient sample 8849. Cells from the bm harvest were analysed regarding the presence of the t(9;22) reciprocal translocation. The BCR gene at chromosome 22q11.2 is visualised with a green fluorophore labelled DNA probe and the ABL1 gene on chromosome 9q34 with a red fluorophore labelled DNA probe. The one green and one red signal represent the intact chromosomes 9 and 22 respectively while the yellow fusion signal becomes visible upon t(9;22) translocation which co-locates the green and red signal. **b)** Cells from M6, transplanted with unsorted cells of patient A67 show two non translocated chromosomes 9 (green) and 22 (red) and 3 Philadelphia chromosomes (yellow fusion signal). A secondary transplantation was carried out with $CD34^+CD19^{low}$ and $CD34^+CD19^{high}$ fractions on representative mice M14 and M17 respectively. As cells in the primary transplantation (M6), both cell fractions reconstitute ALL cells harbouring one normal chromosome 9, one normal chromosome 22 and 3 Philadelphia chromosomes (green, red and 3 yellow fusion signals).

An unsorted limiting dilution experiment was done with diagnostic material from patient M120. M357 was transplanted with the highest cell concentration of 1 x 10^4 cells and engrafted with human cells in which two Philadelphia chromosomes were detected by FISH analysis. The primary sample of this patient was also used for a primary sort regarding CD10 expression levels. M376 was transplanted with 2 x 10^3 $CD19^+CD10^{high}$ cells and in addition to the expectedly enlarged spleen at the time of harvest, it also

presented with obviously ALL infiltrated enlarged kidneys. FISH analysis of single cell suspensions of the spleen and kidney showed two Philadelphia chromosomes as well.

3.4.8.2 Lymphoblast morphology

Additional slides of the same diagnostic patient samples and cells recovered from bm harvests of engrafted mice that were used for FISH analysis (section 3.4.8.1 above) were stained according to the May-Grünwald-Giemsa (MGG) protocol to analyse lymphoblast morphology. The French-American-British (FAB) Cooperative Working group classified ALL morphology in 3 groups L1-L3. L1 morphology is characterised by rather small and monomorphic cells with a high nuclear to cytoplasmic ratio. The thin rim of cytoplasm around a dominant round or clefted dark blue nucleus stains pale blue. Around 90% of childhood ALL present with L1 morphology. Cells of a L2 type represent 5 to 15% of ALL cases and have a more variable size with more cytoplasm and prominent nucleoli. L3 morphology is exclusively found in mature B ALL (around 1% to 2% of cases) and exhibits large cytoplasmic vacuoles in a dark cytoplasm (Silverman, 2009). Lymphoblasts of primary diagnostic patient material (patients WB51, HV101, 2003 and 4917) as well as human cells of the bm harvests of engrafted primary, secondary and tertiary transplantation experiments (mice M1, M6, M14, M17, M43, M45, M64, M122, M183, M187, M357, M437, M525, M539, M551, M553, M609 and M611) all showed typical undifferentiated FAB L1 morphology.

3.5 Discussion

Work described in this chapter was mainly intended to establish a robust murine xenotransplantation assay that would be able to address the stem cell properties of self-renewal and capability to generate the heterogeneous lineages of cancer cells in ALL. The transplantation technique needed to be learned and carried out on a small initial number of mice and a flow cytometry based method had to be developed to reliably detect human chimerism in bm from mouse bmps or bm harvests.

In order to set up flow cytometry, bm cells of mouse origin were available, but original ALL patient samples could not be used for that as they were reserved for the actual xenotransplantation experiments. Human lymphoblasts, that had been passaged through mice and harvested in abundance, for example from mouse spleens, understandably were also not present at the initial stages of the project. The presumably best substitute for ALL blasts, normal human bm cells, were also not accessible due to ethical constraints. PBMCs were therefore utilized as an alternative. In contrast to mouse bm cells that showed considerable unspecific binding of anti-human mAbs, PBMCs did not seem to take up anti-mouse mAbs to a great extent. The problem with unspecific binding of anti-human mAbs to mouse bm cells could be solved by staining the mouse cells with two antibodies that labelled the vast majority of mouse leucocytes (mCD45) and cells of the mouse erythroid lineage (mTER-119). Cells of mouse origin then could simply be gated out, eliminating those that were bound by antibodies against human surface antigens from further analysis. This first gating algorithm (see section 3.4.3) was based on a histogram that showed MFI of PE-Cy7, the fluorochrome that was conjugated to both mCD45 and mTER-119 and all events with an MFI of 10^3 or higher were excluded from further analysis. This greatly simplified the dependent dot plots and meant that human lymphoblasts could subsequently be identified much more easily. This method proved to be very specific for visualising human cells and also already very sensitive as

engraftment below 1% could clearly be identified. However, it became obvious from bmps and bm harvest of the first xenotransplantation experiments that human ALL blasts apparently exhibited a much higher and more variable degree of unspecific binding of anti-mouse mAbs than did normal PBMCs. Consequently, more cells of human origin were gated out by applying the histogram gating method resulting in either not detecting human engraftment of a very low level at all or in determining erroneous and generally too low percentages of human chimerism. Additionally, the variable degree of unspecific mouse mAb binding led to the exclusion of very changeable proportions of human cells for further analyses when the rather inflexible histogram gating algorithm was applied. This analysis bias, inherent in the histogram based method, urged the need to develop a new gating strategy that would include cells of human origin as completely as possible in further analysis steps. The resultant standardised dot plot based gating algorithm described in section 3.4.5, was able to include human cells to a much higher extent for determining engraftment levels. A retrospective comparison between the histogram and the dot plot based gating strategies on a test panel of various xenotransplantation experiments showed a considerably higher human chimerism with differences ranging from 0.02% to 66.7% with a mean increase of 16.26%. Capturing more cells of human origin also resulted in a higher sensitivity of the dot plot gating algorithm making it the method of choice for this project.

The first two xenotransplantation experiments were kept as simple as possible with high numbers of unsorted human ALL cells injected intrafemorally. This resulted in successful engraftment of both leukaemias but of the first transplanted sample (8849), only one out of 3 mice that could be analysed reconstituted the leukaemia despite 1 x 10^6 cells used for transplantation. Of the second transplanted sample (A67, 3 x 10^5cells/mouse) all mice did engraft but engraftment kinetics were inconsistent. With high cell numbers transplanted, it could be expected that engraftment kinetics would be

similar but at least, that if engraftment occurs it would do in all mice. However, as these experiments were the first that were performed, preparation of the samples and the mice as well as the actual transplantation process took considerably longer than later, when it became a routine procedure. It is also possible that some of the if injections partly or completely failed at a time when the procedure still had to be established. On the other hand, stem cell frequency could indeed have been very low in general but higher in the second sample (A67) with the consequence that individual mice received a different and very small number of leukaemic stem cells or none at all leading to failure to engraft.

Bone marrow punctures of mice were effective in monitoring human engraftment in mice without having to kill them. Analysing cells obtained from bmps could be assessed for engraftment and mice subsequently could be left alive in order to develop an overt ALL with high levels of engraftment and a final bm harvest of a high number of human cells for further experiments. However, successive bmps sometimes resulted in lower percentages of human blasts at a later time point as compared to the previous bone marrow sample. Reference to clinical literature provides a possible explanation for these decreased blast counts. It is known from consecutive human sternal bone marrow punctures that due to compartmentalization of the sternum, haemorrhage resulting from a previous puncture can occupy the relatively small marrow space and affect the results of subsequent aspirates from the same site (Bierman and Kelly, 1956). The mouse femur contains a very small volume from which the bone marrow samples are taken. Even with the most cautious puncture, a relatively high negative pressure has to be applied to release cells from this compartment. This inevitably leads to variable volumes of the aspirates and the resulting cavity will consecutively be filled with blood. As a consequence following samples may well contain less human blasts. It is not possible to circumvent this problem as in mice the femur is already the bone with the highest volume that is easily accessible. Additionally, blasts are vulnerable cells and the force

which has to be applied to aspirate them potentially leads to damage of a variable amount of cells, especially as they are drawn through a needle with a small diameter. Variable amounts of cell debris in the analysis additionally lead to a bias in determination of engraftment levels.

The problems for analysis inherent in bmps would probably be less pronounced in analysis of peripheral blood lymphoblasts and also the number of venesections is not restricted unlike bmps, which can only be performed up to 5 times on one mouse. However, although lymphoblasts could be detected in peripheral blood of most mice that were already bone marrow engrafted, this was not the case for all mice and positive engraftment could potentially been missed if only peripheral blood was analysed. Serial analysis of the bm therefore was the preferred mode to assess human engraftment levels. Additionally, ALL primarily is a malignant disease of bm origin and orthotopic xenotransplantation as well would lead to initial proliferation of blasts in the initially injected femur making it the most likely site to detect early and low chimerism. Data obtained from mice transplanted with different ALLs by Kerrie Wilson, a fellow PhD student, additionally demonstrated a much higher frequency of mice in which bone marrow engraftment not necessarily went along with detection of human chimerism in peripheral blood samples taken from the animals. These findings confirmed that flow cytometry performed on bm aspirates would be more sensitive in identifying human ALL engraftment.

From early *post mortem* examinations, enlargement of the spleen and sometimes the liver and kidneys was observed. In order to assess whether this enlargement was caused by infiltration of human lymphoblasts, single cell suspensions of these organs were analysed by flow cytometry. All organs proved to be colonised with human cells.

Spleens of bm engrafted mice were subsequently analysed regularly and served as a rich source of cells for further experiments. Furthermore, these infiltration patterns demonstrated that the transplanted ALL samples repopulated mice with a leukaemia that highly reproduced the clinical pattern typically found in humans.

All engrafted human material harvested from mice was analysed regarding the expression of the human surface antigens CD10, CD19, CD20 and CD34. The early transplantation experiments showed that the leukaemia reconstituted in the mouse xenoenvironment closely resembled the immunophenotypic pattern determined for the original patient sample. However, only unsorted samples were transplanted with high cell numbers and it could have been possible that cells with a different immunophenotype were reconstituted by different cells within the transplanted sample only.

Additional evidence that human cells engrafted in mice were definitely malignant lymphoblasts and not of normal human haematopoietic origin was provided by morphological and FISH analyses of examples of bm samples taken from mice transplanted with BCR/ABL1 positive patient samples. Morphologic examination showed an abundance of typical leukaemic blasts in the bm and FISH analysis visualised the Philadelphia chromosome in all examined samples. Human cells with two non-translocated chromosomes 9 and 22 each could not be identified, clearly demonstrating that engrafted human cells were exclusively lymphoblasts.

3.6 Conclusion

Initial ALL xenotransplant experiments on NSG mice were performed successfully with reconstitution of the full leukaemia in bm and a infiltration of blasts into spleens and other organs of the recipients and flow cytometry analysis proved to be a robust method to answer the questions of engraftment and pattern of surface antigen expression.

With a robust murine xenotransplantation assay and a method that reliably identified cells of human origin in a mouse background with a high sensitivity and specificity powerful tools were established to address questions surrounding the frequency and identity of leukaemic stem cells in ALL.

CHAPTER 4: DIVERSITY OF CANCER STEM CELLS IN ACUTE LYMPHOBLASTIC LEUKAEMIA

Chapter 4: Diversity of cancer stem cells in acute lymphoblastic leukaemia

4.1 Introduction

Leukaemias account for the most common form of cancer in children and approximately one third are of the acute lymphoblastic form. With multiagent chemotherapy given according to sophisticated treatment protocol schedules cure rates of now 80% 5 year EFS are achieved (Möricke et al., 2008; Pui et al., 2004). However, due to the high incidence, ALL still causes 30% of all deaths from cancer (CancerResearchUK, 2010). The most common reason for treatment failure is relapse of the disease originating from malignant blasts that evade chemotherapy. In order to reconstitute the full ALL, a remaining blast cell must combine the two defining characteristics of cancer stem cells; the ability to self-renew and to give rise to all different cells comprising the tumour. Current treatment strategies are generally more effective at killing proliferating cells. Stem cells, however, only infrequently undergo cell division making them a poor target for cell cycle dependent cytotoxic agents. Improvements in ALL survival have been achieved by risk group stratification with adaption of chemotherapy and better supportive care than by finding ways to specifically eradicate the cells that essentially are responsible for reviving the ALL. In order to develop a targeted treatment option against cancer stem cells, they first have to be identified and their underlying biology elucidated.

In AML, rare leukaemic cells with stem cell abilities and their descendants, forming the major part of the malignant clone but not being able to reconstitute the leukaemia, could be identified in immunodeficient mouse xenotransplantation models. The leukaemic

stem cells were characterised by an immature immunophenotype ($CD34^{+}CD38^{-}$), resembling that of normal early haematopoietic cells and were found to be a very rare entity at the apex of a hierarchically organised tumour (Bonnet and Dick, 1997; Lapidot et al., 1994).

The hierarchical model for AML is widely accepted and unchallenged, however, for ALL, conflicting results regarding frequency and identity of LSCs have been published in the past and the debate whether the hierarchical or the stochastic model applies to ALL is still ongoing. In an experiment setup, nearly identical to that used by John Dick´s group for work done on AML, an early study on high risk Philadelphia chromosome positive ALL suggested that the hierarchical model would hold true for ALL as well, with stem cell properties found only in 0.2 to 41 in one million leukaemic cells. In an attempt to characterise these stem cells, the authors reported that only cells of a $CD34^{++}CD38^{-}$ but not of the $CD34^{+}CD38^{+}$ immunophenotype were able to transfer the leukaemia to intravenously transplanted irradiated NOD/SCID mice, as already established for AML. These LICs therefore resembled early, not yet lineage committed normal haematopoietic cells and the hierarchical model was considered to be applicable on ALL as well (Cobaleda et al., 2000).

Philadelphia chromosome positive ALL belongs to the high risk group of ALLs and hence it was necessary to investigate whether or not other types of ALL would lead to similar conclusions. In a study reported by Cox et al which included standard risk ALLs with the ETV6/RUNX1 translocation, hyperdiploidy or no abnormal cytogenetics, primary limiting dilution experiments on unsorted cells confirmed a low frequency of stem cells within the bulk leukaemic population with 5×10^{5} to 1×10^{7} cells being necessary to transfer the disease onto irradiated NOD/SCID mice after intravenous injection. In order to differentiate between immature and more mature blast fractions they sorted according to the expression of the surface markers CD10 and CD19 which

are upregulated earliest in the pre-B-I cell stage of normal haematopoietic development. Upon transplantation, they found that only the immature subfractions ($CD34^+CD10^-$ and $CD34^+CD19^-$) were able to initiate ALL in the murine xenotransplant setting and that with down to 7 x 10^4 cells per mouse far fewer cells were necessary to achieve this compared to unsorted lymphoblasts of the same samples (Cox et al., 2004). The hierarchical model was confirmed for standard risk ALL with this study and ALL was seen as a disease which is initiated by transformation of early, not lineage restricted HSCs that retain their stem cell properties to establish and maintain the leukaemia.

The data presented by Cobaleda and Cox were in agreement but soon after their publication, work by Castor and colleagues was published which could not confirm that LSCs in human ALL were confined to immature populations. Initially they found that blasts of 6 ETV6/RUNX1 positive standard risk ALL were indeed detectable in the immunophenotypically immature subfraction of $CD34^+CD38^-$ cells but they went on and additionally characterised this subfraction towards its expression of the lineage restricting surface marker CD19. What they found was an aberrant but due to expression of CD19 apparently more mature population of $CD34^+CD38^-CD19^+$ lymphoblasts that does not have an immunophenotypic correlate in normal haematopoiesis but as well contained the ETV6/RUNX1 fusion. Furthermore, the $CD34^+CD38^-CD19^-$ immature cell populations of the patients did not harbour the t(12;21) translocation. Albeit on grounds of an aberrant immunophenotype, this finding suggested, that the leukaemia originated in an already committed B-cell developmental stage. Further substantiation for this came from the fact, that in addition to the $CD34^+CD38^-CD19^-$ compartment, myeloid restricted $CD34^+CD33^+CD19^-$ progenitor cells as well were devoid of the ETV6/RUNX1 fusion. In xenotransplantation experiments with tail vein injection into irradiated NOD/SCID and NOD/Scid $B2m^{-/-}$ recipients the aberrant population then proofed its capability to reconstitute the leukaemia. In additional experiments, the

aberrant $CD34^+CD38^-CD19^+$ compartment could also be detected in p190 and p210 BCR/ABL1 positive ALLs, but while the immature $CD34^+CD38^-CD19^-$ compartment was not included in the malignant clone for minor breakpoint Ph^+ cases, identical to the situation in ETV6/RUNX1 positive ALL, in major breakpoint Ph^+ ALL the t(9;22) translocation could also be detected in $CD34^+CD38^-CD19^-$ as well as myeloid committed $CD34^+CD33^+CD19^-$ cells. Most interestingly, for both subtypes of Ph^+ ALL it was the more mature $CD34^+CD38^-CD19^+$ fraction only, that could transfer the leukaemia *in vivo* (Castor et al., 2005). Despite not addressing the question of stem cell frequency, these findings could not be brought into accordance with the proposition that LICs in ALL are restricted to an immature and not lineage restricted stage in a developmental hierarchy.

In another study it was demonstrated that transduction of ETV6/RUNX1 into human cord blood cells resulted in the development of the aberrant $CD34^+CD38^-CD19^+$ clone, but it was also shown, that while this population did cause leukaemia in intratibially transplanted NOD/SCID mice, cells of the presumably little more mature $CD34^+CD38^+CD19^+$ fraction did not and this pointed out that in ALL a hierarchy still might exist albeit with LICs not being restricted to very immature and not lineage restricted cell subfractions (Hong et al., 2008).

However, in contrast to findings of Hong et al, evidence that $CD34^+CD38^+CD19^+$ as well as $CD34^+CD38^-CD19^+$ lymphoblasts and also early multipotent $CD34^+CD38^-CD19^-$ cells have stem cell properties was provided by Kong and colleagues. The group demonstrated successful serial engraftment for two high risk MLL positive and one high presenting blast count B-cell precursor ALL by iv injection of candidate cells into irradiated newborn NSG mice through the facial vein (Kong et al., 2008). Other developmental stages of the B-cell hierarchy or stem cell frequency were not addressed,

but the study again shifted the threshold of LIC containing fractions further towards progressed developmental stages.

Immunophenotypically B-lineage restricted lymphoblasts with stem cell properties were also found by le Viseur et al. In addition to $CD34^{+}CD19^{-}$ and $CD34^{+}CD19^{+}$ cells, also already $CD34^{-}CD19^{+}$ and even $CD19^{+}CD20^{low/-}$ or $CD19^{+}CD20^{+}$ blasts engrafted in NOD/SCID mice treated with anti-CD122 antibodies or NSG mice after intrafemoral transplantation, suggesting that a hierarchical model based on immunophenotypes could not be sustained (le Viseur et al., 2008).

The low frequency of LIC established by earlier studies was also put in question little later, when a limiting dilution study by Morisot and colleagues demonstrated that at least 1 of 1000 but often as few as 1 in 100 or even 1 in10 unsorted leukaemic cells had the ability to reconstitute the leukaemia in iv transplanted irradiated NSG mouse recipients (Morisot et al., 2010).

Studies addressing the questions of how far lymphoblasts progressed through immunophenotypically defined maturation stages until they lose their stem cell characteristics or how frequent LICs are with different findings are difficult to compare. Outcomes of experiments performed in these studies highly depend on the experimental setup itself. The recipient mouse strains in particular, with pronounced disparities in the level of immunosuppression, have a great impact on the chance of any cell population to engraft. Furthermore, the transplantation technique applied influences the number of cells that has to be injected to achieve repopulation. During the time in which these studies were carried out and published, mouse strains with increasing immunosuppression were developed and new transplantation techniques established. As a consequence, NOD/SCID mice, with or without anti-CD122 treatment to deplete residual immunity from NK-cells, NOD/Scid $B2m^{-/-}$ mice or the NSG strain were used.

Mice were transplanted at different ages and intravenously either into tail veins or facial veins but also orthotopically into femora or tibiae. Finally, the different studies could not access identical patient ALL samples. This created a high number of parameters potentially being responsible for different findings.

One factor that was not taken into account previously but could have influenced experiments profoundly was exhibited recently. Usually antibodies for purification by FACS were considered to have no effect on engraftment ability of transplanted cells. However, when this general assumption was tested by Taussig et al, they found that in particular antibodies directed against CD38 could label cells in a way that they would be cleared via an mAb Fc receptor mediated process by the residual immune function of the host. This effect was demonstrated to be more pronounced in less immunocompromised mice like those of the NOD/SCID strain than in the more recently developed NSG mice. Additionally, NK-cell depletion or orthotopic transplantation methods were attenuating this effect (Taussig et al., 2008). CD38 played an essential role for sorting of candidate stem cell populations in the above mentioned studies published by Cobaleda, Cox and Hong. All three used NOD/SCID mice and an iv transplantation technique and in the light of the findings from Taussig et al, not surprisingly, populations with surface expression of CD38 were not able to engraft suggesting that they lacked stem cell properties. Thus the difference in findings, at least for identifying LICs in different immunophenotypic subpopulations could probably be explained. However, clearance of CD38 labelled cells could not be responsible for the differences in stem cell frequencies determined for unsorted leukaemic cells. These probably have to be assigned to the mouse strains and transplantation techniques applied or to the individual biological characteristics of the transplanted leukaemias.

In 2009, Cox et al again addressed the question whether or not stem cell characteristics were restricted to immunophenotypically immature blast populations. For purification of

putative ALL stem cells this time they used the differentiation marker CD133 present on early normal HSCs, in addition to CD38 and CD19 and found that only $CD133^{+}CD19^{-}$ and the $CD133^{+}CD38^{-}$ blast populations engrafted intravenously injected irradiated NOD/SCID (Cox et al., 2009). These findings were in line with their previous study but again could have been biased by the use of CD38 for sorting and the NOD/SCID mouse model with a more residual immunity than more recently introduced mice, like those of the NSG strain.

Nevertheless, despite the indications that the earlier findings could have been biased by the experiment model and the identification of cells with LIC character in already B-cell lineage restricted progenitor cells, there still is no agreement in the research community regarding which stem cell model, the hierarchical or the stochastic, holds true for ALL.

The experiments presented in this chapter will address the questions of blast immunophenotypes connected with stem cell capabilities and their frequencies within the bulk leukaemic population using the most suitable mouse strain available for orthotopic xenotransplantation and by avoiding the use of CD38 antibodies for cell purification.

4.2 Aims

In order to conclusively elucidate the distribution of stem cells in ALL the aims of this study were:

- To address the question if leukaemia repopulating ability is restricted to relatively immature ALL cell populations or can be found on cells already progressed to more mature stages of B-cell development indicated by expression of CD10, CD19, CD20 and loss of CD34.
- To determine if the most mature cell populations that engraft mice are able to self-renew and repopulate several generations of mice in serial transplantation assays.
- To determine the frequency of cells with leukaemia repopulating ability within unsorted and sorted fractions of the ALL.
- To investigate whether or not ALL blast populations from different maturation stages reconstitute the immunophenotypic pattern of the original patient ALL

4.3 Materials and methods

4.3.1 Patient samples

Patient ID	Sex	Material	Cytogenetics	Age at presentation	WCC (x10^9/l) at diagnosis
L784	M	BM	Dicentric 9;20	18	52.2
L858	F	BM	No abnormalities	14	27.0
4917	M	BM	-	9	13.4
L876	F	PB	t(4;11)	<1	258
WB51	M	BM	t(9;22)	15	184
A67	M	BM	t(9;22)	13	230
HV101	F	BM	t(9;22)	3	55.9
M120	M	BM	t(9;22)	16	66.2
2003	M	PB	t(9;22)	15	179
2510	M	BM	t(9;22)	16	44.2
4540a	M	BM	t(9;22)	2	71.0
8849	M	BM	t(9;22)	20	360
9406	M	BM	cryptic BCR/ABL rearrangement	39	-
737c	M	PB	BCR/ABL like*	4	264
758b	M	PB	BCR/ABL like*	5	97.5

Table 5: Clinical features and cytogenetics of patient samples. When available, the sex, the source of material, cytogenetics, age at presentation and initial peripheral white cell count is given.
M male, F female, BM bone marrow, PB peripheral blood, WCC white cell count.

4.3.2 Statistical analysis of limiting dilution experiments

Analysis of limiting dilution experiments was performed using the open access software ELDA (extreme limiting dilution analysis, http://bioinf.wehi.edu.au/software/elda/). In stem cell research limiting dilution experiments aim to quantify the proportion of cells with stem cell activity within a larger cell population and therefore the active stem cell frequency. Generally, a single hit model is assumed in stem cell research. This means, that a single active stem cell is capable of resulting in a positive response. In the experiments, the cell dose usually is diluted down to numbers that fail to result in or lead

to a very low response only, however, sometimes none or all individual experiments result in a positive response. Furthermore, due to the nature of models applied in stem cell research, the number of replicates of experiments is usually low. ELDA accounts for these constraints by combining appropriate statistical techniques. Ninety-five percent confidence intervals are given for the stem cell frequency in each population group and upper or lower bounds are calculated with an exact one-sided 95% confidence interval in case no response is achieved or all individual experiments resulted in a response.

4.4 Results

4.4.1 Blast populations of different maturation stages are endowed with ALL repopulating capability

One important question was to determine if leukaemia propagating abilities are restricted to immature blast populations characterised by the absence of CD10, CD19 and CD20 B-lineage differentiation marker expression or if blasts of progressive immunophenotypic maturity could as well re-establish the leukaemia in mice. Furthermore, it was investigated whether leukaemic cells would lose engraftment abilities upon downregulation of the stem cell marker CD34. NSG mice were transplanted intrafemorally with highly FACS purified blast populations of differing immunophenotypes and monitored for engraftment by flow cytometry of cells from bone marrow aspirates. Successfully engrafted human ALL was analysed regarding the reconstituted surface marker expression pattern.

4.4.1.1 $CD34^{+}CD19^{low}$ and $CD34^{+}CD19^{high}$ ALL cells reconstitute the ALL

Expression of CD19 is initialised relatively early in normal B-cell development at the stage of pre-B-I cells (see section 1.3.3.1 and Figure 3) and maintained throughout maturation up to the stage of mature B-lymphocytes. Hence, cell sorting according to expression of CD19 separates distinct populations at a very early B-cell differentiation phase. The $CD34^{+}CD19^{low}$ subfraction resembles cells in normal haematopoiesis that are not yet lineage committed or represent the very early stage of pro-B cells only while the $CD34^{+}CD19^{high}$ subfraction resembles pre-B-I cells before these become $CD34^{-}$ at the level of pre-B-II large cells. Experiments with sorted blasts according to CD19 expression were performed in order to establish whether or not there is a difference in engraftment capability between very early and all later stages of B-cell development.

Initially, cell sorts were carried out on bm material that had been passaged through a mouse once before. The original patient ALL sample A67 (BCR/ABL1 positive) was intrafemorally transplanted with 1 x 10^6 unsorted cells onto each of 4 mice (M5-M8, experiment KR2). All 4 mice reconstituted the patient ALL and the pattern of human CD surface antigen expression was analysed by flow cytometry in cells from bone marrow punctures and the final bone marrow harvest. In all 4 mice the engrafted leukaemia closely resembled the original patient leukaemia immunophenotype. Terminal engraftment levels of human cells between 14.8% and 75.8% were determined by the dot plot based gating algorithm described in section 3.4.5.

The bone marrow of two mice (M5 and M7) from the primary transplantation was purified into $CD34^+CD19^{low}$ and $CD34^+CD19^{high}$ subfractions with the BD FACS Vantage SE cell sorter. The sort of bm from mouse M5 resulted in a sorting purity of 97.6% for the $CD34^+CD19^{low}$ subfraction and 99.9% for the $CD34^+CD19^{high}$ subfraction and of each 3 NSG mice were if transplanted with 4.0 x 10^3 cells/mouse and 2.0 x 10^4 cells/mouse respectively (experiment 5). Two of the 3 mice transplanted with $CD34^+CD19^{low}$ cells showed bm engraftment of human cells at the time point of the first bone marrow puncture 8 weeks after transplantation. Engraftment levels determined by flow cytometry were 2.7% (M20) and 5.2% (M22). Analysis of bmp2 11 weeks after transplantation failed for technical reasons but the third mouse M21 showed human blasts (2.3%) in peripheral mouse blood two days later. All 3 mice transplanted with $CD34^+CD19^{high}$ were engrafted at the time of bmp1 with human chimerisms of 2.3% (M23), 0.13% (M24) and 17.9% (M25). Transplanted mice survived between 11and 21 weeks and final engraftment levels determined from bm harvests (or a bmp in case a mouse died without a terminal bm harvest) ranged between 0.13% and 34.9%. Both transplanted cell populations reconstituted in the mice with an immunophenotype distribution pattern of cells that resembled that of the original patient sample A67. Flow

cytometry analysis, depicted as contour dot plots of human cells in bm of engrafted mice is illustrated in Figure 19.

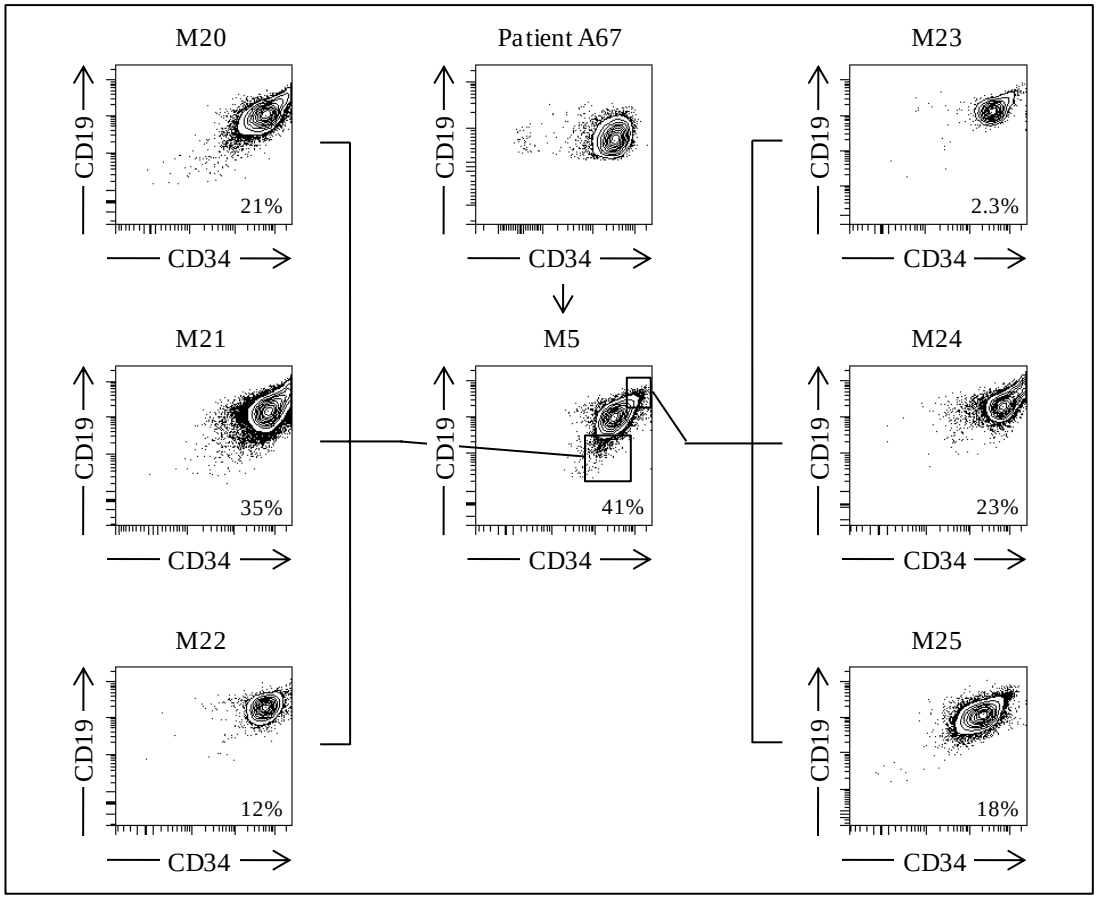


Figure 19: CD34^{+}CD19^{low} and CD34^{+}CD19^{high} sorted blast populations engraft in NSG mice and reconstitute the original ALL immunophenotype (patient A67). Mouse M5 was transplanted with unsorted diagnostic bm from patient A67 and engrafted in bm to 41% human chimerism. Harvested bm was sorted into CD34^{+}CD19^{low} and CD34^{+}CD19^{high} subfractions and retransplanted into new NSG hosts. The mice were monitored for human ALL engraftment using bmps and, unless a mouse died unexpectedly, a terminal bm harvest was carried out once mice became ill. The contour dot plot for mouse M5 shows the immunophenotype for CD19 and CD34 expression and the sorting gates. In the left row of contour dot plots, engraftment in M20 to M22 transplanted with CD34^{+}CD19^{low} cells is depicted and in the right row, engraftment of M23 to M25 transplanted with CD34^{+}CD19^{high} cells. Engraftment levels of the harvested bm or last bmps performed are inserted in the plots. Both transplanted subpopulations reconstitute human cells in NSG mice with an immunophenotype resembling the original transplanted sample.

Purities of the sort on mouse M7 were 96.9% for $CD34^{+}CD19^{low}$ cells and 99.5% for $CD34^{+}CD19^{high}$ cells. Of both populations 2.0 x 10^3 cells were transplanted for each mouse (KR4). Two of 4 mice transplanted with $CD34^{+}CD19^{low}$ cells showed engraftment in aspirates of bmp1 performed 10 weeks after transplantation with 4.0% (M15) and 8.9% (M16) human chimerism. M13 had 0.1% engraftment in peripheral mouse blood 12 weeks after transplantation and in bmp3, 16 weeks post transplantation, 36.9% bm engraftment was determined for this mouse and a first positive engraftment of 0.1% for mouse M14. Of the 3 mice that received $CD34^{+}CD19^{high}$ ALL cells, all were engrafted at 10 weeks post transplantation with 0.06% (M17), 37.8% (M18) and 2.6% (M19) in bm collected from bmp1. Mice from this experiment died or were killed between 13 and 23 weeks after transplantation. Engraftment levels ranged between 2.6% and 52.1%. Both transplanted cell populations again reconstituted the immunophenotypic pattern established for the diagnostic patient sample.

In a third experiment setup it was investigated if the results gained from CD19 sorts performed on human cells that had already grown in mouse bm could be shown to be the same in the original diagnostic patient material. Bm of the Philadelphia chromosome positive sample WB51 was directly sorted and 3.1 x 10^4 $CD34^{+}CD19^{high}$ cells were transplanted into each of 4 mice and 4.0 x 10^3 $CD34^{+}CD19^{low}$ cells into each of 2 mice (KR18). In the first bmp 7 weeks after transplantation, all 6 mice showed engraftment of human cells with levels of 2.2% (M74), 14% (M75), 1.3% (M76) and 7.4% (77) for mice transplanted with $CD34^{+}CD19^{high}$ cells and 0.04% (M78) and 0.05% (M79) for mice transplanted with $CD34^{+}CD19^{low}$ cells. All mice of this experiment survived between 15 and 20 weeks post transplantation until the terminal bm harvest was performed showing high engraftment levels for all mice ranging from 58.6% to 81.9%. Similar to the initial experiments on secondary material from patient A67, original primary patient material WB51 engrafted both the $CD34^{+}CD19^{high}$ and $CD34^{+}CD19^{low}$

subpopulation with an immunophenotype reconstituted in bm of mice resembling that of the presenting patient bm.

In summary, altogether 19 NSG mice were transplanted in 3 different experiments. All mice engrafted the leukaemia to the original patient immunophenotype. The experiment with primary cells of patient WB51 (KR18) reproduced the findings of experiments KR4 and KR5 performed with secondary blasts from patient leukaemia A67. The setup and results of these xenotransplantations are illustrated in Table 6.

Exp. No.	Patient / Leukemia	Injected Cell No.	Injected Cells	No. transplanted	No. engrafted
KR4	A67 sec	2.0×10^3	CD19high	3	3
		2.0×10^3	CD19low	4	4
KR5	A67 sec	2.0×10^4	CD19high	3	3
		4.0×10^3	CD19low	3	3
KR18	WB51 prim	3.12×10^4	CD19high	4	4
		4.0×10^3	CD19low	2	2
Sum				19	19

Table 6: A list summarising xenotransplant experiments with ALL blasts sorted according to CD19 surface antigen expression.

4.4.1.2 $CD19^+CD20^{low}$ and $CD19^+CD20^{high}$ ALL cells reconstitute the ALL

The surface marker CD20 is highly expressed only on cells already progressed to the pre-B-II small cell stage in B-lineage development (see section 1.3.3.1 and Figure 3). Purification of CD20 high expressing cells therefore separates cells of an advanced maturity from all other preceding developmental stages. Several previous publications suggested that only cells up to a certain maturity level are able to repopulate mice and that this essential stem cell characteristic is lost by immunophenotypically more mature precursor cells. Of the CD surface antigens used for cell sorting in this project, $CD20^{high}$ cell populations resemble the most mature stage of normal B-cell development.

Therefore, $CD20^{high}$ expressing cells would be the most likely cells to have lost the ability to repopulate NSG mice. In order to investigate whether or not that is the case, several ALL samples, sorted into $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ subpopulations were if transplanted into mice by intrafemoral injection.

ALL sample A67 was the first to be used for these experiments (KR11). Mouse M6 was initially transplanted with 3.0×10^5 unsorted cells of the original diagnostic ALL sample A67 and engrafted to a final level of 15% human chimerism. Harvested human bm cells from this mouse were sorted according to CD19 and CD20 surface marker expression and both the $CD19^{+}CD20^{low}$ (83.9% purity) and $CD19^{+}CD20^{high}$ (99.9% purity) subpopulations were transplanted into 3 NSG mice each with 2.0×10^4 cells per mouse. Due to technical reasons the first bmp failed and could not be analysed for engraftment. However, analysis of peripheral mouse blood, carried out two days after the first bmp at 9 weeks after transplantation, showed human circulating ALL blasts in all 6 mice. Mouse M44 died unexpectedly after bmp3 but the 5 other mice were killed 14 to 16 weeks after initiation of the experiment when they appeared to become ill from the developing ALL. Terminal bm engraftment levels were between 15% and 72% and did not differ between the subfractions of different maturity with high engraftment levels determined for both groups. Furthermore, the original immunophenotype of the ALL was closely recapitulated in all transplanted mice. Engraftment of bm in mice from this experiment is illustrated in Figure 20.

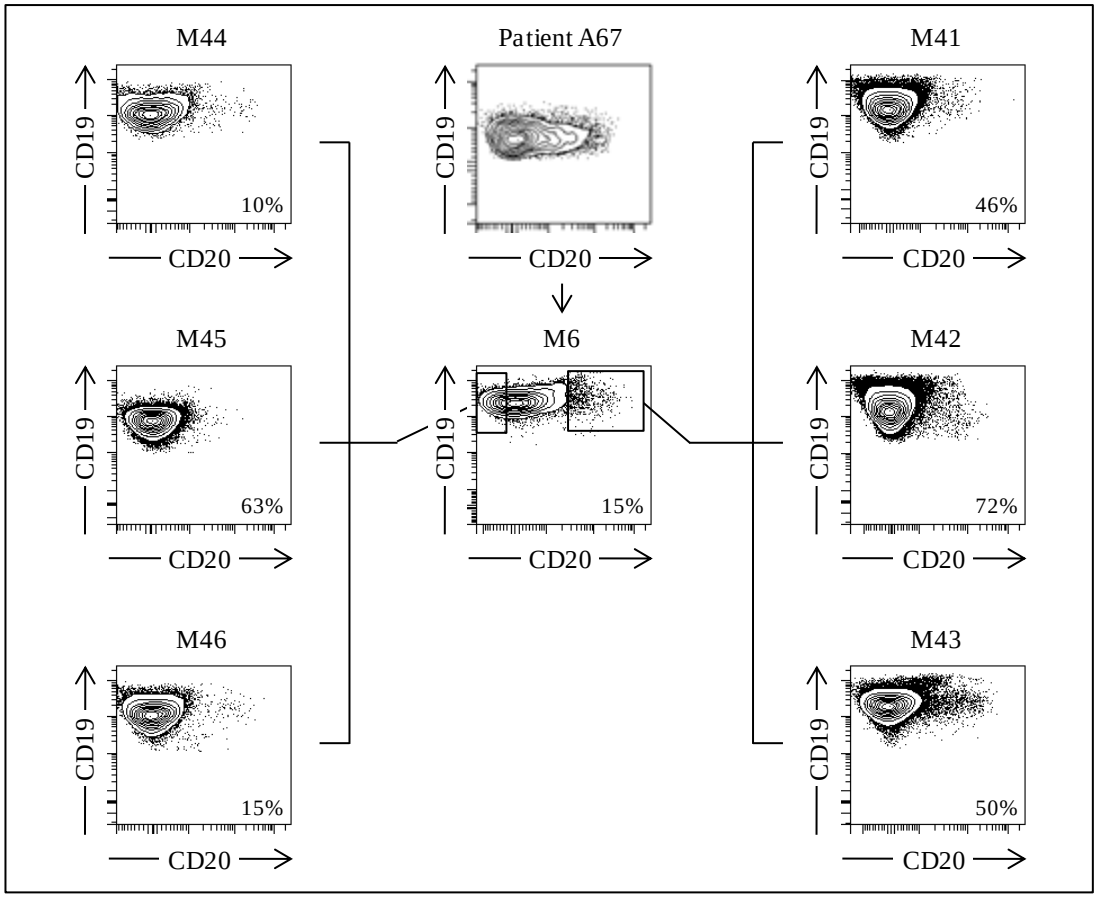


Figure 20: CD19^{+}CD20^{low} and CD19^{+}CD20^{high} sorted blast populations engraft in NSG mice and reconstitute the original ALL immunophenotype (patient A67). Mouse M6 was transplanted with unsorted diagnostic bm from patient A67. Final bm analysis showed 15% human chimerism. Purified bm subfractions with CD19^{+}CD20^{low} and CD19^{+}CD20^{high} immunophenotypes were transplanted into NSG mice and human ALL engraftment was analysed. The contour dot plot for mouse M6 in the centre shows CD19 and CD20 expression profiles of its bm cells and the sorting gates. On the left side bm analyses of bmp3 for M44 and of terminal bm harvests for M45 and M46 show engraftment of CD19^{+}CD20^{low} blasts. On the right side, terminal bm harvests of mice M41 to M43, transplanted with CD19^{+}CD20^{high} cells are depicted. Human chimerisms of the harvested bm or last bmp performed are inserted in the plots. Both transplanted subpopulations reconstitute human cells in NSG mice with an immunophenotype resembling the original transplanted sample.

Following these initial findings, further samples that had been passaged through mice once were transplanted and the question was addressed as to whether cells that have not been passaged through mice would also engraft both sorted subfractions of CD19^{+}CD20^{low} and CD19^{+}CD20^{high} cells and reconstitute the immunophenotypic pattern

of the original ALL. Additionally, the same assay was performed on ALL samples that had already been passaged through mice over two generations.

Altogether, 6 original diagnostic patient samples were sorted according to CD20 expression (MS45, WB51, HV101, 737c, M120 and 758b) with purities for the $CD19^{+}CD20^{low}$ subfractions between 92% and 100% and for the $CD19^{+}CD20^{high}$ subfractions between 99% and 100%. For sample MS45 both fractions were transplanted into 4 mice each with 3.0×10^{3} cells per mouse (KR38). Over the observation time of 34 weeks none of the 8 mice transplanted engrafted human cells. For all other 5 samples human engraftment could be detected. Sample WB51 (KR17) engrafted both the less and more mature subfraction to high levels between 60% and 86%. One mouse transplanted with $CD19^{+}CD20^{high}$ cells was not engrafted at bmp1 and died shortly after due unrelated reasons. Sample HV101 (KR31) as well engrafted both subfractions in all but one mouse. $CD19^{+}CD20^{high}$ cells engrafted from 26% to 78% and $CD19^{+}CD20^{low}$ cells from 26% to 74%. One mouse injected with $CD19^{+}CD20^{high}$ cells died after bmp5 at which it was positive outside limits (pol, see section 3.4.6). Diagnostic bm from patient 737c (KR42) engrafted all mice transplanted with either the $CD19^{+}CD20^{high}$ or $CD19^{+}CD20^{low}$ subfraction. Engraftment levels in the last bone marrow punctures before unexpected death or final bm analyses were between 2.4% and 74%. Samples M120 (KR48) and 758b (KR51) both failed to engraft $CD19^{+}CD20^{high}$ expressing cells. For M120 the $CD19^{+}CD20^{low}$ subfraction engrafted 3 of 4 mice transplanted, albeit with relatively low levels, between 4.4% and 29%. However, $CD19^{+}CD20^{low}$ cells transplanted from ALL sample 758b, resulted in high human chimerism between 29% and 88% in 4 out of 4 mice.

In addition to the first experiment described in this subchapter, another 6 secondary samples from 4 different ALLs (A67, WB51, 2510 and 737c) were sorted and $CD19^{+}CD20^{high}$ and $CD19^{+}CD20^{low}$ cells transplanted with cell numbers of 1,000 cells

or higher per mouse. The second A67 sample that had been passaged through a mouse once engrafted all 3 mice of each purified subfraction to levels between 24% and 75% in bm harvests and 19% in bmp2 of one mouse transplanted with $CD19^{+}CD20^{high}$ cells before early death (KR12). Three separate CD20 sorts were carried out on secondary samples from patient WB51. In the first of these experiments (KR26) all transplanted mice survived for a terminal bm harvest and engraftment analysis. Final engraftment levels were comparable between the both groups transplanted with $CD19^{+}CD20^{high}$ and $CD19^{+}CD20^{low}$ cells and ranged between 31% and 81%. Experiment KR27 as well successfully propagated the ALL in all 6 mice transplanted with the two differentially mature cell populations. Engraftment levels determined in bmps or final bm harvests were between 2.3% and 77%. The third secondary transplant experiment (KR34) on WB51 with cells sorted in regards to CD20 expression led to engraftment of 4 of 4 mice transplanted with $CD19^{+}CD20^{high}$ cells and 3 of 4 mice transplanted with $CD19^{+}CD20^{low}$ cells at the time of the first bmp. Only 3 mice survived for a terminal bm harvest with engraftment levels between 38% and 62%. The mouse that was not engrafted at bmp1 died shortly after and could not be assessed again. One secondary 2510 sample was included in the CD20 sorts and both subpopulations, the $CD19^{+}CD20^{high}$ and the $CD19^{+}CD20^{low}$, were leading to overt ALL in mice. Analysis of harvested bm resulted in human chimerisms between 21% and 69%. While all 4 mice transplanted with $CD19^{+}CD20^{low}$ cells engrafted, 2 of 4 transplanted with $CD19^{+}CD20^{high}$ cells did not. 737c was the last secondary sorting experiment on CD20 (KR55). From this sample, all mice engrafted the $CD19^{+}CD20^{high}$ subfraction and 2 of 4 mice engrafted the $CD19^{+}CD20^{low}$ subfraction. From all mice, bm could be harvested and analysed for human chimerism which ranged from 25% to 67% in the engrafted cases.

Furthermore, CD20 sorts were performed on two tertiary A67 samples with more than 1.0×10^{3} cells of each subfraction transplanted per mouse. In experiment KR28 all 4

mice transplanted with $CD19^{+}CD20^{high}$ cells engrafted to 15% to 68% human chimerism in terminal bm harvests and all 4 mice transplanted with $CD19^{+}CD20^{low}$ cells to 36% to 58%. All harvests were performed between 14 and 17 weeks post transplant. Additionally one tertiary sample from patient WB51 was sorted and $CD19^{+}CD20^{high}$ and $CD19^{+}CD20^{low}$ subfractions transplanted with only 500 cells into 3 mice each (KR45). All 6 mice engrafted but engraftment levels were very low in mice transplanted with $CD19^{+}CD20^{high}$ cells (0.08% to 1.0%) and high in mice transplanted with $CD19^{+}CD20^{low}$ cells (36% to 77%). In this transplantation experiment, observation period was relatively long and mice survived between 24 and 49 weeks.

In summary, altogether 118 NSG mice were transplanted in 16 different experiments. 90 mice engrafted the leukaemia and the immunophenotype, as determined by flow cytometry, always closely resembled that of the original patient ALL, indicating that results were highly reproducible. The setup and results of these xenotransplantations with primary, secondary and tertiary leukaemic blasts sorted according to CD20 surface marker expression are summarised in Table 7.

Exp. No.	Patient / Leukemia	Injected Cell No.	Injected Cells	No. transplanted	No. engrafted
KR11	A67 sec	$CD20^{high}$	2.0×10^4	3	3
		$CD20^{low}$	2.0×10^4	3	3
KR12	A67 sec	$CD20^{high}$	9.0×10^3	3	3
		$CD20^{low}$	9.0×10^3	3	3
KR17	WB51 prim	$CD20^{high}$	1.08×10^4	3	2
		$CD20^{low}$	6.24×10^4	3	3
KR26	WB51 sec	$CD20^{high}$	3.06×10^3	4	4
		$CD20^{low}$	3.06×10^3	4	4
KR27	WB51 sec	$CD20^{high}$	2.0×10^3	3	3
		$CD20^{low}$	2.0×10^3	3	3
KR28	A67 tert	$CD20^{high}$	1.35×10^3	4	4
		$CD20^{low}$	1.35×10^3	4	4
KR29	A67 tert	$CD20^{high}$	2.4×10^3	4	3*
		$CD20^{low}$	2.4×10^3	4	4
KR31	HV101 prim	$CD20^{high}$	3.0×10^3	4	3
		$CD20^{low}$	3.0×10^3	4	4
KR33	2510 sec	$CD20^{high}$	3.0×10^3	4	2
		$CD20^{low}$	3.0×10^3	4	4
KR34	WB51 sec	$CD20^{high}$	1.2×10^3	4	4
		$CD20^{low}$	1.2×10^3	4	3
KR38	MS45 prim	$CD20^{high}$	3.0×10^3	4	0
		$CD20^{low}$	3.0×10^3	4	0
KR42	737c prim	$CD20^{high}$	1.5×10^3	4	4
		$CD20^{low}$	1.5×10^3	4	4
KR45	WB51 tert	$CD20^{high}$	5.0×10^2	3	3
		$CD20^{low}$	5.0×10^2	3	3
KR48	M120 prim	$CD20^{high}$	3.0×10^3	4	0
		$CD20^{low}$	3.0×10^3	4	3
KR51	758b prim	$CD20^{high}$	1.5×10^3	4	0
		$CD20^{low}$	1.5×10^3	4	4
KR55	737c sec	$CD20^{high}$	1.0×10^3	4	4
		$CD20^{low}$	1.0×10^3	4	2
Sum				118	90

Table 7: A list summarising xenotransplant experiments with ALL blasts sorted according to CD20 surface antigen expression.

4.4.1.3 $CD19^+CD10^{low}$ and $CD19^+CD10^{high}$ ALL cells reconstitute the ALL

CD10 is the surface marker that, with the exception of infant *MLL*-rearranged cases, is commonly expressed on ALL blasts and therefore was named common ALL antigen (CALLA). During B cell development it is first expressed at the pre-B-I cell level just before CD19 is up-regulated (see section 1.3.3.1 and Figure 3) and stays positive through all B precursor stages. In normal B lymphopoiesis, proliferative activity is decreasing with higher levels of CD10 expression. Thus ALL samples were as well sorted into $CD19^+CD10^{low}$ and $CD19^+CD10^{high}$ expressing blast populations in order to investigate whether or not there was a difference in leukaemia reconstituting potential.

The first $CD19^+CD10^{low}$ (purity of 98.5%) and $CD19^+CD10^{high}$ (purity of 96.7%) subfractions were transplanted from a secondary sample harvested of mouse M1, which originally was injected with 1.0 x 10^6 unsorted cells from diagnostic material of patient 8849. After 11 weeks the first bmp was performed and all 3 mice transplanted with 3.0 x 10^4 $CD19^+CD10^{high}$ cells showed engraftment (M53 1.9%, M54 0.05% and M55 1.1%) while no cells of human origin could be detected in the 3 mice transplanted with 3.0 x 10^4 $CD19^+CD10^{low}$ cells. Terminal engraftment levels between 26 and 50 weeks post transplantation were 55.5% for M53, 42.4% for M54 and 4.1% for M55 but $CD19^+CD10^{low}$ transplanted mice stayed negative for human chimerism until terminal bm analysis between 48 and 50 weeks post transplantation. The results of this experiment indicated an engraftment deficit for ALL cells with the immunophenotype $CD19^+CD10^{low}$, suggesting a hierarchy in the ALL tumour population. However, this hierarchy would not mirror a physiological haematopoietic hierarchy, where immunophenotypically more mature cells rather than less mature cells have limited or no clonogenic potential. Further transplantation experiments were performed to confirm or contradict this unusual finding. First of all, another sort was performed on the same secondary material. Four mice each were transplanted with $CD19^+CD10^{low}$ and

$CD19^{+}CD10^{high}$ cells. One mouse of each group showed little engraftment in cells from bmp2 19 weeks after transplantation and in final bm harvests, 2 mice of the group transplanted with $CD19^{+}CD10^{low}$ cells (M84 41.5% and M85 0.01%) but still only one mouse of the group transplanted with $CD19^{+}CD10^{high}$ cells (M80 0.56%) was engrafted. Results from this experiment implied that the $CD19^{+}CD10^{low}$ subfraction did not exhibit a repopulation deficit. The contradictory results in experiments performed on the same secondary ALL sample prompted CD10 sorts on more secondary and also primary patient samples. Another 3 secondary ALLs (WB51, 2510 and 737c) and 7 primary ALLs (MS45, L831, L784, HV101, 737c, M120 and 758b) were transplanted with cell numbers of 1,000 of more injected per mouse. Secondary human cells of WB51, harvested with bm from M64 were sorted into the same subfractions and transplanted into 4 mice each (KR21). Three of 4 mice transplanted with $CD19^{+}CD10^{high}$ cells and all 4 mice transplanted with $CD19^{+}CD10^{low}$ cells exhibited engraftment in mouse bm from bmp1 10 weeks after start of the experiment and all 8 mice finally reconstituted the ALL with terminal engraftment levels ranging from 18% to 61% for the $CD19^{+}CD10^{high}$ and from 27% to 67% for the $CD19^{+}CD10^{low}$ transplanted mice respectively. The flow cytometry contour dot plot analysis for this experiment is illustrated in Figure 21.

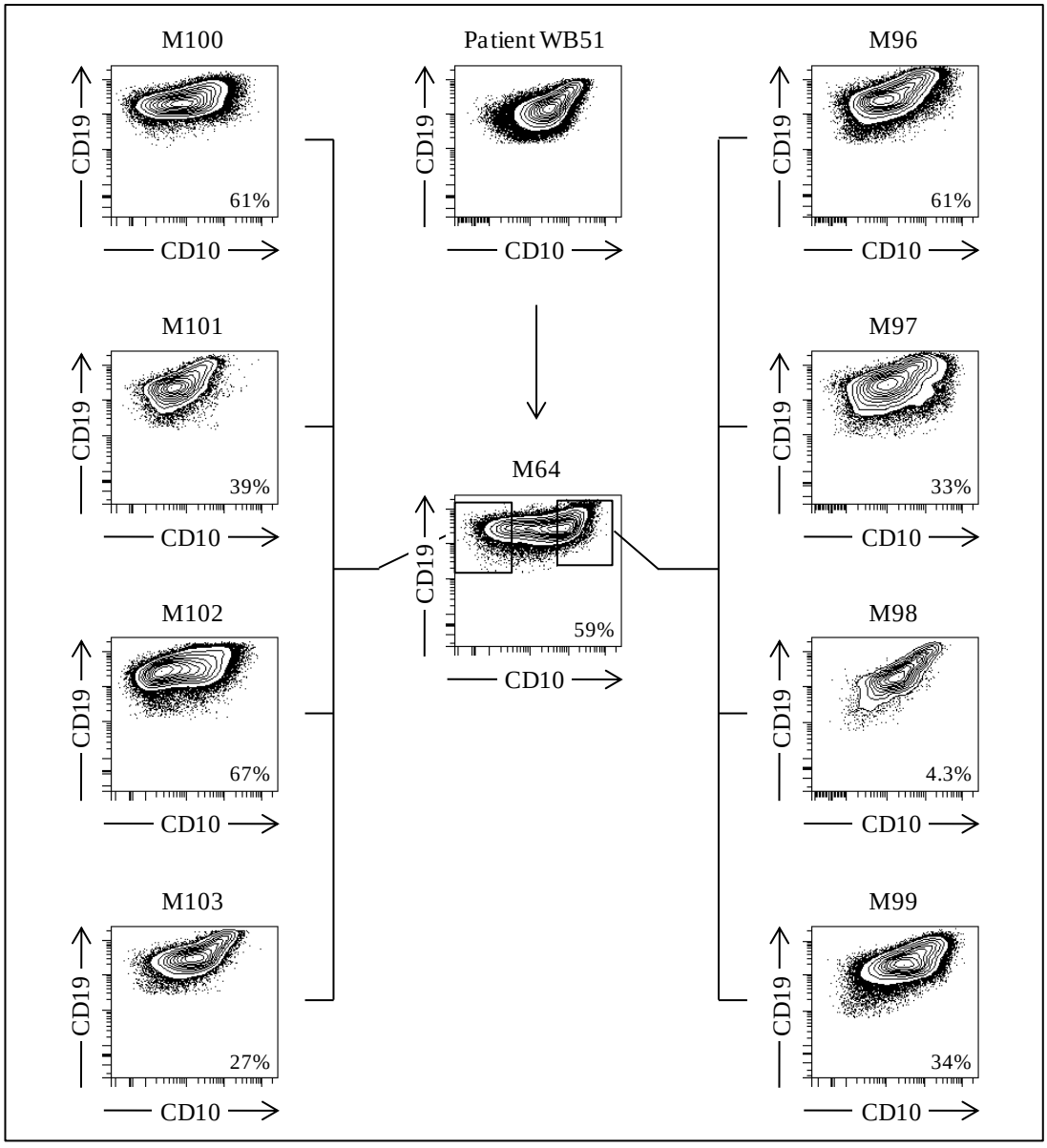


Figure 21: CD19^{+}CD10^{low} and CD19^{+}CD10^{high} sorted blast populations engraft in NSG mice and reconstitute the original ALL immunophenotype (patient WB51). Mouse M64 was transplanted with unsorted diagnostic bm from patient WB51. Final bm analysis showed 59% human chimerism. Purified bm subfractions with CD19^{+}CD10^{low} and CD19^{+}CD10^{high} immunophenotypes were transplanted into NSG mice and human ALL engraftment was analysed. The contour dot plot for mouse M64 in the centre shows CD19 and CD10 expression profiles of its bm cells and the sorting gates. On the left side bm analyses of terminal bm harvests for M100 to M103 show engraftment of CD19^{+}CD10^{low} blasts. On the right side, terminal bm harvests of mice M96 and M97 and bmp2 of M98 and M99, transplanted with CD19^{+}CD10^{high} cells are depicted. Human chimerisms of the harvested bm or last bmp performed are inserted in the plots. Both transplanted subpopulations reconstitute human cells in NSG mice with an immunophenotype resembling the original transplanted sample.

Both subfractions of sorted secondary cells also successfully engrafted from ALL 2510 and 737c. Sample 2510 (KR24) repopulated two of 4 mice of each transplanted subpopulation with final human chimerism of 0.01% and 50% achieved by the $CD19^{+}CD10^{high}$ population and 0.14% and 1.2% by the $CD19^{+}CD10^{low}$ population. ALL 737c again led to ALL in all 8 transplanted mice. Mouse bm harboured human cell proportions ranging from 55% to 82% (mice transplanted with $CD19^{+}CD10^{high}$ cells) and 18% to 73% (mice transplanted with $CD19^{+}CD10^{low}$ cells). Of the transplanted primary samples ALL MS45 and L831 never led to any engraftment during an observation period of up to 37 weeks. Successful engraftment of both subpopulations, however, was demonstrated for the first transplanted primary patient sample L784 (KR22). One of the 4 mice of the group transplanted with the immunophenotypically less mature $CD19^{+}CD10^{low}$ subpopulation died before the first bm analysis presumably for non ALL related reasons but all remaining 7 mice of both populations were engrafted at bmp1 (15 weeks post transplant) already. At the end of the experiment mice transplanted with the more mature $CD19^{+}CD10^{high}$ population were bm engrafted between 20% and 55% and mice transplanted with $CD19^{+}CD10^{low}$ cells between 35% and 68%. Two samples, 737c (KR43) and M120 (KR47), only repopulated mice with human ALL when transplanted with $CD19^{+}CD10^{high}$ populations. Each sample was transplanted in groups of 4 for the $CD19^{+}CD10^{high}$ and the $CD19^{+}CD10^{low}$ subpopulations respectively. For 737c 3 of 4 mice engrafted the leukaemia to levels between 7% and 42% and for M120 two mice engrafted with 9% and 16%. ALL HV101 engrafted one of 4 mice transplanted with $CD19^{+}CD10^{low}$ cells to a low final human chimerism of 0.02% and two of 4 mice transplanted with $CD19^{+}CD10^{low}$ cells to 0.04% and 60%. The last experiment with a primary CD10 sort was performed with sample 758b (KR50). Of this sample all 4 mice transplanted with the $CD19^{+}CD10^{low}$ cell fraction reconstituted human ALL cells in mouse bm to human chimerisms between

21% and 70% and two of 4 mice transplanted with $CD19^{+}CD10^{high}$ cells engrafted human cells in mouse bm with percentages of 0.25% and 1.0%.

The different primary and secondary transplant experiments show that both the immunophenotypically less mature $CD19^{+}CD10^{low}$ and the more mature $CD19^{+}CD10^{high}$ subpopulations are able to reconstitute human ALL cells in the mouse bm xenoenvironment. Between the ALL samples, this ability varied, but taken together, the stem cell characteristic of tumour repopulation ability was not restricted to one population only. Furthermore, both sorted subpopulations reconstituted the ALL to its original immunophenotype, as illustrated for the secondary sample WB51 in Figure 21.

In summary, altogether 78 NSG mice were transplanted in 10 different experiments of which 44 NSG mice engrafted the leukaemia with an immunophenotype mirroring the immunophenotype of the original patient sample. The results of the individual experiments were therefore highly reproducible. The xenotransplantations with primary and secondary human ALL cells sorted according to CD10 expression are illustrated in Table 8.

Exp. No.	Patient / Leukemia	Injected Cell No.	Injected Cells	No. transplanted	No. engrafted
KR13	8849 sec	$CD10^{high}$	3.0×10^4	3	3
		$CD10^{low}$	3.0×10^4	3	0
KR19	8849 sec	$CD10^{high}$	1.44×10^4	4	1
		$CD10^{low}$	2.19×10^4	4	2
KR21	WB51 sec	$CD10^{high}$	1.24×10^4	4	4
		$CD10^{low}$	1.35×10^4	4	4
KR22	L784 prim	$CD10^{high}$	2.61×10^4	4	4
		$CD10^{low}$	6.3×10^3	4	3*
KR24	2510 sec	$CD10^{high}$	4.5×10^3	4	2
		$CD10^{low}$	6.9×10^3	4	2
KR30	HV101 prim	$CD10^{high}$	3.0×10^3	4	2
		$CD10^{low}$	3.0×10^3	4	1
KR43	737c prim	$CD10^{high}$	1.5×10^3	4	3
		$CD10^{low}$	1.5×10^3	4	0
KR47	M120 prm	$CD10^{high}$	2.0×10^3	4	2
		$CD10^{low}$	2.0×10^3	4	0
KR50	758b prim	$CD10^{high}$	1.5×10^3	4	2
		$CD10^{low}$	1.5×10^3	4	4
KR56	737c sec	$CD10^{high}$	1.0×10^3	4	4
		$CD10^{low}$	1.0×10^3	4	4
Sum				78	44

Table 8: A list summarising xenotransplant experiments with ALL blasts sorted according to CD10 surface antigen expression.

4.4.1.4 $CD19^+CD34^{low}$ and $CD19^+CD34^{high}$ ALL cells reconstitute the ALL

The surface marker CD34 commonly is used as an established haematopoietic stem cell marker. It is expressed on normal haematopoietic stem cells and on progenitor cells that are capable of long-term reconstitution of the haematopoietic system and is down-regulated on cells that lack this ability. In the B lineage, CD34 expression is lost during the transition of pre-B-I cells to pre-B-II large cells. In the case of a hierarchical organisation among ALL blast populations, a discrepant ability to reconstitute the malignancy in mice would be likely to be present between subfractions that did or did not express CD34. In order to test this hypothesis, 2 primary patient samples (4540a and

L784), one secondary sample (737c) and one tertiary sample (WB51) were sorted into $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ subfractions and transplanted into NSG mice.

In order to primarily address the question of general ability to engraft the leukaemia, only mice transplanted with 1,000 or more $CD19^{+}CD34^{high}$ or $CD19^{+}CD34^{low}$ cells were evaluated. The first ALL to be transplanted was a tertiary WB51 sample. Bone marrow from mouse M272 was sorted into $CD19^{+}CD34^{high}$ (population purity of 97.9%) and $CD19^{+}CD34^{low}$ (population purity of 97.8%) subpopulations and these were injected into 4 mice each with 1,000 cells per animal. Of the group transplanted with the $CD19^{+}CD34^{high}$ cell fraction one died before the first bmp but the remaining 3 as well as all 4 mice of the group transplanted with $CD19^{+}CD34^{low}$ cells showed positive engraftment of human cells only 9 weeks after initiation of the experiment. Maximum human chimerism at the time of the last available bmp or in terminal bm harvests 16 to 22 weeks post transplantation ranged from 41% to 71% in the group transplanted with $CD19^{+}CD34^{high}$ cells and from 24% to 58% in the group transplanted with $CD19^{+}CD34^{low}$ cells. The contour dot plots in Figure 22 illustrate the engraftment of bm in mice from this experiment.

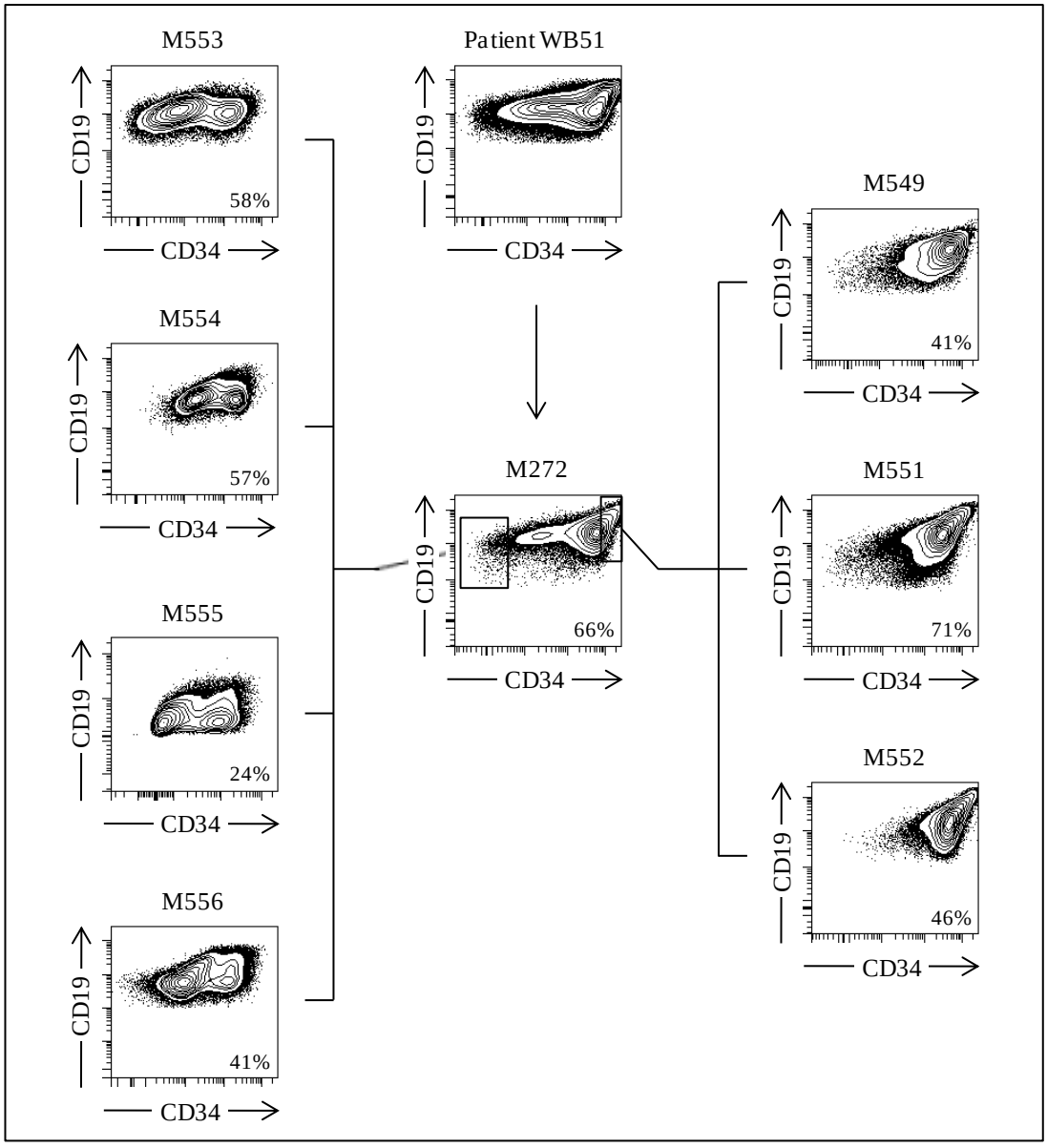


Figure 22: CD19^{+}CD34^{low} and CD19^{+}CD34^{high} sorted blast populations engraft the ALL in NSG mice (patient WB51). Mouse M272 was transplanted with unsorted secondary bm from patient WB51. Final bm analysis showed 66% human chimerism. Purified bm subfractions of this mouse with CD19^{+}CD34^{low} and CD19^{+}CD34^{high} immunophenotypes were transplanted into new NSG mice and human ALL engraftment was analysed. The contour dot plot for mouse M272 in the centre shows CD19 and CD34 expression profiles of its bm cells and the sorting gates. On the left side bm analyses of terminal harvests for M553 to M556 show engraftment of CD19^{+}CD34^{low} blasts. On the right side, terminal bm harvests of mice M549 and M551 and bmp3 of mouse M552, transplanted with CD19^{+}CD34^{high} cells are depicted. Human chimerisms of the harvested bm or last bmp performed are inserted in the plots. Both transplanted subpopulations reconstitute human cells in NSG mice. The immunophenotype resembles the original transplanted sample when CD19^{+}CD34^{low} blasts were transplanted but only a uniform CD19^{+}CD34^{high} blast population is reconstituted from the transplanted CD19^{+}CD34^{high} subfraction.

The finding that both the immunophenotypically less mature $CD19^{+}CD34^{high}$ as well as the more mature $CD19^{+}CD34^{low}$ cells are able to repopulate NSG mice was confirmed in a sorting experiment with secondary 737c ALL cells. The opposing populations were transplanted in groups of 4 mice each and all mice engrafted the leukaemia. The less mature population reconstituted human cells to levels between 6% and 94% and the more mature population to levels between 13% and 78% in last bone marrow punctures or bm harvests.

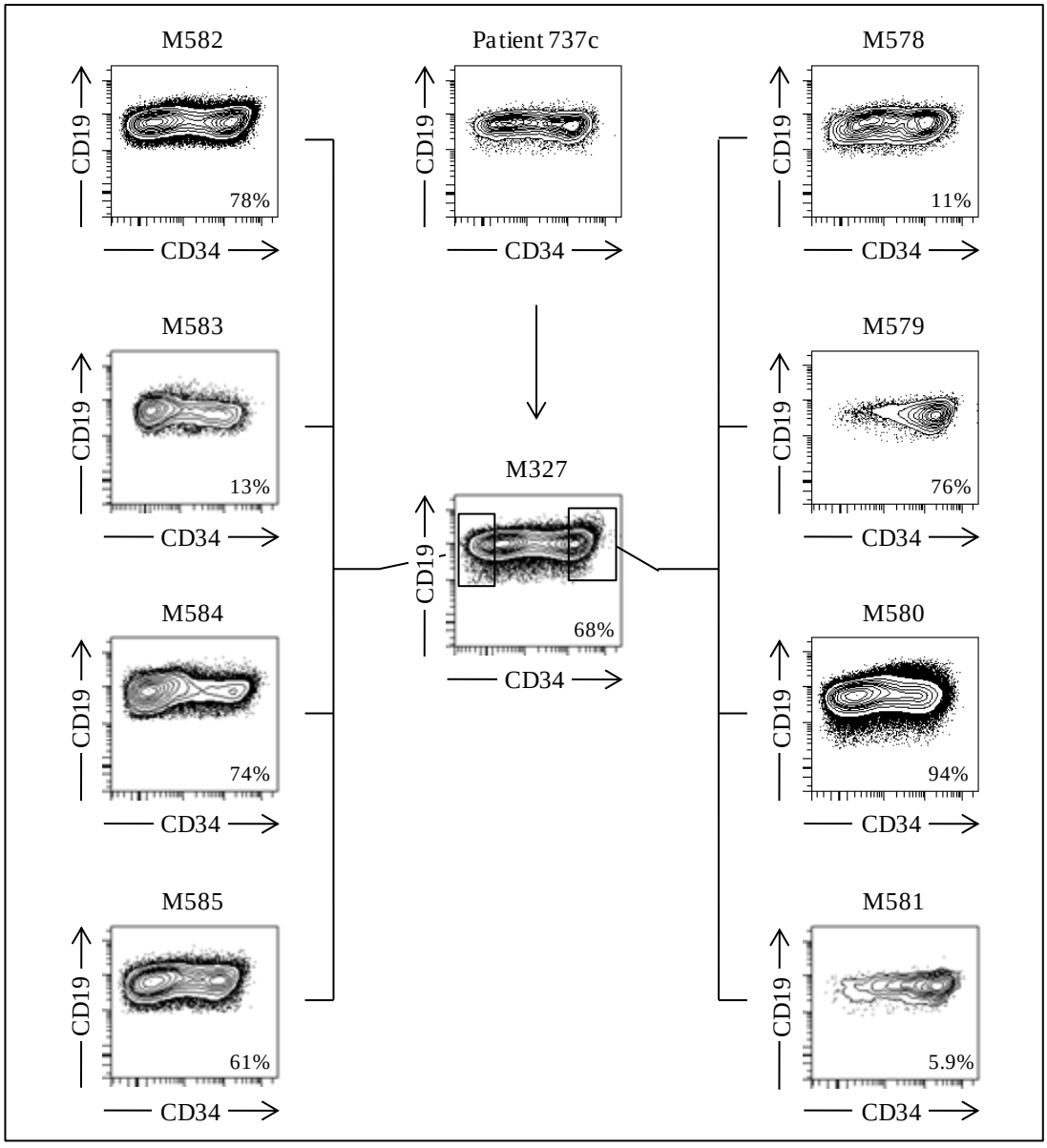


Figure 23: CD19^{+}CD34^{low} and CD19^{+}CD34^{high} sorted blast populations engraft in NSG mice and reconstitute the original ALL immunophenotype (patient 737c). Mouse M327 was transplanted with unsorted diagnostic bm from patient 737c. Final bm analysis showed 68% human chimerism. Purified bm subfractions of this mouse with CD19^{+}CD34^{low} and CD19^{+}CD34^{high} immunophenotypes were transplanted into new NSG mice and human ALL engraftment was analysed. The contour dot plot for mouse M327 in the centre shows CD19 and CD34 expression profiles of its bm cells and the sorting gates. On the left side bm analyses of bmp3 for M583 and of terminal bm harvests for M582, M584 and M585 show engraftment of CD19^{+}CD34^{low} blasts. On the right side, bm analyses of bmp2 for M579 and bmp4 for M581 and of terminal bm harvests for M578 and M580 show engraftment of mice transplanted with CD19^{+}CD34^{high} cells. Human chimerisms of the harvested bm or last bmp performed are inserted in the plots. Both transplanted subpopulations reconstitute human cells in NSG mice with an immunophenotype resembling the original transplanted sample.

To provide further proof that these results were not due to prior passage of cells through one or two generations of mice, primary diagnostic patient samples were as well sorted and transplanted. $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ blast fractions of patient 4540a were transplanted with 1,000 cells per mouse into 5 mice each. Two mice of the group transplanted with $CD19^{+}CD34^{high}$ cells engrafted to levels of 1% (bmp1) and 44% (bm harvest) and one mouse of the group transplanted with $CD19^{+}CD34^{low}$ cells to 59% (bm harvest). The second primary sample L784 was transplanted with 10,000 cells per mouse of each subfraction in groups of 4 mice. All 4 mice that received $CD19^{+}CD34^{high}$ cells engrafted human cells with fractions in the bm from bmps or bm harvests of 17% to 73%. Two of the 4 mice that received $CD19^{+}CD34^{low}$ cells engrafted to human cell levels of 41% and 58% respectively.

All 4 experiments, two primary, one secondary and one tertiary, showed that, unlike in normal haematopoiesis, both CD34 positive and negative cells can reconstitute the ALL in the murine xenotransplant model. 21 of the 34 transplanted mice engrafted the leukaemia. Of note, both sorted cell populations from the samples 737c, 4540a and L784 reconstituted the original immunophenotype of the patient leukaemia. However, patient sample WB51 exhibited a different immunophenotypic engraftment pattern. While mice transplanted with $CD19^{+}CD34^{low}$ cells generated human blast subfractions with $CD19^{+}CD34^{low}$ as well as $CD19^{+}CD34^{high}$ immunophenotypes, mice transplanted with $CD19^{+}CD34^{high}$ did reconstitute the ALL with only $CD19^{+}CD34^{high}$ expressing cells. Table 9 summarises the experiments performed on human ALL blasts sorted according to CD34 surface marker expression.

Exp. No.	Patient / Leukemia	Injected Cell No.	Injected Cells	No. transplanted	No. engrafted
KR59	WB51 tert	CD34high	1.0 x 10^3	4	3*
		CD34low	1.0 x 10^3	4	4
KR60	737c sec	CD34high	1.0 x 10^3	4	4
		CD34low	1.0 x 10^3	4	4
KR61	4540a prim	CD34high	1.0 x 10^3	5	2
		CD34low	1.0 x 10^3	5	1
KR62	L784 prim	CD34high	1.0 x 10^4	4	4
		CD34low	1.0 x 10^4	4	2
Sum				34	21

Table 9: A list summarising xenotransplant experiments with ALL blasts sorted according to CD34 surface antigen expression.

4.4.2 Self-renewal capability across ALL blasts of different immunophenotypic maturity

In addition to the capability to generate the heterogeneous lineages of cells within a malignancy, cancer stem cells must be endowed with the ability to self-renew. Long term self-renewal of candidate leukaemic stem cells was assessed by transplanting sorted populations of the ALL over several generations of NSG mice. Human ALL cells engrafted in mouse bm after transplantation of diagnostic patient material was sorted into populations of different immunophenotypic maturity and re-transplanted into a secondary generation of mice. In case of positive engraftment of these animals, bm from mice that became ill from overt leukaemia was harvested again and a third generation of mice was transplanted after cells had been sorted once more. In the subchapters of 4.4.1, it was demonstrated that all leukaemic subpopulations sorted according to the B lineage development surface markers CD10, CD19 and CD20 or the stem cell marker of normal haematopoiesis CD34 carried the potential to re-constitute the ALL in mice in primary, secondary and tertiary generations of mice.

Self-renewal ability within normal haematopoiesis is progressively lost with later stages of the developmental hierarchy. If ALL was organised in a hierarchy, the stem cell characteristic of self-renewal would have to be confined to subpopulations of ALL cells and lost in others that had progressed in the hierarchy already. The surface marker CD20 is only expressed to a high degree at the level of immature B cells, the latest stage of B precursor cells. If the inability to self-renew would be an attribute found in subpopulations of ALL cells it would be expected in the one mirroring the immunophenotype of immature B cells. In order to investigate whether $CD20^{high}$ expressing lymphatic cells as well as their less mature $CD20^{low}$ expressing counterparts have the ability of long term self-renewal, serial transplantations were performed.

Human ALL cells from mouse M6 that initially was transplanted with unsorted cells of patient A67 (experiment KR2) were sorted according to their CD20 expression. All 3 mice transplanted with $CD19^{+}CD20^{low}$ as well as all 3 mice transplanted with $CD19^{+}CD20^{high}$ cells engrafted the leukaemia and reconstituted the original ALL immunophenotype pattern in experiment KR11 as already described in subchapter 4.4.1.2 and Figure 20. From this sorting experiment, secondary bm of one mouse (M43) with overt leukaemia reconstituted from the $CD19^{+}CD20^{high}$ subpopulation and bm of another mouse (M45) also presenting with leukaemia but reconstituted from the $CD19^{+}CD20^{low}$ population was again sorted into more immature $CD19^{+}CD20^{low}$ and more mature $CD19^{+}CD20^{high}$ candidate stem cell populations and re-transplanted into a third generation of mice (experiments KR28 and KR29). ALL blasts of both sorted populations were again able to reconstitute the full ALL with generation of subpopulations containing less and more mature $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cells. These sorting experiments over 3 generations of mice are demonstrated in Figure 24.

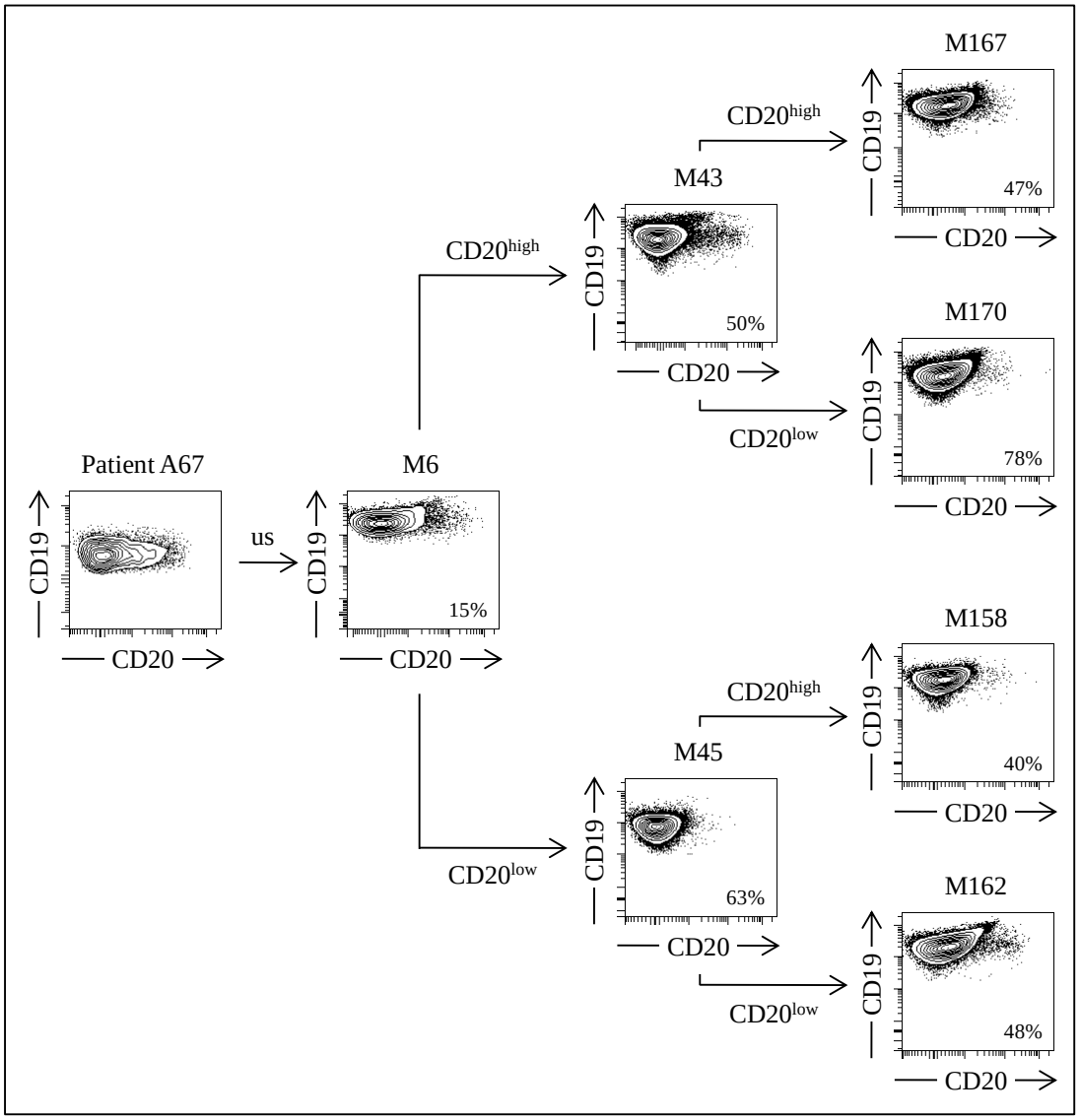


Figure 24: Self-renewal capability of immunophenotypically less mature CD19^{+}CD20^{low} and more mature CD19^{+}CD20^{high} cells (patient A67). ALL sample A67 was transplanted over 3 generations of mice. Candidate ALL stem cells with an immunophenotype resembling immature B cells (CD19^{+}CD20^{high}) or earlier B precursor stages (CD19^{+}CD20^{low}) both reconstitute the leukaemia in NSG mice to the original immunophenotype. Leukaemias engrafted from either subfraction can again be sorted into CD19^{+}CD20^{low} and CD19^{+}CD20^{high} blast subpopulations and once more successfully engraft new animals. This demonstrates extended self-renewal capability inherent in CD19^{+}CD20^{low} as well as CD19^{+}CD20^{high} ALL blast populations.

Sample WB51 was primarily sorted into CD19^{+}CD20^{low} and CD19^{+}CD20^{high} cell fractions and both reconstituted the ALL to the original immunophenotype (experiment KR17). In one secondary experiment (KR27) the leukaemia reconstituted in mouse bm

(M68) transplanted with the more mature $CD19^{+}CD20^{high}$ cells was sorted again regarding CD20 expression and both subfractions were transplanted into 3 new mice each. In this second generation of mice all engrafted the leukaemia and reconstituted the full immunophenotypic pattern of the patient ALL. Accordingly, another secondary experiment (KR34) used bm from mouse M73 which itself was transplanted with less mature primary $CD19^{+}CD20^{low}$ cells for sorting into $CD19^{+}CD20^{low}$ or $CD19^{+}CD20^{high}$ subfractions. Following transplantation of 4 mice for each subfraction resulted in engraftment of all but one mouse transplanted with $CD19^{+}CD20^{low}$ cells. Finally, a third generation of mice was transplanted with $CD19^{+}CD20^{low}$ or $CD19^{+}CD20^{high}$ subfractions sorted from bm of mouse M152, itself engrafted from $CD19^{+}CD20^{high}$ purified cells. In this experiment (KR45) two of 3 mice from each group injected with either $CD19^{+}CD20^{low}$ or $CD19^{+}CD20^{high}$ human subpopulations again reconstituted human ALL cells of the diverse original immunophenotypes.

Bone marrow from mouse M68 (sample WB51), primarily transplanted with $CD19^{+}CD20^{high}$ cells, additionally was used for another secondary transplantation experiment and following this for a tertiary xenotransplantation. In experiment KR39 unsorted bm cells harvested from mouse M68 were used and resulted in reconstitution of the original patient ALL in 4 of 4 mice transplanted with 1.0×10^3 cells. Harvested bm from this secondary transplantation (M272) was then sorted into $CD19^{+}CD34^{low}$ and $CD19^{+}CD34^{high}$ subpopulations and re-transplanted with 1.0×10^3 cells per mouse in groups of 4. One mouse of the group transplanted with $CD19^{+}CD34^{high}$ purified cells could not be analysed for human engraftment due to unexpected early death but all other 7 mice engrafted the ALL.

The serial transplantation experiments over 3 mouse generations with cells of sample A67 and WB51 sorted into subfractions of divergent immunophenotypic maturity ($CD19^{+}CD20^{low}$ or $CD19^{+}CD20^{high}$ expression profile) conclusively demonstrated, that

the defining characteristic of cancer stem cells, extended ability to self-renew, is not limited to earlier developmental stages but can be demonstrated equally in advanced stages of the B lineage.

4.4.3 Frequency of leukaemic stem cells in ALL

In several previous studies, prevalence of ALL cancer stem cells was determined to be restricted to rare and immunophenotypically immature blast populations. Furthermore, of these already rare cells within the bulk leukaemia a still relatively high number had to be transplanted in xenotransplant mouse models to result in positive engraftment. Later studies and the work presented in this thesis led to controversial results by showing that blasts resembling more advanced stages of B cell development, up to the level of immature B cells, were also able to transfer the leukaemia onto mice. If LICs are not restricted to rare subpopulations but also can be found in cell populations that form the bulk tumour mass, it is self evident that the frequency of cells with stem cell activity must be higher than previously thought. Therefore, the frequency of LICs in ALL was assessed by transplanting unsorted as well as sorted blast populations in limiting dilution experiments down to only 10 cells per mouse.

4.4.3.1 Frequency of leukaemic stem cells in unsorted ALL cells

First, the frequency of LICs in unsorted bulk ALL cell populations was assessed. The detection of stem cell activity within blast populations of different maturity according to the immunophenotype described in 4.4.1 and 4.4.2 suggested a higher stem cell frequency in unsorted ALL blasts than determined previously. In order to investigate whether or not that was the case, primary diagnostic patient material as well as secondary and tertiary samples were used for unsorted limiting dilution experiments.

Altogether 9 different primary samples (L876 (KR53), 737c (KR44), L858 (KR35), M120 (KR46), 758b (KR49), 2003 (KR57), 4917 (KR58), HV101 (KR32) and A67 (KR52)) were intrafemorally injected into NSG mice in cell numbers of 1.0×10^3, 1.0×10^2 and 1.0×10^1 cells per mouse. In 4 cases (737c, M120, 758b and A67) one group of mice additionally was transplanted with 1.0×10^4 cells per mouse. All mice transplanted with 1.0×10^4 cells and available for at least one bm analysis showed engraftment of the ALL. When 1.0×10^3 unsorted blasts were transplanted into mice, the 9 primary patient samples resulted in engraftment of all (737c, L876) or a fraction of transplanted mice (L858, M120, 758b, 2003 and 4917). For samples 758b, 2003 and 4917 transplantation of 1.0×10^3 cells additionally was the limiting cell dose for leukaemia reconstitution. Mice transplanted with 100 or 10 cells without exception failed to show human chimerism in any bm analysis. Patient sample HV101 was injected with 5.0×10^2 instead of 1.0×10^3 unsorted cells per mouse as the maximum dose only and this resulted in engraftment of two out of 4 transplanted mice. In this experiment 1.0×10^2 (4 mice) or 1.0×10^1 (4 mice) injected cells also failed to engraft any of the NSG mice, therefore establishing 5.0×10^2 as the limiting cell dose. For the primary samples 737c, L876, L858, M120 and A67 engraftment of mice was achieved with 1.0×10^2 transplanted cells. However, for all of these samples this was also the limiting cell dose as no mouse transplanted with 1.0×10^1 cells only showed engraftment. Engraftment rates with the limiting cell dose varied between samples. While 737c and A67 each engrafted all 5 transplanted mice, L876 engrafted 4 out of 5, L858 two out of 5 and M120 one out of 5 transplanted mice. The calculated LIC frequencies for the primary samples determined to be between one in approximately 40 to one in 3×10^3.

Two patient samples (WB51 (KR39 and KR40) and HV101 (KR54)) were transplanted unsorted in limiting dilution after one passage through mice. For sample WB51 two separate limiting dilution experiments were performed. The first (KR39) used unsorted

bm cells from M68, a mouse that initially was transplanted with $CD19^{+}CD20^{high}$ sorted primary cells and terminally engrafted to 86% human chimerism. From this limiting dilution all 4 mice transplanted with 1.0×10^3 cells and all 5 mice transplanted with 1.0×10^2 cells successfully reconstituted the human ALL. Of the 5 mice injected with 1.0×10^1 one died before a first bm analysis was performed but of the remaining 4 mice two still successfully engrafted. In the bm of one of the mice that was counted as not engrafted (M281), a cluster of cells was detected in the terminal bm flow cytometry analysis that resembled cells of human origin. However, the acquired cell number was too low for this sample to be counted as successfully engrafted (positive outside limits, see section 3.4.6). The second limiting dilution on secondary cells from WB51 (KR40) used unsorted cells from M65, a mouse initially transplanted with unsorted primary cells and a terminal engraftment level of 81%. Of the 3 mice transplanted with 1.0×10^3 cells one died before the first bmp but both remaining mice engrafted. 5 mice were transplanted with 1.0×10^2 cells and in this group all animals reconstituted the ALL. Another 5 mice were transplanted with 1.0×10^1 cells. One mouse of that group could also not be analysed for bm engraftment due to early death but 3 of the 4 remaining mice engrafted human cells. HV101 was the other sample used to transplant unsorted secondary cells in limiting dilution. Cells for this experiment came from harvested bm of mouse M187 which itself was transplanted with purified $CD19^{+}CD20^{low}$ expressing cells and exhibited human chimerism of 74% at terminal analysis. Four mice were transplanted with 1.0×10^3 unsorted cells and 5 mice each with 1.0×10^2 and 1.0×10^1 cells respectively. All but one mouse of the group transplanted with 1.0×10^1 cells only repopulated the NSG mice with human ALL cells. Again, the one not engrafted mouse (M468) did show signs of engraftment but failed to match the criteria to be counted as positively engrafted. Unlike in the experiment performed with diagnostic patient material (KR32), 1.0×10^2 as well as 1.0×10^1 unsorted cells were capable of

transferring the leukaemia onto mice. However, the experiments are difficult to compare as the primary patient sample was transplanted with 5.0 x 10^2 unsorted cells per mouse as the maximum cell dose and not 1.0 x 10^3. Contour dot plots for the secondary unsorted limiting dilution experiment on sample HV101 are depicted in Figure 25.

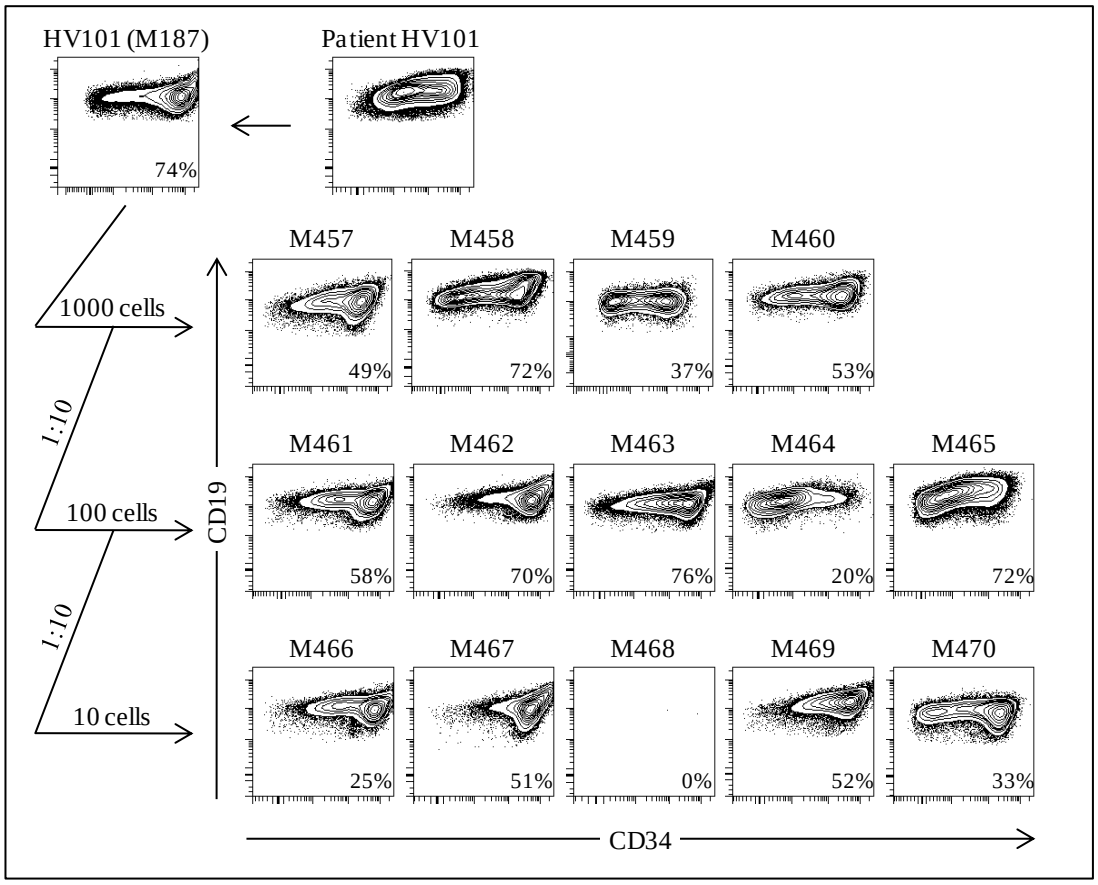


Figure 25: Secondary unsorted limiting dilution experiment on sample HV101. Mouse M187 was initially transplanted with $CD19^+CD20^{low}$ cells of diagnostic patient sample HV101. Unsorted bone marrow from this mouse subsequently was transplanted on secondary mice in limiting dilutions of 1.0 x 10^3, 1.0 x 10^2 and 1.0 x 10^1 cells per mouse. All mice transplanted with 1.0 x 10^3 and 1.0 x 10^2 cells reconstituted the original patient ALL. Of the 5 mice transplanted with 1.0 x 10^1 cells per mouse one failed to engraft. Bone marrow of the other 4 mice exhibited an immunophenotypic expression pattern comparable to that of bm from mice transplanted with higher cell numbers and the original patient material.

The only tertiary limiting dilution experiment with unsorted cells was performed with sample A67 (KR41, bm of mouse M130, terminal engraftment of 73%). Before being used for limiting dilution, the sample was transplanted over two generations of mice, both times without prior sorting. The 3 mice transplanted with 1.0×10^3 and all 5 mice transplanted with 1.0×10^2 unsorted cells reconstituted the ALL in mice and 1.0×10^1 cells were sufficient to initiate the leukaemia in 3 out of 5 transplanted mice. As in primary and secondary limiting dilution assays, less than 1.0×10^1 cells were not transplanted. While the limiting cell number in the experiment (KR52) with the primary patient sample A67 was determined to be 1.0×10^2 cells, the tertiary A67 sample was capable to transfer the ALL on mice when only 1.0×10^1 cells were transplanted.

Table 10 summarises all limiting dilution experiments carried out with unsorted blast populations and provides calculated LIC frequencies.

Patient ID	Risk group	Transplant	Transplanted blast population	Number of cells transplanted (number of mice engrafted/number of mice transplanted)			LIC frequency (ELDA)
				1000	100	10	
737c	High risk (BCR/ABL1 like)	Primary	Unsorted	4/4	5/5	0/5	42.2
L876	High risk t(4;11)	Primary	Unsorted	4/4	4/5	0/5	77.5
L858	High risk (MRD high)	Primary	Unsorted	4/5	2/5	0/5	463
M120	High risk t(9;22)	Primary	Unsorted	1/4	1/5	0/5	1880
758b	High risk (BCR/ABL1 like)	Primary	Unsorted	2/3	0/5	0/5	1203
2003	High risk t(9;22)	Primary	Unsorted	1/3	0/4	0/5	2922
4917	Standard risk	Primary	Unsorted	1/3	0/4	0/5	2922
WB51	High risk t(9;22)	Secondary	Unsorted	6/6[#]	10/10[#]	5/8[#]	10.69
HV101	High risk t(9;22)	Primary	Unsorted	2/4[*]	0/4	0/4	949
		Secondary	Unsorted	4/4	5/5	4/5	6.73
A67	High risk t(9;22)	Primary	Unsorted	4/4	5/5	0/5	42.2
		Tertiary	Unsorted	3/3	5/5	3/5	11.40

Table 10: A list of all primary, secondary and tertiary ALL samples transplanted in unsorted limiting dilution experiments. While the limiting cell dose for primary patient material was 1.0×10^2 or 1.0×10^3 cells, the two secondary and the tertiary limiting dilution engrafted mice even when only 1.0×10^1 cells were transplanted orthotopically. The frequencies of ALL LICs in unsorted blast populations were calculated using the ELDA programme.

[*] mice were transplanted with 5.0×10^2 cells per mouse. [#] combined engraftment numbers of two separate experiments.

Unsorted limiting dilution experiments were as well performed on sample MS45. As for the experiments with sorted cell populations, mice transplanted from this diagnostic patient sample never showed any signs of engraftment during the observation period of 36 weeks.

4.4.3.2 Frequency of leukaemic stem cells in $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ ALL subpopulations

The xenotransplantation experiments performed with ALL blasts highly purified according to their CD20 surface antigen expression level described in subchapter 4.4.1.2 showed that both the immunophenotypically less mature $CD19^{+}CD20^{low}$ as well as the more mature $CD19^{+}CD20^{high}$ cell population in principle were able to reconstitute the original leukaemia in NSG mice. Additionally, limiting dilution assays with unsorted ALL cells demonstrated that between 1×10^{3} and 1×10^{1} cells only can be sufficient to transfer the ALL onto mice. The earlier CD20 sorting experiments were transplanted with cell numbers between 3.06×10^{3} and 6.24×10^{4} cells per mouse and therefore all in cell doses higher than the limiting cell dose for engraftment of unsorted ALL blast populations. In order to investigate the limiting cell doses for engraftment of $CD19^{+}CD20^{low}$ and the $CD19^{+}CD20^{high}$ cell fractions respectively and to determine whether or not ALL stem cells are present at the same frequency in the less and more mature populations, limiting dilution experiments were performed transplanting purified $CD19^{+}CD20^{low}$ and the $CD19^{+}CD20^{high}$ cells.

The first limiting dilution on CD20 sorted cells used a secondary WB51 sample (experiment KR27). Mouse M68 initially was transplanted with $CD19^{+}CD20^{high}$ diagnostic patient cells and engrafted in bm to 86% as determined by flow cytometry analysis. Recovered bone marrow of this mouse was purified into $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ expressing blast populations and subsequently these were transplanted each in cell numbers of 2.0×10^{3}, 5.0×10^{2} and 1.0×10^{2} into groups of 3 mice. 10 weeks after the start of the experiment, mice transplanted with doses of 2.0×10^{3} showed engraftment in all 3 mice transplanted with $CD19^{+}CD20^{low}$ cells (mean engraftment 12.3%) and also in all 3 mice transplanted with $CD19^{+}CD20^{high}$ cells (mean engraftment 15.9%). All mice transplanted with 5.0×10^{2} sorted cells also engrafted with mean

engraftment levels of 8.9% for mice transplanted with the $CD19^{+}CD20^{low}$ and of 28.0% for mice transplanted with the $CD19^{+}CD20^{high}$ subpopulation. Two of the 3 mice transplanted with 1.0 x 10^2 $CD19^{+}CD20^{low}$ cells engrafted to bm levels of 0.04% and 3.0% but only two mice transplanted with 1.0 x 10^2 $CD19^{+}CD20^{high}$ cells were alive at the time point of the first bmp at which none of them showed human chimerism. However, both mice showed engraftment of 0.04% and 0.3% respectively at bmp2, 15 weeks after transplantation and the third mouse of the group transplanted with 1.0 x 10^2 $CD19^{+}CD20^{low}$ cells was first positive for human cells at a level of 0.2% after 21 weeks at bmp3. From the group transplanted with 2.0 x 10^3 $CD19^{+}CD20^{high}$ cells a terminal bm harvest could be carried out only on one mouse (M143). Flow cytometry on bm revealed a human chimerism of 76.5%. Mice 144 and M145 of the same group were found dead in the weeks after bmp1 and bmp2 respectively when bm engraftment levels were still low. Of the group transplanted with 2.0 x 10^3 $CD19^{+}CD20^{low}$ cells mice M140 and M141 were harvested after 15 and 19 weeks *post* bone marrow transplantation with bm engraftment levels of 18.0% and 28.5% respectively. Mouse M142 died after bmp2 had revealed 3.9% human cells. However, all mice transplanted with 5 x 10^2 cells of either group survived until they became ill from overt leukaemia. Experiments with mice transplanted with $CD19^{+}CD20^{low}$ cells were terminated after 18 weeks (M150) and 20 weeks (M149 and M151) and terminal bm engraftment levels were determined to be 78.4%, 82.1% and 79.5% respectively with a mean engraftment of 80.0%. The group of mice transplanted with $CD19^{+}CD20^{high}$ cells was harvested slightly earlier after 18 weeks (M146 and M148) and 20 weeks (M147) with lower engraftment levels of 67.7%, 31.6% and 66.6% respectively and a mean engraftment of 55.3%. Two mice each from the groups that received 1.0 x 10^2 cells only could be analysed regarding terminal engraftment of human cells. M152 and 154, transplanted with the more mature blast population, were harvested after 31 and 25 weeks with engraftment levels of 46.7% and

39.4% and mice M156 and M157, transplanted with more immature blasts, engrafted to 42.9% and 70.7% after 19 and 33 weeks respectively. One mouse of each group died relatively early in the course of the experiment. M155 of the $CD19^{+}CD20^{low}$ injected group died unexpectedly after only 0.22% human ALL cells were detected in bmp2 after 15 weeks and M153, injected with $CD19^{+}CD20^{high}$ cells, was found dead 6 weeks after transplantation. Reliable *post mortem* analysis of the bm was not possible due to extensive cell decay but flow cytometry analysis of a single cell suspension of the spleen revealed a small cluster of events of likely human origin. However, the previously set thresholds were not met and the mouse could not be counted as positively engrafted. Dot plots of all mice transplanted in this first limiting dilution experiment with cells sorted according to CD20 expression level are illustrated in Figure 26.

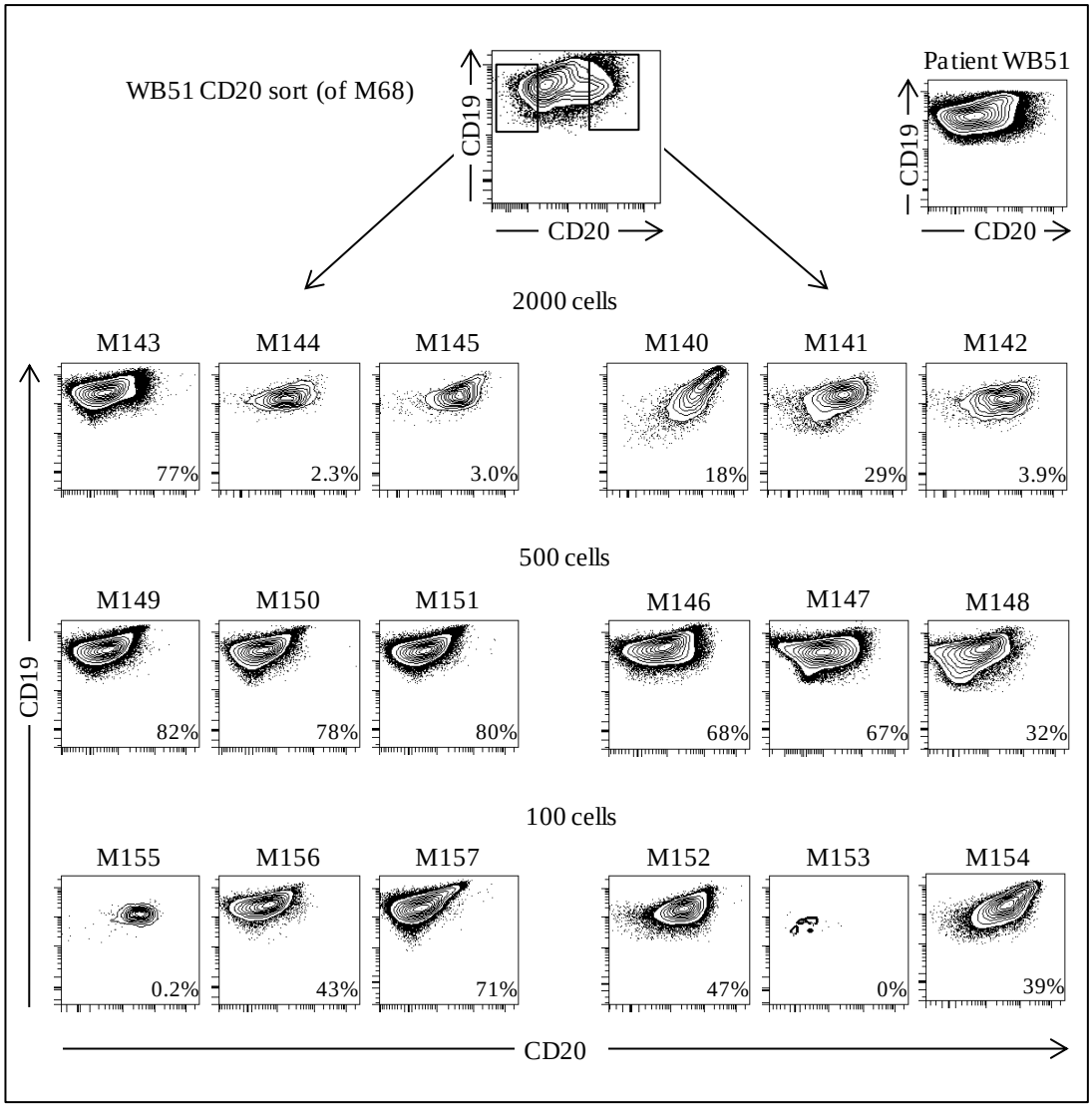


Figure 26: Limiting dilution experiment on secondary sorted $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cells of sample WB51. Mouse M68 was initially transplanted with $CD19^{+}CD20^{high}$ purified cells of diagnostic patient bm sample WB51. Recovered bm of this mouse sorted into $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ subfractions subsequently was transplanted on secondary mice in limiting dilutions of 2.0×10^3, 5.0×10^2 and 1.0×10^2 cells per mouse. All mice transplanted with 2.0×10^3 and 5.0×10^2 cells reconstituted the original patient ALL. Of the 3 mice transplanted each with 1.0×10^2 $CD19^{+}CD20^{low}$ cells all engrafted the ALL. One mouse transplanted with 1.0×10^2 $CD19^{+}CD20^{high}$ cells died early in the course of the experiment before possible engraftment, while the two other mice of that group did show engraftment. Bone marrow of all engrafted mice exhibited an immunophenotypic expression pattern comparable to that of the sorted bm from mouse M68 and the original patient material.

Another two leukaemias (2510 and 737c) were transplanted in sorted secondary limiting dilution experiments, KR33 and KR55 respectively. Mouse M30 was initially transplanted with 1.3 x 10^6 bm cells of diagnostic patient sample 2510 and engrafted to 56.5% human chimerism in the mouse bm from the terminal harvest (KR7). This bm was sorted according to CD20 surface antigen expression and $CD19^+CD20^{low}$ and $CD19^+CD20^{high}$ purified cell fractions were both retransplanted with 3.0 x 10^3 and 3.0 x 10^2 cells in groups of 4 mice each. 3.0 x 10^3 $CD19^+CD20^{low}$ cells reconstituted the ALL in all 4 transplanted mice with terminal engraftment levels of 68% (M206), 21% (M207), 28% (208), 51% (M209) and a mean engraftment of 42%. Of the group transplanted with 3.0 x 10^2 cells of this population only one successfully engrafted to a maximum bm level of 56% human cells in bmp2. Mice that received the $CD19^+CD20^{high}$ subfraction engrafted two of the 4 mice transplanted with 3.0 x 10^3 cells to levels of 67% (M203) and 47% (M204) and none of the 4 mice transplanted with 3.0 x 10^2 cells. The other leukaemia sample used for a secondary CD20 sort and limiting dilution transplantation was 737c. Recovered bm from mouse M315, originally transplanted with 1.5 x 10^3 $CD19^+CD20^{low}$ purified diagnostic patient cells (KR42) and engrafted to 69% human chimerism in the terminal bm analysis was sorted into $CD19^+CD20^{low}$ and $CD19^+CD20^{high}$ subpopulations. Of either fraction 1.0 x 10^3 and 1.0 x 10^2 cells were injected into 4 secondary mice and 1.0 x 10^1 cells were injected into 5 secondary mice each. Mice transplanted with $CD19^+CD20^{low}$ cells engrafted two of the 4 mice that received a cell dose of 1.0 x 10^3 to final engraftment levels of 40% (M475) and 54% (M478) all other mice failed to reconstitute the leukaemia. Mice transplanted with $CD19^+CD20^{high}$ cells reconstituted the ALL in all 4 mice injected with 1.0 x 10^3 cells to final engraftment levels of 25% (M471), 67% (M472), 60% (M473) and 65% (M474) with a mean engraftment of 54% and in two of the 4 mice injected with 1.0 x 10^2 cells to

final engraftment levels of 71% (M481) and 64% (M482) while mice injected with 1.0 x 10^1 did not result in a measurable chimerism.

Additionally, tertiary cells of ALL WB51 were transplanted in numbers of 5.0 x 10^2 and 1.0 x 10^1 cells of either $CD19^+CD20^{low}$ or $CD19^+CD20^{high}$ purified cells into 3 and 4 mice each respectively (KR45). The preceding primary as well as the secondary mouse was transplanted with $CD19^+CD20^{high}$ sorted cells and engrafted to 86% (M68, KR17, injected with 1.08 x 10^4 cells) 47% (M152, KR27, injected with 1.0 x 10^2 cells). The 3 mice transplanted with 5.0 x 10^2 $CD19^+CD20^{low}$ cells showed terminal engraftment levels of 65% (M346), 36% (M347) and 77% (M348) with a mean engraftment of 59% and the 3 mice transplanted with 5.0 x 10^2 $CD19^+CD20^{high}$ had engraftment levels of 1.0% (M343), 0.08% (M344) and 0.3% (bmp3 of M345) with a mean engraftment of 0.46%. Mice transplanted with 1.0 x 10^1 cells only of either purified subfraction did not reconstitute human cells in the bm.

Despite some inter-patient variability the secondary and tertiary limiting dilution xenotransplantation experiments on ALL blasts sorted according to the expression of the surface marker CD20 indicate that leukaemic stem cells are present at the same frequency in the less and more mature blast $CD19^+CD20^{low}$ and the $CD19^+CD20^{high}$ populations respectively. This LSC frequency is also comparable to the frequency in unsorted blasts. This is summarised in Table 11.

Patient ID	Risk group	Transplant	Trans-planted blast population	Number of cells transplanted (number of mice engrafted/number of mice transplanted)				LIC frequency (ELDA)
				1000-3000	300-500	100	10	
737c	High risk (BCR/ABL 1 like)	Secondary	CD20high	4/4	-	2/4	0/5	165.8
			CD20low	2/4	-	0/4	0/5	1676
2510	High risk t(9;22)	Secondary	CD20high	2/4	0/4		-	4950
			CD20low	4/4	1/4		-	780
WB51	High risk t(9;22)	Secondary	CD20high	3/3	3/3	2/2	-	>49.6
			CD20low	3/3	3/3	3/3	-	>308
		Tertiary	CD20high	-	3/3	-	0/4	137.5
			CD20low	-	3/3	-	0/4	137.5

Table 11: A list of secondary and tertiary ALL samples transplanted in limiting dilution experiments of $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cells. The limiting cell dose for secondary and the tertiary transplant experiments of $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cells was determined to lie between 1.0 and 5.0 x 10^{2} cells. The frequencies of ALL LICs in the sorted blast populations were calculated using the ELDA programme.

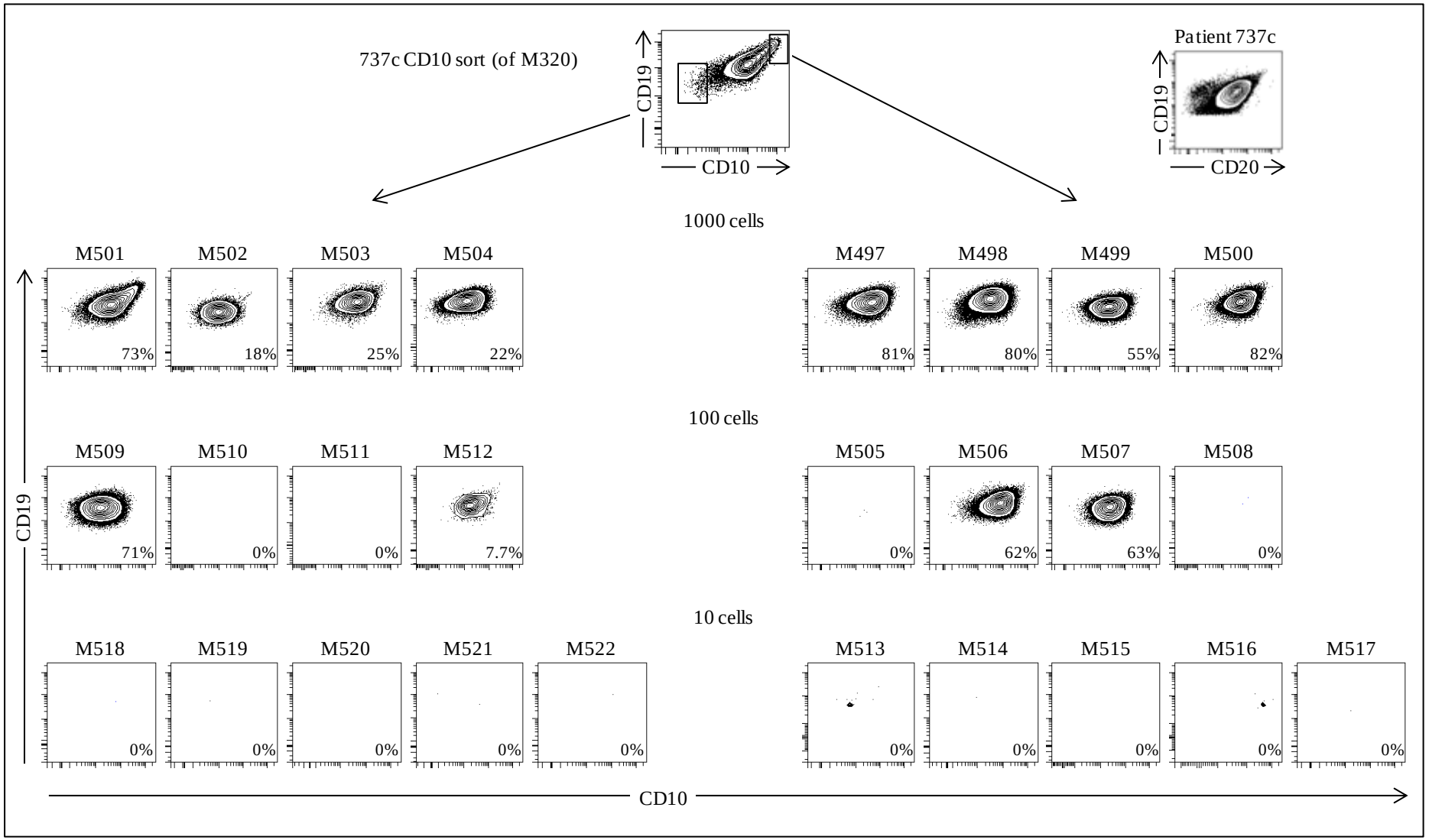
4.4.3.3 Frequency of leukaemic stem cells in $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ ALL subpopulations

The CD10 surface antigen is first expressed on pre-B-I cells in the B lineage and stays positive through all further developmental stages including immature B cells (see subchapters 1.3.3.1, 1.3.3.2 and Figure 3). Cell characterisation according to CD10 expression therefore separates the early, less differentiated pro-B cells from all following more mature B precursor cells which have a less pronounced proliferative potential than their predecessor. Experiments with $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ purified cell populations transplanted in numbers exceeding 1,000 cells per mouse described in subchapter 4.4.1.3 showed that blasts with high expression of CD10 generally were not inferior in the ability to repopulate NSG mice with the ALL than immunophenotypically less mature blasts with no or only low expression of CD10. However, in order to compare the stem cell frequencies in subpopulations of $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ expressing cells with each other as well as with that

of unsorted bulk leukaemic populations, a limiting dilution xenotransplantation experiment was performed on secondary CD10 sorted cells from sample 737c (KR56). $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ purified cells of bm recovered from mouse M320, initially transplanted with 1.5 x 10^3 $CD19^{+}CD10^{high}$ cells and engrafted to 42% human chimerism in the terminal bm analysis were transplanted into NSG mice with 1.0 x 10^3 cells per mouse (n=4), 1.0 x 10^2 cells (n=4) and 1.0 x 10^1 cells (n=5). The first bmp 8 weeks after transplantation identified human ALL cells in two out of 4 mice that received 1.0 x 10^3 $CD19^{+}CD10^{low}$ cells at levels of 0.42% (M502) and 0.09% (M503) and in 3 out of 4 mice that received the same number of $CD19^{+}CD10^{high}$ cells at levels of 4.1% (M497), 16% (M 498) and 0.73% (M500) with a mean engraftment of 6.9%. No human cells could be detected in mice transplanted with less cells of either cell fraction at the time point of bmp1. However, one mouse transplanted with 1.0 x 10^2 $CD19^{+}CD10^{low}$ cells unexpectedly died before the first bm analysis. In the second bmp 7 weeks later, 3 of the 4 mice transplanted with 1.0 x 10^3 $CD19^{+}CD10^{low}$ cells and all 4 mice transplanted with 1.0 x 10^3 $CD19^{+}CD10^{high}$ cells were engrafted with mean engraftment levels of 16% and 70% respectively. Additionally two mice each were engrafted of both groups transplanted with 1.0 x 10^2 cells. Mice that received $CD19^{+}CD10^{low}$ cells engrafted to 2.3% (M509) and 0.01% (M512) and mice that received $CD19^{+}CD10^{high}$ cells to 0.02% (M506) and 0.13% (M507) human chimerism. Another 8 weeks later, the fourth mouse transplanted with 1.0 x 10^3 $CD19^{+}CD10^{low}$ cells also showed human cell engraftment at a level of 0.3% (M504). No further mouse transplanted with 1.0 x 10^2 cells or any of the 10 mice transplanted with 1.0 x 10^1 cells showed human engraftment in the observation period up to 46 weeks. Engraftment levels at terminal bm harvest or the last available bmp for mice transplanted with 1.0 x 10^3 cells of the $CD19^{+}CD10^{low}$ subfraction were 73% (M501), 18% (M502), 25% 8M503) and 22% (M504) with a mean engraftment of 35%. Of mice transplanted with

1.0 x 10^3 $CD19^+CD10^{high}$ cells engraftment levels were 81% (M497), 80% (M498), 55% (M499) and 82% (M500) and the mean engraftment level was 75%. Highest engraftment levels for mice transplanted with 1.0 x 10^2 $CD19^+CD10^{low}$ cells were 71% (M509) and 7.7% (M512) and for mice transplanted with 1.0 x 10^2 $CD19^+CD10^{high}$ cells 62% (M506) and 63% (M507) respectively. Both fractions were able to reconstitute the ALL in NSG mice when 1.0 x 10^2 cells only were transplanted but neither cell fraction resulted in human chimerism when individual mice received 1.0 x 10^1 cells. Similar to the findings in the limiting dilution experiments with unsorted blasts and ALL blasts sorted with respect to expression of CD20, the limiting dilution experiment with secondary cells sorted according to CD10 expression does not suggest, that the frequency of LSCs is different in the two subfractions. Figure 27 depicts all contour dot plots and Table 12 summarises the results of the limiting dilution experiment on CD10 sorted cells from patient 737c.

Figure 27 (page 206): Limiting dilution experiment on secondary sorted $CD19^+CD10^{low}$ and $CD19^+CD10^{high}$ cells of sample 737c. Mouse M320 was initially transplanted with $CD19^+CD10^{high}$ purified cells of diagnostic patient bm sample 737c. Recovered bm of this mouse sorted into $CD19^+CD10^{low}$ and $CD19^+CD10^{high}$ subfractions subsequently was transplanted on secondary mice in limiting dilutions of 1.0 x 10^3, 1.0 x 10^2 and 1.0 x 10^1 cells per mouse. All mice transplanted with 1.0 x 10^3 cells of either subfraction reconstituted the original patient ALL. In the groups transplanted with 1.0 x 10^2 cells per mouse 2 of 4 mice each engrafted the ALL. None of the mice that received only 1.0 x 10^1 cells of $CD19^+CD10^{low}$ or $CD19^+CD10^{high}$ sorted cells did show any engraftment. Bone marrow of the engrafted mice exhibited an immunophenotypic expression pattern comparable to that of the sorted bm from mouse M320 and the original patient material.


737c CD10 sort (of M320)
CD19
CD10
Patient 737c
CD19
CD20
1000 cells
M501
73%
M502
18%
M503
25%
M504
22%
M497
81%
M498
80%
M499
55%
M500
82%
100 cells
M509
71%
M510
0%
M511
0%
M512
7.7%
M505
0%
M506
62%
M507
63%
M508
0%
10 cells
M518
0%
M519
0%
M520
0%
M521
0%
M522
0%
M513
0%
M514
0%
M515
0%
M516
0%
M517
0%
CD19
CD10

Patient ID	Risk group	Transplant	Transplanted blast population	Number of cells transplanted (number of mice engrafted/number of mice transplanted)			LIC frequency (ELDA)
				1000	100	10	
737c	High risk (BCR/ABL1 like)	Secondary	CD10high	4/4	2/4	0/5	165.8
			CD10low	4/4	2/3	0/5	118.1

Table 12: A table summarising the results of the secondary limiting dilution experiment of $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ cells on ALL sample 737c. The limiting cell dose for this secondary transplant experiment of $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cells was determined to be 1.0×10^{2} cells for both subpopulations. The frequencies of ALL LICs in the sorted blast populations were calculated using the ELDA programme.

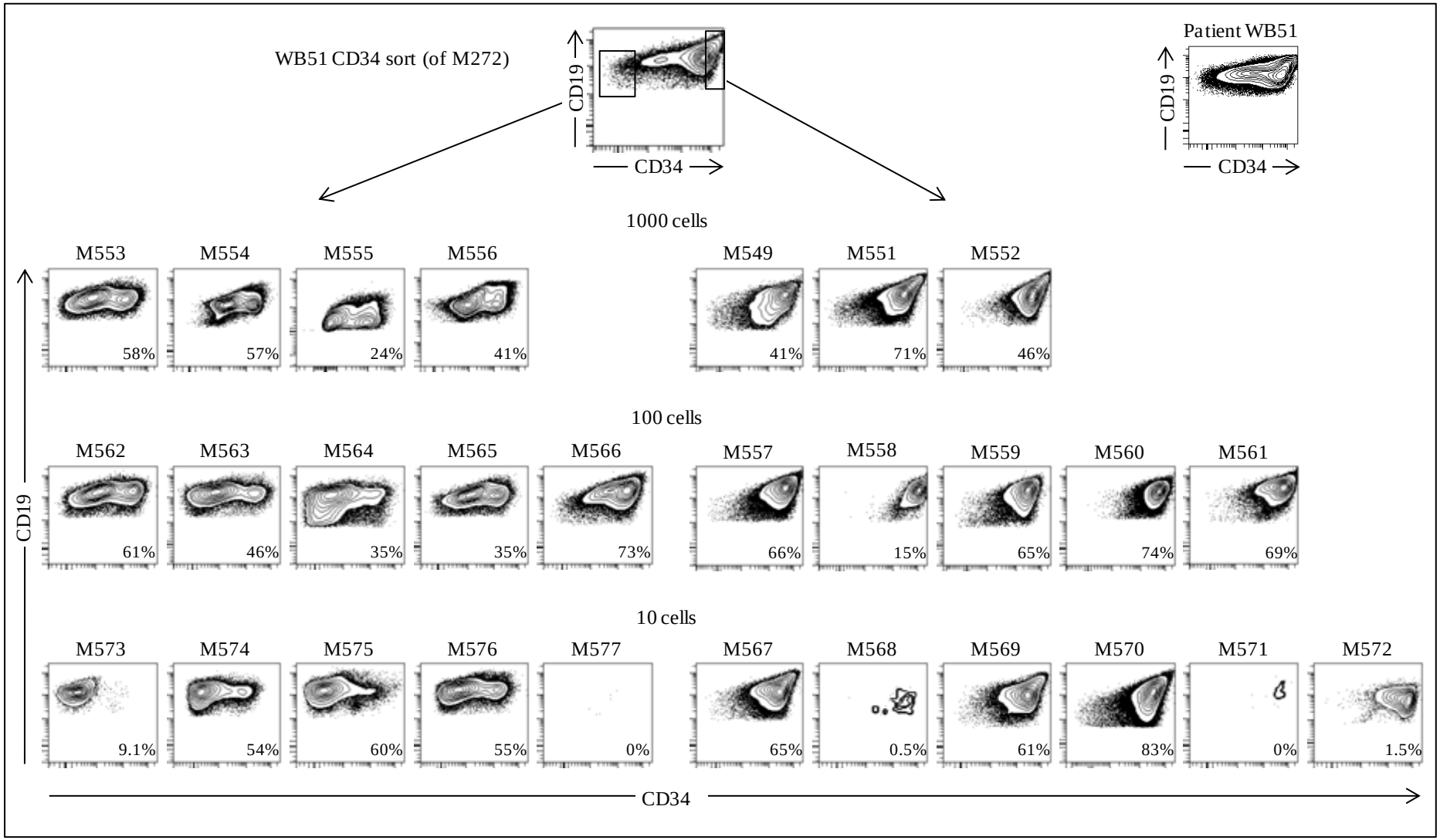
4.4.3.4 Frequency of leukaemic stem cells in $CD19^{+}CD34^{low}$ and $CD19^{+}CD34^{high}$ ALL subpopulations

In normal bm CD34 expressing cells represent only a minor fraction of all haematopoietic cells but these cells constitute the apex of the haematopoietic hierarchy, endowed with extensive proliferative and self-renewal capacity which consecutively is lost on down-regulation of this surface marker. In the B lineage of lymphoid development, CD34 is present on the cell surface of pro-B cells and pre-B I cells but lost on the transition to pre-B II large cells as described in section 1.3.3.1 and Figure 3. It also has been shown that $CD34^{+}$ cell subfractions in AML are highly enriched in stem cells and some studies claim the same for ALL. Transplanting 1.0×10^{3} or more $CD19^{+}CD34^{high}$ or $CD19^{+}CD34^{low}$ purified ALL blasts led to engraftment in NSG mice after orthotopic injection as described in subchapter 4.4.1.4 above but this allows for no conclusion regarding the frequency of stem cells within the different cell fractions. In order to determine whether or not in ALL LICs are enriched in CD34 expressing cell fractions, $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ sorted blast populations were transplanted in limiting dilutions.

The first limiting dilution experiment with CD34 sorted cells was performed on a tertiary WB51 bm sample recovered from mouse M272. This mouse had a final human bm chimerism of 66% after it was transplanted with 1.0 x 10^3 unsorted cells of mouse M68 which itself was injected with 1.08 x 10^4 $CD19^+CD20^{high}$ cells in a primary sorting experiment and engrafted to a bm level of 86%. $CD19^+CD34^{high}$ or $CD19^+CD34^{low}$ purified cell populations were transplanted into groups of NSG mice with 1.0 x 10^3 (n=4), 1.0 x 10^2 (n=5) and 1.0 x 10^1 (n=5) cells respectively. One mouse of the group transplanted with 1.0 x 10^3 $CD19^+CD34^{high}$ cells was lost for analysis due to early death before bmp1 at 9 weeks after the start of the experiment but the remaining 3 were already engrafted to levels of 11% (M549), 1.2% (M550) and 31% (M552) with a mean engraftment of 43%. All 4 mice transplanted with 1.0 x 10^3 $CD19^+CD34^{low}$ cells were as well engrafted to 4.8% (M553), 4.7% (M554), 0.3% (M555) and 0.5% (M556) with a mean engraftment of 2.6%. Low bm engraftment was also found in both groups transplanted with 1.0 x 10^2 cells. Mice M557 and M558 from the $CD19^+CD34^{high}$ transplanted group reconstituted 0.16% and 0.04% human cells in mouse bm and mice M562, M564 and M566 from the $CD19^+CD34^{low}$ transplanted group 0.67%, 0.1% and 0.08% (mean engraftment 0.28%) respectively. Mice receiving 1.0 x 10^1 sorted cells only led to low level engraftment at bmp1 in the group transplanted with $CD19^+CD34^{high}$ but not with $CD19^+CD34^{low}$ cells. Mouse M568 showed 0.4% human cells in flow cytometry and M569 0.13%. At the time of bmp2, 3 weeks later, bm of all 3 mice (M559, M560 and M561) transplanted with 1.0 x 10^2 $CD19^+CD34^{high}$ cells that were not engrafted at bmp1 contained human cells while no further mouse transplanted with 1.0 x 10^2 $CD19^+CD34^{low}$ cells was engrafted. In the groups transplanted with 1.0 x 10^1 cells two more mice (M567 and M570) reconstituted human blasts from $CD19^+CD34^{high}$ cells and a first mouse (M573) also from $CD19^+CD34^{low}$ cells. Another 6 weeks later (bmp3), one additional mouse (M565) engrafted in the group transplanted with 1.0 x 10^2

$CD19^{+}CD34^{low}$ cells and one each (M572 and M574) transplanted with 1.0 x 10^{1} $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ cells respectively. After 23 weeks (bmp4) the last mouse (M563) that received 1.0 x 10^{2} $CD19^{+}CD34^{low}$ cells was engrafted and one more (M575) that received 1.0 x 10^{1} $CD19^{+}CD34^{low}$ cells. In the final bmp, 32 weeks *post* transplantation, engraftment was detected in a fourth mouse (M576) transplanted with 1.0 x 10^{1}. Mean engraftment levels calculated from terminal bm harvests or a bmp in case bm from a harvest was not available, were 54% and 45% for groups transplanted with 1.0 x 10^{3}, 58% and 56% for groups transplanted with 1.0 x 10^{2} and 53% each for groups transplanted with 1.0 x 10^{1} $CD19^{+}CD34^{high}$ or $CD19^{+}CD34^{low}$ purified cell populations respectively. In this tertiary limiting dilution experiment, the limiting cell dose that was able to reconstitute the human ALL in mice was 1.0 x 10^{1} cells for both sorted subfractions. Despite both the $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ cell populations being able to transfer the leukaemia onto NSG mice, there was a difference in the pattern of immunophenotypic reconstitution. While $CD19^{+}CD34^{low}$ purified ALL cells led to an engrafted ALL immunophenotype resembling that of the sorted and transplanted sample and the original patient ALL with blasts of low and high CD34 expression, $CD19^{+}CD34^{high}$ purified cells did not generate CD34 low expressing cells in the bm of engrafted mice but only reconstituted the immunophenotype of the injected cells. This is illustrated in Figure 28 by contour dot plots of all mice from this experiment.

Figure 28 (page 211): Limiting dilution experiment on tertiary sorted $CD19^{+}CD34^{low}$ and $CD19^{+}CD34^{high}$ cells of sample WB51. Mouse M272 was initially transplanted with unsorted secondary cells of sample WB51. Recovered bm of this mouse sorted into $CD19^{+}CD34^{low}$ and $CD19^{+}CD34^{high}$ subfractions subsequently was transplanted on tertiary mice in limiting dilutions of 1.0×10^{3}, 1.0×10^{2} and 1.0×10^{1} cells per mouse. One mouse (M550) transplanted with 1.0×10^{3} $CD19^{+}CD34^{high}$ cells died before first bm analysis but all other mice transplanted with 1.0×10^{3} or 1.0×10^{2} cells of the opposing subfractions reconstituted the original patient ALL. Transplantation of only 1.0×10^{1} cells per mouse resulted in engraftment of 4 out of 5 mice in the group injected with $CD19^{+}CD34^{low}$ cells and 5 out of 6 mice injected with $CD19^{+}CD34^{high}$ cells. The bm of mice transplanted with $CD19^{+}CD34^{low}$ sorted cells reconstituted an immunophenotypic expression pattern comparable to that of the sorted bm from mouse M272 and the original patient material. Of note, all mice transplanted with $CD19^{+}CD34^{high}$ sorted cells only reconstituted an ALL with the transplanted immunophenotype but did not reconstitute a $CD19^{+}CD34^{low}$ blast population.

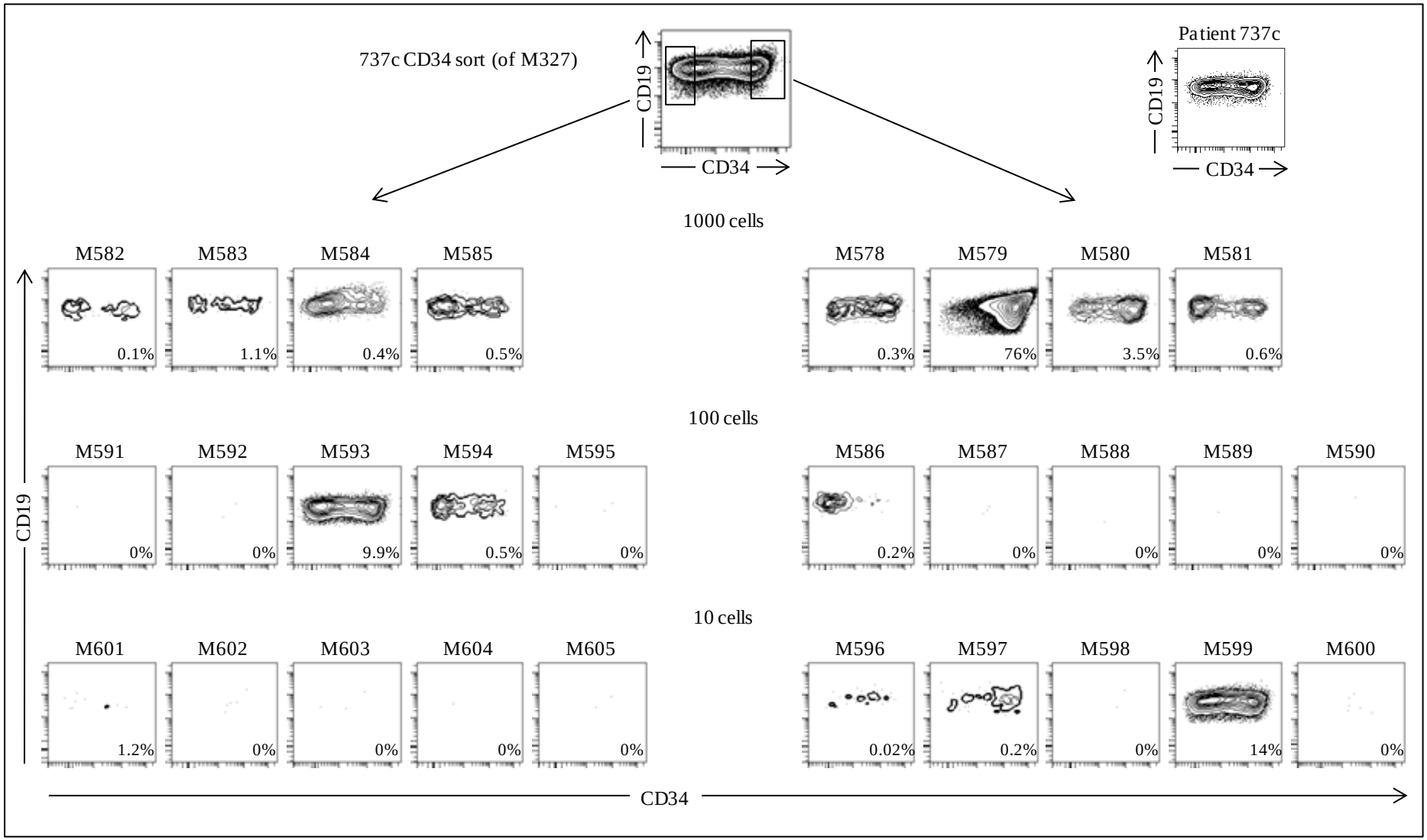

WB51 CD34 sort (of M272)
CD19
CD34
Patient WB51
CD19
CD34
1000 cells
M553
M554
M555
M556
M549
M551
M552
58%
57%
24%
41%
41%
71%
46%
100 cells
M562
M563
M564
M565
M566
M557
M558
M559
M560
M561
61%
46%
35%
35%
73%
66%
15%
65%
74%
69%
10 cells
M573
M574
M575
M576
M577
M567
M568
M569
M570
M571
M572
9.1%
54%
60%
55%
0%
65%
0.5%
61%
83%
0%
1.5%
CD19
CD34

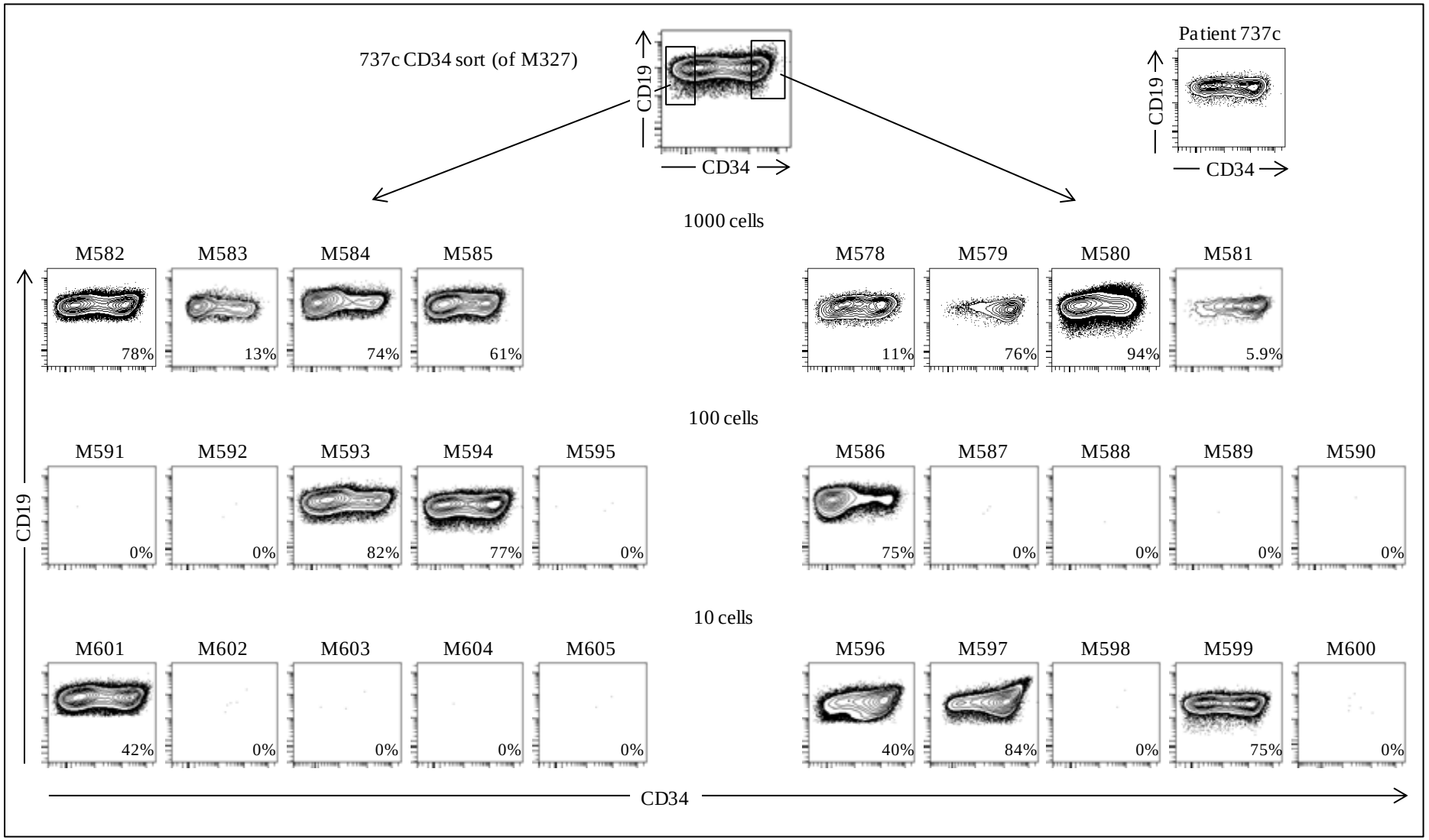
Another CD34 sorted limiting dilution experiment was carried out with secondary cells of sample 737c. Mouse M327 was originally transplanted with 1.0 x 10^4 unsorted patient ALL bm cells and reconstituted the leukaemia to 68% in mouse bm. The bm was sorted into $CD19^+CD34^{high}$ and $CD19^+CD34^{low}$ subfractions and both were transplanted into mice in numbers of 1.0 x 10^3 (n=4), 1.0 x 10^2 (n=5) and 1.0 x 10^1 (n=5) cells per mouse. All 4 mice transplanted with 1.0 x 10^3 $CD19^+CD34^{high}$ cells engrafted the ALL to levels of 11% (M578), 76% (M579), 94% (M580) and 6% (M581) with a mean engraftment of 47% and the same number of $CD19^+CD34^{low}$ cells was sufficient to engraft all 4 transplanted mice to levels of 78% (M582), 13% (M583), 74% (M584) and 61% (M585) with a mean engraftment of 57%. In the groups transplanted with 1.0 x 10^2 cells per mouse, one reconstituted the ALL from $CD19^+CD34^{high}$ purified cells with a human chimerism of 75% (M586) and two mice from $CD19^+CD34^{low}$ cells with 82% (M593) and 77% (M594). For both subfractions 1.0 x 10^1 cells only were again the limiting cell dose to transfer the leukaemia into NSG mice. 3 of the 5 mice that received $CD19^+CD34^{high}$ blasts engrafted to 40% (M596), 84% (M597) and 75% (M599) (mean engraftment 66%) and one of the 5 mice that received $CD19^+CD34^{low}$ blasts engrafted to 42% (M601). In this experiment, both subfractions were able to reconstitute the leukaemia to the original immunophenotype of the sorted sample and the patient ALL material independent of the cell number transplanted. Additionally it was determined if the sorted subfractions preferentially would first reconstitute one immunophenotype, their own or probably the immunophenotypically less mature regardless of the transplanted population, and consecutively generate the opponent one or if the full ALL immunophenotypic pattern would be re-established from early engraftment on. Therefore, contour dot plots of mouse bm at the time point of first flow cytometric signs of engraftment were analysed. The immunophenotype expression pattern of these contour dot plots already resembled the one finally gained for bm at the time of terminal

analysis when mostly overt ALL was present. Contour dot plots for first and terminal engraftment in mouse bm are depicted in Figure 29 and Figure 30.

Figure 29 (page 214): Limiting dilution experiment on secondary sorted CD19^{+}CD34^{low} and CD19^{+}CD34^{high} cells of sample 737c, early engraftment. Mouse M327 was initially transplanted with unsorted primary cells of sample 737c. Recovered bm of this mouse sorted into CD19^{+}CD34^{low} and CD19^{+}CD34^{high} subfractions subsequently was transplanted on secondary mice in limiting dilutions of 1.0 x 10^3, 1.0 x 10^2 and 1.0 x 10^1 cells per mouse. All mice transplanted with 1.0 x 10^3 cells of one of the two subfractions reconstituted the original patient ALL. Transplantation of 1.0 x 10^2 cells resulted in engraftment of 2 out of 5 mice in the group injected with CD19^{+}CD34^{low} cells and 1 out of 5 mice injected with CD19^{+}CD34^{high} cells whereas transplantation of 1.0 x 10^1 cells resulted in engraftment of 1 out of 5 and 3 out of 5 mice respectively. Already in bmps with the first detectable engraftment in the mouse xenoenvironment both the D19^{+}CD34^{low} and CD19^{+}CD34^{high} immunophenotype is reconstituted irrespective of the transplanted subfraction. Furthermore, the bm of all mice reconstituted an immunophenotypic expression pattern comparable to that of the sorted bm from mouse M327 and the original patient material.

Figure 30 (page 215): Limiting dilution experiment on secondary sorted CD19^{+}CD34^{low} and CD19^{+}CD34^{high} cells of sample 737c, final engraftment. Dot plots of the final engraftment levels of mice of the same experiment as in **Figure 27** are depicted. Compared to the earliest recorded engraftment, the immunophenotypic expression pattern of bm blasts from mice transplanted with the CD19^{+}CD34^{low} or the CD19^{+}CD34^{high} subfraction does not alter significantly in highly engrafted mice. This holds true irrespective of the cell number transplanted per mouse.


737c CD34 sort (of M327)
CD19
CD34
Patient 737c
CD19
CD34
1000 cells
M582
M583
M584
M585
0.1%
1.1%
0.4%
0.5%
M578
M579
M580
M581
0.3%
76%
3.5%
0.6%
100 cells
M591
M592
M593
M594
M595
0%
0%
9.9%
0.5%
0%
M586
M587
M588
M589
M590
0.2%
0%
0%
0%
0%
10 cells
M601
M602
M603
M604
M605
1.2%
0%
0%
0%
0%
M596
M597
M598
M599
M600
0.02%
0.2%
0%
14%
0%
CD19
CD34


737c CD34 sort (of M327)
CD19
CD34
Patient 737c
CD19
CD34
1000 cells
M582
78%
M583
13%
M584
74%
M585
61%
M578
11%
M579
76%
M580
94%
M581
5.9%
100 cells
M591
0%
M592
0%
M593
82%
M594
77%
M595
0%
M586
75%
M587
0%
M588
0%
M589
0%
M590
0%
10 cells
M601
42%
M602
0%
M603
0%
M604
0%
M605
0%
M596
40%
M597
84%
M598
0%
M599
75%
M600
0%
CD19
CD34

In order to exclude that engraftment of the subpopulation of $CD19^{+}CD34^{high}$ cells more closely resembling normal HSCs but also the subpopulation with the more mature progenitor immunophenotype $CD19^{+}CD34^{low}$ was not an artefact caused by prior passage through one or two generations of mice, two primary CD34 sorts on samples 4540a and L784 were transplanted in limiting dilutions. $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ purified blasts of sample 4540a were injected into the femora of 4 mice each with 1.0×10^{3} cells per mouse and of 5 mice each with 1.0×10^{2} or 1.0×10^{1} cells per mouse. Two mice that received 1.0×10^{3} $CD19^{+}CD34^{high}$ sorted cells engrafted to 1.1% (M606, died after bmp1) and 44% (M609) and one mouse (M611) that received 1.0×10^{3} $CD19^{+}CD34^{low}$ sorted cells engrafted to 59%. In the groups transplanted with 1.0×10^{2} cells, 3 mice injected with the $CD19^{+}CD34^{high}$ subpopulation reconstituted human blasts to 9% (M616), 22% (M617) and 45% (M620) and the mean engraftment was 25%. Flow cytometry on all other mice did not detect human cells. In this experiment the limiting cell dose for engraftment of $CD19^{+}CD34^{high}$ expressing cells was 1.0×10^{2} and for $CD19^{+}CD34^{low}$ expressing cells 1.0×10^{3} per mouse. However, both subfractions were able to repopulate mice with an immunophenotypic expression pattern mirroring the diagnostic ALL sample. Cells sorted according to CD34 marker expression from diagnostic bm of L784 (Figure 31) were transplanted in numbers of 1.0×10^{4} (n=4), 1.0×10^{3} (n=4), 1.0×10^{2} (n=5) and 1.0×10^{1} ($CD19^{+}CD34^{high}$ n=5 and $CD19^{+}CD34^{low}$ n=6) cells per mouse. All 4 mice transplanted with 1.0×10^{4} $CD19^{+}CD34^{high}$ cells engrafted human cells (M636 17%, M637 32%, M638 19% and M639 73%; mean engraftment 35%) as did 2 of the 4 mice transplanted with $.0 \times 10^{4}$ $CD19^{+}CD34^{low}$ cells (M642 58% and M643 41%. 3 of 4 mice each engrafted human cells when transplanted with 1.0×10^{3} $CD19^{+}CD34^{high}$ or $CD19^{+}CD34^{low}$ cells to 48% (M644), 3% (M645) and 21% (M647) (mean engraftment 24%) and 14% (M648), 0.2% (M649, died after bmp2) and 63% (M651) (mean engraftment 26%) respectively. From

the groups that received 1.0 x 10^2 sorted cells, one injected with the $CD19^+CD34^{low}$ subfraction engrafted to a terminal bm level of 52% human ALL cells (M657). Neither subfraction reconstituted the leukaemia when 1.0 x 10^1 CD34 sorted cells were transplanted only. Therefore the limiting cell dose for the $CD19^+CD34^{high}$ cell population was 1.0 x 10^3 and for the $CD19^+CD34^{low}$ cell population 1.0 x 10^2 cells per mouse. Engrafted mice again presented with the complete original patient ALL immunophenotype.

Similar to the limiting dilution experiments on blasts sorted according to CD20 expression and again with some inter-patient variability the 4 limiting dilution experiments on primary, seconday and tertiary $CD19^+CD34^{high}$ and $CD19^+CD34^{low}$ sorted ALL blasts from 4 different patient samples demonstrate, that leukaemic cells resembling less mature developmental stages of physiologic B-cell development do not harbour a higher frequency of LSCs than presumably more mature blasts.

Figure 31 (page 218): Limiting dilution experiment on primary sorted $CD19^+CD34^{low}$ and $CD19^+CD34^{high}$ cells of sample L784. Diagnostic patient bm was sorted into $CD19^+CD34^{low}$ and $CD19^+CD34^{high}$ subfractions and subsequently transplanted on primary mice in limiting dilutions of 1.0 x 10^3, 1.0 x 10^2 and 1.0 x 10^1 cells per mouse. Of mice transplanted with 1.0 x 10^3 $CD19^+CD34^{low}$ cells 2 out of 4 and of mice transplanted with $CD19^+CD34^{high}$ cells 4 out of 4 engrafted respectively. Mice transplanted with 1.0 x 10^2 cells of the same subfractions engrafted each 3 out of 4 mice. 5 mice each were injected with only 1.0 x 10^1 cells per mouse and all but one transplanted with cells from the $CD19^+CD34^{low}$ subfraction did not show any human engraftment. The bm of all engrafted mice irrespective of the transplanted cell subfraction reconstituted an immunophenotypic expression pattern comparable to that of the sorted bm from sample L784.

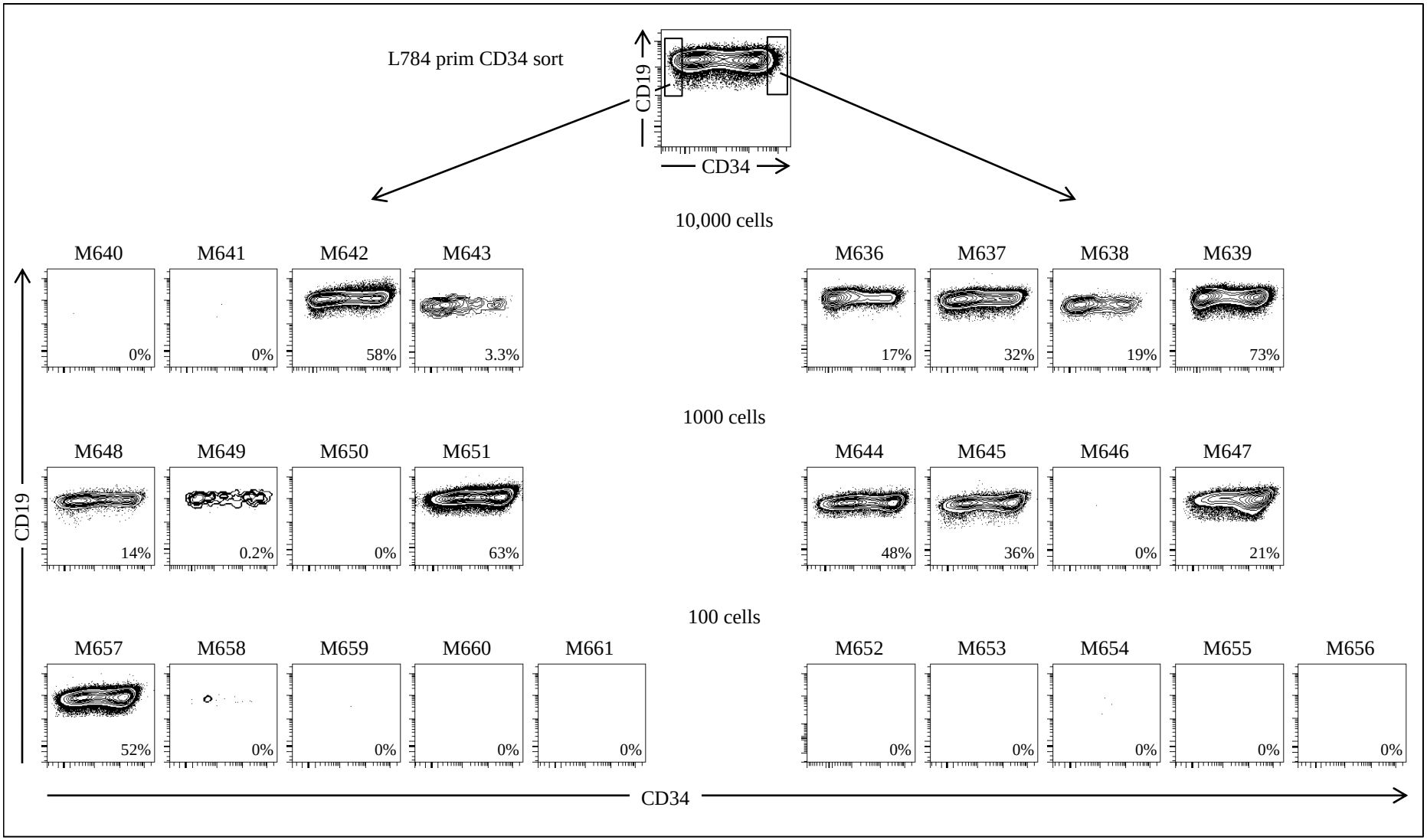

L784 prim CD34 sort
CD19
CD34
10,000 cells
M640
M641
M642
M643
M636
M637
M638
M639
0%
0%
58%
3.3%
17%
32%
19%
73%
1000 cells
M648
M649
M650
M651
M644
M645
M646
M647
14%
0.2%
0%
63%
48%
36%
0%
21%
100 cells
M657
M658
M659
M660
M661
M652
M653
M654
M655
M656
52%
0%
0%
0%
0%
0%
0%
0%
0%
0%
CD19
CD34

Table 13 summarises limiting dilution experiments carried out with CD34 sorted cells of all primary, secondary and tertiary ALL samples.

Patient ID	Risk group	Transplant	Transplanted blast population	Number of cells transplanted (number of mice engrafted/number of mice transplanted)			LIC frequency
				1000	100	10	
L784	Standard risk	Primary	CD34high	3/4	0/5	0/5	1:929
			CD34low	3/4	1/5	0/6	1:662
4540a	High risk t(9;22)	Primary	CD34high	2/5	3/5	0/4	1:839
			CD34low	1/5	0/5	0/5	1:5034
737c	High risk (BCR/ABL1 like)	Secondary	CD34high	4/4	1/5	3/5	1:120
			CD34low	4/4	2/5	1/5	1:143.7
WB51	High risk t(9;22)	Tertiary	CD34high	3/3	5/5	5/6	1:6.10
			CD34low	4/4	5/5	4/5	1:6.73

Table 13: A table of primary, secondary and tertiary ALL samples transplanted in limiting dilution experiments of $CD19^{+}CD34^{low}$ and $CD19^{+}CD34^{high}$ purified cells. The limiting dilution dose of 1.0 x 10^{1} $CD19^{+}CD34^{low}$ as well as $CD19^{+}CD34^{high}$ cells was sufficient to transfer the ALL in secondary and the tertiary transplant experiments onto NSG mice. In the primary transplantation experiment on sample L784, 1.0 x 10^{2} cells were able to reconstitute the leukaemia from $CD19^{+}CD34^{low}$ cells and 1.0 x 10^{3} cells from $CD19^{+}CD34^{high}$ cells. For primary sample 4540a it was the other way round. The frequencies of ALL LICs in the sorted blast populations were calculated using the ELDA programme.

4.4.4 ALL immunophenotypic reconstitution patterns originating from diverse transplanted subpopulations

During the hierarchically organised haematopoietic development from early stem cells through progenitor cells of the diverse lineages to the mature effector cells, cells acquire or lose surface epitopes and exhibit a specific surface marker expression pattern that correlates to their maturation stage. This process is not reversible and daughter cells will not re-acquire an immunophenotype less mature than that of their predecessor. The

occurrence of CD surface markers in physiological B-lineage development is described in section 1.3.3.1 and depicted in Figure 3. However, cancer stem cells, in addition to the capability to self-renew are required to fulfil a second defining characteristic; the ability to differentiate into all diverse cell phenotypes found within a tumour. In the subchapters above it has been shown that all subfractions of cells sorted according to the expression of CD19, CD10, CD20 and CD34 were able to transfer the ALL onto NSG mice when transplanted orthotopically into the hosts' femora. Generally, the transplanted immunophenotypic subpopulations also led to the generation of human cells of the not transplanted immunophenotype in the mouse xenoenvironment when dot plots were compared. Immunophenotypically less mature cell populations therefore were able to reconstitute immunophenotypically more mature cell populations but in contrast to the haematopoietic system more mature ALL subfractions were also able to reconstitute less mature ALL subpopulations. The only exception was the tertiary limiting dilution experiment on CD34 sorted cells from sample WB51 (KR59) where $CD19^{+}CD34^{high}$ purified cells failed to reconstitute an immunophenotypically less mature population of $CD19^{+}CD34^{low}$ cells in mouse bm.

Portraying flow cytometry data of surface marker expression in histograms visualises the distribution of cells with low and high expression of the respective CD antigen. In order to compare the distribution of cells in the bm of mice transplanted with different sorted subpopulations with each other and with the parent sample, histograms were created for each individual bm sample and then overlaid. Expression of CD19 showed the smallest variation within leukaemic samples and was generally high. When cells with the lowest and highest expression of CD19 were purified and transplanted, the CD19 expression patterns of engrafted mice were similar to each other and to the sample that was sorted into these two subfractions (M5, A67, experiment KR5). CD10 expression varied from very low to very high among cells of patient WB51. This variation was fully

reconstituted in the bm of mice transplanted with $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ cells purified from bm of mouse M64 (KR21). However, the mean expression level of CD10 was lower in bm of the transplanted mouse generation and mice transplanted with the $CD19^{+}CD10^{low}$ subfraction generally had a little lower level of CD10 expression than the mice transplanted with $CD19^{+}CD10^{high}$ cells. When bm of mouse M6 (A67) was sorted into $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ cell populations and transplanted into NSG mice (KR11), the CD20 expression pattern of all engrafted mice was nearly identical but the mean expression level was little lower than in the parent sample. The surface marker CD34 had a different expression pattern. The primary patient sample L784 contained two major populations of blasts, one with high expression and another with low expression of CD34. Both populations were able to reconstitute the opposing population in transplanted mice with variable contribution of the two populations to the bulk leukaemia (KR62). This was also the case for the CD34 sort on secondary cells (M327) from 737c (KR60). Both the $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ subfraction, reconstituted itself as well as the contrasting subfraction in transplanted mice and the two populations were also variable in size. An exception to this was the CD34 sort on sample WB51 (KR59). Bone marrow of mouse M272 had a small population of $CD19^{+}CD34^{low}$ and a large population of $CD19^{+}CD34^{high}$ expressing cells. When this sample was sorted according to CD34 expression levels, the $CD19^{+}CD34^{low}$ subfraction reconstituted cells with low and high CD34 expression with the number of $CD19^{+}CD34^{low}$ cells exceeding the number of $CD19^{+}CD34^{high}$ reconstituted cells. The purified $CD19^{+}CD34^{high}$ subfraction, however, failed to reconstitute a population of $CD19^{+}CD34^{low}$ cells and only replicated itself in engrafted mice. Histogram overlays of the CD19, CD10, CD20 and CD34 sorts are depicted in Figure 32.

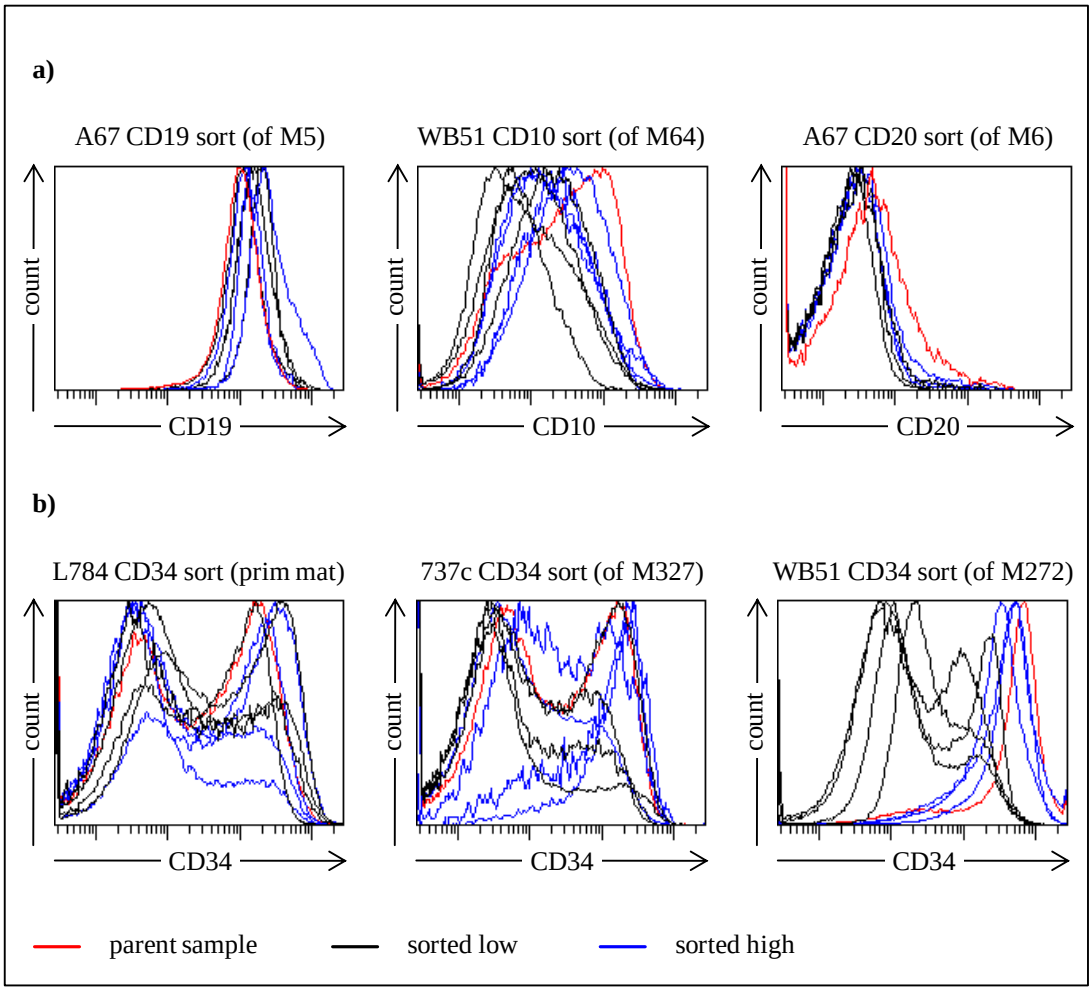


Figure 32: Histogram overlays of the sorted ALL sample and engrafted bm from mice transplanted with the low or high expressing fraction of the specific surface antigen. a) CD19, CD10 and CD20 show a Gaussian distribution of surface antigen expression. The smallest variation is present in CD19 expression and is reconstituted in the transplanted mouse generation by $CD34^{+}CD19^{low}$ and $CD34^{+}CD19^{high}$ cell populations. CD10 expression presents a wide variation. This is fully reconstituted in bm of transplanted mice by both sorted fractions, $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$, however, there is a little shift of mean CD10 expression towards the transplanted immunophenotype. The CD20 expression spectrum is also re-established in transplanted mice with a little lower mean expression compared to the parent sample similar for mice transplanted with $CD19^{+}CD20^{low}$ or $CD19^{+}CD20^{high}$ subfractions. **b)** The CD34 antigen divides the bulk ALL into one population with lower and one with higher CD34 expression. Both populations are generally reconstituted in mice by $CD19^{+}CD34^{low}$ or $CD19^{+}CD34^{high}$ purified fractions with variable size (left and middle image). The single exception was a tertiary sort of WB51 from which the $CD19^{+}CD20^{high}$ subfraction only reconstituted daughter cells of the same immunophenotype.

4.4.5 Engraftment kinetics of cell populations of diverse immunophenotypes

In the subchapters above it could be shown that blasts of different maturity according to the surface marker expression of CD10, CD19, CD20 and CD34 were able to engraft the leukaemia and reconstitute the complete immunophenotypic expression profile of the original ALL. Furthermore, it could be demonstrated that diverse sorted blast subfractions harboured self-renewal capability and that the stem cell frequencies within the different transplanted immunophenotypes did not vary considerably. Despite being similar in regards of these decisive stem cell characteristics, it still could be possible that the blast immunophenotype affects the engraftment kinetics of leukaemic blasts. In order to address this question the human xenograft chimerism caused by CD10, CD19, CD20 and CD34 sorted blasts over time was determined by flow cytometric analysis of sequential bmps. For sufficient numbers of mice at each time point, engrafted mice transplanted with comparable numbers of purified human leukaemic blasts were pooled for the investigation of engraftment kinetics. When possible, engraftment kinetics were determined for mice transplanted with relatively low numbers of sorted cells close to the limiting dilution as well as for mice transplanted with higher numbers for each subpopulation.

Among all xenotransplantations with CD10 sorted cells only one limiting dilution experiment was performed. Secondary $CD19^{+}CD10^{high}$ and $CD19^{+}CD10^{low}$ cells of sample 737c were transplanted with 100 cells per mouse into 4 mice each and in both groups two mice engrafted the leukaemia (experiment KR56). However, the development of the ALL in the transplanted mice followed a similar time pattern in both subgroups. More mice were transplanted with cell numbers between 1.0×10^3 and 2.0×10^3 cells per mouse. Altogether 16 mice engrafted with $CD19^{+}CD10^{high}$ blasts and also 16 mice engrafted with $CD19^{+}CD10^{low}$ blasts from the primary samples 737c (KR43),

M120 (KR47), 758b (KR50) and one secondary sample of 737c (KR56) were pooled and mean engraftment was calculated for sequential bmps. Again, detection of first engraftment and propagation of the ALL in mice showed a similar time course when higher numbers of cells were transplanted. The engraftment kinetics of $CD19^{+}CD10^{high}$ and $CD19^{+}CD10^{low}$ blasts are depicted in the graphs of Figure 33.

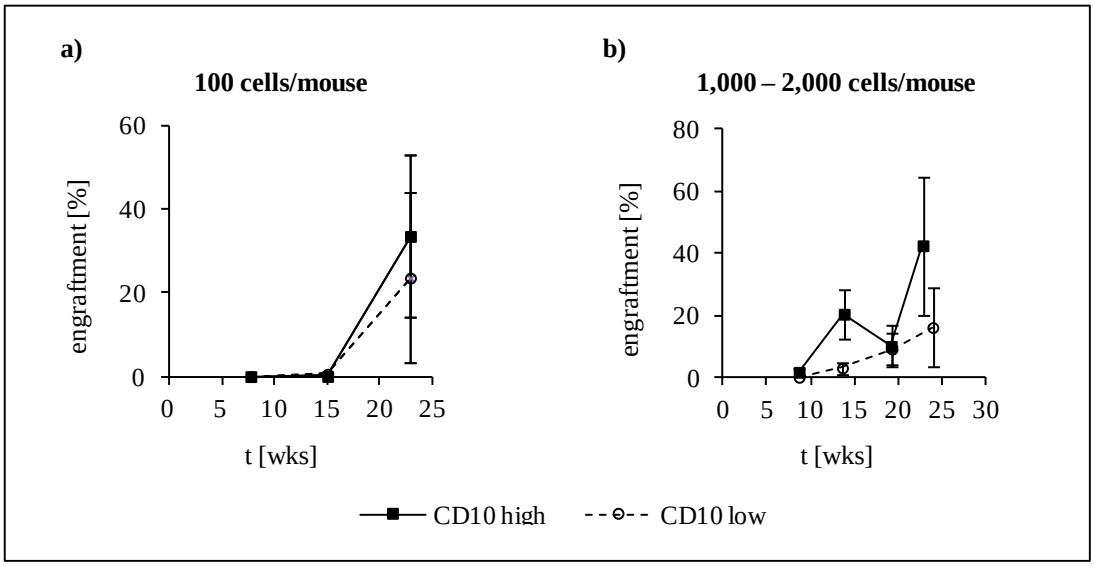


Figure 33: Engraftment kinetics of $CD19^{+}CD10^{high}$ and $CD19^{+}CD10^{low}$ purified ALL subpopulation as assessed by sequential bone marrow punctures. a) Only one experiment was performed with 100 CD10 sorted cells transplanted per mouse on sample 737c (KR56). Two mice each engrafted from injected $CD19^{+}CD10^{high}$ or $CD19^{+}CD10^{low}$ blast subfractions. **b)** Engrafted mice transplanted with 1.0 x 10^3 to 2.0 x 10^3 cells per mouse from the limiting dilution experiments on samples 737c (KR43 and KR56), M120 (KR47) and 758b (KR50) were pooled (16 mice in the $CD19^{+}CD10^{high}$ as well as in the $CD19^{+}CD10^{low}$ group) in order to increase the number of mice in each transplanted subgroup. $CD19^{+}CD10^{high}$ as well as $CD19^{+}CD10^{low}$ purified cells need a similar period of time to initiate first detectable human engraftment in NSG mice and for major propagation of the ALL. Error bars represent the standard error of the mean (SEM).

Patient samples sorted according to the surface marker expression of CD19 were not transplanted in limiting dilution assays. Therefore engrafted mice from all transplantations injected with between 2.0 x 10^3 and 3.0 x 10^4 cells from the primary

sample WB51 (KR18) and the secondary experiments on A67 (KR4 and KR5) were analysed together. Altogether 10 mice that received the $CD34^{+}CD19^{high}$ subfraction and 9 mice that received the $CD34^{+}CD19^{low}$ subfraction could be used for the calculations. There is no clear difference in the engraftment kinetics of mice transplanted with either of the sorted blast subpopulations. This is illustrated in Figure 34.

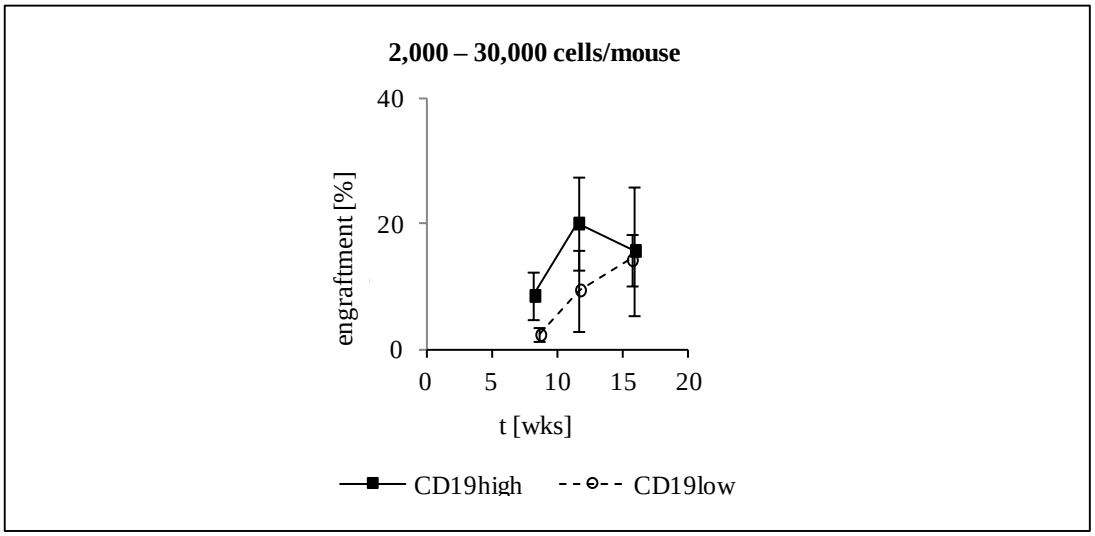


Figure 34: Engraftment kinetics of $CD34^{+}CD19^{high}$ and $CD34^{+}CD19^{low}$ purified ALL subpopulation as assessed by sequential bone marrow punctures. No limiting dilution experiments were performed on CD19 sorted cells with cell numbers of less than 2.0×10^3 transplanted per mouse. Engrafted mice from the experiments on samples A67 (KR4 and KR5) and WB51 (KR18) transplanted with 2.0×10^3 to 3.0×10^4 cells per mouse were pooled (10 mice for the $CD34^{+}CD19^{high}$ and 9 mice for the $CD34^{+}CD19^{low}$ subfraction) in order to increase the number of mice in each transplanted subgroup. $CD34^{+}CD19^{high}$ and $CD34^{+}CD19^{low}$ purified cells need a similar period of time to initiate detectable leukaemic growth and to propagate the human ALL in NSG mice. Error bars represent the standard error of the mean (SEM).

CD20 was the surface marker that was used for sorting in many experiments as it separates leukaemic blasts that resemble lymphoid precursor stages experiments were performed to determine whether or not the nearly mature blast populations would need a longer time period for reconstituting the leukaemia in mice than their less mature

counterparts and if there was a difference in these engraftment kinetics when lower or higher numbers of cells were transplanted. In limiting dilution experiments, 10 mice each engrafted when transplanted with 1.0 x 10^2 to 5.0 x 10^2 $CD19^+CD20^{high}$ and $CD19^+CD20^{low}$ cells respectively. These were mice transplanted from secondary experiments on WB51 (KR27), 2510 (KR33) and 737c (KR55) and a tertiary experiment on WB51 (KR45). Considerably more mice were transplanted and engrafted with cell numbers ranging from 1.0 x 10^3 to 3.0 x 10^3 sorted cells. Thirty-one of the engrafted mice received $CD19^+CD20^{high}$ sorted cells and 39 engrafted mice received $CD19^+CD20^{low}$ cells. These mice were pooled from primary experiments on samples HV101 (KR31), 737c (KR42), M120 (KR48) and 758b (KR51), secondary experiments on WB51 (KR26, KR27 and KR34), 2510 (KR33) and 737c (KR55) and tertiary experiments on A67 (KR28 and KR29). There is no clear difference in engraftment kinetics of mice transplanted with lower or higher numbers of cells of the $CD19^+CD20^{high}$ or the $CD19^+CD20^{low}$ immunophenotype. This is visualised in Figure 35.

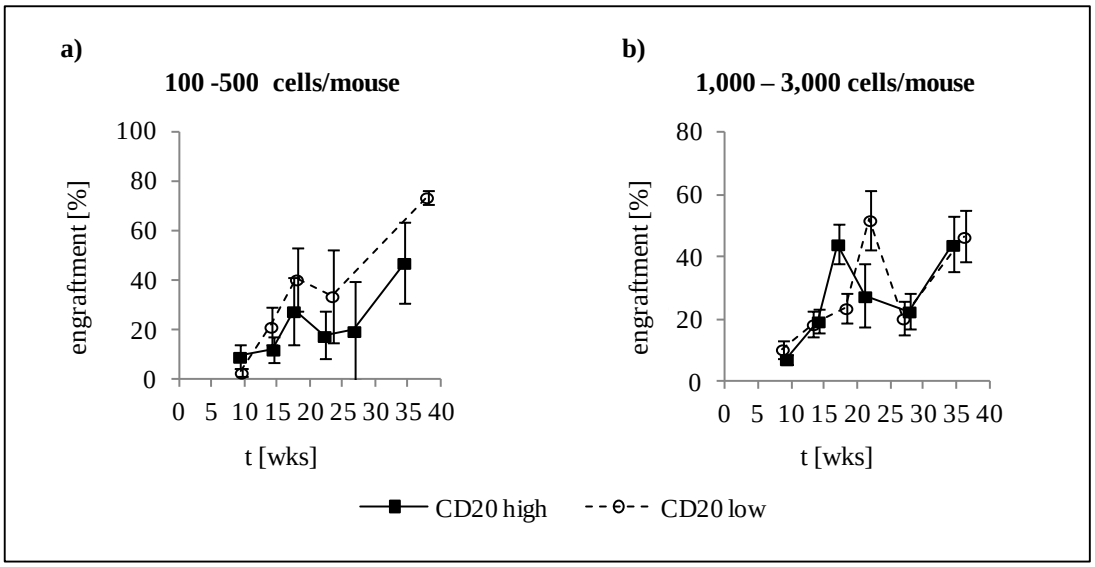


Figure 35: Engraftment kinetics of CD19^{+}CD20^{high} and CD19^{+}CD20^{low} purified ALL subpopulation as assessed by sequential bone marrow punctures. a) Engrafted mice transplanted with only 1.0 to 5.0 x 10^2 cells per mouse from the limiting dilution experiments on samples WB51 (KR27 and KR45), 2510 (KR33) and 737c (KR55) were pooled (10 mice each for the CD19^{+}CD20^{high} as well as for the CD19^{+}CD20^{low} subfraction) in order to increase the number of mice in each transplanted subgroup. **b)** Engrafted mice transplanted with 1.0 to 3.0 x 10^3 cells per mouse from the limiting dilution experiments on samples WB51 (KR26, KR27 and KR34), A67 (KR28 and KR29), HV101 (KR31), 2510 (KR33), 737c (KR42 and KR55), M120 (KR48) and 758b (KR51) were pooled (31 mice for the CD19^{+}CD20^{high} and 39 for the CD19^{+}CD20^{low} subfraction) in order to increase the number of mice in each transplanted subgroup. Regardless of the cell number transplanted per mouse CD19^{+}CD20^{high} as well as CD19^{+}CD20^{low} purified cells need a similar mean period of time to initiate first detectable human engraftment in NSG mice and for major propagation of the ALL. Error bars represent the standard error of the mean (SEM).

The surface marker CD34 is an established stem cell marker of the haematopoietic system. However, the work described in this thesis did not confirm that it can be used as a stem cell marker in ALL. Still, it could be possible that proliferation activity and therefore engraftment kinetics between CD34 high and CD34 low expressing blast populations would be different. This question was addressed by comparing the progress of engraftment of CD19^{+}CD34^{high} and CD19^{+}CD34^{low} purified cell fractions over time transplanted in smaller and larger cell numbers. When only 1.0 x 10^2 cells were transplanted per mouse from the primary patient samples 4540a (KR61) and L784

(KR62), the secondary sample 737c (KR60) or the tertiary sample WB51 (KR59), altogether 9 mice engrafted when injected with $CD19^{+}CD34^{high}$ and 8 mice when injected with $CD19^{+}CD34^{low}$ expressing lymphoblasts. The time needed for first detectable engraftment as well as for major expansion of the leukaemia in the xenotransplant environment was comparable. The same held true when 1.0×10^{3} sorted cells per mouse were transplanted from the same samples. In these experiments 11 mice reconstituted the ALL from the $CD19^{+}CD34^{high}$ and 12 mice from the $CD19^{+}CD34^{low}$ subfraction respectively. The mean engraftment levels of these mice over time are depicted in the graphs of Figure 36.

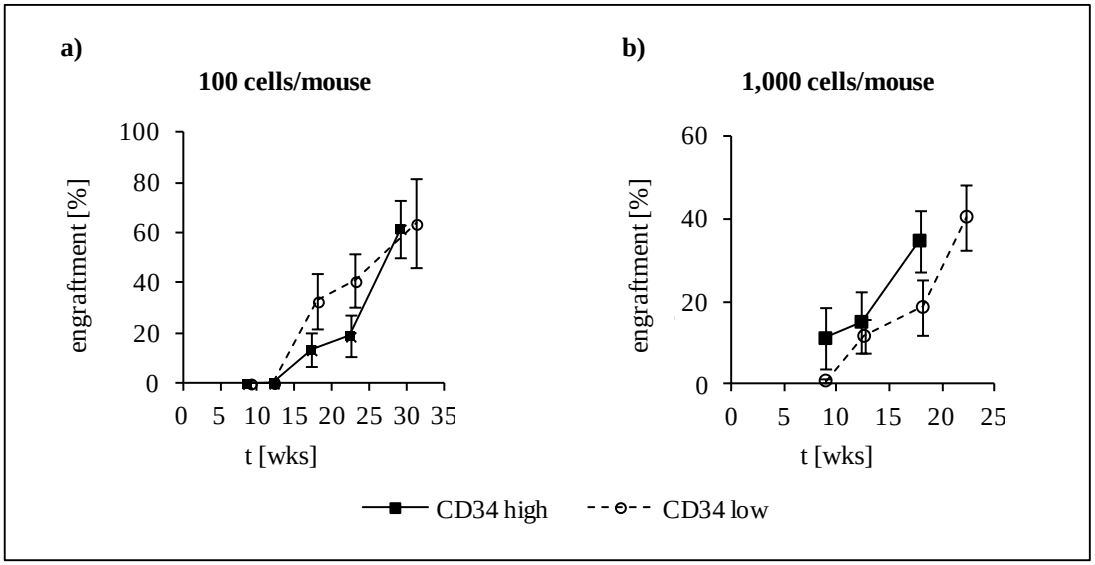


Figure 36: Engraftment kinetics of CD19^{+}CD34^{high} and CD19^{+}CD34^{low} purified ALL subpopulation as assessed by sequential bone marrow punctures. a) Engrafted mice transplanted with only 1.0 x 10^2 cells per mouse from the limiting dilution experiments on samples WB51 (KR59), 737c (KR60), 4540a (KR61) and L784 (KR62) were pooled (9 mice for the CD19^{+}CD34^{high} and 8 mice for the CD19^{+}CD34^{low} subfraction) in order to increase the number of mice in each transplanted subgroup. **b)** Engrafted mice transplanted with 1.0 x 10^3 cells per mouse from the same experiments were as well pooled (11 mice for the CD19^{+}CD34^{high} and 12 mice for the CD19^{+}CD34^{low} subfraction) in order to increase the number of mice in each transplanted subgroup. Regardless of the cell number transplanted per mouse CD19^{+}CD34^{high} as well as CD19^{+}CD34^{low} purified cells need a similar mean period of time to initiate first detectable human engraftment in NSG mice and for major propagation of the ALL. Error bars represent the standard error of the mean (SEM).

Despite some variability, taken together the data provide convincing evidence that all sorted subpopulations engrafted with similar kinetics. In summary, there was no enrichment for stem cell activity in any of the populations tested, demonstrating the absence of a stem cell hierarchy in ALL.

4.5 Discussion

Work described in this chapter aimed to identify and characterise leukaemic stem cells in ALL. It could be demonstrated that sorted blast populations of diverse maturity according to the immunophenotype have the ability to reconstitute the leukaemia in the murine xenotransplantation assay and that this potential is not restricted to immature blast populations as suggested in several publications. The re-established leukaemias closely mirrored the immunophenotypic expression pattern of the transplanted sample and the original patient sample respectively, regardless of the sorted subfraction transplanted. Furthermore, evidence was provided that the frequency of LICs in the bulk leukaemia, as well as in subfractions purified according to the immunophenotype, is much higher than previously thought, with only 1.0×10^3 down to 1.0×10^1 cells being sufficient to transfer the ALL onto NSG mice.

The ability of blast subpopulations that resemble distinct maturation stages of physiologic haematopoietic development to reconstitute the ALL in the xenogenic mouse environment was determined for 15 different ALL samples. ALL bone marrow cells were sorted according to their expression of the surface antigens CD19, CD10, CD20 and CD34. Using these markers it is possible to purify cell populations of distinct stages within the B cell lineage. In B cell development populations with a high expression of CD20 represent immature B cells, the most mature stage of B precursor cells. The first stages in the B lineage, pro-B cells and pre-B-I cells, are characterised by expression of CD34 with pro-B cells additionally lacking CD10 and CD19 on the cell surface. All sorted subpopulations without exception, from immunophenotypically early $CD34^+$ blasts to the most mature $CD20^{high}$ expressing stages, successfully transferred the leukaemia onto NSG mice, therefore demonstrating leukaemia initiation ability.

Furthermore, the engrafted leukaemias generally reconstituted the full immunophenotype pattern of the parent sample. This constitutes one defining characteristic of cancer stem cells ie the capability to give rise to all heterogeneous cell populations that comprise the original tumour. When more immature blast populations of the immunophenotypes $CD34^{+}CD19^{low}$, $CD19^{+}CD10^{low}$, $CD19^{+}CD20^{low}$ or $CD19^{+}CD34^{high}$ were injected into mice, they reconstituted themselves as well as the more mature $CD34^{+}CD19^{high}$, $CD19^{+}CD10^{high}$, $CD19^{+}CD20^{high}$ or $CD19^{+}CD34^{low}$ subpopulations. This is in line with normal haematopoietic development. Less mature cell populations reproduce cells of identical maturity but also differentiate into downstream developmental stages. In normal haematopoiesis this process is not reversible and more mature haematopoietic cells do not dedifferentiate to generate less mature cell clones. However, work presented in this thesis proved a different behaviour for B precursor ALL cells. Transplantation of more mature $CD34^{+}CD19^{high}$, $CD19^{+}CD10^{high}$, $CD19^{+}CD20^{high}$ or $CD19^{+}CD34^{low}$ subpopulations in addition to reconstituting cells of an identical immunophenotype also generated less mature $CD34^{+}CD19^{low}$, $CD19^{+}CD10^{low}$, $CD19^{+}CD20^{low}$ and $CD19^{+}CD34^{high}$ cells, thereby reconstituting the whole original ALL immunophenotype.

Several scenarios could lead to these observations. One is that the more mature cells are able to dedifferentiate to the less mature developmental stage. After this process they proliferate to reproduce a cell clone of identical less mature cells but partly also differentiate again to give rise to more mature progeny. Another possibility would be that immunophenotypically more mature cells directly produce less mature cells following cell division. The parent cell itself then would not have to dedifferentiate in order to produce more immature progeny. Furthermore, it also could be possible that surface marker expression on ALL blasts, unlike in haematopoiesis or AML, does not relate to the maturational stage but that expression of all investigated antigens, CD19,

CD10, CD20 and CD34, is random and can be up or down regulated on individual cells. However, unpublished work from our group (manuscript submitted) does not support this alternative. Still, the observation that in the earliest detectable bm engraftments generally the final immunophenotype is already established, even when only less than 0.1% of mouse bm cells are of human origin, favours the two latter possibilities. However, it can not be excluded that a dedifferentiation takes place at a time point, when the ALL is not detectable using flow cytometry.

It was clearly demonstrated that all subfractions sorted according to the expression of the CD surface antigens 19, 10, 20 and 34 from several ALL samples could re-establish the complete original immunophenotype in transplanted mice but there was one individual exception. $CD19^{+}CD34^{high}$ cells from the tertiary CD34 sort on ALL WB51 did successfully engraft NSG mice but only reconstituted their own immunophenotype while $CD19^{+}CD34^{low}$ cells reconstituted the full original variety of CD34 surface marker expression. This was somehow surprising and it is difficult to find an explanation for this phenomenon. CD34 expressing cells are more immature in their surface marker expression profile than cells that do not express CD34. Therefore, in this case, the more mature subpopulation was able to dedifferentiate and reconstitute the more immature subpopulation while less mature cells failed to differentiate to reconstitute more mature cells. However, this observation potentially indicates that rather mature blast subfractions do not need to dedifferentiate in order to proliferate and are by themselves able to reconstitute the ALL albeit of reduced immunophenotypic variety.

The other hallmark of CSCs, self-renewal, was also investigated. In serial transplantation experiments up to 3 generations of mice were transplanted with ALL subpopulations of different maturity and reconstituted the malignancy. Of note, even when the most mature cell population according to surface marker profile ($CD19^{+}CD20^{high}$) was purified from diagnostic patient bm, it was able to maintain the

disease in three mouse generations over a cumulative period of up to 78 weeks. However, the tertiary CD20 sort of WB51 resulted in generally low engraftment levels in transplanted mice. This CD20 sort was performed on bm from a mouse initially transplanted with $CD19^{+}CD20^{high}$ patient cells, an immunophenotypically more mature cell fraction that arguably could have less self-renewal activity than more immature cell populations. However, when tertiary cells of the same leukaemia were sorted into $CD19^{+}CD34^{high}$ and $CD19^{+}CD34^{low}$ subpopulations transplanted mice engrafted to high levels. This tertiary CD34 sort was performed on material that as well initially was transplanted with $CD19^{+}CD20^{high}$ cells and then was used for an unsorted limiting dilution experiment in a second generation of mice. High engraftment levels were achieved in this tertiary limiting dilution experiment, indicating, that $CD19^{+}CD20^{high}$ cells do not lose self-renewal capacity when serially transplanted over more than two generations of mice. Another reason for the low engraftment level of mice from the tertiary experiment with CD20 sorted cells could be that they were all injected with low numbers of 5.0×10^{2} cells only and were harvested as they were approaching the end of their physiological life span. It can not be excluded that these mice eventually would have engrafted to high levels.

Furthermore, engraftment kinetics of opposing subpopulations sorted according to the surface marker expression of CD10, CD20 and CD34 were compared and found to be similar even at lower transplanted cell numbers. This suggests that LIC frequency is not different between sorted subfractions of more immature and more mature immunophenotypes but that stemness is evenly distributed across all maturational stages. It also suggests that the biological behaviour of assumed stem cells within these subpopulations resembles each other in respect to stem cell quiescence and proliferative activity.

Stem cells in haematopoiesis or AML constitute a rare subset. Several studies on ALL indicated that the situation is similar for ALL stem cells. However, demonstrating leukaemia reconstitution potential in all investigated subpopulations of different maturity according to CD surface antigen expression suggested that LICs might be more abundant than proposed by studies that detected LICs only in rare and immature blast populations. This created the desire to scrutinize the overall LIC frequency in ALL in the orthotopic NSG mouse model. Therefore, unsorted limiting dilution experiments were performed on several samples with transplanted cell doses down to 1.0×10^1 cells per mouse. The determined LIC frequencies revealed inter-patient variability but for all samples 1.0×10^3 cells were sufficient to initiate ALL at least in some transplanted mice. In the experiments with primary patient material, several samples were additionally able to cause leukaemia in mice from only 1.0×10^2 injected cells but this was the limiting cell dose. When secondary or tertiary blasts were transplanted, 1.0×10^1 cells were sufficient to transfer the ALL onto the majority of mice. The general tendency that higher transplanted cell numbers led to earlier detectable engraftment of human cells and earlier onset of overt leukaemia supports the assumption that indeed more than one stem cell was present among these higher numbers of injected cells. Otherwise mice transplanted with a lower number of cells and successful engraftment would have been expected to reconstitute the leukaemia in the same time manner.

Using the orthotopic NSG mouse model may be one reason to explain why LIC frequencies across several ALL samples were found to be higher than those determined in other studies that applied the iv transplantation technique on less immunocompromised mice. Additionally, the high sensitivity and specificity of the developed gating strategy in detecting human cells in the mouse xenoenvironment may also have contributed to the difference of LIC frequencies in this work compared to that of other groups.

The reproducibly found high LIC content in various unsorted ALL samples raised important questions. The earlier experiments with sorted subpopulations usually were performed by transplanting relatively high cell doses in the range of 1.4×10^3 and 6.2×10^4 per mouse, cell doses that were higher than the limiting cell numbers required for engraftment of unsorted blasts. Taking this into account, although being present in subpopulations of dissimilar maturity, the frequency of LICs in these specific subpopulations could be significantly different. Therefore, one question was to determine whether or not LIC frequencies were comparable between unsorted bulk leukaemia cells and sorted cell fractions with a differentially mature surface marker profile. The other question arose from the accuracy of cell sorting and its potential influence on experiment outcome. Despite high sorting purities, usually in excess of 95%, contamination of more mature blast subfractions with a substantial number of less mature cells and vice versa could not be completely ruled out. This created the possibility that human ALL engraftment of mice was not initiated by the presumed sorted subfraction but indeed originated from the few cells of the contaminating opposing immunophenotype. In order to address these issues, sorted cell subpopulations according to the CD surface antigens 10, 20 and 34 were transplanted in limiting dilutions as well. As already seen for limiting dilution experiments on unsorted cells, there also was inter-patient variability in LIC frequencies in sorted populations and secondary or tertiary transplanted cells generally were able to transfer the leukaemia onto mice with lower numbers of injected cells as primary patient samples. However, all sorted subpopulations were able to engraft the leukaemia at least in some mice when 1.0×10^2 cells only were transplanted. For CD20 and CD34 sorts, there were examples where 10 times more cells were needed to engraft the immunophenotypically more mature cell population than the more immature population but equally, for both sorts, other samples needed 10 times more cells to engraft the more immature population than

the more mature one. A possible explanation for this lies in the small group size of 4 to 5 mice transplanted with each subfraction of which only 1 to 3 engrafted. Higher numbers of mice injected with the different subpopulations would probably have resulted in engraftment of both subpopulations in some of the transplanted mice. Taken together, the limiting dilution experiments on sorted ALL blast subfractions strongly suggest that LIC frequency is evenly distributed across an extended spectrum of immunophenotypical maturation stages. Additionally, the finding that as few as 1.0 x 10^1 or 1.0 x 10^2 cells proved to be sufficient to engraft the ALL from purified blast populations, regardless of the immunophenotypic maturity level, made it unlikely that engraftment originated from contaminating cells of the complementary immunophenotype.

4.6 Conclusion

Work presented in this chapter indicates that acute lymphoblastic leukaemia can be reconstituted to the complete original immunophenotype and maintained over extended periods of time from blast subpopulations of different maturity according to surface marker expression profile. Leukaemia initiating cells additionally were frequent in the sorted subpopulations with 1.0×10^1 to 1.0×10^2 cells being sufficient to reconstitute the leukaemia from secondary or tertiary material and 1.0×10^2 to 1.0×10^3 sorted cells being sufficient to reconstitute the leukaemia from primary patient samples. These findings strongly argue against a hierarchical organisation being applicable for ALL like for normal haematopoiesis or AML and suggest that leukaemic stem cells in ALL best fit the stochastic stem cell model.

CHAPTER 5: GENERAL DISCUSSION

Chapter 5: General Discussion

5.1 Summary and Discussion

Improving treatment outcome for patients with different malignancies remains a huge challenge in cancer medicine and greatly depends on revealing and understanding the biology of a specific cancer in depth. Since the hazard to the organism from cancer largely derives from its uncontrolled growth, it is essential to find out which cells within the tumour drive unrestrained proliferation, what proportion of the tumour do they constitute for and what distinguishes them from other cells in the tumour without this capability. Dependent on the answers to these questions two main models are generally applied in cancer biology, the stochastic model, in which every cell within the cancer potentially is able to maintain the complete tumour and the hierarchical model, in which only a rare subset of cells is responsible for sustaining the tumour and gives rise to progeny that itself loses this ability but still has limited proliferative potential. These tumour maintaining cells are the cancer stem cells that by definition are able to self-renew and to differentiate into the diverse lineages that make up the complete malignancy.

Leukaemias are commonly believed to mirror the classical hierarchical organisation well established for physiological haematopoiesis and therefore the hierarchical cancer stem cell model is favoured for haematologic malignancies. However, due to conflicting results, there is a continuing debate about the frequency and identity of stem cells in acute lymphoblastic leukaemia which divides opinions in regard to which model has to be applied.

The work presented in this thesis had the main aim to determine whether or not stem cells in B precursor ALL are rare and restricted to an immunophenotype that resembles

early stages in physiologic B cell development or if they are more abundant and not restricted to immunophenotypically defined immature blast subfractions. Identification of stem cells is highly dependent on the ability of the applied model to serve as a host and to provide as similar as possible support for candidate cancer stem cells as the genuine organism. Therefore, a prerequisite was to chose and establish a robust xenotransplantation model in order to provide the most ideal conditions for the engraftment of leukaemic stem cells. Additionally, it was necessary to reliably detect engrafted cells in the murine xenoenvironment with a high sensitivity as well as specificity.

The generally accepted gold standard to address the stem cell characteristics of self-renewal and differentiation in cell populations of human origin is the orthotopic xenotransplantation assay (Clevers, 2011; Nolta and Jordan, 2005). However, the technical aspects of the applied transplantation procedure as well as the read out of results and even more importantly the characteristics of the transplanted mouse strain have an essential impact on experiment outcome. The orthotopic intra-bone marrow LIC injection model was selected for this project as it was shown to have a profoundly higher sensitivity in detecting normal haematopoietic stem cells in the murine model than the traditionally applied intravenous transplantation procedure (Kushida et al., 2001; Mazurier et al., 2003; McDermott et al., 2010; Wang et al., 2003; Yahata et al., 2003). The NSG mouse strain was regarded as the most suitable host for human ALL blasts. In addition to the most profound immunodeficiency in comparison to other mouse strains NSG mice harbour the advantage of a relatively long lifespan as they are less prone to develop lymphomas (Shultz et al., 2005). This was of particular importance for experiments with very low numbers of cells transplanted and consequently longer times needed for engraftment. The different available mouse strains and the advantages of the orthotopic intrafemoral transplantation approach for a project on LICs were introduced

and discussed in more detail in chapter 3. Flow cytometry was used for detection of human cells in the murine xenoenvironment. As antibodies to human surface CD antigens of interest showed some unspecific binding to cells of murine origin, antibodies directed against the white and red cell lineage of mouse cells were used in order to differentiate between human and mouse cells with more accuracy. With the developed and consequently improved gating algorithm it finally was possible to reliably detect one cell of human origin in 1.0 x 10^4 mouse bm cells. An even higher sensitivity probably only would have been possible by using RQ-PCR based methods as used for MRD monitoring in the clinical setting, however, this was not regarded as being necessary as it would have been questionable to count a human chimerism of less than one in 1.0 x 10^4 bone marrow cells as engraftment of human ALL. Furthermore, in addition to higher costs this method is much more time consuming and results would not be available the same day but rather be delayed for a couple of days. On the other hand a timely recognition of high bm engraftment was desirable and necessary in order to harvest the respective mouse and not lose it due to death from overt leukaemia. Another point is that with MRD monitoring alone it would not have been possible to draw a conclusion about the specific immunophenotype of the respective reconstituted ALL. In contrary a molecular analysis of bm engraftment, the flow cytometry based method satisfyingly combined a sufficient sensitivity with the opportunity to determine the reconstituted leukaemic immunophenotype in one step.

ALL is a malignancy that arises within the haematopoietic system and like the formation of blood cells it develops within the bm. Therefore, in order to detect engraftment at the earliest possible time point and lowest levels, cells from mouse bone marrow punctures were scrutinised rather than peripheral blood where blast numbers can be still low or undetectable even when blasts already represent a high percentage of cells within the bm. Analysing mouse bm samples reliably led to the detection of human ALL cells,

however, in the course of an experiment, sometimes engraftment levels in a later bmp were found to be lower than those determined in an earlier bmp. Several explanations can be given for this observation. The distribution of ALL blasts in the bone cavity can be heterogeneous with remaining islands of mouse blood formation. Depending on the part of bm aspirated in the bmp, the percentage of human cells can vary. Aspiration of bm also requires the application of a considerable negative pressure in the syringe and it is impossible to exactly control the amount of bm taken from the punctured bone. Removal of large parts of ALL blasts may well lead to a lower number of blasts found in the following bmp and while the leukaemia has spread through the organism already and repopulated further bones or other organs blast content in the initially transplanted and punctured bone cavity has potentially been artificially decreased by the previous bmp. Additionally, a bmp causes a trauma to the bone and its cavity. Posttraumatic bleeding with a resulting haematoma can consecutively lead to fibrosis of the bone and indeed, at the time of bm harvest, sometimes nearly completely solidified bones were observed. Attempts to aspirate cells from bones with only a small remaining bm cavity will lead to aspiration of larger amounts of contaminating mouse blood cells resulting in determination of lower human chimerisms. In future experiments, the influence of bmps on human blast engraftment levels potentially can be avoided by non-invasive blast detection methods like bioluminescence of lentivirally transduced blast populations, a technique which also holds a great promise in elucidating exact modalities of systemic involvement in ALL. However, ideally these methods would detect every single ALL blast anywhere in the mouse body but it is questionable if it is ever possible to achieve such a high sensitivity. Furthermore, an influence of the processes involved in lentiviral transduction as well as the inserted lentivirus itself on the engraftment capability of the leukaemia can not ultimately be ruled out. Nevertheless, systemic spread in mice closely mirroring ALL disease manifestation in humans could already be proved with the

applied NSG mouse model. In post mortem analyses a regular involvement of the spleen was detected and also infiltration of the liver or the kidneys was demonstrated in this study. Additionally, brains of a fraction of mice were collected and blast infiltration was demonstrated by another laboratory (work performed by Dr. Christina Halsay, Glasgow; unpublished data). These findings indicate that NSG mouse xenotransplantation experiments are an excellent model for ALL.

The data presented in this thesis provide good evidence that a variety of blast populations that resemble different stages of B cell development according to CD surface marker expression harbour leukaemic stem cells. Furthermore, it was shown that LIC frequencies and engraftment kinetics between subpopulations of different maturity were comparable. These findings strongly suggest that ALL is not organised in a hierarchy but follows the stochastic stem cell model.

In contrast to the insights presented here, other studies published before and during the work for this thesis found much lower frequencies of LICs in ALL that additionally are restricted to rather immature B cell precursor stages and therefore proposed the hierarchical stem cell model for ALL. However, as already pointed out, the mouse model used in these studies has a substantial impact on the outcome; therefore, conclusions drawn in the published studies have to be interpreted cautiously in the context of the individually applied xenotransplantation model and therefore will be discussed in more detail.

Cobaleda and colleagues took a very similar approach for ALL samples that had been successful in identifying AML stem cells. In initial limiting dilution transplantation experiments of unsorted MNC they found a LIC frequency of between 0.2 and 41 in one million ALL cells. This suggested that the SL-IC was a rare entity within the bulk tumour population. To identify the SL-IC they sorted leukaemic samples into

$CD34^+CD38^-$ and $CD34^+CD38^+$ fractions and achieved engraftment in mice only from the less mature $CD34^+CD38^-$ fraction regardless of the immunophenotypic composition of the bulk leukaemia. This finding was identical to the situation already established for normal and AML stem cells. On blasts from engrafted mice they additionally found the B-cell lineage marker CD19, indicating that the transplanted cells were able to differentiate into the B-precursor lineage but no myeloid differentiation could be observed in methylcellulose assays. Cells with the $CD34^+CD38^-$ immunophenotype were also reconstituted in the murine environment over serial transplantations proving self-renewal capacity of this subfraction. However, all samples were transplanted into irradiated NOD/SCID mice which are now known to have considerably more residual immunity than the NSG mice used for this thesis. In addition, the route of transplantation was by intravenous injection, a technique recognised to be inferior to orthotopic transplantation regarding engraftment success. Finally, the transplanted material consisted of samples only from Ph^+ ALL patients making it difficult to draw general conclusions for ALL (Cobaleda et al., 2000). The limitations of the transplantation model used might not only explain the inferior engraftment success of this study in comparison to the orthotopic NSG mouse model but also the results of another project that subsequently extended the search for LSC to standard risk ALL. The patient cohort of this study consisted of 16 ALLs of the pre-B and c-ALL subtypes. Most were of the standard risk group having no apparently abnormal cytogenetic constitution, being hyperdiploid or containing the ETV6/RUNX1 translocation. Only one sample was from a relapsed, and therefore high risk, ALL. In initial transplantation experiments with again intravenous injection of unsorted samples into irradiated NOD/SCID mice 14 out of the 16 ALL successfully engrafted. A minimum of 5×10^5 cells were injected but most samples only engrafted when one million or more cells were transplanted. They then purified patient samples into immunophenotypic subgroups

according to their expression of the stem cell marker CD34 and the B-lineage markers CD10 and CD19 and repeated the transplantation experiments. Engraftment was exclusively found in mice injected with the $CD34^+CD10^-$ and $CD34^+CD19^-$ subfractions. No other population containing $CD34^-$, $CD34^+CD10^+$ or $CD34^+CD19^+$ blasts was able to transfer the leukaemia onto mice, even when more than 10 times more cells were injected. For the engrafted subpopulations, self-renewal capability of SL-ICs was demonstrated in serial transplantations. The authors concluded that, comparable to the Ph^+ ALL, SL-IC in standard risk ALL are restricted to an immature cell fraction that have not yet undergone lineage restriction and that a hierarchical organisation exists in ALL progenitor cells (Cox et al., 2004).

Taken together, these two studies suggested that the target cells for leukaemic transformation were immature, not lineage restricted, HSC and that ALL was hierarchically organised, regardless of prognostic factors. In line with these findings, Cox *et al* published another study in 2009. For purifying candidate stem cell populations in childhood ALL this time they used the CD133 mAb in combination with either CD19 or CD38 mAbs. From 10 ALL samples of different prognostic subgroups only the $CD133^+CD19^-$ and the $CD133^+CD38^-$ subfractions reconstituted the original leukaemia in irradiated NOD/SCID mice after intravenous injection. From this study they derived confirmation of their previous finding that ALL SL-IC exclusively are part of uncommitted early stem cell compartment and do resemble HSC (Cox et al., 2009). However, this study as well was performed with NOD/SCID mice and intravenous transplantation at the time when NSG mice were already available and evidence for superiority of orthotopic intrafemoral or intratibial injection was published.

Despite using the same model with irradiated NOD/SCID mice and intravenous cell transplantation another group did not confirm the finding of only non-lineage restricted blast populations being able to transfer the ALL onto host mice. Castor *et al* detected a

blast population that had aberrant $CD34^{+}CD38^{-}CD19^{+}$ expression, an immunophenotype that is not part of normal haematopoiesis but with expression of CD19 surface antigen nevertheless was B-lineage restricted already. This $CD34^{+}CD38^{-}CD19^{+}$ subfraction was able to reconstitute and serially transfer the ALL of ETV6/RUNX1 and p190 BCR/ABL1 positive samples. Of note, both translocations were not detected in the $CD34^{+}CD38^{-}CD19^{-}$ immature cell populations of the diagnostic patient samples. In contrast, in ALL with the p210 BCR/ABL1 fusion the $CD34^{+}CD38^{-}CD19^{-}$ compartment and even myeloid cell lineages were involved but again LIC activity was only found in $CD34^{+}CD38^{-}CD19^{+}$ purified blasts. In conclusion, LSC in all investigated types of ALL were only part of the lymphoid committed precursor cell compartment (Castor et al., 2005). However, CD19 still is expressed early in B cell development and more mature blast populations with expression of CD20 were not investigated. The study also did not address the question of LIC frequency and even with the challenging data provided it still could not be concluded that ALL was not organised in a hierarchy.

In support of the findings of Castor *et al*, Hong *et al* demonstrated tumour propagating abilities in B-lineage restricted lymphocytic blasts as well. They purified the same abnormal $CD34^{+}CD38^{low/-}CD19^{+}$ cells from ETV6/RUNX1 positive c-ALLs. From four patient samples this population engrafted and reproduced itself in primary and secondary generations of NOD/SCID mice after intravenous transplantation, providing evidence for self-renewal (Hong et al., 2008). Again, more mature blast populations or LIC frequency were not addressed.

More studies continued to provide support for the hypothesis that ALL blast populations with an already committed B-precursor immunophenotype have leukaemia propagating abilities. Kong *et al* intravenously injected purified populations, not only of the aberrant $CD34^{+}CD38^{-}CD19^{+}$ but also of the $CD34^{+}CD38^{+}CD19^{+}$ immunophenotype which resembles a physiological B-cell precursor, into NOD/SCID/IL2rγ^{null} (NSG) mice.

Remarkably, using the more immunocompromised mouse strain, now both subfractions were able to transfer the patient ALL onto the mice with a typical ALL dissemination pattern and to show self-renewal by serial transplantation. The samples used were from higher risk paediatric patients, one defined by a high presenting blast count and two by the presence of the MLL-rearrangement. However, again in contrast to the studies performed earlier, only normal haematopoietic engraftment was achieved from the immature $CD34^+CD38^+CD19^+CD10^-$ compartment (Kong et al., 2008). The decisive contribution of this work regarding the dispute over the differentiation level of LIC in ALL is that it not only determined LIC in the abnormal $CD34^+CD38^-CD19^+$ population but also in the $CD34^+CD38^+CD19^+$ fraction, that truly resembles a committed stage in the physiological lymphoid hierarchy. Additionally, supporting the hypothesis that immonophenotypically B-lineage committed blasts harbour LICs, le Viseur *et al* published the observation that not only $CD34^+CD19^-$ and $CD34^+CD19^+$ but interestingly also developmentally further progressed $CD34^-CD19^+$ and $CD19^+CD20^{low/-}$ or $CD19^+CD20^+$ blasts serially reconstituted the patient leukaemia. This study used mice and transplantation techniques most favourable of xenoengraftment known to date. In contrast to earlier studies, additionally candidate LSC populations were orthotopically injected into the bone marrow of the mice femora. The hosts consisted of NOD/SCID mice in which residual natural killer cells (NK-cells) were additionally depleted by anti-CD122 treatment for the majority of experiments or NSG mice that naturally lack any NK-cells. However, only cell numbers of 2.0×10^3 per mouse or higher were sufficient for engraftment of NOD/SCID mice and limiting cell doses in NSG mice, used for few experiments only, were not determined (le Viseur et al., 2008).

Another recent study did not address the question of the immunophenotypic identity of a potential ALL stem cell but aimed to establish the frequency of LIC within the unsorted bulk ALL population. The high frequency that was found in their intravenous NSG

mouse transplantation model, with 1 to 100 blasts being sufficient to transfer the leukaemia, provided additional support that LIC could not be restricted to the very small compartment of immature blast immunophenotypes (Morisot et al., 2010).

The comparison of these studies confirms the superiority of the orthotopic NSG mouse model to the intravenous NOD/SCID mouse model as already established for normal haematopoiesis in that it was capable to engraft immunophenotypically more mature blast populations and detected a higher frequency of LICs. In line with that, the limiting dilution experiments on intrafemorally transplanted NSG mice in this thesis demonstrate a much higher frequency of LSC´s in unsorted and sorted populations of ALL than the studies performed on NOD/SCID mice. Furthermore, purified blast populations more mature in the surface marker expression profile as well were able to engraft the leukaemia to the full original immunophenotype. Reconstitution of the immunophenotype of the original leukaemia sample was demonstrated before in the intravenous NOD/SCID xenotransplant model with the engrafted immature cell fraction giving rise to normally lineage restricted and more mature immunophenotypes (Cobaleda et al., 2000; Cox et al., 2009; Cox et al., 2004). However, the presented work additionally demonstrated that more mature blast populations are able to reconstitute the full immunophenotype of the original ALL sample. This suggests that ALL blasts do not follow regulations of normal haematopoiesis where development usually is unidirectional- probably with the exception of CD34 expression which interestingly has been found to be reversible on human bone marrow cells capable of long-term multilineage engraftment (Zanjani et al., 2003).

In addition to the applied xenotransplantation model, another contributing factor for not seeing engraftment from some sorted subpopulations or lower transplanted cell numbers in the above mentioned studies could lie in the observation time of the transplanted mice. This varied considerably between groups and sometimes was less than 10 weeks

and therefore probably not long enough to allow for engraftment of all investigated subpopulations. However, this is not too likely to be the case as engraftment kinetics of differentially mature blast subpopulations in this study were comparable when ALL samples were sorted according to CD10, CD20 or CD34 expression.

A very important reason why in some studies immunophenotypically more mature ALL subpopulations did not engraft in intravenously injected NOD/SCID mice was recognized more recently. Identifying normal or malignant populations of cells with stem cell characteristics in general involves purification of subfractions according to their expression of surface marker antigens. Subfractions of cells can either be flow sorted by labelling different CD surface antigens with mAbs conjugated to distinguishable fluorescent dyes or separated with the help of columns and magnetic beads bound to specific mAbs. Until recently the impact of cell labelling with mAbs gained little attention within the research community and they were generally considered to be neutral in regards to the outcome of the experiment. Work by Dominique Bonnet´s group recently investigated the possibility of antibody mediated clearance of transplanted cells by residual immunity of different mouse strains. They found that especially certain antibodies directed against CD38 had profound inhibitory effects on engraftment of normal haematopoietic as well as AML cells. This inhibitory effect was mediated by the Fc receptor of the mAb and could be reduced by additional immunosuppression with unspecific IVIG or specific anti-CD122 antibodies. The inhibitory effect was also reduced by orthotopic injection of cells into the bone marrow, avoiding clearance of iv injected cells by residual peripheral immune cells. Furthermore, antibody mediated clearance was less pronounced in more immunodeficient mouse strains (NSG and NOD/SCID-$\beta 2m^{-/-}$ mice) as compared to NOD/SCID mice with higher residual innate immunity due to production of NK cells (Taussig et al., 2008). CD38 expression has been an important surface marker in discriminating stem cells in the

haematopoietic system and in leukaemias from cells that presumably lacked stem cell defining properties. The above described studies that used CD38 in their sorting strategies have to be seen in this context. Clearance of candidate stem cells that are labelled with anti-CD38 mAbs can explain why earlier studies failed to engraft a $CD34^{+}CD38^{+}$ subfraction of blasts but it can not explain, why the subset of cells negative for the CD34 surface marker expression, in contrast to the CD34 positive subset, did not engraft in an identical experimental setup when the leukaemia was only purified according to CD34 expression. Additionally, the influence of antibodies bound to surface antigens on stem cell readout can not explain the higher frequency of LICs in unsorted blast populations found in this work compared to frequencies published from other laboratories. A likely explanation for both observations again would be the higher degree of immune suppression in the NSG mice as opposed to the NOD/SCID mice in combination with the injection mode.

The work from this PhD project demonstrated that a wide variety of subpopulations within the ALL purified according to the immunophenotype is able to reconstitute the leukaemia in mice therefore demonstrating stem cell characteristics. In other experiments it was proved that only very low numbers of blasts were necessary for engraftment of mice and consequently it could be argued that engraftment of presumably more mature subfractions of blasts was indeed initiated by small numbers of contaminating less mature ALL cells. In limiting dilution experiments on sorted subpopulations this was found to be unlikely. Engraftment of all paired subfractions sorted according to expression of CD10, CD20 or CD34 was initiated by comparable and very low cell numbers down to between 1.0×10^{2} and 1.0×10^{1} cells per mouse where contaminating cells become negligible. Furthermore, engraftment kinetics were similar, which would be very unlikely in the case that engraftment from one population originated only from a very small number of contaminating cells. Still, it can not be

completely ruled out that engraftment kinetics or determined LIC frequencies in sorted subpopulations were influenced by the bound sorting antibodies as described for the CD38 surface antigen. However, the argument of contribution to or exclusive origin of engraftment from contaminating cells of the opposing immunophenotype is additionally weakened by the tertiary CD34 sorting experiment (KR59) in which the transplanted $CD19^{+}CD34^{high}$ cells only gave rise to a population of the identical immunophenotype, not merely in mice transplanted with just 1.0×10^{1} cells but also when transplanted with 1.0×10^{3} cells. Any contaminating $CD19^{+}CD34^{low}$ LIC would have led to the reconstitution of the whole original immunophenotype as seen in mice transplanted with the $CD19^{+}CD34^{low}$ subfraction. Nevertheless, contamination can not be ruled out definitely for every single experiment that was performed for this thesis. Finding a solution for this problem is difficult with the currently available cell sorting options. A second round of cell sorting would probably increase purities further but as well have a negative impact on the viability of blasts which in reverse would then influence the likelihood of engraftment. The ideal solution in the xenotransplantation setting would be to orthotopically transplant single cells of a highly purified and viable ALL blast population. However, this is technically very difficult to achieve and it is most likely that the number of mice needed would yet increase again. Nevertheless, unlike earlier less immunocompromised mice, the NSG mouse model already seems to be sensitive enough to be permissive for single cell engraftment at least for ALL blasts (Morisot et al., 2010).

In the presented study, much higher LIC frequencies for unsorted and sorted ALL subpopulations than previously reported were determined in an improved xenotransplantation model but it is very likely that these frequencies are still an underestimation and do not match reality. The defining properties of self-renewal and differentiation capability of normal and cancer stem cells can currently only be assayed

in the potentially hostile animal xenotransplantation models. Only candidate cells that can adapt and engraft in the animal host do also have the chance to prove self-renewal and to spawn the more differentiated progeny cells that comprise the tumour, the two hallmarks of CSCs. This is the problem with probably the highest impact on all stem cell research; the xenotransplant setting. Many mechanical steps are involved from the preparation of purified candidate populations to the process of transplantation, when the transplanted cells finally come across a significantly different microenvironment. Both soluble factors and factors bound to membranes in the microenvironment play an important and critical role in tumour growth as does accessibility to blood circulation, the availability and composition of growth factors and the extracellular matrix provided by the host (Gupta et al., 2009; Hanahan and Weinberg, 2011). All this is likely to have a significant impact on the seeding efficiency of human malignant cells in the murine xenoenvironment and one major step to improve this xenotransplantation mouse model could be to further humanise the NSG mouse strain probably most importantly in respect to the bone marrow niche with its nurturing cells and growth factors. Still, the possibility that candidate human CSCs populate the murine xenotransplant model but would not reconstitute a tumour in the appropriate human environment seems unlikely but can not definitely be ruled out. However, the contribution of different cells with stem cell character within a cancer to the re-established tumour in the mouse could well be different than would be the case in humans.

However, the extent of residual immune function in mice certainly plays a crucial role for the sensitivity of the xenotransplant assay. NOD/SCID mice have been used in many studies and led to invaluable progress in the field of stem cell research, however, with increasing immune suppression of more recently developed mouse strains like the NSG mice, normal haematopoietic stem cells and CSCs could be identified in subpopulations that evaded detection previously. Another caveat of the xenotransplantation models is

that it is possible that only dominant subclones read out thereby imitating a hierarchical organisation (Adams and Strasser, 2008; Shackleton et al., 2009). However, the xenotransplantation model as developed and used in the work for this thesis with the use of severely immunocompromised NSG mice, highly FACS purified candidate cancer stem cells and the orthotopic injection method as well as the analytical techniques and strategies applied can be used not only for malignant diseases arising from the bone marrow. The same model most likely can be applied to most or all other cancer entities, including solid tumours or even non-malignant diseases in order to help elucidating the origin, progression or physiology.

To circumvent inter-species barriers syngenic animal transplant models have been established. The second major advantage of this approach is that the necessity of immune suppression can be avoided. Applying this strategy on Myc-induced T-cell ALL in clonal zebrafish, a frequency of self-renewing cells of up to 16% was found in the bulk T-cell ALL mass by transplant experiments (Smith et al., 2010). Similar experiments were performed with syngenic mice. In an elegant study, re-transplanting of pre-B/B lymphoma cells from Eμ-myc transgenic mice into non-irradiated histocompatible sibling mice resulted in the development of disseminated lymphoma in just over one month even when only 10 cells were transplanted (Kelly et al., 2007). High frequencies of LSCs (approximately one in 3 to 4 cells) have also been found for MLL/AF9 induced AML in a syngenic mouse model and in contrast to the human mouse xenotransplant findings, the large majority was found in already myeloid lineage restricted cell fractions and not only in the very small stem and progenitor cell populations (Somervaille and Cleary, 2006). Opposed to that xenotransplantation assays have determined the leukaemia initiating cell in human AML as being much less abundant with approximately 1 in 10^6 blasts (Hope et al., 2004). Taking into account that there will be biological differences between murine and human AML this still points out

that the sensitivity for AML LIC detection decreases hugely in an interspecies setting and most likely that also holds true for ALL.

Analysis of the results of xenotransplant assays also needs to consider the limitations and caveats of the transplantation technique. Not every cell that potentially has HSC or CSC characteristics will find its way into the appropriate niche following transplantation and the efficiency of engraftment is influenced by the transplantation procedure. Two different groups demonstrated that after injection into the tail vein of NOD/SCID mice only around 5% of human progenitor cells from bm, umbilical cord blood (UCB) and peripheral blood stem cells (PBSC) home to the bone marrow within 24 hours. The majority of cells are most likely to be sequestered in organs with dense capillary networks such as the lungs and the liver (Cashman and Eaves, 2000; van Hennik et al., 1999). This low seeding efficiency of human cells in the murine xenoenvironment automatically must lead to a significant underestimation of stem cell frequencies. In order to improve seeding efficiencies, orthotopic transplant models that place human cells into the closest possible proximity to their niche have been developed and shown to be very efficient. However, even when cells are transplanted intrafemorally, they are, not directly delivered into the niche. Drilling a hole into the bone sets a trauma with consecutive bleeding and it is very likely that some of the delivered cells will be caught in a resulting blood clot or be carried away by continuing bleeding. This will lead to a reduced seeding efficiency and subsequently to an experiment bias with underestimation of LIC frequency.

On the other hand, it has been shown for AML and ALL samples that the detected SL-IC frequency, as determined by limiting dilution experiments in the xenotransplantation model, raises after a first passage through the mouse by a factor of approximately 10 (Cobaleda et al., 2000; Lapidot et al., 1994). This observation forms the basis of criticism to assays where first unsorted ALL samples were transplanted into mice and

recovered bm of engrafted mice was used for secondary transplantation experiments with sorted cell subfractions. It has been suspected that this results in a preselection of blasts with the ability to repopulate primary mice (Cox et al., 2009). This argument has to be looked at in more detail and used with caution. It may well be possible that primary diagnostic patient blasts are heterogeneous in the ability to engraft the xenoenvironment of mice and this may explain the disparity of numbers of cells necessary for mouse engraftment in primary and secondary or tertiary experiments. However, unsorted primary patient cells reconstituted the full immunophenotype of the original ALL and thereafter blast populations of different maturity according to the immunophenotype were sorted, transplanted and successfully reconstituted the ALL in secondary mice. A preselection of cells can not explain why the whole variety of differentially mature cells was able to transfer the leukaemia. It can only question the frequency of stem cells within the populations. Additionally, cells will go through a process of adaption during growth in mice but adaption is a universal response in biology to changed environments and conditions. These are certainly present in a xenotransplant murine model and some transplanted cells may settle in locations with more favourable conditions for adaption in the new environment than others which then fail to read out as LICs. Additionally, strong support that the engrafted ALL in mice largely resembles the original patient leukaemia and that it is not an *in vivo* cell line that is created upon first transplantation into mice was provided by the studies of Anderson *et al* and Notta *et al*. They proved that not only one but different subclones of the patient leukaemia are reconstituted in the recipient mice (Anderson et al., 2011; Notta et al., 2011). However, there are other reasons that can explain the differences found for stem cell frequencies between primary and secondary samples at least to some degree. The viability and fitness to engraft of transplanted cells may well be considerably different as well. Patient samples are often collected in a diagnostic bmp in one hospital and then

sent to the study centre, ideally over night. However, transport still takes a considerable amount of time and samples are exposed to varying temperatures and mechanic forces before they are frozen down. Samples are then again often shipped to research centres on dry ice before being transplanted. After thawing cells may still appear to be alive according to Trypan blue exclusion but it is known that blasts usually are not able to survive outside the body for extended periods of time or to maintain their proliferation ability. Secondary or tertiary bm samples on the other hand are collected and frozen down with minimal delay or even re-transplanted immediately. As this involves less people than the multiple steps in which diagnostic patient material is dealt with, these processes are self evidently also more standardised. In fact, stem cell frequencies in leukaemias of mouse origin have been found to be considerably higher in primary but syngenic mouse models. One reason for that in addition to the advantages of a syngenic mouse model could also be that the mouse with the original leukaemia and the recipient of transplanted material were dealt with in the same lab by the same people and that harvested material could be re-transplanted without delay.

Applying the orthotopic xenotransplantation model with NSG mice a LIC frequency for several ALL samples somewhere between one in 1.0×10^3 and less than one in 1.0×10^1 could be demonstrated. Despite this high proportion of LICs in the leukaemic population and the still likely underestimation of LIC frequency even in the most advanced xenotransplant setting, it could potentially be possible that a hierarchical organisation existed if only unsorted bulk ALL populations were considered. However, convincingly demonstrating comparable LIC frequencies in defined subpopulations that by surface antigen expression resemble different stages of physiological B cell development strongly suggests that the hierarchical stem cell model can not be applied to ALL.

Otherwise, like in AML, LICs should at least be found more frequently if not exclusively within immunophenotypically less mature blast subpopulations.

The proposition that ALL, unlike AML or normal myeloid haematopoietic development, follows the stochastic stem cell model potentially can be explained by normal lymphatic cell development. Despite a tight regulation, B and T cells do not lose the ability of clonal expansion during their progress through different maturation stages and this characteristic probably is maintained in malignancies of the lymphoid lineage. Interestingly, the insights gained from this study on ALL have been suggested for normal lymphoid development already many years ago by one of the pioneers of stem cell biology. At the meeting of the American Society of Hematology (ASH) in 1982, McCulloch stated in the Henry Stratton Lecture that "a minimum conclusion to be reached from a comparison of myelopoiesis and lymphopoiesis is that a firm linkage between differentiation and loss of proliferative capacity is not a general characteristic of hemopoiesis. The implication is that separate genetic mechanisms may exist for the regulation of the two processes" (McCulloch, 1983). The assumption is that in contrast to the hierarchically organised myeloid lineage, the ability of clonal expansion is maintained at all maturational levels of lymphoid precursor cells.

The findings described in this PhD thesis potentially have important implications for ALL therapy. It is generally accepted that relapse arises from cancer stem cells that survive the standard cytotoxic chemo- and radiotherapy. There is also hope that if it was possible to directly and effectively target these cancer stem cells with more specific treatment patient outcome would improve significantly. It is also generally acknowledged that cancers are heterogeneous regarding the composition of cells. However, if stem cell properties can be found across all or most cells comprising the

malignancy as shown here for ALL, theoretically all of these cancer cells could be potential survivors of therapy and eventually would be able to initiate relapse. This gives a new aspect that needs to be considered for the design of future studies on ALL. As it is already possible to achieve remission in most cases of ALL, it is self evident that current treatment regimens successfully eradicate most cells that have stem cell properties. Again, the remaining question is which are the cells that eventually give rise to relapse and what characterizes them? Is it a certain and defined subset of the cells that have been found to be tumour propagating cells or is it a stochastic survival of any random phenotype? Furthermore, is there a difference in early and late relapse? Some answers to these questions could be given by determining the cycling characteristic of different subpopulations which is believed to have a significant impact on the success of treatment that is mainly based on antiproliferative drugs. This has been done in *in vitro* experiments performed by Hirt and colleagues. They investigated proliferation kinetics in different immunophenotypic subgroups of paediatric ALLs and demonstrated varying proportions of blasts in S-phase by assessing bromodeoxyuridine labelling indices. They found that the main $CD19^+$ subfraction of blasts was much faster cycling compared to the very small populations of $CD19^-CD10^-$ and $CD19^-CD34^-$ leukaemic cells which remained in a mainly quiescent status (Hirt et al. 2011). These results indicate that despite possessing stem cell abilities, leukaemic subpopulations of different immunophenotypes are differently amenable for current chemotherapeutic regimens. Despite not addressing cycling kinetics directly, the *in vivo* experiments in this PhD project can not confirm the findings of Hirt *et al*. Engraftment kinetics of rather immature and more mature subpopulations sorted according to CD10, CD20 or CD34 surface marker expression were comparable indicating that average cycling times of transplanted cells must be similar. Although the cycling status of cells within one sorted subpopulation could still be heterogeneous with parts of cells being quiescent and other

parts actively proliferating it then would be likely that the fractions of quiescent and cycling cells would be comparable between subpopulations. Therefore, a stochastic survival of cells of different immunophenotypically defined maturity is more realistic and elucidating the extrinsic factors that have an impact on stem cell protection from conventional therapy and their long term survival will be of invaluable importance. On the other hand, despite the finding in this work that all sorted blasts of different immunophenotypes do reconstitute the ALL with similar kinetics, it could still be possible, that some immunophenotypes are more sensitive to chemotherapy than others. Still, as all different blast immunophenotypes do reconstitute the complete original immunophenotype of the patient leukaemia, the immunophenotypes that are potentially easier to eradicate would be immediately reconstituted during recovery phases of chemotherapy regimens by the other immunophenotypes that are less responsive to the applied chemotherapy. If that would be the case, one approach could be to force the leukaemic blasts with yet unknown strategies to express only the immunophenotype that is more amenable to chemotherapy. A following project to this work could aim to assess the sensitivity of leukaemias reconstituted from different sorted blast populations to the chemotherapeutic drugs commonly used in ALL treatment regimens. Groups of mice could be transplanted with different blast immunophenotypes of ALL subtypes. The most important drugs used in the treatment of precursor B-ALL, steroids (Prednisone, Dexamethasone), Vincristine, L-Asparaginase, Cyclophosphamide, Methotrexate, Anthracyclines (Daunorubicine, Doxorubicine), Cytarabine, 6-Mercaptopurine could then be applied in adapted doses to the transplanted mice starting at the time of transplantation or at the time when the leukaemia is already engrafted and the mouse shows the full picture of overt systemic ALL. This would provide data about drugs that are more relevant in reducing the tumour burden in initial treatment and drugs that could be more efficient in a maintenance therapy that aims at potentially remaining blasts that

potentially are the origin of relapse. Furthermore, in a second step, combinations of drugs could be used and synergistic effects could be evaluated. The findings of these experiments could then potentially have an impact on the adaption of current treatment regimens. Application of single drugs or drug combinations is most likely to reduce the tumour burden and it can also be expected that transplanted, engrafted and then treated mice go into complete morphological or even molecular disease remission. However, alike in patients it is not likely that this remission is stable in nature and it must be assumed that the leukaemia will eventually relapse unless a treatment regimen with the different blocks of remission induction, consolidation and maintenance is applied. This provides an interesting model of disease relapse. The relapsed leukaemia then can be genetically compared to the initial ALL using expression arrays and conclusions regarding the clonal evolution can be drawn. The relatively long life span of the NSG mice would probably even allow investigating secondary and tertiary relapses as well. Furthermore, it would be interesting to address differences of relapses that araise in the bone marrow, the brain and in male mice potentially also in the testes. Examining the brains of mice from experiments of this work already could prove leukaemic infiltration of the meninges in NSG mice (work performed by Dr. Christina Halsay, Glasgow; unpublished data) and this provides an excellent opportunity to investigate the blasts that remain in this sanctuary site more closely.

The work for this thesis included several *BCR/ABL1* positive ALL and the clinical picture of the ALL reconstituted in NSG mice closely resembles that of the ALL in human beings. Again, this makes the NSG xenotransplant model an ideal setting in order to investigate the effects of the different tyrosine kinase inhibitors in pre-clinical studies. In a first setting, Imatinib, Dasatinib and Nilotinib could be applied to mice transplanted with unsorted *BCR/ABL1* positive ALL blasts but surely, the effect on different subpopulations sorted according to the immunophenotype could be tested as well.

Furthermore, chemoresistance is a common problem faced in cancer therapy. The NSG model would also allow testing for the development of chemoresistance. In serial transplantation experiments it could be established if clones evolve that are resistant to the applied tyrosine kinase inhibitors. It then would be of major importance to characterise these clones by expression arrays. This could contribute to elucidate the mechanisms of clonal evolution in Philadelphia chromosome positive ALL.

The Philadelphia chromosome is regarded to play a major role in malignant transformation. However, secondary events are necessary before unlimited growth of ALL blasts is initiated but also, during clonal evolution a leukaemia eventually can become independent of this chromosomal translocation (t(9;22)). The NSG mouse model could also be used to elucidate the steps that are involved in this process and sufficient amounts of material of BCR/ABL1 positive ALL was generated and conserved in liquid nitrogen during the work for this thesis. Short hairpin RNA (sh-RNA) can be efficiently used to silence the expression of the BCR/ABL1 RNA via RNA interference leaving the ALL blasts devoid of the BCR/ABL1 protein. Plasmids containing a specific sh-RNA directed against BCR/ABL1 transcripts can be delivered to Philadelphia chromosome positive ALL blasts by viral vectors and these blasts then can be transplanted into mice. Primary patient material but also material that was passaged through mice already over one or two generations could be used for these experiments. Again it would be interesting to investigate whether or not there are differences in engraftment between the primary, secondary or tertiary leukaemia. Furthermore, this experimental setup could be used not only on ALL that were serially transplanted into mice or that relapsed in mice treated with chemotherapy but probably even more interestingly on primary material of patients at the time points of initial presentation with the ALL and blasts from relapse after chemotherapy. It is not unlikely that blasts of the primary leukaemia biologically behave differently compared to blasts

from relapse in the way that they became independent of the BCR/ABL1 fusion protein. Similar experiments could as well be performed on leukaemias with the *ETV6/RUNX1* (*TEL/AML1*) translocation and on leukaemias with *MLL* rearrangements or other translocations. These experiments would aim at engraftment potential of the various blast populations but it would be also interesting to investigate how an overt leukaemia behaves in the case of withdrawel of BCR/ABL1, ETV6/RUNX1 or MLL rearranged fusion proteins. The development of inducible vectors containing sh-RNA directed at the individual transcripts would be the solution for these experiments. Transduced ALL blasts would be transplanted applying the NSG mouse model and once the leukaemia was established in the mouse the sh-RNA would be induced by application of usually Doxocycline. The effect on the leukaemia after deprivation of fusion proteins could then be assayed again in primary and serially transplaned human ALL samples.

However, important conclusions regarding effects of the current treatment protocols can be drawn from the data of this work. The stochastic distribution and high frequency of stem cells in ALL can potentially already explain the necessity for prolonged maintenance chemotherapy as all cells need to be reached, especially those in cell cycle quiescence or others residing in sanctuary sites like the CNS or the testes. The ability of potentially long periods of cycle quiescence, if present, additionally could be a difference between lymphatic and myeloid malignancies and lymphoblasts, like the potential of clonal expansion, could have taken this over from their normal lymphocyte counterparts which apply this ability in the memory B or T cells.

On the other hand, the proposition that lymphoid clonal expansion is regulated in a different way than malignant proliferation of myeloid cells holds attractive prospects for the development of more specific and better tolerable therapeutic strategies. Elucidating the intracellular pathways that are activated in lymphoid clonal expansion but not involved in the self-renewal of adult stem cells opens a new and broad field of research

and could potentially lead to the discovery of druggable targets. Previously, it was difficult to investigate physiologic or malignant lymphoid development. Unlike with myeloid lineages, there were no *in vitro* assays that enabled growth of lymphoid progenitors. The developed NSG xenotransplant mouse assay now provides the most ideal means to date to model the involved stages of the lymphoid hierarchy. If it now would be possible to elucidate and specifically target the pathways responsible for uncontrolled malignant lymphoid proliferation, myeloid development potentially will not be affected. This would lead to an invaluable improvement in ALL therapy away from more or less uniform and generally cytotoxic chemotherapy towards a specifically targeted and ultimately even tailored patient specific treatment regimen. Current antiproliferative treatment modalities result in sometimes prolonged phases of neutropenia that put patients at high risk for opportunistic infections which potentially can be lethal. Concomitant anaemia increases the probability of infections and mucositis with loss of integrity of the gut lining, another side effect of unspecific antiproliferative agents, provides entry sites for pathogens to the human organism. Avoiding these side effects on ALL patients would lower the risks of chemotherapy considerably. Additionally, the probability of secondary malignancies caused by the unspecific antiproliferative chemotherapy also potentially could be diminished. Knowledge about the involved mechanisms of lymphoid clonal expansion would not only provide a chance for a great step forward in the treatment of lymphoblastic leukaemias but as well could potentially be implemented in treatment strategies for other lymphoid malignancies, lymphoproliferative diseases or even autoimmune diseases, especially if it would be possible to modulate and not only interrupt the involved pathways.

5.2 Overall Conclusions

Using the most advanced xenotransplantation model the data presented in this PhD thesis provide strong indication that leukaemia initiating cells in acute lymphoblastic leukaemia are by far more frequent than previously thought and equally present in subfractions of diverse maturity according to CD surface antigen expression profile. Therefore the long defended assumption that ALL is organised in a hierarchy can not be maintained and now clear evidence is given that the stochastic stem cell model has to be applied to ALL.

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APPENDICES

Appendix 1- Raw Data

Raw data of mouse experiments

The following 22 pages contain a list of all mouse xenotransplant experiments carried out as part of this PhD project. For each experiment essential information is provided regarding the original patient sample or the engrafted mouse sample used for transplant, the sorted immunophenotype and the respective sorting purity as well as the number of transplanted cells per mouse. Furthermore, the level of leukaemic engraftment and the time after transplantation in weeks for each bone marrow puncture and the final bone marrow harvest is provided. Altogether 62 individual experiments with 672 transplanted mice are listed.

Exp. No. experiment number, Wks weeks, BMP bone marrow puncture, * positive outside limits, # sample contained a large amount of cell debris, § mouse was found dead

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR1	M1	8849 prim	unsorted	1.0×10^6	n/a	8	0.039	10	0.134	13	0.087	16	4.0	21	7.8	25	75.7
	M2					8	0	10	0	13	0	16	0	21	0	49	0
	M3					8	0	10	0	13	0	16	0			>16	
	M4															<8	
KR2	M5	A67 prim	unsorted	3.0×10^5	n/a	9	0.008*	12	1.4	15	5.2	16	4.2			16	40.6
	M6					9	0	12	0.057	15	0.041	16	0.32			18	14.8
	M7					9	2.1	12	3.7							14	74.8
	M8					9	0.51	12	0.064	15	8.6	16	3.2	19	22.5	19	75.8
KR3	M9	HV102 prim	unsorted	3.0×10^5	n/a	9	0	12	0							45	0
	M10					9	0	12	0							45	0
	M11					9	0	12	0							45	0
	M12					9	0									>9	
KR4	M13	A67 sec (of M7)	$CD34^+CDC19^{low}$	2.0×10^3	96.9%	10	0	13	failed	16	36.9	19	0.466			20	7.0
	M14					10	0	13	failed	16	0.11	19	0.174	23	1.9	23	36.9
	M15					10	4	13	failed							13	16.8
	M16					10	8.9	13	failed	16	13.4					17	$0^{\#}$
	M17		$CD34^+CDC19^{high}$	2.0×10^3	99.5%	10	0.059	13	failed	16	1.0	19	3.3			20	46.9
	M18					10	37.8	13	failed	16	14.9	19	0.087			19	52.1
	M19					10	2.6	13	failed							13	$0.68^{\#\S}$
KR5	M20	A67 sec (of M5)	$CD34^+CDC19^{low}$	4.0×10^3	97.6%	8	2.7	11	failed	15	18.0	17	21.4	20	9.8	21	$1.2^{\#}$
	M21					8	0	11	failed	15	3.6	17	14.5	20	34.8	20	34.9
	M22					8	5.2	11	failed	15	9.6	17	1.1			18	11.5
	M23		$CD34^+CDC19^{high}$	2.0×10^4	99.9%	8	2.3	11	failed	15	1.8					>15	
	M24					8	0.133	11	failed							11	22.6
	M25					8	17.9	11	failed							>11	
KR6	M26	A67 sec (of M5)	unsorted	1.0×10^5	n/a	8	10.5	11	failed	15	1.6					>15	
	M27					8	14.3	11	failed							11	33.5
	M28					8	0	11	failed	15	13.2					>15	
KR7	M29	2510 prim	unsorted	1.3×10^6	n/a	15	14	21	16.8	25	25.3					25	30.9
	M30					15	3.4	21	15.3	25	55.8					25	56.5
	M31					15	1.3	21	12.4	25	24.1					25	30.6
KR8	M32	9406 prim	unsorted	2.5×10^6	n/a	15	0	21	0	25	0	32	0.0023*			54	0
	M33					15	4.6	21	3.7	25	13.2	32	0.057			45	14.5
	M34					15	20	21	2.2	25	3.7					26	21.1

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR9	M35	HS39 prim	unsorted	3.0×10^3	n/a	8	0	15	0	21	0	26	0			>26	
	M36					8	0	15	0	21	0	26	0			31	0
	M37					8	0									>8	
KR10	M38	HV127 prim	unsorted	3.0×10^4	n/a	8	0	15	0	21	0	26	0			49	0
	M39					8	0	15	0	21	0	26	0			31	0
	M40															4	0
KR11	M41	A67 sec	$CD19^+CD20^{high}$	2.0×10^4	83.9%	9	failed	13	4.1							14	46.2
	M42	(of M6)				9	failed	13	6.8							14	72.2
	M43					9	failed	13	0.071	15	14.9					16	49.6
	M44		$CD19^+CD20^{low}$	2.0×10^4	99.9%	9	failed	13	1.9	15	10.0					>13	
	M45					9	failed	13	2.3							14	62.5
	M46					9	failed	13	5.4	15	5.5					15	15.2
KR12	M47	A67 sec	$CD19^+CD20^{high}$	9.0×10^3	89.7%	8	24.3	12	50.5	15	7.4					19	61.4
	M48	(of M8)				8	1.6	12	18.5							>12	
	M49					8	21.5	12	9.0	15	22.5					18	69.8
	M50		$CD19^+CD20^{low}$	9.0×10^3	99.5%	8	42	12	3.5	15	10.5					19	74.8
	M51					8	18.1	12	43.9							18	32.8
	M52					8	23.1	12	50.6							18	24.1
KR13	M53	8849 sec	$CD19^+CD10^{high}$	3.0×10^4	96.7%	11	1.9	13	1.6	17	0.533	22	1.6	34	0.65	50	55.5
	M54	(of M1)				11	0.054	13	0.045	17	1.1	22	0.054			26	42.4
	M55					11	1.1	13	0.118	17	1.1	22	1.1	34	0.016*#	36	4.1
	M56		$CD19^+CD10^{low}$	3.0×10^4	98.5%	11	0	13	0	17	0	22	0	34	0	48	0
	M57					11	0	13	0	17	0	22	0	34	0	50	0.0027*
	M58					11	0	13	0	17	0	22	0	34	0	50	0
KR14	M59		unsorted	2.4×10^5	n/a	11	0.079	13	0.17	17	1.4	22	8.4			>22	
KR15	M60	A67 sec	unsorted	2.0×10^6	n/a	9	3.6	13	7.2							13	20.3
	M61	(of M6)				9	16.9	13	33.5	17	4.0					>14	
	M62															<9	
	M63															<9	
KR16	M64	WB51 prim	unsorted	9.8×10^4	n/a	7	11.4									11	58.6
	M65					7	19.4	11	8.4							16	80.9
	M66					7	43.3	11	12.8							14	51.7
	M67					7	27.8	11	7.7							14	55.5

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR17	M68	WB51 prim	$CD19^{+}CD20^{high}$	1.08×10^{4}	91.9%	7	1.1	11	7.2	17	12.8					19	86.4
	M69					7	0.53	11	33.2							16	77.7
	M70					7	0									>7	
	M71		$CD19^{+}CD20^{low}$	6.24×10^{4}	99.9%	7	0.54	11	7.6							15	72.0
	M72					7	2.9	11	12.6							15	69.5
	M73					7	1.7	11	11.1							15	60.3
KR18	M74	WB51 prim	$CD34^{+}CDC19^{high}$	3.12×10^{4}	99.3%	7	2.2	11	8.5							16	76.0
	M75					7	14	11	14.8							16	76.9
	M76					7	1.3	11	11.8	16	45.4					17	71.5
	M77					7	7.4	11	1.4							15	58.6
	M78		$CD34^{+}CDC19^{low}$	4.0×10^{3}	99.0%	7	0.043	11	0.43	16	23.7					20	74.0
	M79					7	0.047	11	10.5	16	8.9					19	81.9
KR19	M80	8849 sec (of M1)	$CD19^{+}CD10^{high}$	1.44×10^{4}	98.4%	8	0.012*	12	0.013*	19	0.615	23	0.096			41	0.556
	M81					8	0	12	0	19	0	23	0			23	0
	M82					8	0	12	0							14	0
	M83					8	0	12	0	19	0	23	0			26	0
	M84		$CD19^{+}CD10^{low}$	2.19×10^{4}	99.4%	8	0	12	0.016*	19	0.01	23	0.117			41	41.5
	M85					8	0	12	0	19	0	23	0			41	0.012
	M86					8	0	12	0	19	0	23	0			41	0
	M87					8	0	12	0	19	0	23	0			41	0
KR20	M88	8849 sec (of M1)	$CD19^{+}CD10^{high}$	1.44×10^{4}	98.4%	7	0	12	0							22	0
	M89					7	0	12	0							>12	
	M90					7	0	12	0							18	0
	M91					7	0	12	0							>12	
	M92		$CD19^{+}CD10^{low}$	2.19×10^{4}	99.4%											<7	
	M93					7	0	12	0							27	0
	M94					7	0	12	0							27	0
	M95					7	0	12	0							27	0
KR21	M96	WB51 sec (of M64)	$CD19^{+}CD10^{high}$	1.24×10^{4}	98.1%	10	0.01*	15	2.4							18	61.1
	M97					10	0.388	15	25.3							18	33.4
	M98					10	1.8	15	4.3							18	30.9
	M99					10	0.2	15	33.9							18	18.1
	M100		$CD19^{+}CD10^{low}$	1.35×10^{4}	99.4%	10	1.2									14	61.3
	M101					10	13.2	15	12.9							18	38.8
	M102					10	8.2	15	3.7							21	66.9
	M103					10	34.9	15	2.6							15	26.6

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR22	M104	L784 prim	$CD19^{+}CD10^{high}$	2.61×10^{4}	98.3%	15	46.3	20	6.2	29	0.27	39	7.1			41	20.3
	M105					15	61.4	20	43.8	29	4.3					36	33.6
	M106					15	12.3	20	17.2	29	13.3					33	55.2
	M107															14	35.1
	M108		$CD19^{+}CD10^{low}$	6.3×10^{3}	99.8%	15	0.266	20	22.2							25	35.5
	M109					15	15.5	20	3.0	29	16.0					>29	
	M110															<15	
	M111					15	36.6	20	17.6	29	46.4					33	67.8
KR23	M112	L831 prim	$CD19^{+}CD10^{high}$	6.0×10^{3}	98.3%	13	0	18	0							>18	
	M113					13	0	18	0							36	0
	M114					13	0	18	0							>13	
	M115					13	0	18	0							>13	
	M116		$CD19^{+}CD10^{low}$	6.0×10^{3}	99.9%	13	0	18	0							36	0
	M117					13	0	18	0							36	0
	M118					13	0	18	0							36	0
	M119					13	0	18	0							23	0
KR24	M120	2510 sec (of M31)	$CD19^{+}CD10^{high}$	4.5×10^{3}	98.5%	13	0	18	0	21	0	27	0	36	0	46	0
	M121					13	0	18	0.082	21	0	27	0	36	0	49	0.013
	M122					13	0.017*	18	0.097	21	0.412	27	3.4	36	16.5	39	50.3
	M123					13	0	18	0	21	0	27	0	36	0	49	0
	M124		$CD19^{+}CD10^{low}$	6.9×10^{3}	98.8%	13	0	18	0	21	0	27	0	36	0	49	0
	M125					13	0	18	0	21	0	27	0	36	0	49	0
	M126					13	0.004*	18	53.6	21	9.2	27	19.7			30	1.2
	M127					13	5.1	18	41.1	21	18.9	27	30.4	36	0.066	48	0.142
KR25	M128	A67 sec (of M7)	unsorted	6.0×10^{4}	n/a	13	1.1									>13	
	M129					13	74.2									15	#§
	M130					13	8.9									15	73.3
	M131					13	10.6									15	30.5
KR26	M132	WB51 sec (of M68)	$CD19^{+}CD20^{high}$	3.06 x 103	93.8%	10	8.2	15	32.5	18	63.8					18	30.6
	M133					10	9.4	15	49.9							16	57.4
	M134					10	8.1	15	23.6	18	16.9					20	62.8
	M135					10	6.4	15	1.5	18	9.4					20	59.4
	M136		$CD19^{+}CD20^{low}$	3.06 x 103	99.8%	10	0.119	15	33.9	18	23.6					21	63.3
	M137					10	14.5									15	72.0
	M138					10	20.4									15	81.4
	M139					10	0.38	15	42.0	18	61.9					21	64.5

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR27	M140	WB51 sec (of M68)	$CD19^{+}CD20^{high}$	2.0×10^{3}	94.2%	10	37.1									15	18.0
	M141					10	10.4	15	23.4	21						19	28.5
	M142					10	0.167	15	3.9	21						>15	
	M143		$CD19^{+}CD20^{low}$	2.0×10^{3}	99.8%	10	32.4									15	76.5
	M144					10	2.3									>10	
	M145					10	2.1	15	3.0							17	#§
	M146		$CD19^{+}CD20^{high}$	5.0×10^{2}	94.2%	10	18.9	15	37.4							18	67.7
	M147					10	33.7	15	39.0							19	66.6
	M148					10	31.4	15	20.4							18	31.6
	M149		$CD19^{+}CD20^{low}$	5.0×10^{2}	99.8%	10	16.5	15	46.4							20	82.1
	M150					10	10	15	33.2							18	78.4
	M151					10	0.062	15	31.1							20	79.5
	M152		$CD19^{+}CD20^{high}$	1.0×10^{2}	94.2%	10	0	15	0.039	21	17.8	30	53.7			31	46.7
	M153															6	#§
	M154					10	0	15	0.3	21	16.8					25	39.4
	M155		$CD19^{+}CD20^{low}$	1.0×10^{2}	99.8%	10	0.041	15	0.22							>15	
	M156					10	3	15	47.7							19	42.9
	M157					10	0	15	0	21	0.24	30	64.7			33	70.7
KR28	M158	A67 tert (of M45)	$CD19^{+}CD20^{high}$	1.35×10^{3}	90.3%	9	0	14	60.8							15	40.0
	M159					9	0	14	51.4							16	57.7
	M160					9	11	14	20.8							16	68.0
	M161					9	11									14	15.4
	M162		$CD19^{+}CD20^{low}$	1.35×10^{3}	100.0%	9	3	14	44.4							16	47.6
	M163					9	4.2	14	4.6							17	58.1
	M164					9	40.6	14	29.6							17	36.4
	M165					9	14.9	14	4.8							17	54.6
KR29	M166	A67 tert (of M43)	$CD19^{+}CD20^{high}$	2.4×10^{3}	92.3%											<8	
	M167					8	0	13	0.048							17	47.3
	M168					8	0.188	13	21.1							17	47.6
	M169					8	11.1	13	1.5							>13	
	M170		$CD19^{+}CD20^{low}$	2.4×10^{3}	98.5%	8	0.063	18	12.9							22	78.4
	M171					8	61	18	16.4							>18	
	M172					8	47.6	18	13.3							>18	
	M173					8	0.01*	18	0.13							22	50.0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR30	M174	HV101 prim	$CD19^{+}CD10^{high}$	3.0×10^{3}	100.0%	8	0	14	0							>14	
	M175					8	0.004*	14	0.012*	20	2.9	27	16.9	34	43.2	42	60.4
	M176					8	0	14	0	20	0	27	0.006*	34	0.04	>34	
	M177					8	0	14	0	20	0	27	0	34	0	46	0
	M178		$CD19^{+}CD10^{low}$	3.0×10^{3}	100.0%	8	0	14	0	20	0	27	0	34	0	50	0
	M179					8	0	14	0	20	0	27	0			30	0.021
	M180					8	0	14	0	20	0	27	0	34	0	44	0
	M181					8	0	14	0	20	0	27	0	34	0	50	0
KR31	M182		$CD19^{+}CD20^{high}$	3.0×10^{3}	99.3%	8	0	14	0	20	0	27	0	34	0.184	50	77.7
	M183					8	0	14	0.015	20	4.2	27	25.8	34	42.9	38	64.1
	M184					8	0	14	0.015	20	0.0071*	27	14.7	34	26	50	
	M185					8	0	14	0	20	0	27	0	34	0.0066*	>34	
	M186		$CD19^{+}CD20^{low}$	3.0×10^{3}	99.3%	8	0.24	14	27.9	20	30.7	27	54.6			32	66.2
	M187					8	0	14	0.026	20	36.2	27	36.4	34	21.8	40	73.6
	M188					8	0.0056*	14	0.15	20	7.0	27	12.8			31	65.8
	M189					8	0.027	14	5.7	20	33.8	27	15.6			31	25.8
KR32	M190		unsorted	5.0×10^{2}	n/a	8	0	14	0	20	0.007*					>20	
	M191					8	0	14	0	20	0.155	27	29.2			>27	
	M192					8	0	14	0	20	0	27	0	34	0	47	0
	M193					8	0	14	0	20	0.023	27	8.7	34	48.9	44	57.6
	M194			1.0×10^{2}		8	0	14	0	20	0	27	0	34	0	50	0
	M195					8	0	14	0	20	0	27	0	34	0	50	0
	M196					8	0	14	0	20	0	27	0	34	0	50	0
	M197					8	0	14	0	20	0	27	0	34	0	50	0
	M198			1.0×10^{1}		8	0									>8	
	M199					8	0	14	0	20	0	27	0	34	0	>34	
	M200					8	0	14	0	20	0	27	0	34	0	50	0
	M201					8	0	14	0	20	0					23	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR33	M202	2510 sec (of M30)	$CD19^{+}CD20^{high}$	3.0×10^{3}	94.5%	8	0	12	0	17	0	27	0			>27	
	M203					8	0.71	12	9.7	17	1.7	27	5.2			30	66.6
	M204					8	15.8	12	14.4	17	8.1					25	46.5
	M205					8	0	12	0	17	0	27	0	34	0	49	0
	M206		$CD19^{+}CD20^{low}$	3.0×10^{3}	99.2%	8	28.6	12	12.1	17	4.9					21	68.9
	M207					8	9.4	12	52.8	17	d	27	9.4	34	0.43	34	21.4
	M208					8	25.5	12	2.2	17	0.71					26	28.0
	M209					8	32	12	1.7	17	16.2					26	50.9
	M210		$CD19^{+}CD20^{high}$	3.0×10^{2}	94.5%	8	0	12	0	17	0	27	0	34	0	49	0
	M211					8	0	12	0	17	0	27	0	34	0	>34	
	M212					8	0	12	0	17	0	27	0	34	0	44	0
	M213					8	0	12	0	17	0	27	0	34	0	49	0
	M214		$CD19^{+}CD20^{low}$	3.0×10^{2}	99.2%	8	0.033	12	55.6	17	31.2					>17	
	M215					8	0	12	0	17	0	27	0	34	0	45	0
	M216					8	0	12	0	17	0	27	0	34	0	44	0
	M217					8	0	12	0	17	0	27	0	34	0	45	0
KR34	M218	WB51 sec (of M73)	$CD19^{+}CD20^{high}$	1.2×10^{3}	95.0%	11	8.8									>11	
	M219					11	14.3									14	37.7
	M220					11	2.1									16	56.4
	M221					11	33.6									>11	
	M222		$CD19^{+}CD20^{low}$	1.2×10^{3}	99.7%	11	2.6									>11	
	M223					11	34.7									>11	
	M224					11	3.9									16	61.5
	M225					11	0									>11	

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR35	M226	L858 prim	unsorted	1.0×10^3	n/a	12	0	17	0	20	0	29	0	36	0	57	0.14
	M227					12	0	17	0	20	0.0024*	29	0.048	36	0.305	44	9.5
	M228					12	0.004*	17	6.2	20	3.9	29	0.50			36	1.9
	M229					12	0	17	0	20	0	29	0	36	0	57	86.7
	M230					12	0	17	0	20	0					>20	
	M231			1.0×10^2		12	0	17	0	20	0	29	0	36	0	38	0
	M232					12	0	17	0	20	0	29	0	36	0	57	0.0018*
	M233					12	0	17	0	20	0	29	0	36	0	57	57.4
	M234					12	0	17	0	20	0	29	0	36	0	57	0.0036*
	M235					12	0	17	0	20	0	29	0	36	0	46	17.4
	M236			1.0×10^1		12	0	17	0	20	0	29	0	36	0	57	0.0079*
	M237					12	0	17	0	20	0	29	0	36	0	57	0.0022*
	M238					12	0	17	0	20	0	29	0	36	0	57	0.0023*
	M239					12	0	17	0	20	0	29	0	36	0	57	0.0038*
	M240					12	0	17	0	20	0	29	0	36	0	57	0.0039*
KR36	M241	MS45 prim	unsorted	1.0×10^3	n/a	9	0	17	0	25	0					36	0
	M242					9	0	17	0	25	0					36	0
	M243					9	0	17	0	25	0					36	0
	M244					9	0	17	0	25	0					36	0
	M245			1.0×10^2		9	0	17	0	25	0					36	0
	M246					9	0	17	0	25	0					36	0
	M247					9	0	17	0	25	0					36	0
	M248					9	0	17	0	25	0					36	0
	M249					9	0	17	0	25	0					36	0
	M250			1.0×10^1		9	0	17	0	25	0					36	0
	M251					9	0	17	0	25	0					36	0
	M252					9	0	17	0	25	0					36	0
	M253					9	0	17	0							17	0
	M254					9	0	17		25	0					36	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR37	M255	MS45 prim	$CD19^{+}CD10^{low}$	1.2×10^{3}	90.2%	9	0	17	0	25	0					37	0
	M256					9	0	17	0	25	0					37	0
	M257					9	0	17	0	25	0					37	0
	M258					9	0	17	0	25	0					37	0
	M259		$CD19^{+}CD10^{high}$	1.2×10^{3}	96.9%	9	0	17	0	25	0					37	0
	M260					9	0	17	0	25	0					37	0
	M261					9	0	17	0	25	0					37	0
	M262					9	0	17	0	25	0					37	0
KR38	M263		$CD19^{+}CD20^{high}$	3.0×10^{3}	98.1%	9	0	17	0	25	0					34	0
	M264					9	0	17	0	25	0					34	0
	M265					9	0	17	0	25	0					34	0
	M266					9	0	17	0	25	0					34	0
	M267		$CD19^{+}CD20^{low}$	3.0×10^{3}	99.8%	9	0	17	0	25	0					34	0
	M268					9	0	17	0	25	0					34	0
	M269					9	0	17	0	25	0					34	0
	M270					9	0	17	0	25	0					34	0
KR39	M271	WB51 sec (of M68)	unsorted	1.0×10^{3}	n/a	10	2	17	69.7							17	48.5
	M272					10	17.8	17	76.9							17	65.5
	M273					10	3.2	17	60.5							18	70.3
	M274					10	18.7	17	69.7							18	59.0
	M275			1.0×10^{2}		10	2.2	17	32.9							20	72.5
	M276					10	0.0026*	17	0							20	0.25
	M277					10	1.3	17	12.2							20	54.4
	M278					10	0	17	23.2							20	#
	M279					10	0	17	0.023	22	4.0	26	36.7			29	74.2
	M280			1.0×10^{1}												<10	
	M281					10	0	17	0	22	0	26	0	30	0	50	0.0049*
	M282					10	0	17	1.2							22	14
	M283					10	0	17	0	22	0	26	0	30	0	46	f.d.
	M284					10	0.017	17	59.0							21	68.0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR40	M285	WB51 sec	unsorted	1.0×10^3	n/a											<12	
	M286	(of M65)				12	42.2									12	55.3
	M287					12	47.3									14	55.8
	M288			1.0×10^2		12	37.1									15	33.1
	M289					12	23.1									15	55.3
	M290					12	13.4									13	1.3 d
	M291					12	1.1									15	35.2
	M292					12	5.2									15	57.6
	M293			1.0×10^1												<12	
	M294					12	0	17	0	21	0	25	0	37	61.5	39	76.6
	M295					12	0.046									>12	
	M296					12	8	17	11.7							>17	
	M297					12	8.1									>12	
KR41	M298	A67 tert	unsorted	1.0×10^3	n/a	12	39.8									13	68.4
	M299	(of M130)				12	50.8									12	33.5
	M300					12	23.1									13	36.6
	M301			1.0×10^2		12	27.4									15	52.3
	M302					12	44.4									15	51.1
	M303					12	0.808									>12	
	M304					12	38.1									15	62.2
	M305					12	1.7[#]									15	52.0
	M306			1.0×10^1		12	11.8									>12	
	M307					12	0.25	17	0.50[#]							20	68.4
	M308					12	0	17	0	21	0.27	25	8.9			28	55.3
	M309					12	0	17	0	21	0	25	0	37	0	64	0
	M310					12	0	17	0	21	0	25	0	37	0	64	0
KR42	M311	737c prim	$CD19^+CD20^{high}$	1.5×10^3	99.0%	12	4	15	44.7							19	70.6
	M312					12	17.3	15	38.6							19	73.8
	M313					12	2.6	15	2.4							>15	
	M314					12	16.9	15	58.6							16	56.8
	M315		$CD19^+CD20^{low}$	1.5×10^3	98.9%	12	29.7	15	7.0	19	18.6					23	68.9
	M316					12	[#]	15	13.1							>15	
	M317					12	1.1[#]									12	24.6
	M318					12	23.5	15	20.8							>15	

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR43	M319	737c prim	$CD19^{+}CD10^{high}$	1.5×10^{3}	97.2%	12	0.112	15	4.8	19	27.4	24	8.3			>24	31.2
	M320					12	62.7									12	42.4
	M321					12	0.025	15	0	19	0	24	0			29	0.0034*
	M322					12	12.4	15	1.3	19	7.3					>19	
	M323		$CD19^{+}CD10^{low}$	1.5×10^{3}	98.7%	12	0	15	0	19	0	24	0	31	0.0042*	44	
	M324					12	0	15	0	19	0	24	0	31	0	59	0
	M325					12	0	15	0	19	0	24	0	31	0	59	0
	M326					12	0	15	0	19	0	24	0	31	0	49	0
KR44	M327		unsorted	1.0×10^{4}	n/a	12	32.6									13	67.5
	M328					12	23.4									13	43.6
	M329			1.0×10^{3}		12	12	15	28.8							16	49.2
	M330					12	13.1	15	16.0							>15	
	M331					12	47.1									12	23.4
	M332					12	46.8	15	42.0							16	37.5
	M333			1.0×10^{2}		12	0.176	15	3.9							>15	
	M334					12	3.3	15	55.4							19	4.7
	M335					12	0.016*	15	0.208	19	11.9	24	1.7	31	0.323	33	65.1
	M336					12	0	15	0	19	0	24	0	31	0	47	0.104#
	M337					12	1.3	15	33.4							19	69.5
	M338			1.0×10^{1}		12	0	15	0	19	0	24	0	31	0	63	0
	M339					12	0	15	0	19	0	24	0	31	0	>31	
	M340					12	0	15	0	19	0	24	0	31	0	63	0
	M341					12	0	15	0	19	0	24	0	31	0	63	0
	M342					12	0	15	0	19	0	24	0	31	0	47	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR45	M343	WB51 tert (of M152)	$CD19^{+}CD20^{high}$	5.0×10^{2}	95.6%	9	0	13	0	17	0					24	1.04
	M344					9	0	13	0	17	0	29	0			39	0.076
	M345					9	0	13	0	17	0.302					29	
	M346		$CD19^{+}CD20^{low}$	5.0×10^{2}	99.8%	9	0	13	0.014*	17	9.9					24	64.8
	M347					9	0	13	0.015*	17	0.073					25	36.2
	M348					9	0	13	0	17	0.044	29	0.117	41	9.4	46	76.5
	M349		$CD19^{+}CD20^{high}$	1.0×10^{1}	95.6%	9	0	13	0	17	0	29	0	41	0	56	0
	M350					9	0	13	0	17	0	29	0	41	0	43	0
	M351					9	0	13	0							14	
	M352					9	0	13	0	17	0	29	0	41	0	56	0
	M353		$CD19^{+}CD20^{low}$	1.0×10^{1}	99.8%	9	0	13	0	17	0	29	0	41	0	61	0
	M354					9	0	13	0	17	0	29	0	41	0	47	0
	M355					9	0	13	0	17	0	29	0	41	0	61	0
	M356					9	0	13	0	17	0	29	0	41	0	46	0
KR46	M357	M120 prim	unsorted	1.0×10^{4}	n/a	9	0	15	0.0035*	19	0.154	26	4.9	40	43.1	43	43.6
	M358					9	0	15	0.027	19	0.595	26	2.0	40	8.5	43	32.5
	M359					9	0	15	0.096	19	0 d	26	8.9	40	18.6	40	33.1
	M360					9	0	15	0.071	19	0.242	26	0.30			27	
	M361			1.0×10^{3}		9	0	15	0	19	0	26	0			30	0
	M362					9	0	15	0	19	0	26	0	40	0	58	0
	M363					9	0	15	0	19	0	26	0	40	0	46	0
	M364					9	0	15	0	19	0	26	0	40	5	58	29.1
	M365			1.0×10^{2}		9	0	15	0	19	0	26	0	40	0	55	
	M366					9	0	15	0	19	0	26	0	40	0	56	0
	M367					9	0	15	0	19	0	26	0.19	40	2.6	44	0.012*
	M368					9	0	15	0	19	0	26	0	40	0	58	0
	M369					9	0	15	0	19	0	26	0	40	0	58	0
	M370			1.0×10^{1}		9	0	15	0	19	0	26	0	40	0	>40	
	M371					9	0	15	0	19	0	26	0	40	0	58	0
	M372					9	0	15	0	19	0	26	0	40	0	58	0
	M373					9	0	15	0	19	0	26	0	40	0	58	0
	M374					9	0	15	0	19	0	26	0	40	0	58	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR47	M375	M120 prim	$CD19^{+}CD10^{high}$	2.0×10^{3}	99.6%	9	0	15	0	19	0	26	0	40	0	>40	
	M376					9	0.029	15	10.1	19	5.2	26	22.1			34	15.5
	M377					9	0.072	15	0.008*	19	0.01*	26	4.5	40	7.5	58	9.1
	M378					9	0	15	0	19	0	26	0	40	0	58	0
	M379		$CD19^{+}CD10^{low}$	2.0×10^{3}	97.4%	9	0	15	0	19	0	26	0	40	0	>40	
	M380					9	0	15	0	19	0	26	0	40	0	58	0
	M381					9	0	15	0	19	0	26	0	40	0	58	0
	M382					9	0	15	0	19	0	26	0	40	0	58	0
KR48	M383		$CD19^{+}CD20^{high}$	3.0×10^{3}	96.3%	9	0	15	0	19	0	26	0	40	0	49	0
	M384					9	0	15	0	19	0	26	0			39	
	M385					9	0	15	0	19	0	26	0	40	0	>40	
	M386					9	0	15	0	19	0	26	0	40	0	58	0
	M387		$CD19^{+}CD20^{low}$	3.0×10^{3}	98.0%	9	0	15	0	19	0	26	0.039			35	4.4
	M388					9	0	15	0	19	0.045	26	28.7	40	7.5	>40	
	M389					9	0	15	0	19	0	26	0.083	40	10	52	11.1
	M390					9	0	15	0	19	0	26	0	40	0	58	0
KR49	M391	758b prim	unsorted	1.0×10^{4}	n/a											<9	
	M392					9	0	13	0	20	7.6					28	50.8
	M393					9	0.004 *	13	0 d	20	0.129	34	3.5	42	19.8	48	61.1
	M394					9	0	13	0 d	20	0.002*	34	0.009*	42	0.012	48	0.398
	M395			1.0×10^{3}												8	0
	M396					9	0	13	0	20	0	34	0			42	
	M397					9	0.005*	13	0.042	20	4.5	34	68.3	42	37.7	51	64.1
	M398					9	0.006*	13	0	20	0.051	34	1.3	42	52.1	51	
	M399			1.0×10^{2}		9	0	13	0	20	0	34	0	42	0	51	0
	M400					9	0	13	0	20	0	34	0	42	0	51	0
	M401					9	0	13	0	20	0	34	0			36	
	M402					9	0	13	0	20	0	34	0	42	0	50	0
	M403					9	0	13	0	20	0	34	0	42	0	50	0
	M404			1.0×10^{1}		9	0	13	0	20	0	34	0			40	0
	M405					9	0	13	0	20	0	34	0	42	0	51	
	M406					9	0	13	0	20	0	34	0	42	0	52	0
	M407					9	0	13	0	20	0	34	0	42	0	50	
	M408					9	0	13	0	20	0	34	0	42	0	52	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR50	M409	758b prim	$CD19^{+}CD10^{high}$	1.5×10^{3}	99.4%	9	0	13	0.0071*	20	0.0026*	34	0.071	42	0.149	52	1.03
	M410					9	0	13	0.011*	20	0.249					>20	
	M411					9	0	13	0	20	0	34	0	42	0	52	0
	M412					9	0	13	0	20	0	34	0	42	0	44	
	M413		$CD19^{+}CD10^{low}$	1.5×10^{3}	100.0%	9	0.087	13	0.056	20	0.073	34	35.6			39	37.7
	M414					9	0.46	13	1.3	20	36.7	34	45.5	42	0	48	20.8
	M415					9	0	13	0.056	20	3	34	68.3			42	
	M416					9	0.03	13	0.59	20	54.9	34	0.42			40	69.7
KR51	M417		$CD19^{+}CD20^{high}$	1.5×10^{3}	98.4%	9	0	13	0	20	0	34	0	42	0	>42	
	M418					9	0	13	0	20	0	34	0	42	0	>42	
	M419					9	0									11	
	M420					9	0	13	0	20	0	34	0	42	0	>42	
	M421		$CD19^{+}CD20^{low}$	1.5×10^{3}	100.0%	9	0.057	13	0.244	20	3.2	34	87.7	42	0.156	51	
	M422					9	0.024	13	4.1	20	72.8	34	82.5	42	0.012*	52	84.2
	M423					9	0.087	13	0.258	20	7.8	34	86.0	42	0.039	52	64.1
	M424					9	0	13	0	20	0	34	0.0073*	42	0.045	52	28.8
KR52	M425	A67 prim	unsorted	1.0×10^{4}	n/a	12	1.4	15	54.3							15	77.9
	M426					12	8.2	15	3.0							15	31.1
	M427					12	0.26	15	33.5							15	1.8
	M428					12	9.4	15	11.0							15	66.1
	M429			1.0×10^{3}		12	5.0	15	16.3							18	74.5
	M430					12	36.0	15	55.1							18	66.8
	M431					12	2.3	15	0.034#							18	56.0
	M432					12	2.2	15	27.2							18	23.6
	M433			1.0×10^{2}		12	0	15	0.013	18	24.5	26	23.8			29	75.5
	M434					12	2.7	15	12.0	18	39.1					22	62.7
	M435					12	0.068	15	8.7	18	13.9					21	31.7
	M436					12	0.18	15	25.3							>15	
	M437					12	5.2	15	70.4	18	0#					22	73.1
	M438			1.0×10^{1}		12	0	15	0	18	0	26	0	33	0	46	0
	M439					12	0	15	0	18	0					19	0
	M440					12	0	15	0	18	0	26	0	33	0	46	0
	M441					12	0	15	0	18	0	26	0	33	0	46	0
	M442					12	0	15	0	18	0	26	0	33	0	46	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR53	M443	L876 prim	unsorted	1.0 x 10^3	n/a	9	0.023	13	0.033	20	0.089					25	0.064
	M444					9	0	13	0.133	20	74.4					22	3.4
	M445					9	0	13	0.028	20	0.63	27	0			35	0
	M446					9	0	13	0.345	20	36.3	27		39		25	26.4
	M447			1.0 x 10^2		9	0	13	0	20	0	27	0	39		31	0
	M448					9	0	13	0.008*	20	15.7	27	2.1			31	3.1
	M449					9	1.03	13	47.5							19	62.0
	M450					9	0.699	13	66.9	20	47.1	27	44.5			32	86.8
	M451					9	0.37	13	3.1	20	2.1	27	12.7			32	68
	M452			1.0 x 10^1		9	0	13	0	20	0	27	0	39	0	40	0
	M453					9	0	13	0	20	0	27	0	39	0	40	0
	M454					9	0	13	0	20	0	27	0	39	0	40	0
	M455					9	0	13	0	20	0	27	0	39	0	40	0
	M456					9	0	13	0	20	0	27	0	39	0	40	0.014
KR54	M457	HV101 sec	unsorted	1.0 x 10^3	n/a	9	15.2	13	17.3	19	58.7					19	49.1
	M458	(of M187)				9	53.1	13	36.1							18	71.7
	M459					9	23.1	13	60.3							16	37.2
	M460					9	25.8	13	43.1							16	52.9
	M461			1.0 x 10^2		9	0.542	13	35.1	19	30.5					23	57.8
	M462					9	0	13	2.9	19	77.5					25	70.0
	M463					9	0.85	13	45.7	19	51.2					23	76.0
	M464					9	0.197	13	19.7							>13	
	M465					9	0.017*	13	0.02							18	71.6
	M466			1.0 x 10^1		9	0.003*	13	0	19	0.321	27	28.0	39	29.7	40	25.4
	M467					9	0.027	13	8.6	19	23.5					25	51.2
	M468					9	0	13	0	19	0	27	0	39	0.013	40	0.0019*
	M469					9	0	13	0	19	0	27	0.20	39	8	40	51.5
	M470					9	0	13	0.013	19	47.1	27	72.7			28	32.5

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR55	M471	737c sec (of M315)	$CD19^{+}CD20^{high}$	1.0×10^{3}	97.5%	8	0	15	0	23	0.534	30	11.3			36	25
	M472					8	0	15	7.3	23	35.5	30	32.8			36	67.2
	M473					8	0.01*	15	1.5	23	13.1	30	19.4			36	60.3
	M474					8	0	15	8.6	23	73.0	30	49.0			36	64.5
	M475		$CD19^{+}CD20^{low}$	1.0×10^{3}	98.4%	8	0	15	0.213	23	0.017	30	9.1			36	39.6
	M476					8	0	15	0	23	0	30	0	37	0	43	0
	M477					8	0	15	0	23	0	30	0	37	0	43	0
	M478					8	0	15	1.5	23	22.0	30	0.291			36	53.6
	M479		$CD19^{+}CD20^{high}$	1.0×10^{2}	97.5%	8	0	15	0	23	0	30	0	37	0	45	0
	M480					8	0	15	0	23	0	30	0	37	0	39	
	M481					8	0.206	15	25.2	23	52.9	30	2.9#			33	70.5
	M482					8	0	15	0	23	0	30	2.3			36	64.4
	M483		$CD19^{+}CD20^{low}$	1.0×10^{2}	98.4%	8	0	15	0	23	0	30	0	37	0	39	0
	M484					8	0	15	0	23	0					>23	
	M485					8	0	15	0	23	0	30	0	37	0	46	0
	M486					8	0	15	0	23	0					29	
	M487		$CD19^{+}CD20^{high}$	1.0×10^{1}	97.5%	8	0	15	0	23	0	30	0	37	0	46	0
	M488					8	0	15	0	23	0	30	0	37	0	46	0
	M489					8	0	15	0	23	0	30	0			31	0
	M490					8	0	15	0	23	0	30	0	37	0	43	0
	M491					8	0	15	0	23	0					>23	
	M492		$CD19^{+}CD20^{low}$	1.0×10^{1}	98.4%	8	0	15	0	23	0	30	0	37	0	46	0
	M493					8	0	15	0	23	0	30	0	37	0	46	0
	M494					8	0	15	0	23	0	30	0	37	0	46	0
	M495					8	0	15	0	23	0					29	
	M496					8	0	15	0	23	0	30	0	37	0	46	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR56	M497	737c sec (of M320)	$CD19^{+}CD10^{high}$	1.0×10^{3}	97.3%	8	4.1	15	67.6							18	80.7
	M498					8	15.8	15	84.2							21	80.1
	M499					8	0	15	54.6							>15	
	M500					8	0.728	15	73.3							21	81.9
	M501		$CD19^{+}CD10^{low}$	1.0×10^{3}	99.1%	8	0	15	11	23	79.2					24	73.4
	M502					8	0.424	15	5	23	17.8					26	
	M503					8	0.093	15	31.4							19	24.6
	M504					8	0	15	0	23	0.293					27	21.7
	M505		$CD19^{+}CD10^{high}$	1.0×10^{2}	97.3%	8	0	15	0	23	0	30	0	37	0	>37	
	M506					8	0	15	0.016	23	71.5	30	14.0			36	61.8
	M507					8	0	15	0.126	23	62.7	30	2.2			34	
	M508					8	0	15	0	23	0	30	0	37	0	46	0
	M509		$CD19^{+}CD10^{low}$	1.0×10^{2}	99.1%	8	0	15	2.3	23	70.7					29	
	M510					8	0	15	0	23	0	30	0	37	0	46	0
	M511															4	0
	M512					8	0	15	0.011	23	0.042	30	0.779	37	0.695	46	7.7
	M513		$CD19^{+}CD10^{high}$	1.0×10^{1}	97.3%	8	0	15	0	23	0	30	0	37	0	46	0
	M514					8	0	15	0	23	0	30	0	37	0	46	0
	M515					8	0	15	0	23	0	30	0	37	0	46	0
	M516					8	0	15	0	23	0	30	0	37	0	46	0
	M517					8	0	15	0	23	0	30	0	37	0	43	0
	M518		$CD19^{+}CD10^{low}$	1.0×10^{1}	99.1%	8	0	15	0	23	0	30	0			37	0
	M519					8	0	15	0	23	0	30	0	37	0	46	0
	M520					8	0	15	0	23	0	30	0	37	0	46	0
	M521					8	0	15	0	23	0	30	0	37	0	>37	
	M522					8	0	15	0	23	0					30	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR57	M523	2003 prim	unsorted	1.0×10^3	n/a											3	
	M524					8	0	14	0	24	0					24	0
	M525					8	0	14	0	24	2.7					27	21.0
	M526					8	0	14	0	24	0					28	0
	M527			1.0×10^2		8	0	14	0			33	0			42	0
	M528					8	0	14	0			33	0			42	0
	M529					8	0	14	0			33	0			42	0
	M530					8	0	14	0			33	0			42	0
	M531			1.0×10^1		8	0	14	0			33	0			42	0
	M532					8	0	14	0			33	0			39	0
	M533					8	0	14	0			33	0			42	0
	M534					8	0					33				>8	
	M535					8	0	14	0			33	0			42	0
KR58	M536	4917 prim	unsorted	1.0×10^3	n/a	8	0	14	0	24	0	33	0			>33	
	M537					8	0	14	0	24	0	33	0			36	0
	M538															5	0
	M539					8	0.0028*	14	0.976	24	4.6					27	20.3
	M540			1.0×10^2		8	0	14	0							>14	
	M541					8	0	14	0			33	0			39	0
	M542					8	0	14	0							29	0
	M543					8	0	14	0							22	
	M544			1.0×10^1		8	0	14	0			33	0			42	0
	M545					8	0	14	0			33	0			39	0
	M546					8	0	14	0			33	0			36	0
	M547					8	0	14	0			33	0			42	0
	M548					8	0	14	0			33	0			42	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR59	M549	WB51 tert (of M272)	$CD19^{+}CD34^{high}$	1.0×10^{3}	97.9%	9	11.2	12	59#							16	41.1
	M550															2	
	M551					9	1.2	12	19#	18	67.8					19	71.2
	M552					9	30.8	12	49.72#	18	46.1					22	
	M553		$CD19^{+}CD34^{low}$	1.0×10^{3}	97.8%	9	4.8	12	0.175#	18	29.4					22	57.5
	M554					9	4.7	12	29.7#	18	0.877#					21	56.9
	M555					9	0.262	12	3.4#	18	53					21	23.9
	M556					9	0.533	12	0.986#	18	21.7					21	41.1
	M557		$CD19^{+}CD34^{high}$	1.0×10^{2}	97.9%	9	0.158	12	0.917#	18	57	23	0.049#			30	65.7
	M558					9	0.037	12	0.008*#	18	0.108	23	0.013			31	14.8
	M559					9	0	12	0.123#	18	36.3	23	67.5			26	64.7
	M560					9	0	12	0.011#	18	23.2	23	45.3			27	74.2
	M561					9	0	12	0.0189#	18	1	23	0.804			31	68.5
	M562		$CD19^{+}CD34^{low}$	1.0×10^{2}	97.8%	9	0.673	12	0.498#	18	6.2	23	20.4			26	60.7
	M563					9	0	12	0#	18	0	23	0.034			31	45.9
	M564					9	0.102	12	1.31#	18	65.2	23	62.7			23	34.4
	M565					9	0	12	0#	18	67.7	23	34.6			>23	
	M566					9	0.076	12	0#	18	62.5	23	28.3			26	73.4
	M567		$CD19^{+}CD34^{high}$	1.0×10^{1}	97.9%	9	0	12	30.7	18	59					19	65.3
	M568					9	0.41	12	0.146	18	0.034#					19	0.474
	M569					9	0.127	12	25.7	18	7.9					19	61.4
	M570					9	0	12	0.059	18	27.2	23	35.4			28	83.3
	M571					9	0	12	0	18	0.0032*	23	0.0086*			28	0.009*
	M572					9	0.096*	12	0	18	3.1					22	1.5
	M573		$CD19^{+}CD34^{low}$	1.0×10^{1}	97.8%	9	0	12	0.04	18	40.5	23	9.1			23	
	M574					9	0	12	0.014*	18	1.3	23	9			26	53.6
	M575					9	0	12	0	18	0	23	0.34	32	26.1	42	59.8
	M576					9	0	12	0	18	0	23	0	32	2.3	42	54.8
	M577					9	0	12	0	18	0	23	0	32	0	42	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR60	M578	737c sec (of M327)	$CD19^{+}CD34^{high}$	1.0×10^{3}	98.5%	9	0.322	12	0.133							17	10.9
	M579					9	75.8	12	14.8							15	
	M580					9	3.5	12	8.4	18	3.7#					19	94.0
	M581					9	0	12	0.556	18	5.9	23	2.2	32		25	
	M582		$CD19^{+}CD34^{low}$	1.0×10^{3}	98.8%	9	0.138	12	29.9	18	9					22	77.7
	M583					9	0.132	12	6.9	18	13.1					20	
	M584					9	0	12	0	18	0.4	23	56.4			27	74.4
	M585					9	0.538	12	15	18	0.031#	23	9			26	60.8
	M586		$CD19^{+}CD34^{high}$	1.0×10^{2}	98.5%	9	0	12	0	18	0	23	0.178	32	83.1	42	74.8
	M587					9	0	12	0	18	0	23	0	32	0	47	0
	M588					9	0	12	0	18	0	23	0	32	0	>32	
	M589					9	0	12	0	18	0	23	0	32	0	35	
	M590					9	0	12	0	18	0	23	0	32	0	47	0
	M591		$CD19^{+}CD34^{low}$	1.0×10^{2}	98.8%	9	0	12	0	18	0	23	0	32	0	47	0
	M592					9	0	12	0	18	0	23	0			>23	
	M593					9	0	12	0	18	9.9					31	81.6
	M594					9	0	12	0.456	18	49.9	23	77			>23	
	M595					9	0	12	0	18	0.0055*	23	0#			31	0.0018*
	M596		$CD19^{+}CD34^{high}$	1.0×10^{1}	98.5%	9	0.006*	12	0.024	18	13.8					22	40
	M597					9	0	12	0.15	18	83.9					>18	
	M598					9	0	12	0	18	0	23	0#			31	0
	M599					9	14	12	54.4	18	74.5	23	62.9			25	
	M600					9	0	12	0	18	0.013*	23	0#			26	0
	M601		$CD19^{+}CD34^{low}$	1.0×10^{1}	98.8%	9	0	12	0	18	0.029*	23	1.2			30	42.2
	M602					9	0	12	0	18	0	23	0	32	0	47	0
	M603					9	0	12	0	18	0	23	0	32	0	40	0
	M604					9	0	12	0	18	0	23	0	32	0	39	
	M605					9	0	12	0	18	0	23	0	32	0	47	0

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR61	M606	4540a prim	$CD19^{+}CD34^{high}$	1.0×10^{3}	93.8%	8	1.1									8	0
	M607					8	0	16	0	22	0	28	0	36	0	0	
	M608					8	0	16	0	22	0	28	0	36	0	0	
	M609					8	0	16	0.35	22	30.5	28	39.6			30	43.8
	M610					8	0	16	0	22	0					28	
	M611		$CD19^{+}CD34^{low}$	1.0×10^{3}	100.0%	8	0.003 *	16	36.9	22	33.8					24	58.9
	M612					8	0	16	0	22	0	28	0.005*	36	0	0	
	M613					8	0	16	0	22	0	28	0	36	0	0	
	M614					8	0	16	0	22	0	28	0	36	0	0	
	M615					8	0	16	0	22	0	28	0	36	0	0	
	M616		$CD19^{+}CD34^{high}$	1.0×10^{2}	93.8%	8	0.047	16	0.455	22	13.3	28	58.1			29	8.9
	M617					8	0.07	16	0.025	22	10	28	44.2			29	22.2
	M618					8	0	16	0	22	0	28	0	36	0	0	
	M619					8	0	16	0	22	0					23	
	M620					8	0	16	3.1	22	33.1					27	45.1
	M621		$CD19^{+}CD34^{low}$	1.0×10^{2}	100.0%	8	0	16	0	22	0	28	0	36	0	0	
	M622					8	0	16	0	22	0	28	0	36	0	0	
	M623					8	0	16	0	22	0	28	0	36	0	0	
	M624					8	0	16	0							16	0
	M625					8	0	16	0							13	0
	M626		$CD19^{+}CD34^{high}$	1.0×10^{1}	93.8%	8	0	16	0							20	
	M627					8	0	16	0	22	0	28	0	36	0	0	
	M628															6	
	M629					8	0	16	0	22	0	28	0	36	0	0	
	M630					8	0	16	0	22	0	28	0	36	0	0	
	M631		$CD19^{+}CD34^{low}$	1.0×10^{1}	100.0%	8	0	16	0	22	0	28	0	36	0	0	
	M632					8	0	16	0							22	
	M633					8	0	16	0	22	0	28	0	36	0	0	
	M634					8	0	16	0	22	0	28	0	36	0	0	
	M635					8	0	16	0	22	0	28	0	36	0	0	

Exp. No.	Mouse ID.	Patient / Leukemia	Injected Cells	Injected Cell No.	Population purity (%)	Wks post transplant	BMP1 Result	Wks post transplant	BMP2 Result	Wks post transplant	BMP3 Result	Wks post transplant	BMP4 Result	Wks post transplant	BMP5 Result	Wks post transplant	Final Engraftment
KR62	M636	L784 prim	$CD19^{+}CD34^{high}$	1.0×10^{4}	97.7%	9	0.019	13	0.48	19	18.6	25	15			29	17.3
	M637					9	0.19	13	40.9	19	0.064#	25	26.1			27	31.9
	M638					9	0.005*	13	4.8	19	18.7	25	1.3			0	
	M639					9	0	13	0.87	19	6.5	25	8.4			29	72.5
	M640		$CD19^{+}CD34^{low}$	1.0×10^{4}	99.6%	9	0	13	0	19	0	25	0	33	0	0	
	M641					9	0	13	0	19	0	25	0	33	0	0	
	M642					9	0	13	0	19	2.6	25	31.4			29	58.3
	M643					9	0	13	0.16	19	40.6	25	0.21#	33	3.3	36	
	M644		$CD19^{+}CD34^{high}$	1.0×10^{3}	97.7%	9	0	13	0	19	15.5	25	0 d	33	1.4	36	47.8
	M645					9	0	13	0.0069*	19	13.5	25	9.3	33	35.7	36	2.7
	M646					9	0	13	0	19	0	25	0	33	0.007*	0	
	M647					9	0.005*	13	0.048	19	52.3	25	0.023#			27	20.5
	M648		$CD19^{+}CD34^{low}$	1.0×10^{3}	99.6%	9	0.014	13	4.1	19	3.9	25	8.3	33	13.8	36	13.7
	M649					9	0.0024*	13	0.22							13	
	M650					9	0	13	0	19	0	25	0	33	0	0	
	M651					9	0.019	13	13.2	19	55.6	25	0#			27	63.3
	M652		$CD19^{+}CD34^{high}$	1.0×10^{2}	97.7%	9	0	13	0							>13	
	M653					9	0	13	0	19	0	25	0	33	0	0	
	M654					9	0	13	0	19	0	25	0			29	0
	M655					9	0	13	0	19	0	25	0	33	0	0	
	M656					9	0	13	0	19	0	25	0	33	0	0	
	M657		$CD19^{+}CD34^{low}$	1.0×10^{2}	99.6%	9	0	13	0.0046*	19	1.2	25	63.2			26	52.3
	M658					9	0	13	0	19	0	25	0.003*	33	0	0	
	M659					9	0	13	0	19	0	25	0	33	0	0	
	M660					9	0	13	0	19	0	25	0	33	0	0	
	M661					9	0	13	0	19	0	25	0	33	0	0	
	M662		$CD19^{+}CD34^{high}$	1.0×10^{1}	97.7%	9	0	13	0	19	0	25	0	33	0	0	
	M663					9	0	13	0	19	0	25	0			33	0
	M664					9	0	13	0	19	0	25	0	33	0	0	
	M665					9	0	13	0	19		25				14	
	M666					9	0	13	0	19	0	25	0	33	0	0	
	M667		$CD19^{+}CD34^{low}$	1.0×10^{1}	99.6%	9	0	13	0	19	0	25	0	33	0	43	
	M668					9	0	13	0	19	0	25	0	33	0	0	
	M669					9	0	13	0	19	0	25	0	33	0	0	
	M670					9	0	13	0	19	0	25	0	33	0	0	
	M671					9	0	13	0	19	0	25	0	33	0	0	
	M672					9	0	13	0	19	0	25	0	33	0	0	

Appendix 2 – Presentations

Only presentations in direct relation to the work of this PhD project on international conferences are attached.

ASH Abstract 1348 – Poster Presentation

The American Society of Hematology, 50th Annual Meeting

December 6th-9th 2008, Moscone Convention Center, San Francisco, California

In Childhood ALL, Both Blasts With a CD20-/low and a CD20high Immunophenotype Have the Ability to Transfer the Leukemia onto Immune-deficient NOD/scid y-/- Mice

Kerrie Wilson, Klaus Rehe, Simon Bomken, Julie Irving and Josef Vormoor

There is an ongoing controversy as to whether cancer is always maintained by a rare population of highly specialized cancer stem cells or whether cancer-propagating cells may be more abundant in some cancer types. We have previously shown that in a heterogeneous group of childhood ALL different blast populations, regardless of their expression of the progenitor/stem cell marker CD34 or the lymphoid differentiation antigen CD19, contain leukemia-initiating activity (Cancer Cell 2008, 14(1), 47-58). By profiling B cell transcription factor expression, these different populations appeared to mirror stages of normal B cell development. Here we extend our experiments to another lymphoid differentiation marker, CD20, to provide further evidence that ALL blasts at different stages of maturation possess the ability to re-initiate the leukaemia.

Unsorted bone marrow cells from 3 different ALL patients (L754, L736 and A67) were transplanted into 12 primary mice. Bone marrow was harvested from leukemic mice and flow sorted candidate populations (CD19+CD20-/low and CD19+CD20high) were re-transplanted into 52 secondary and 8 tertiary mice. As expected from our previous experiments both CD19+CD20-/low and CD19+CD20high cells were able to re-establish the disease in unconditioned NOD/scid y-/- mice (see table). Leukemic engraftment ranged from 0.5 to 73% as determined by flow cytometry on bone marrow

aspirates. Both populations re-established the complete phenotype of the original leukemia including CD20-/low and CD20high blasts. These results were confirmed by directly sorting primary ALL blasts from pt. #L754 without prior passage in the mice. Cell purity after flow sorting was high (81-99%) and low numbers of cells engrafted (5000 for both CD19+CD20-/low and CD19+CD20high). The three patients reflected different ALL subtypes (A67: high risk ALL/t(9;22), L754:high hyperdiploid/MRD high risk; and L736 intermediate risk ALL: high WBC/MRD low risk).

Patient	Transplant	Mice	Population	Cell dose	Engrafted
L736	Secondary	9	CD20high	10.000-100.000	6
		11	CD20-/low		10
	Tertiary	4	CD20high	10.000	4
		4	CD20-/low		1
L754	Primary	4	CD20high	100.000	2
		4	CD20-/low		3
	Secondary	11	CD20high	5.000-100.000	9
		11	CD20-/low		7
A67	Secondary	6	CD20high	9.000-20.000	6
		6	CD20-/low		6

In conclusion, these results confirm our previous observation that ALL blasts irrespective of the expression of lymphoid differentiation markers are able to engraft immune-deficient mice. Therefore, leukemia-propagating cells in childhood ALL may be more abundant than previously thought.

In Childhood ALL, Both Blasts with a CD20^{Low} and a CD20^{High} Immunophenotype Have the Ability to Transfer the Leukemia Onto Immunodeficient NOD Scid γ$^{-/-}$ (NOG) Mice

Kerrie Wilson[1], Klaus Rehe[1], Simon Bomken[1], Marian Case[1], Lenny Shultz[2], Julie Irving[1] and Josef Vormoor[1]

[1]Northern Institute for Cancer Research, Paul O'Gorman Building, Medical School, Newcastle University, Newcastle upon Tyne, United Kingdom, NE2 4HH and [2] The Jackson Laboratory, Bar Harbor, Maine, USA. **Email: Kerrie.Wilson@ncl.ac.uk**

Newcastle University

Abstract Number: 1348

Introduction

There is an ongoing controversy as to whether cancer is always maintained by a rare population of highly specialized cancer stem cells or whether cancer-propagating cells may be more abundant in some cancer types. In AML evidence had consistently suggested that only immunophenotypically immature cells possess SCID-repopulating ability indicative of cancer stem cell ability (1), however, this may be different for different types of leukemia. We have previously shown that in a heterogeneous group of childhood ALL different blast populations, regardless of their expression of the progenitor/stem cell marker CD34 or the lymphoid differentiation antigen CD19, contain leukemia-initiating activity (2). Here we extend our experiments to another lymphoid differentiation marker, CD20, to provide further evidence that ALL blasts at different stages of maturation possess the ability to re-initiate the leukemia.

Methods

Bone marrow samples from patients with acute lymphoblastic leukemia (n=3) were transplanted into immunodeficient mice (n=12). All 3 leukemias (2 high risk and 1 standard risk) engrafted in primary animals and bone marrow was harvested and flow sorted into populations of candidate leukemic stem cells; CD19^{+}CD20^{Low} and CD19^{+}CD20^{High} which were then serially transplanted into secondary and tertiary recipient mice (n=62). Bone marrow from 1 patient (L754) was directly flow sorted into these populations and transplanted into primary recipients (n=8).

Results

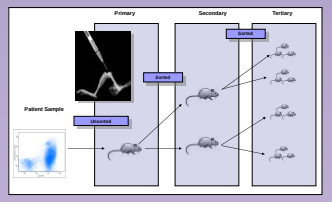


Figure 1 – The leukemic stem cell potential of candidate populations is assayed by serial transplantation in immunodeficient NOG mice

Results

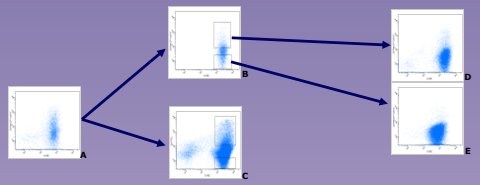


Figure 2 – Both CD19^{+}CD20^{High} and CD19^{+}CD20^{Low} populations transfer the leukemia. Unsorted cells **(A)** from patient A67 engrafted primary mice (**B**/**C**). Flow sorted cells from the primary mice were re-transplanted (9x10^{3} cells/mouse) and the original immunophenotype was recapitulated from both sorted fractions.

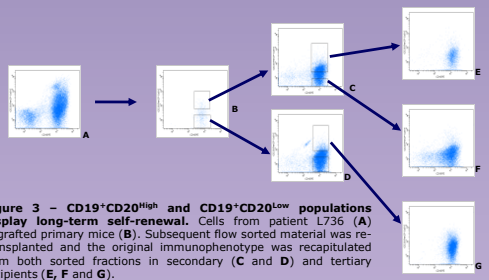


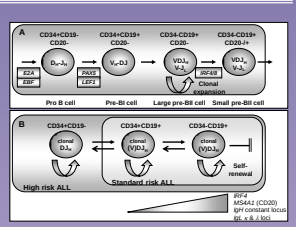
Figure 3 – CD19^{+}CD20^{High} and CD19^{+}CD20^{Low} populations display long-term self-renewal. Cells from patient L736 (**A**) engrafted primary mice (**B**). Subsequent flow sorted material was re-transplanted and the original immunophenotype was recapitulated from both sorted fractions in secondary (**C** and **D**) and tertiary recipients (**E, F** and **G**).

Patient	Transplant	Mice	Population	Cell Dose	Engrafted
L736 Dic(9;20)	Secondary	9	CD19^{+}CD20^{High}	10,000-100,000	6
		11	CD19^{+}CD20^{Low}		10
	Tertiary	4	CD19^{+}CD20^{High}	10,000	4
		4	CD19^{+}CD20^{Low}		1
L754 (High Hyperdiploid)	Primary	4	CD19^{+}CD20^{High}	100,000	2
		4	CD19^{+}CD20^{Low}		3
	Secondary	11	CD19^{+}CD20^{High}	5,000-100,000	9
		11	CD19^{+}CD20^{Low}		7
A67 t(9;22)	Secondary	6	CD19^{+}CD20^{High}	9,000-20,000	6
		6	CD19^{+}CD20^{Low}		6

Table 1 – Overview of all mice transplanted with CD19^{+}CD20^{High} and CD19^{+}CD20^{Low} cells

Figure 4 – Our new ALL stem cell model.

Our previous gene expression studies (2) indicate that the different leukemic populations mirror the corresponding stages of normal B cell development.



Conclusions

- Sorted fractions with both a more immature (CD19^{+}CD20^{Low}) and a more mature immunophenotype (CD19^{+}CD20^{High}) engrafted immunodeficient NOG mice.
- Both populations were able to reconstitute the complete leukemic immunophenotype.
- These findings are supported by recent evidence demonstrating that both CD34^{+}CD38^{-}CD19^{+} and CD34^{+}CD38^{+}CD19^{+} cells initiate leukemia in immunodeficient mice (3).
- In conclusion, these results confirm our previous observation that ALL blasts irrespective of the expression of lymphoid differentiation markers are able to engraft immunodeficient mice. Therefore, leukemia-propagating cells in childhood ALL may be more abundant than previously thought.

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Acknowledgements

This work was supported by grants from the North East of England Children's Cancer Research Fund (NECCR) and the JGW Patterson foundation. There are no conflicts of interest to disclose.

ASH Abstract 1421 – Poster Presentation

The American Society of Hematology, 51st Annual Meeting

December 5th-8th 2009, Ernest N. Morial Convention Center, New Orleans, Louisiana

Disease Propagating Blasts in Standard and High Risk Acute Lymphoblastic Leukemia are Frequent and of Diverse Immunophenotype

Klaus Rehe, Kerrie Wilson, Hesta McNeill, Martin Schrappe, Julie Irving and Josef Vormoor

Conflicting results in the field of cancer stem cells have reignited debate regarding the frequency and identity of cells with the ability to self-renew and to propagate the complete phenotype of the malignancy. Initially it was suggested by different studies that cancer stem cells represent only a small minority of the malignant population and that the immunophenotypes of these cells resemble a rather immature type in the cell hierarchy. More recent data from our own and other groups have challenged these findings by demonstrating that cells at different maturity levels within the leukemic hierarchy have cancer stem cell abilities and that the frequency of the leukemia maintaining cell is higher than previously thought (Cancer Cell 2008, 14(1), p47-58). We use an *in vivo* NOD/scid IL2Rγnull (NSG) mouse intra-femoral transplant model to determine the clonogenicity of sorted candidate leukemic stem cell populations, characterized by specific immunophenotypes. We selected the surface markers CD10 and CD20, in order to differentiate between rather immature and more mature cells. Furthermore we carried out limiting dilution experiments on sorted (CD20) and unsorted leukemic blasts to investigate the frequency of the proposed leukemic stem cells. Flow sorted ALL blasts of CD19+CD20low and CD19+CD20high as well as of

CD19+CD10low and CD19+CD10high immunophenotype were transplanted into NSG mice. Sorts were performed on primary patient material and on leukemic blasts that had been harvested following prior passage in mice. Different subtypes of ALL were included (high risk: BCR/ABL (t9;22) positive (patients L4967, L4951, L49101, L8849, L2510), high hyperdiploid/MRD positive high risk (L754, L835), intermediate risk: high WBC/MRD negative (L736, L784), age >10 years (L803)). CD20 sorts were performed on primary patient material (L4951, L49101, L754, L835 and L776), on secondary samples harvested from engrafted primary mice (L4967, L4951, L2510, L736 and L754) and on tertiary samples harvested from engrafted secondary mice (L4967 and L736). In total 151 mice were transplanted, with 122 showing engraftment in consecutive bone marrow punctures or in bone marrow harvests. CD10 sorts were performed on primary patient material (L784 and L49101) and on secondary samples harvested from engrafted primary mice (L4951, L8849, L2510 and L803) with 31 out of 52 mice transplanted with sorted material showing engraftment as seen with CD20 sorted cells. Blasts of all selected immunophenotypes were able to engraft the leukemia in unconditioned NSG mice as determined by 5 color flow cytometry. In particular, sorted cells of both fractions were able to reconstitute the complete phenotype of the leukemia. Harvested cells from engrafted mice could then be re-sorted into high and low antigen expressing fractions and successfully re-engrafted on secondary and tertiary mice. Cell purities of transplanted cells were usually higher than 90% (range 67-100%). The ability of all populations to serially engraft mice demonstrates long-term self-renewal capacity. Two additional patients were used in the limiting dilution assays (high WBC/t(4;11) high risk (L826); low WBC/MRD negative low risk (L792)) and experiments were performed on primary unsorted and secondary sorted material. Cell numbers necessary for ALL engraftment differed between individual leukemias but as little as 100 cells proved to be

sufficient in one unsorted and in both the CD19+CD20low and CD19+CD20high fractions (Table 1). Mice transplanted with 10 cells only are still under observation.

Patient	Transplant	Population	Cell dose	Mice engrafted/ transplanted
L4951	Secondary	CD20 high	500	3/3
		CD20 low		3/3
		CD20 high	100	3/3
		CD20 low		3/3
L2510	Secondary	CD20 high	3,000	2/4
		CD20 low		4/4
		CD20 high	300	0/4
		CD20 low		1/4
L49101	Primary	Unsorted	500	3/4
			100	0/4
L792	Primary	Unsorted	1,000	5/5
			100	1/5
L826	Primary	Unsorted	1,000	3/4
			100	0/4

Table 1

In conclusion we present strong evidence that leukemia-propagating cells are much more prevalent than previously thought and that blasts of diverse immunophenotype are able to serially reconstitute the complete leukemia in immune-deficient mice.

Disease Propagating Blasts in Standard and High Risk Acute Lymphoblastic Leukemia are Frequent and of Diverse Immunophenotype

Abstract Number: 1421

Klaus Rehe[1], Kerrie Wilson[1], Hesta McNeill[1], Martin Schrappe[2], Julie Irving[1] and Josef Vormoor[1]

[1]Northern Institute for Cancer Research, Paul O'Gorman Building, Newcastle University, Newcastle upon Tyne, UK, NE2 4HH and
[2]Department of Pediatrics, University Medical Center Schleswig-Holstein, Campus Kiel, 24105 Kiel, Germany **Email: k.t.rehe@ncl.ac.uk**

Introduction

Conflicting results in xenograft transplantation models have reignited the debate on the existence and nature of leukemic stem cells (LSC) (1). While some studies suggested that acute lymphoblastic leukemia is maintained by a rare LSC population, some of these results may have been an artifact of the xenograft model itself: Bonnet and her group have recently shown that conventional NOD/scid mice are able to clear human cells coated with an anti-CD38 antibody (2), falsely suggesting that these CD38$^+$ populations lack the ability to engraft. Using anti-CD122 pretreated NOD/scid or the novel NSG mice, we have demonstrated that leukemic blasts at different levels of maturation have the ability to re-establish the disease in *vivo* (3), challenging the stem cell model in ALL. Here, we confirm these results by showing that leukemic blasts irrespective of the expression of the lymphoid differentiation markers CD10 and CD20 possess leukemia-initiating potential. More importantly, leukemia-initiating cells are frequent and 10-100 blasts are sufficient to re-grow the leukemia, even in a xeno-environment.

Results

Patient	Transplant	Risk category	Population	Cell dose	Mice engrafted/ transplanted	Time to engraftment	Status
L792	Primary	Standard risk	Unsorted	1,000	5/5	<10	-
				100	1/5	<10	alive
				10	0/5	-	alive
L826	Primary	High risk t(4;11)	Unsorted	1,000	3/4	<13 - <32	-
				100	1/4	<50	-
				10	0/4	-	-
L737c	Primary	High risk (bcr/abl like ALL)	Unsorted	1,000	4/4	<12	alive
				100	3/5	<12 -	alive
				10	0/5	-	alive
L49101	Primary	High risk t(9;22)	Unsorted	500	3/4	<20	alive
				100	0/4	-	alive
				10	0/4	-	alive
L858	Primary	High risk (poor responder)	Unsorted	1,000	2/5	<16 -	alive
				100	0/5	-	alive
				10	0/5	-	alive
L4951	Secondary	High risk t(9;22)	Unsorted	1,000	7/7	<10	-
				100	10/10	<10 - <20	-
				10	7/10	<12 -	alive
L754	Secondary	High risk (HeH)	CD20 high	1,000	4/4	<12 - <21	alive
			CD20 low		3/4	<12	alive
			CD20 high	100	3/5	<12 - <21	alive
			CD20 low		2/4	<21	alive
L4951	Secondary	High risk t(9;22)	CD20 high	500	3/3	<10	-
			CD20 low		3/3	<10	-
			CD20 high	100	3/3	<15	-
			CD20 low		3/3	<10 - <20	-
L2510	Secondary	High risk t(9;22)	CD20 high	3,000	2/4	<8 -	-
			CD20 low		4/4	<8 -	-
			CD20 high	300	0/4	-	alive
			CD20 low		1/4	<12 -	alive
L4967	Tertiary	High risk t(9;22)	Unsorted	1,000	3/3	<12	-
				100	5/5	<12	-
				10	2/5	<12 -	alive

Table 1: Summary of all mice transplanted at limiting dilutions.

Methods

Sorted and unsorted blasts from standard/intermediate (n=5) or high risk (n=9) acute lymphoblastic leukemias were serially transplanted via intrafemoral injection in unconditioned NSG mice using our standard protocol (3). Flow sorting was based on the expression of the B cell precursor surface antigens CD10 and CD20. Limiting dilution experiments were performed with 10 - 10,000 sorted and unsorted cells.

Results

Population	Number of mice in total (primary/secondary/tertiary)	Cell Number	Engrafted mice in total (primary/secondary/tertiary)
CD19$^+$CD10^{high}	26 (8/18/0)	3,000-26,000	17 (65%) (6(75%)/11(61%))
CD19$^+$CD10^{low}	26 (8/18/0)	3,000-22,000	14 (54%) ((3(38%)/11(61%))
CD19$^+$CD20^{high}	92 (19/59/14)	100-100,000	72 (78%) (11(58%)/47(80%)/14(100%))
CD19$^+$CD20^{low}	91 (19/58/14)	100-100,000	77 (85%) (16(84%)/50(86%)/11(79%))

Table 2: Summary of all mice transplanted with sorted cell populations.

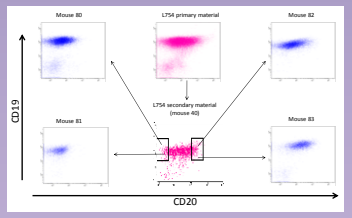

Figure 1: Both CD19$^+$CD20^{high} and CD19$^+$CD20^{low} populations engraft NSG mice.

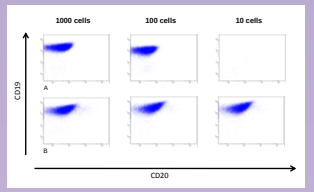

Figure 2: Limiting dilutions experiments with unsorted cells from a standard risk patient L792 **(A)** and unsorted cells from high risk patient L4967 **(B)**

Results

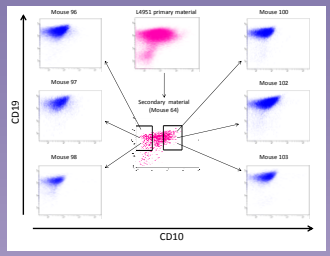

Figure 3: Both CD19$^+$CD10^{high} and CD19$^+$CD10^{low} populations engraft NSG mice.

Conclusions

- Sorted fractions irrespective of the expression of CD10 or CD20 engraft immunodeficient NSG mice.
- All populations are able to re-establish the complete immunophenotype of the original leukemia.
- Both fractions of CD10 or CD20 sorted cells serially engraft in primary, secondary and tertiary (CD20 sort) mice.
- Limiting dilution experiments show that 10 – 100 ALL blasts are often sufficient to reinitiate the leukemia in a xeno-environment.
- In summary, leukemia-propagating cells in childhood ALL are abundant and appear not to be restricted to a specific immunophenotype.

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Acknowledgements

This work was supported by grants from the North of England's Children's Cancer Research Fund (NECCR), Leukaemia Research (LR) and the JGW Patterson foundation. There are no relevant conflicts of interest to disclose.

Poster Presentation

IBFM Bi-Annual Childhood Leukaemia Symposium,

4-6th October 2010, Maritim Pine Beach resort, Antalya, Turkey

In standard and high risk acute lymphoblastic leukemias disease propagating cells are abundant and not confined to certain immunophenotypic fractions

Klaus Rehe, Kerrie Wilson, Hesta McNeill, Martin Schrappe, Julie Irving and Josef Vormoor

In childhood acute lymphoblastic leukemia (ALL) conflicting reports regarding frequency and identity of the leukemia-propagating cell (LPC) highlight the need for further investigations. We aim to address these questions of frequency and clonogenicity of sub-populations at different maturational stages, using an *in vivo* xenograft model.

ALL's from different risk groups (according to treatment protocol criteria) were included (high risk: BCR/ABL positive (6), BCR/ABL-like (2), high hyperdiploid/MRD positive high risk (2), intermediate risk: high WBC/MRD negative (2), age >10 years (1) and standard risk (1)). Blasts were transplanted intra-femorally into NOD/scid IL2Rγnull mice and engraftment patterns in bone marrow were investigated using 5 color flow cytometry. To determine the frequency of LPCs we performed limiting dilution experiments (10 to 10,000 cells/mouse) on primary patient material or leukemic cells previously passaged through mice once or twice (secondary/tertiary material). To investigate the immunophenotypic identity of candidate LPC's we used sorted blast populations of $CD19^{+}CD10^{low}$, $CD19^{+}CD10^{high}$, $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ representing different immunophenotypic maturation stages for serial transplantations.

Cell numbers successfully engrafting mice in limiting dilution experiments differed between individual leukemias, however, 10 cells only were sufficient for two unsorted bcr-abl positive leukemias.

Primary, secondary and tertiary cells sorted into $CD19^{+}CD20^{low}$ and $CD19^{+}CD20^{high}$ populations were serially transplanted into 159 mice in total, of which 125 engrafted. $CD19^{+}CD10^{low}$ and $CD19^{+}CD10^{high}$ sorted primary and secondary samples engrafted 34 out of 60 mice transplanted. Engraftment and reconstitution of the complete ALL phenotype was observed in all sorted populations. Successful serial engraftment of these diverse populations demonstrated long-term self-renewal capacity.

In conclusion strong evidence suggested leukemia-propagating cells were much more frequent than previously thought and that blasts of diverse maturational stages were able to serially reconstitute the complete leukemia in a xenotransplant model.

In Acute Lymphoblastic Leukemia, stemness is frequent and ubiquitous

Klaus Rehe[1], Kerrie Wilson[1], Hesta McNeill[1], Monique den Boer[2], Martin Schrappe[3], Julie Irving[1] & Josef Vormoor[1]

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Introduction

In acute lymphoblastic leukemia (ALL) no definitive leukemia-initiating cell has been identified as conflicting results from xenograft models persist (1). While the acute myeloid leukemic stem cell phenotypically resembles the rare normal hematopoietic stem cell, studies demonstrating this is also true for ALL may be flawed, as it has recently been shown that coating of human cells with anti-CD38 antibody causes residual immune clearance in some immunodeficient mice (2). In addition, methodological refinements now can demonstrate much higher frequencies of stem cells in cancers such as melanoma (3). Using NK depleted NOD/scid or the severely immuno-suppressed NSG mice, we previously demonstrated that leukemic blasts at different levels of maturation have the ability to re-establish the disease *in vivo,* already supporting the stochastic model of cancer stem cells (4). Here, we provide further evidence that stemness is prevalent in leukemic blasts irrespective of the expression of lymphoid differentiation markers CD10, CD20 and CD34. More importantly, leukemia-initiating cells are frequent in these subgroups and 10-100 blasts are sufficient to repopulate the leukemia in a xeno-environment, suggesting that the real frequency is even higher.

Results

Patient	Risk category	Blast origin	Blast population	Number of mice engrafted/ Number of mice trasplanted			Status	Calculated frequency (ELDA)
				Number of cells transplanted				
				3000-1000	500-100	10		
L4967	High risk t(9;22)	Primary	Unsorted	4/4	5/5	0/5	alive	41.7
		Tertiary	Unsorted	3/3	5/5	3/5	alive	10.9
L792	Standard risk	Primary	Unsorted	5/5	1/5	0/5	-	304
L870	High risk (MRD high)	Primary	Unsorted	3/4	2/4	0/5	alive	629
L826	High risk t(4;11)	Primary	Unsorted	3/4	0/4	0/4	-	888
L829	High risk (HeH)	Primary	Unsorted	3/5	0/4	0/5	alive	1251
L2510	High risk t(9;22)	Secondary	CD20high	2/4	0/4	-	-	4949
			CD20low	4/4	1/4	-	-	779
L4951	High risk t(9;22)	Secondary	Unsorted	6/6	10/10	5/10	-	14.24
		Secondary	CD20high	3/3	6/6	-	-	>41.7
			CD20low	3/3	6/6	-	-	>41.7
L49101	High risk t(9;22)	Primary	Unsorted	3/4	0/4	0/4	-	524
L858	High risk (MRD high)	Primary	Unsorted	4/5	2/5	0/5	alive	463
L737c	High risk (bcr/abl like)	Primary	Unsorted	4/4	4/5	0/5	alive	77.0
L754	High risk (HeH)	Secondary	CD20high	4/4	4/4	-	-	>41.7
			CD20low	3/3	3/4	-	-	72.1
		Tertiary	CD20high	6/8	1/8	0/8	alive	740
			CD20low	6/7	1/9	0/8	alive	590
L758b	High risk (bcr/abl like)	Primary	Unsorted	2/4	0/5	0/5	alive	1727
L49120	High risk t(9;22)	Primary	Unsorted	2/4	2/5	0/5	alive	812

Table 1: Summary of mice transplanted at limiting dilutions.

Methods

Sorted and unsorted blasts from standard/intermediate (n=5) or high risk (n=9) ALL's were serially transplanted via intrafemoral injection in unconditioned NSG mice using our standard protocol (3). Flow sorting was based on the expression of the B cell precursor surface antigens CD10 and CD20 and the hematopoietic stem cell antigen CD34. Limiting dilution experiments were performed with 10 - 10,000 sorted and unsorted cells.

Results

Population	Number of mice in total (primary/secondary/tertiary)	Cell Number	Engrafted mice in total (primary/secondary/tertiary)
CD19$^+$CD10^{high}	41 (23/18/0)	3,000-26,000	28 (68%) (16(70%)/12(67%))
CD19$^+$CD10^{low}	40 (22/18/0)	3,000-22,000	21 (53%) (10(45%)/11(61%))
CD19$^+$CD20^{high}	113 (30/58/25)	100-100,000	88 (78%) (17(57%)/48(83%)/23(92%))
CD19$^+$CD20^{low}	112 (30/57/25)	100-100,000	98 (88%) (26(87%)/51(89%)/21(84%))

Table 2: Summary of all mice transplanted with sorted cell populations.

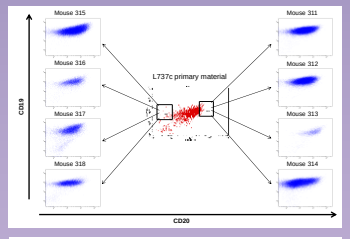

Figure 1: Both CD19$^+$CD20^{high} and CD19$^+$CD20^{low} populations engraft NSG mice.

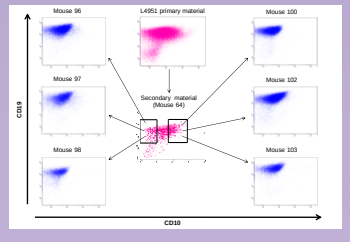

Figure 2 Both CD19$^+$CD10^{high} and CD19$^+$CD10^{low} populations engraft NSG mice.

Results

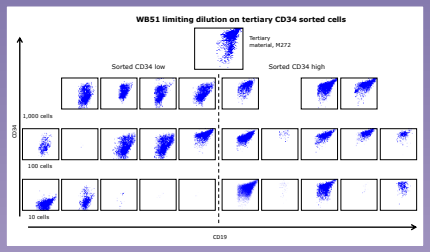

Figure 3: : Limiting dilutions experiments with tertiary CD34 sorted cells from a bcr/abl positive patient (L4951)

Conclusions

- Sorted fractions irrespective of the expression of CD10, CD20 or CD34 engraft immunodeficient NSG mice.
- All populations are able to re-establish the complete immunophenotype of the original leukemia.
- Both fractions of CD10 or CD20 sorted cells serially engraft in primary, secondary and tertiary (CD20 sort) mice.
- Limiting dilution experiments show that 10 – 100 ALL blasts (sorted and unsorted) are often sufficient to reinitiate the leukemia in a xeno-environment.
- In summary, leukemia-initiating cells in childhood ALL are abundant and appear not to be restricted to a specific immunophenotype.

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4. Le Viseur C, Hotfilder M, Bomken S, Wilson K et al. In childhood acute lymphoblastic leukemia, blasts at different stages of immunophenotypic maturation have stem cell properties. Cancer Cell 2008; 14:37-48

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ASH Abstract 92 – Oral Presentation

The American Society of Hematology, 52nd Annual Meeting

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In acute lymphoblastic leukemia stemness is frequent and ubiquitous

Klaus Rehe, Kerrie Wilson, Simon Bomken, Hesta McNeill, Martin Stanulla, Monique den Boer, Julie Irving, Olaf Heidenreich and Josef Vormoor

Research on cancer stem cells, cells that self-renew and reconstitute the full phenotype of the original malignancy, has yielded controversial results regarding their frequency and identity for many cancers. The hierarchical stem cell model has been well established in some malignancies such as acute myeloid leukemia and states that only rare, immunophenotypically immature blasts harbor stem cell activity, resembling a normal physiological hierarchy. The opposing stochastic model proposes that stemness in cancer cells is supported by extrinsic stimuli and that a substantial fraction of malignant cells have this potential. Continued optimization of *in vivo* xenotransplantation modeling recently caused a paradigm shift for some cancers, for example in malignant melanoma where stem cell activity was found in as many as 1 in 4 cells. For acute lymphoblastic leukemia (ALL) we and others previously challenged the hierarchical model by demonstrating that both immature and more mature leukemic blasts contain self-renewal properties (Cancer Cell 2008, 14(1), p47-58). In this study we address the frequency of leukemic stem cells in the bulk leukemia and also, more specifically, in subpopulations of different blast maturity by using unsorted and highly purified flow sorted cell fractions. Primary patient material as well as leukemic blasts harvested from engrafted mouse bone marrow (secondary and tertiary material) were

sorted for their CD10, CD20 or CD34 expression followed by orthotopic intrafemoral transplantation into severely immunocompromised NOD/scid IL2Rγnull (NSG) mice. Engraftment of transplanted CD19+CD10low and CD19+CD10high, CD19+CD20low and CD19+CD20high and CD19+CD34low and CD19+CD34high blast populations was monitored by 5 color flow cytometry using material from consecutive bone marrow punctures, final bone marrow harvests and/or single cell suspensions from spleens. Primary ALL samples from 15 high risk (BCR/ABL positive (n=8), BCR/ABL like ALL (n=2), high hyperdiploid/MRD positive (n=2), MRD positive (n=1), MLL/AF4 (n=2)), 3 intermediate risk (high WBC/MRD negative (n=2), age >10 years (n=1)) and 3 standard risk (n=3) patients were included. Cells sorted into CD19+CD10low and CD19+CD10high fractions were transplanted from primary patient material (n=4, HR; n=1, SR) and from secondary samples (n=4, HR; n=1; IR) with cells from one HR patient used at limiting dilutions. As few as 100 sorted cells of either fraction were sufficient to repopulate the leukemia. CD19+CD20high and CD19+CD20 low fractions from primary (n=7, HR; n=1, IR), secondary (n=5, HR; n=1, IR) and tertiary material (n=2, HR; n=1, IR) engrafted NSG mice. Limiting dilutions were performed on secondary (n=4, HR) and tertiary material (n=2, HR). Cell numbers required for engraftment varied between leukemias with as few as 100 cells being sufficient to cause engraftment.

Limiting dilution experiments using CD19+CD34high and CD19+CD34low fractions from secondary (n=1, HR) and tertiary (n=1, HR) material yielded engraftment with as few as 10 CD19+CD34high and 100 CD19+CD34low cells. Similarly, unsorted primary (n=11, HR; n=2, IR), secondary (n=2, HR) and tertiary material (n=1, HR) required as few as 10 cells for leukemic reconstitution. Taken together, both unsorted and sorted blasts of all immunophenotypes and transplanted with low numbers were able to reconstitute the complete original phenotype of the patient leukemia. All limiting

dilutions were transplanted down to 10 cells per mouse and those mice not engrafted yet are still under observation. Furthermore, the ability to self-renew was demonstrated by serial transplantation. Finally, we compared expression of self-renewal associated genes (BMI1, EZH2, HMGA2, MEIS1, TERT) in CD19+CD34low and CD19+CD34high fractions of 5 HR and 1 SR samples with that in cord blood. Interestingly, expression of these genes was not dependent on the CD34 status of the leukemic cells, whereas HMGA2, MEIS1 and TERT were upregulated in CD34+ cord blood cells. In summary we provide strong evidence for the stochastic cancer stem cell model in B precursor ALL by demonstrating that (i) a broad spectrum of blast immunophenotypes exhibit stem cell characteristics and (ii) that this stemness is highly frequent among ALL cells.

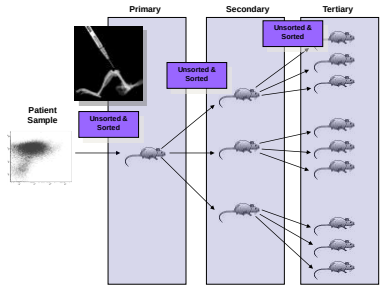
Newcastle Cancer Centre
at the Northern Institute for Cancer Research

In Acute Lymphoblastic Leukaemia,
Stemness is Frequent and Ubiquitous

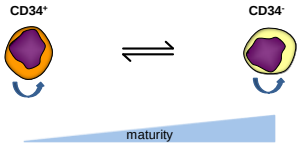
Klaus Rehe

Newcastle University

Transplant Model



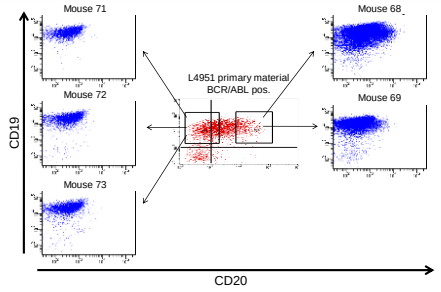
Malleability of Leukaemic Blasts



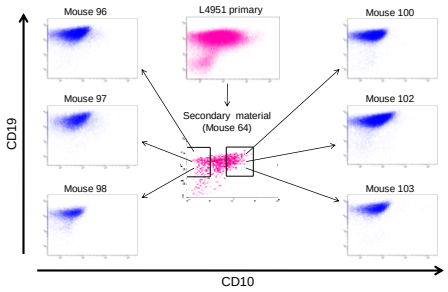
Cancer Cell,14(1): 47-58, 2008

Is malleability restricted to CD34 expression?

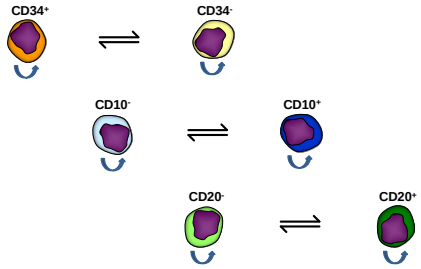
Engraftment of CD20 Sorted Cells



Engraftment of CD10 Sorted Cells



Malleability of Leukaemic Blasts



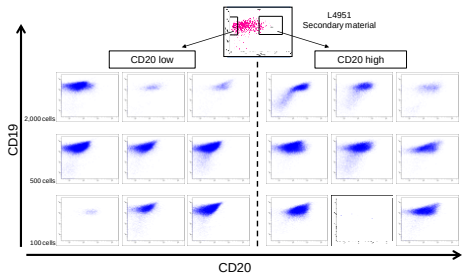
How frequent is the leukaemic stem cell?

Stem Cell Frequency in Unsorted Blasts

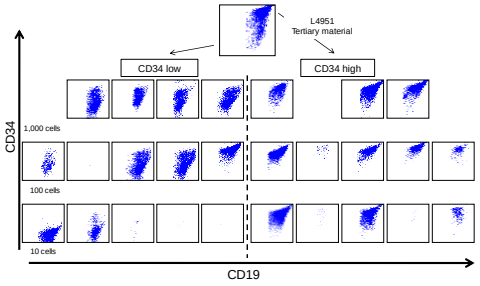
Patient	Blast origin	Number of mice engrafted/ Number of mice transplanted		
		Number of cells transplanted		
		1000	100	10
L4967	Primary	4/4	5/5	0/5
	Tertiary	3/3	5/5	3/5
L792	Primary	5/5	1/5	0/5
L870	Primary	3/4	2/4	0/5
L826	Primary	3/4	0/4	0/4
L876	Primary	4/4	4/5	0/5
L829	Primary	3/5	0/4	0/5
L2003	Primary	1/3	0/4	0/4
L4917	Primary	1/3	0/4	0/5
L4951	Secondary	6/6	10/10	5/10
L49101	Primary	3/4	0/4	0/4
	Secondary	4/4	5/5	4/5
L858	Primary	4/5	4/5	5/5 *
L737c	Primary	4/4	4/5	0/5
L758b	Primary	2/4	0/5	0/5
L49120	Primary	1/4	2/5	0/5

How frequent is the leukaemic stem cell in defined subpopulations?

Limiting Dilution of CD20 Sorted Cells



Limiting Dilution of CD34 Sorted Cells

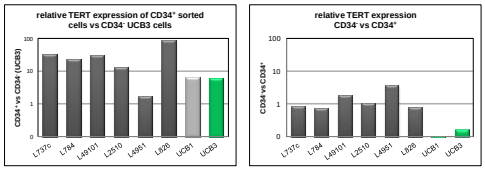


Stem Cell Frequency in Subpopulations

Patient	Blast origin	Blast population	Number of mice engrafted/ Number of mice transplanted		
			Number of cells transplanted		
			1000	100	10
L2510	Secondary	CD20high	2/4	0/4	-
		CD20low	4/4	1/4	-
L4951	Secondary	CD20high	3/3	6/6	-
		CD20low	3/3	6/6	-
	Tertiary	CD34high	3/3	5/5	5/6
		CD34low	4/4	4/5	4/5
	Tertiary	CD20high	-	2/3	0/4
		CD20low	-	3/3	0/4
L737c	Secondary	CD10high	4/4	2/4	0/5
		CD10low	4/4	2/4	0/5
	Secondary	CD20high	4/4	1/4	0/5
		CD20low	2/4	0/4	0/5
	Secondary	CD34high	4/4	1/5	4/5
		CD34low	4/4	3/5	1/5
L754	Secondary	CD20high	4/4	4/4	-
		CD20low	3/3	3/4	-
	Tertiary	CD20high	6/8	1/8	0/8
		CD20low	6/7	1/9	0/8

Do subpopulations share a common self-renewal programme?

TERT - Leukaemic Stemness Marker



Simon Bomken, unpublished results

Conclusions

- In acute lymphoblastic leukaemia, stem cells are frequent
- The frequency of stem cells is comparable between different subpopulations
- Preliminary data suggest a self-renewal programme common to leukaemic subpopulations

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Leukaemia & Lymphoma Research
BEATING BLOOD CANCERS

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Appendix 3 - Publication