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The Significance of the Microenvironment for the Development of p185 BCR-ABL Triggered Leukemia: A Comparison of Mouse Models in Neonatal and Adult Recipients

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Meiner Familie

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1. Introduction

In 2010 over 11,500 people in Germany were diagnosed with leukemia, 6% of whom were children. In the same year 7,200 people succumbed to the disease. For the year 2014 the prognosis was that 12,100 new cases of leukemia would be diagnosed. In humans the four most common forms are acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) as well as and chronic lymphocytic leukemia (CLL) (Robert-Koch-Institut, 2013).

Human clinical studies show that there are substantial differences between adults and infants in leukemic development. Horvath and colleagues described that chronic myeloid leukemia (CML) is only found in 2% of diseased children, whereas it is developed in 15-20% of over 20-year-old patients (Horvath et al., 2011). In Inaba et. al. (2013), it was found that ALL can be seen both in children and adult patients (Inaba et al., 2013). However, children with ALL have a significantly better relative survival rate than adult patients (Pulte et al., 2014).

To understand the development of the disease and to develop new therapies and understand therapy resistance, animal experiments are necessary in both human and veterinary medicine. Combining insights gained in animal models and patient data allows the study of translational aspects as well as improves the chances that the field advances by the respective scientific efforts. Thus, to gain an advantage against human leukemic diseases, effective but strictly controlled animal experiments, which also help researchers to gain an understanding and therapeutic methodology for veterinary medicine, are necessary.

The aim of this thesis was to develop a new, effective mouse model for BCR-ABL-induced leukemia using intrahepatical (i.h.) transplantation of retroviral transduced

fetal liver cells into newborn recipients, and to compare the disease developments with those of adult recipients.

2. Literature Review

2.1. The Niche

In 1978 the term “niche” was proposed for the first time by R. Schofield in his publication “The relationship between the spleen colony-forming cell and the hematopoietic stem cell.” He described the niche as an anatomical compartment in which the stem cell is observed in association with other cells, which determine its behavior (Schofield, 1978). It was indeed found that the maintenance of hematopoietic stem cells (HSCs) through self-renewal, survival, differentiation and proliferation is dependent on HSC-niche interaction (Fuchs et al., 2004; Morrison et al., 2008; Nagasawa et al., 2011; Takizawa et al., 2012). The niche is thought to consist of discrete anatomical sites. On the one hand, there is the endosteal niche, where HSCs are located close to osteoblasts of trabecular bone and incoming arterioles, and on the other hand there is the vascular niche, where stem cells reside close to the sinusoid endothelium and perivascular cells (Frassoni et al., 1982; Nilsson et al., 2001). Many groups have described a variety of cell types that are thought to be part of the niche and that are the critical components of either the endosteal or the vascular regions (Heissig et al., 2002; Wilson et al., 2007). Near the endosteum, for example, there were found spindle-shaped N-cadherin+ CD45- osteoblasts (SNO) (Zhang et al., 2003), or immature early B-cell factor 2+ osteoblasts (IEO) (Kieslinger et al., 2010). Nestin+ mesenchymal stem cells (MSCs) are found throughout the bone marrow and do not appear to be restricted to either niche (Mendez-Ferrer et al., 2010). On the perivascular side there are non-myelinating Schwann cells (Yamazaki et al., 2011) and CXCL-12 abundant reticular cells (CAR) (Sugiyama et al., 2006) and SCF-secreting LepR+ perivascular cells (Ding et al., 2012).

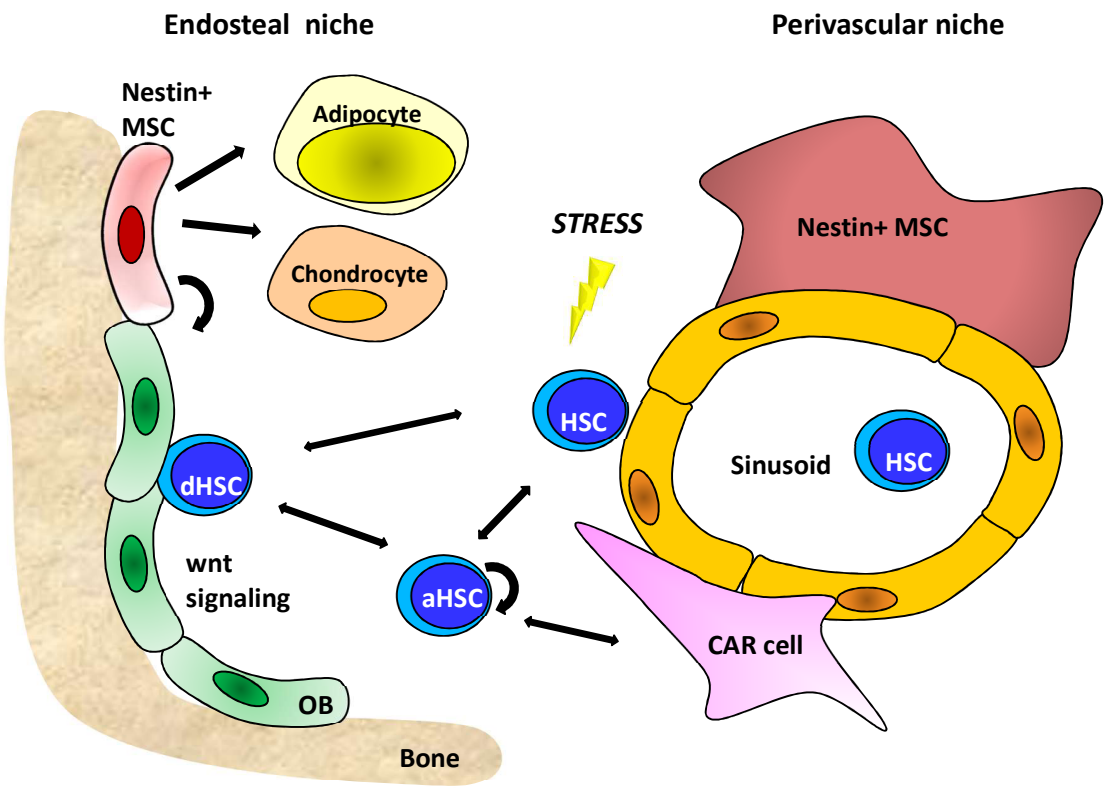

Figure 1: *Model of endosteal and perivascular niche.*

Differentiation of mesenchymal stem cells (MSCs) into osteoblasts (OBs), adipocytes or chondrocytes. HSCs reside in a dormant state (dHSC) in the endosteal region through OBs and Nestin+ MSCs. Factors of Wnt signaling regulate HSC quiescence, while dormant HSCs, can be activated (aHSC) by responding to hematopoietic stress such as wounding, infection, irradiation or leukemia. Self-renewal of active HSCs is promoted by the components of the perivascular niche, the CAR cells. Model adapted from Ehninger et.al., 2011 (Ehninger et al., 2011) and recently published by Schreck, C. and Bock, F., Grziwok, S. 2014 (Schreck C. et al., 2014).

2.2. Hematopoiesis

To understand the processes of a hematopoietic pathology, it is necessary to first understand the normal physiology of hematopoiesis. As Sinowatz and his colleagues describe, hematopoiesis is one of the most complex mechanisms in mammalians that has been shown to self-renew and to adapt to certain circumstances for the lifetime of the animal. Hematopoiesis is a hierarchical system that works very effectively and flexibly and produces billions of blood cells each day (Hees; et al., 1992). At the very top of this hierarchical system is the LT (long-term) hematopoietic stem cell (LT-HSC), which resides in postembryonic mammals mostly in the red bone marrow (BM) of big bones such as the cranium, sternum, ribs, hip and spine bones as well as the humerus and femur. HSCs are unique somatic stem cells, of which only a single cell is necessary to rebuild the complete hematopoietic system of one mouse after lethal irradiation; this is thanks to the ability of LT-HSCs to self-renew and the fact that other progenitor and mature cells are produced, depending on the needs of the body, for a lifetime. Without self-renewal, stem cells would be exhausted, resulting in severe cytopenia. LT-HSCs give rise to the so-called MPPs (Multipotent Progenitors) or ST (short-term) HSCs. MPPs lose their self-renewal ability and are set on the path of proliferation and differentiation. As the name says, these cells do not repopulate mice for a lifetime, but only for short periods of time. Their ongoing differentiation gives rise to lineage-committed lineages, such as the LMPP (lympho-myeloid potential progenitors), the CLPs (common lymphoid progenitors), and CMPs (common myeloid progenitors). The latter give further rise to GMPs (granulocyte-monocyte progenitors) and MEPs (megakaryocyte-erythrocyte progenitors), bi-potent progenitors with an irreversible commitment of differentiation towards pro-inflammatory granulocytes and monocytes/macrophages (GMPs), oxygen-transporting erythroid cells and megakaryocytes/platelets (MEPs), lymphoid pre T-, B-cells (CLPs) and natural killer (NK) cells. MEPs develop into erythrocytes and finally thrombocytes. Erythrocytes include hemoglobin and take care of the transportation of oxygen and CO_2 in and out of the capillaries of the lungs and arterial capillaries of every tissue

and organ in the body. Thrombocytes are responsible for adhesion on lesions and are thus responsible for hemostasis. GMPs differentiate into granulocytes and monocytes that belong to the congenital/innate immune system, whereas CLPs generate B- and T-cells, the operators of the adaptive immune system (Ackermann et al., 2015; Engelhard et al., 2009). As malignancies such as leukemia often have similar signaling pathways to physiological transformations, the understanding of these mechanisms is highly important (Reya et al., 2001).

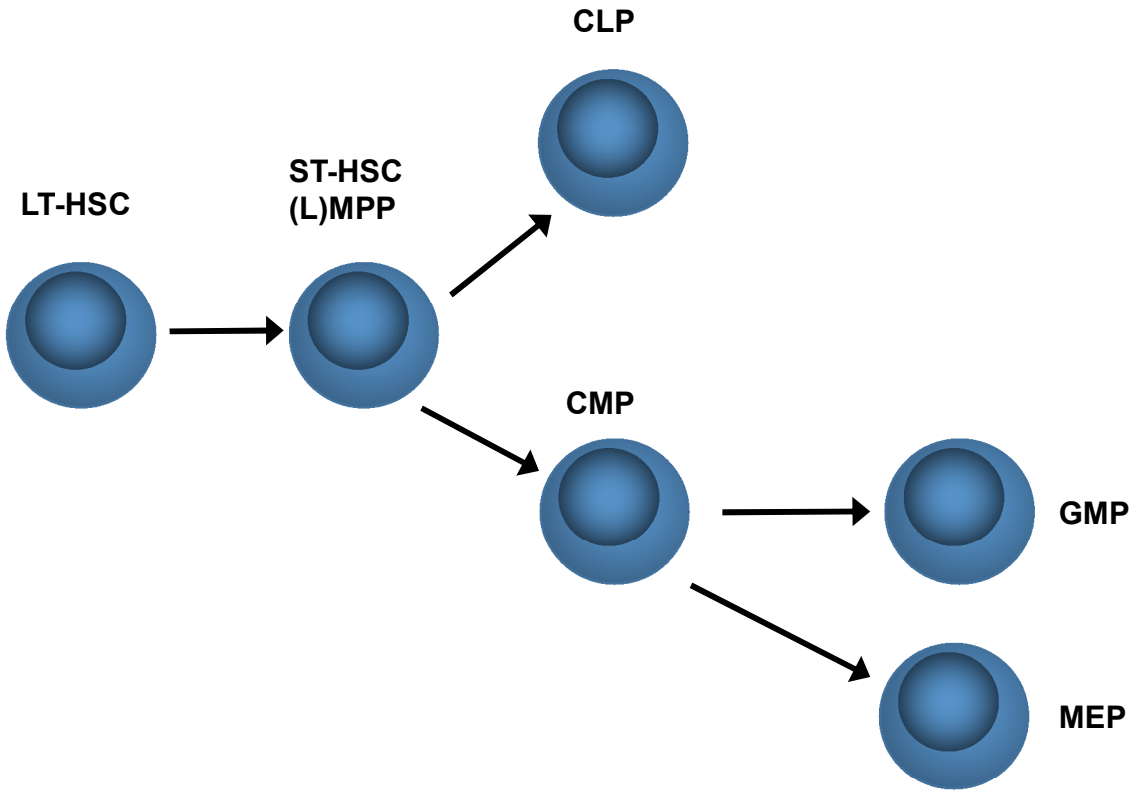

Figure 2: *Hematopoietic hierarchy.*

Model according to (Renstrom et al., 2010). HSC: hematopoietic stem cell (LT: long-term; ST: short-term); MPP: multipotent progenitor; LMPP: lympho-myeloid potential progenitor; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocyte–monocyte progenitor; and MEP: megakaryocyte–erythrocyte progenitor.

2.2.1. Embryonic Hematopoiesis

The first evidence of blood cells was demonstrated in the 1970s when Moore and Metcalf described these cells to be located in “blood islands” in the yolk sac as well as blood vessels that originate from primitive haemangioblasts that, later in embryonic development, form the blood circulation system (Moore and Metcalf 1970). The first definitive HSCs with the ability to repopulate adult recipients can be found in mice in the aorta-gonad-mesonephros-area (AGM), 10-10.5 days after fertilization during the mid-gestational time, which is called the mesodermal period (Muller et al., 1994; Oostendorp et al., 2002). Prior to that period, only primitive non-white blood cells (erythrocytes or megaloblasts) are produced. In the following hepato-lienal period (in mice: later than E11.5 dpc) the fetal liver (FL) and spleen (SP) take over most of the definitive embryonic hematopoiesis and white blood cells expand tremendously. The medullar period takes place in the BM and is the last prenatal step for the hematopoietic development from where it serves the organism throughout its life (Al-Drees et al., 2015; Mercier et al., 2012; Schorr et al., 2006). At postnatal hematopoiesis only granulocytes and erythrocytes are produced in the red BM, whereas lymphocytes are mostly produced in lymphatic organs such as the thymus, spleen or lymph nodes (Al-Drees et al., 2015; Hees;Sinowatz, 1992). In 2006 Michelle B. Bowie detected differences between fetal HSCs and adult HSCs as they alter in their method of self-renewal, gene expression characteristics and differentiated cell output. Supposedly, there exists a so-called “fetal phenotype,” which lasts about three weeks after birth; only one week later fetal HSCs and adult HSCs cannot be distinguished anymore (Bowie et al., 2007).

2.2.2. Adult Hematopoiesis

As already described in more detail in chapter 2.2, hematopoiesis constitutes a hierarchical system starting with multipotent stem cells, which have the ability to self-renew as well as the ability to produce progenitor cells that differentiate to more

committed hematopoietic cells and finally result in mature cells (Hees;Sinowatz, 1992; Mikkola et al., 2006). The main duty of adult hematopoiesis is to produce various cells starting from erythrocytes for oxygenation up to platelets and lymphoid cells and control the daily changes in the organism that may occur as reactions to aging, wounding or diseases. The most important location in this age is the bone marrow, especially the metaphysis of the long bones. What is outstanding for the BM in aged mammals is the growing appearance of adipocytes, which were first seen simply as room fillers and additional energy reservoir for emergencies, but recently were identified as also playing an important role in the activation of hematopoiesis as well as osteogenesis. Additionally, the spleen can react to stress and produce HSCs as well (Al-Drees et al., 2015).

2.3. Leukemia: Definition and Types

The word leukemia originates from the Greek word “leuchaima” and means “white blood.” Leukemia defines the malignant development of HSCs in the bone marrow (Richard W. Nelson et al., 2006). There are two main subtypes of leukemia: myelogenous and lymphocytic leukemia. In myelogenous leukemia, granulocytes and monocytes are malignantly transformed, while in lymphocytic leukemia, based on the amount of involved cell populations, one can distinguish between B-cell or T-cell leukemia. Besides these clear subtypes, there is also leukemia with involvement of both myeloid and lymphoid cells, which is called mixed-type leukemia.

The lymphocytes are characterized by adaptive B- or T-cell receptors (BCR, TCR), which allow the cells to adapt to different immunological challenges. The name of the antibody-producing B-cells originates from the avian “bursa fabricii” where these cells were found first (Ackerman et al., 1959). Mouse experiments showed the existence of pre-B-cells in the BM of mammals as well as in FL (LeBien, 1998; LeBien et al., 2008). TCR-expressing T-cells, on the other hand, are “thymus

derived" cells. The thymus is an organ that is highly active during childhood and degenerates for the most part by adulthood. In the thymus, T-cell precursors from the BM are positively and negatively selected for expression of TCRs, which do not recognize self-antigens. Both B- and T-cells exist in different stages as well as discrete subpopulations, all of which can, in theory, give rise to leukemia. In addition, lymphocytes also reside in lymph nodes, and malignant lymphocytes that preferably populate these immunological organs are called lymphomas.

There are many different types of leukemia and lymphomas, which are characterized mostly through classification of the patient's symptoms and histological findings. These malignancies can be roughly divided into two main groups, namely acute forms, which have a very rapid and aggressive development and can be rapidly lethal if untreated, and chronic diseases, in which a leukemia or lymphoma slowly grows (evolves) into an acute form (examples are CLL, MDS (myelodysplastic syndrome) and CML). The chronic malignancies are neither easy to recognize nor to diagnose, since for a long time, patients often do not show conspicuous symptoms - sometimes even no symptoms at all - and they have a much longer etiopathology than acute forms (Morse et al., 2002; Richard W. Nelson Couto, 2006).

2.4. BCR-ABL Oncogene[1]

Chronic myelogenous leukemia was recognized early as a disease originating from cells with an unusually small chromosome (Baikie et al., 1960), the so-called "Philadelphia chromosome (Ph) translocation" or 22q11 translocation in chronic myelogenous leukemia. The name originates from Peter Nowell and David Hungerford, who discovered the chromosome in 1960 in Philadelphia (Nowell et

[1] In this thesis, besides this chapter, the spelling of *Bcr-Abl* gene will be the same as for BCR-ABL protein and capital letters will be used throughout in this work for this word.

al., 1960). Subsequently it was discovered that *Bcr*, named after the 22q break point cluster region (BCR) and Abelson tyrosine kinase, or *Abelson Musine Leukemia Viral Oncogene Homolog1* (ABL) located on chromosome 9q, together form the chimeric BCR-ABL fusion oncogene (Wells et al., 1996) a translocation of BCR that constitutively activates the ABL tyrosine kinase and misallocates it to the cytoplasm (Guo et al., 1998; Wells et al., 1996). Over time, by inducing genetic instability, the presence of the Philadelphia chromosome pushes the disease from a chronic and nonlethal type to an acute blast crisis that leads in general to death (Guo et al., 1998). So far, different exon-exon fusions of the *Bcr* and *Abl* genes have been detected as well as several breakpoints which cause a variety of BCR-ABL fusion proteins (p185, p190, p210, and p230) (Deininger et al., 2000; Wong et al., 2001). In ***Figure 3*** a scheme adapted to Deininger et. al. with the most common breakpoints can be seen. The most frequent breakpoint within the *Abl* gene occurs between the two exons Ib and Ia, but can also be seen upstream of exon Ib or downstream of exon Ia. In the *Bcr* part there exists a major breakpoint (M-*bcr*) between exons 12 and 16 which results in a b3a2 and/or b2a2 junction, encoding a p210 BCR-ABL fusion protein that is mainly associated with CML (He Y. et al., 2002). The p185 BCR-ABL fusion protein is caused by a transcript containing the junctions e1a2 and BCR breaks at a minor breakpoint (m-*bcr*), which is located between the two alternative exons e2´and e2 in the first intron of BCR and is mainly associated with ALL especially in children (Melo, 1996). However, 30% of the cases of Ph+ ALLs also show the p210 fusion (Chan et al., 1987; Clark et al., 1987; Rafiei et al., 2015). Another fusion protein is p 230 BCR-ABL, whose breakpoint lies in the *µ-bcr* region and which has the very rare junctions e19a2. It is said to cause a form of rare chronic neutrophilic leukemia (Pane et al., 1996). In this thesis, I will use the p185 e1a2 BCR-ABL as a fusion oncogene to induce a hematopoietic malignancy as it is known to play an important role in childhood leukemia. (Hermans et al., 1987; Kurzrock et al., 1987; Melo, 1996).

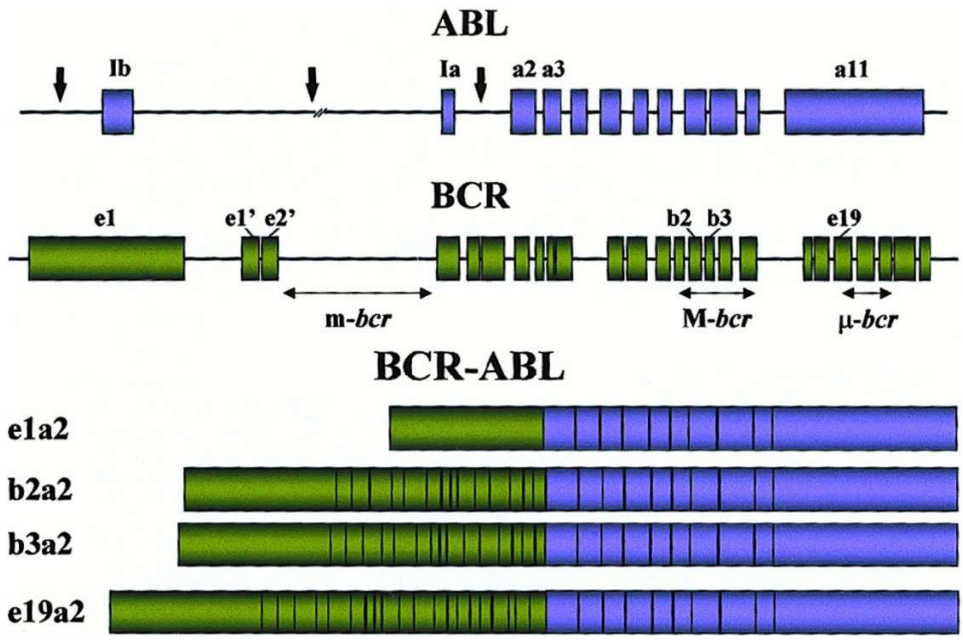

Figure 3: *Scheme of the locations of breakpoints in ABL and BCR genes.*

Illustration adopted from the review of Deininger and his colleagues, Blood (Deininger et al., 2000).

2.5. Green Fluorescent Protein (GFP)

The vector I will use throughout this thesis not only encodes the p185 BCR-ABL fusion oncogene, but also the green fluorescent protein (GFP). Osamu Shimomura first described it in 1962 when it was discovered in a jellyfish (*Aeqourea victoria*) found on the West Coast of North America (Shimomura et al., 1962). It took him over 19 years to collect enough material to identify the structure of this protein. By 1992 a full sequence of GFP was finally presented. Martin Chalfie was the first researcher to combine the GFP with *Escherichia coli* to produce green fluorescing cell cultures. From then on, GFP was used as a protein marker because of its characteristic bright and glowing green luminescence, and by that time, other colors of luminescence could be produced on the basis of these findings (Sanders et al., 2009). In 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien received the Nobel Prize in Chemistry for their exceptional findings on this protein, as it is a key

for *in vivo* science (Shimomura, 2009). The most remarkable characteristic of GFP is that neither an exogenous substance nor a cofactor are necessary for the use of this marker (Haldar et al., 2009). In this thesis, I use GFP to track FL cells transduced by the retroviral vectors and therefore to visualize the expression of BCR-ABL. After optical excitation within a wavelength of 395nm and 475nm, cell populations can be visualized by measuring the fluorescence.

2.6. The Newborn Mouse as a Model for Leukemia in Research

Since the beginning of the use of mouse models in research around 1900, the importance of the mouse as a laboratory animal has grown enormously. For leukemic research, this interest exploded in 1902 when Abbie Lathrop found out by accident that there was a coincidence between family genetics and cancer. This connection led her to develop inbred strains (Steensma et al., 2010). With the development of chemotherapies by Paul Ehrlich in the beginning of the twentieth century, first mouse models for cancer research were developed. One of the biggest milestones was the establishment of the first transplantable tumor system in mice in 1910 by George Clowes (DeVita et al., 2008). The first research on HSCs in newborn microenvironment was performed in 1997, when Yoder found that yolk sac HSCs are able to renew the hematopoietic system of newborn recipients, but not of adult recipients (Yoder et al., 1997a). In the work of Arora et al. from 2014 it is also described that there are differences in the ability of HSCs from different age groups to engraft, depending on the age of the recipients. They found out that HSCs from E.9.5 to E.10.5 are more likely to engraft in neonates, whereas HSCs from E.14.5 engraft better in adult recipients. Even the organ used plays an important role, as FL HSCs give a better result in engraftment in NBs than BM does (Arora et al., 2014). These findings underline the need to differentiate between adult and neonatal mouse models for leukemia. Efforts were undertaken to understand

critical risks and circumstances in the development of childhood leukemia, described e.g., by McCormick (McCormick et al., 2004). In 2004, Traggiai described a new xenotransplantation model for intrahepatic transplantation of cord blood in newborn mice (Traggiai et al., 2004) which was the basis for Silke Gitzelmann's work to establish a transplantation model for FL cells in neonates (Gitzelmann, 2011). Although many models for BCR-ABL-induced leukemia in adult recipients exist and have been used for years, there is no model for BCR-ABL-induced leukemia in NB mice available yet. To improve research in this direction I used this thesis as a basis for developing a p185 BCR-ABL leukemic transplantation model for childhood leukemia research in neonatal mice recipients.

2.7. Aims of the Thesis and Experimental Approach

The first aim of this thesis is to develop a new mouse model for p185 BCR-ABL leukemia, which will help understand the specific origins and impacts of leukemia in juvenile organisms. This model could be a good representative model for Ph+ childhood leukemia. Current models of childhood leukemia study the disease either in transgenic models, in which frequently found oncogenes are expressed in hematopoietic cells throughout embryonic development, or adult marrow cells are transfected with oncogenes and infused in adult recipients. My goal is to establish an alternative for the second method, where the donor cells are fetal liver cells and the recipients are newborn animals, thus, more closely resembling the situation in childhood leukemia.

Furthermore, I aim to use the model of leukemia in juvenile animals to determine what specific type of leukemia is induced by the expression of p185 BCR-ABL in fetal liver cells and to find out whether the study of leukemogenesis in neonatal recipients may bring novel insights in the development of childhood leukemia.

My last aim is to compare leukemogenesis in newborn and adult recipient mice to study whether the ontology of the recipient microenvironment plays a significant role in the outcome of leukemogenesis.

3. Animals, Materials and Methods

3.1. Animals

3.1.1. Mice Strains

For my research, I used mice as laboratory animals, as their genetics are well known, their reproduction rate is high and they have a fast maturation. In particular, I used inbred strains of 129S2/svHsd (129) and C57BL/6.J (B6) from Harlan Laboratories in Rossdorf, Germany. An inbred is produced using at least 20 consecutive generations of sister x brother or parent x offspring matings, or is traceable to a single ancestral pair in the 20th or subsequent generation (The Jackson LaboratoryJackson, 2014). The 129 inbred mouse was developed by Dunn in 1928 from a cross of coat color stocks and a chinchilla stock from Castle (Harlan Harlan-Laboratories, 2015). Together with the BALB/c mice, B6 mice are probably the two most popular strains used in medical research. B6 mice were developed in 1921 by C.C. Little from a brother-sister pair from Miss Abbie Lathrop's stock (Steensma et al., 2010). It is the most popular genetic background for mutants and is a long-lived strain with few tumors. The B6 mice do carry a congenic abnormality, as some sub-strains (most notably the B6) show a functional deletion of the Nnt gene (nicotinamide nucleotide transhydrogenase), a gene involved in mitochondrial respiration.

Through cross-breeding of 129 and B6 mice, I created a system that takes into account a more diverse gene pool than the single inbred strains. The resulting (129xB6)F1 hybrids are more robust, and not as susceptible to inbreeding depression. The increased gene pool in F1 hybrids also decreases sensitivity to stress, which may lead to better survival rates in experimental settings. As the B6 is known to be aggressive, the influence of 129 is also useful in order to better handle the animals. All mice for this thesis were bred directly in the center for

preclinical research (Zentrum für präklinische Forschung (ZPF), Klinikum rechts der Isar, München, Germany).

Name	Company
C57BL/6.J	Harlan Laboratories, Rossdorf, Germany
129S2/SvHsd	Harlan Laboratories, Rossdorf, Germany
(129S2 x C57BL/6.J) F1	Breeding in Zentrum für Präklinische Forschung (ZPF)

Table 1: *Mice strains.*

3.1.2. Housing

Mice were kept according to Federation of European Laboratory Animal Science Association's (FELASA) recommendations in the center for preclinical research (ZPF) of the Technical University in Munich. Animals were hold in micro isolators with Tecniplast individually ventilated cages (Tecniplast-IVC) under specific pathogen-free (SPF) conditions in "Type I Superlong" cages (592cm²). There was a light/dark interval of 12 hours, including a phase of crepuscular light. Every item used in the SPF rooms such as cages, materials like pellets, water bottles, or even nutrition and drinking water was autoclaved and sterilized before brought through the lock. As litter, wooden pellets were used, which were changed up to two times a week.

As described in the *Guide for the Care and Use of Laboratory Animals*, natural behavior is the precondition for a successful animal experiment (Academies, 2010). For that reason environmental enrichment (EE) and social environment (SE) were optimized as much as possible. EE in the units was accomplished through pellets

as described above and cellulose nestlets were used to support the mice's need to dig and to nest. Every cage contains houses for hiding and avoiding other mice in case of a dominance fight and grids to climb on to have the possibility to manage their need for activity. As mice are not able to see the color red as humans do, but see it as black, their houses are transparent and red, so that the rodents still have the feeling of security but the scientists and the personnel are able to properly survey them without disturbing them. As the laboratory animals need to have a proper SE, female mice were not housed singly but in a group of a minimum of two to five females per cage. Male individuals were held either in a group of other males in case they were raised together or alone, as males are also known to be loners. Up to an age of three weeks, male rodents could possibly be socialized with other males; after that age they were not grouped anymore because of the danger of fights. For pairing, one male was paired with one female, always in a fresh and properly kept cage so that both individuals had same territorial conditions. Pups were nursed three weeks and then relocated. Nutrition was given trough autoclaved powdered rodent nutrition pellets, including Fa. Altromin ad libitum as well as autoclaved water ad libitum, which was changed twice a week. Young mice, right after being separated from their mother, were additionally served one spoonful of oat flakes. The animal experiments were approved by the Regierung Oberbayern under the Reference Number (55.2-1-54-2532-85-10) and took place under the realization of European Directive 2010/63/EU.

3.1.3. Health Monitoring and Surveillance of Laboratory Animals.

Laboratory animals were supervised on the basis of the FELASA guidelines (FELASA; et al., 2014) and reforms of the animal protection laws in Germany on the basis of the European Directive 2010/63/EU. These criteria are based on standards for determination on the animal stress in laboratory animal experiments.

All mice were supervised by qualified ZPF personnel, myself and other laboratory members, at least daily. There was a constant exchange of information between the scientists and the mouse facilities personnel. Through supervision in this manner the endpoint of an experiment could be defined as soon as pain and deterioration in physical condition was classified as moderate or severe, according to the scoring criteria shown in ***Table 3.*** The experiment was stopped and the mouse was euthanized for analysis. As the table below shows, I used defined criteria to evaluate the distress and pain level of the subject. I also used the physiological criteria of the Bethesda proposals of classification of non-lymphoid and lymphoid hematopoietic neoplasms in mice (Kogan et al., 2002; Morse et al., 2002). At the latest as the score level reached a maximum of 20 points the experiment was stopped immediately.

I General Condition (Behavior and Posture):		**Score**
Physiological:	- typal interest/curiosity, percipience of surroundings, reaction to contact, typal sleeping habits, social contacts	0
Mildly reduced:	- arched back, slightly limited movements or hyperkinetic, enforced respiration, sunken flanks, reduced interest about environment	5
Moderately reduced:	- strongly arched back, apathetic, cold body surface, gasping	10
Severely reduced:	- lateral position, agonal respiration	20

II Appearance / Nutrition Conditions		**Score**
Physiological:	- sleek and silky fur, good nutritional conditions, no signs of dehydration, body weight reduction <5%	0

II Appearance / Nutrition Conditions		Score
Mildly reduced:	- mild dehydration (grade 1 = 3-6% of body mass), scruffy fur, body weight reduction 5-10%	5
Moderately reduced:	- moderate dehydration (grade 2 = 6-8% of body mass), strongly ruffled fur, body weight reduction 11-20%	10
Severely reduced:	- severe dehydration (grade 3 > 8% of body mass), body weight reduction >20%	20

III Blood Check		Score
Physiological:	- leucocytes in peripheral blood < 15 x 10^3/µl	0
Mildly reduced:	- leucocytes in peripheral blood 15 - 30 x 10^3/µl	5
Moderately reduced:	- leucocytes in peripheral blood 30 - 50 x 10^3/µl	10
Severely reduced:	- leucocytes in peripheral blood > 50 x 10^3/µl	20

III Visual Evidence of an Infection		Score
Physiological:	- no visible effects	0
Mildly Reduced:		0
Moderately reduced:	- slightly swollen face, slight signs of anemia	10
Severely reduced:	- strongly swollen face, signs of anemia, ascites	20

Table 2: *Criteria for evaluation of damage, distress and pain of laboratory animals.*

Stress level	Classification	Consequences	Score
Stress level 0	No distress	No consequences	0
Stress level 1	Mild distress	Careful surveillance	1-9
Stress level 2	Moderate distress	Additional care (infusions, vitamins, analgesics if necessary/possible) and increased surveillance	10-19
Stress level 3	Severe distress	End of the experiment	20

***Table 3**: No-go criteria for laboratory animal experiments.*

3.2. Materials

3.2.1. Apparatuses

Type of device	Name	Manufacturer
Animal blood counter	Scil Vet Abc™	Scil Vet Academy, Viernheim, Germany
Cell incubator	Hera Cell 240	Heraeus Instruments, Hanau, Germany
Centrifuge	Megafuge 3.0 RS , Multifuge 3S Biofuge fresco	Heraeus Instruments, Hanau, Germany
Counting chamber	Neubauer-improved	Paul Marienfeld GmbH, Lauda Königshofen, Germany
Flow Cytometer	CyAn ADP Lx P8	Beckman Coulter, US

Type of device	Name	Manufacturer
Ice machine	S.-No: 061244	Ziegra Eismaschinen, Isernhagen, Germany
Laminar flow hood	ANTAES 48/72	BIOHIT, Germany
Linear accelerator	Mevatron KD2	Siemens, Erlangen, Germany
Microscope	Axiovert 25	Carl Zeiss, Jena, Germany
NanoDrop	ND-1000 UV/Vis-spectrophotometer	NanoDrop Technologies, Wilmington, DE, US
Precision scales	PLJ 2100-2M	Kern & Sohn GmbH, Balingen, Germany
Radiation unit	Gulmay	Gulmay, Suwanee, US
Thermal cycler	PTC 100 Peltier	Bio-Rad, Philadelphia, US
Thermomixer	comfort	Eppendorf AG, Hamburg, Germany
Vortex	IKA® MS1 minishaker	Werke & Co., Staufen im Breisgau, Germany
Water bath	SUB	Grant Instruments Ltd., Cambridgeshire, UK

Table 4: *Apparatuses.*

3.2.2. Consumables

Item	Manufacturer
Blood lancets Supra	Megro GmbH & Co KG, Wesel, Germany
Cell culture flasks Cellstar 125ml/250ml/550ml	Greiner Bio-One GmbH, Frickenhausen, Germany

Item	Manufacturer
Cell culture dish, 10 mm, growth-enhanced treated	Corning Inc., Corning, US
Cell culture dish, 10 cm, growth-enhanced treated	TPP Techno Plastic Products AG, Trasadlingen, Schweiz
Cell culture plates Cellstar 6/12/24/48/96 well	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell strainer 70nm	Greiner Bio-One GmbH, Frickenhausen, Germany
Disposable bags	Carl Roth, Germany
Filter vacuum driven disposable bottle top filter Steritop	Millipore Co., Billerica, US
Filters 0.45/30/70/100µm	BD FalconTM, BD Biosciences, Heidelberg, Germany
Filter tips TipOne 10/100/200/1000µl	Greiner Bio-One GmbH, Frickenhausen, Germany
Hamilton syringe; gastight; 1710	Hamilton, Bonaduz, Switzerland
Hamilton needle; 6/pk	Hamilton, Bonaduz, Switzerland
Microcentrifuge safe-lock tubes 1.5/2ml	Eppendorf AG, Hamburg, Germany
Monoject blunt cannula needles	Kendall Healthcare, US
Needles, 100 Sterican, 27/30 gauge	B. Braun Melsungen AG, Melsungen, Germany
Petri Dish with vents 10mm	Greiner Bio-One GmbH, Frickenhausen, Germany

Item	Manufacturer
Polypropylene centrifuge tubes 15/50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Serological pipets , 2/5/10/25/50 ml	BD FalconTM, BD Biosciences, Heidelberg, Germany
S-Monovette® Blood Collection System	Sarstedt AG & Co., Nümbrecht, Germany
Syringes BD Plastipak™ 1 ml BD	Heidelberg, Germany
Syringes with needle, Sub-Q, 1 ml	BD, Franklin Lakes, US
Syringes single-use omnifix 3/5/10/20/30 ml	B. Braun Melsungen AG, Melsungen, Germany

Table 5: *Consumables.*

3.2.3. Chemicals

Item	Manufacturer
Albumin fraction V ≥ 98% bovine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ACK lysis buffer	Invitrogen GmbH, Darmstadt, Germany
α- MEM,	Invitrogen GmbH, Darmstadt, Germany
Ampicillin	Sigma Aldrich, Taufkrichen, Germany, Catalog No. 21063029
Borgal® 24% ad. us.vet.	MSD, Animal Health GmbH, Germany
Dimethylsulfoxide	SERVA Electrophoresis GmbH, Heidelberg, Germany, Catalog No. 39757.01

Item	Manufacturer
DMEM	Invitrogen GmbH, Darmstadt, Germany
Dulbecco`s PBS (DPBS)	PAA, Cölbe/ Invitrogen GmbH, Darmstadt, Germany
Ethanol, 99.8%	AppliChem, Darmstadt, Germany
Fetal calf serum	PAA, Cölbe / Biochrom, Berlin, Germany
Formalin solution 10%,	Sigma Aldrich, Taufkirchen, Germany
Glutamax	Invitrogen GmbH, Darmstadt, Germany, Catalog No. A1286001
HBSS	Invitrogen GmbH, Darmstadt, Germany,
HEPES	Invitrogen GmbH, Darmstadt, Germany,
Horse serum (HS)	PAA, Cölbe / Biochrom, Berlin, Germany
IMDM	Invitrogen GmbH, Darmstadt, Germany, Catalog No. 31980022 Gibco
Isofluran, Forene® 100%	Abbott GmbH & Co. KG , Taufkirchen, Germany
Isopropanol	Sigma-Aldrich, Taufkirchen, Germany
LB-Agar	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Lipofectamine 2000	Invitrogen GmbH, Darmstadt, Germany
ß-Mercaptoethanol	Invitrogen GmbH, Darmstadt, Germany
mIL3, mIL6, mSCF	R&D Systems, Wiesbaden, Germany
NaCl >99.5% p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Opti-MEM reduced serum medium	Invitrogen GmbH, Darmstadt, Germany, Catalog No.11058021
Penicillin/Streptomycin (Pen/Strep)	Invitrogen GmbH, Darmstadt, Germany

Item	Manufacturer
Peptone	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Polybrene®	Sigma Aldrich, Taufkirchen, Germany
Propidium-Jodid (PI)	Invitrogen GmbH, Darmstadt, Germany
Puromycin	Invitrogen, Darmstadt, Germany
Sterile water 1l	B. Braun Melsungen AG, Melsungen, Germany
Tryptan blue	Invitrogen, Darmstadt, Germany
0.5% Trypsin- EDTA (10x)	Invitrogen GmbH, Darmstadt, Germany
UltraPureDNase/RNase-FreeDistilled water	Invitrogen, Darmstadt, Germany

Table 6: *Chemicals used.*

3.2.4. Antibodies, Drugs, Enzymes and Other Reagents

Antigen	Clone	Fluorochrome	Manufacturer
CD4	GK1.5	PE-Cy5	eBioscience, San Diego, CA, US
CD8a	53-6.7	PE-Cy5	eBioscience, San Diego, CA, US
CD11b	M1/70	APC-eFluor®; 780	eBioscience, San Diego, CA, US
CD127 (IL7R)	A7R34	PE; APC; APC-eFluor® 780	eBioscience, San Diego, CA, US

Antigen	Clone	Fluorochrome	Manufacturer
CD45R (B220)	RA3-6B2	PE-Cy7	eBioscience, San Diego, CA, US
CD34	RAM34	FITC; eFluor®647	ebioscience, San Diego, CA, US
CD117 (KIT)	2B8	PE; APC	ebioscience, San Diego, CA, US
Gr-1(Ly- 6G)	RB6-8C5	eFluor450®	ebioscience, San Diego, CA, US
Sca-1	D7	PE-Cy7	ebioscience, San Diego, CA, US
Biotinylated antimouse Gr-1(Ly-6G)	RB6-8C5		ebioscience, San Diego, CA, US
Biotinylated antimouse B220	RA3-6B2		ebioscience, San Diego, CA, US
Biotinylated antimouse CD3e	145-2C11		ebioscience, San Diego, CA, US
Biotinylated antimouse TER-119	TER-119		ebioscience, San Diego, CA, US
Biotinylated antimouse CD11b	M1/70		ebioscience, San Diego, CA, US

Table 7: *Primary antibodies for flow cytometry.*

Reagent	Conjugate	Manufacturer
Streptavidin	eFluor450®; PE-Cy5.5	Invitrogen, Darmstadt, Germany

Table 8: *Secondary antibodies for flow cytometry.*

Name	Manufacturer
MaxiPrep Kit	Qiagen Inc., Hilden Germany
MiniPrep Kit	Qiagen Inc., Hilden Germany

Table 9: *Kits.*

3.2.5. Buffer, Media and Solutions

Name	Ingredients
FACS buffer	500ml DPBS 0.5% BSA
HF2 buffer:	100ml HBSS (10x) 20ml FCS 10ml HEPES 10ml Penicillin/Streptomycin 860ml H_2O
Phoenix-medium	500ml DMEM 50ml FCS, inactivated
BBMM-medium	325ml IMDM (1x) 150ml FCS 2.5g BSA 5ml L-Glutamin 2.5ml Penicillin/Streptavidine (100x) 1ml ß-Mercaptoethanol (50mM)
Freezing medium	FCS 10% Dimethylsulfoxide (DMSO)

Name	Ingredients
Trypsin solution (1x, 50ml)	5ml 10x Trypsin 45ml DPBS

Table 10: *Home-made buffer media and solutions.*

3.2.6. Cell Lines

Name	Company
***Phoenix™ ecotropic* helper-free retroviral producer cells**	G Nolan, Stanford, US

Table 11: *Cell lines used*

3.2.7. Bacteria

Name	Company
Escherichia coli DH5α	Thermo Fischer Scientific, Germany

Table 12: *Bacteria used.*

3.2.8. Expression Vectors

Name	Company
pMIG-p185 BCR-ABL	Kindly provided by Bubnoff group (Miething et al., 2003b)
pMIG	Kindly provided by Bubnoff group (Miething et al., 2003b)

Table 13: *Expression vectors used.*

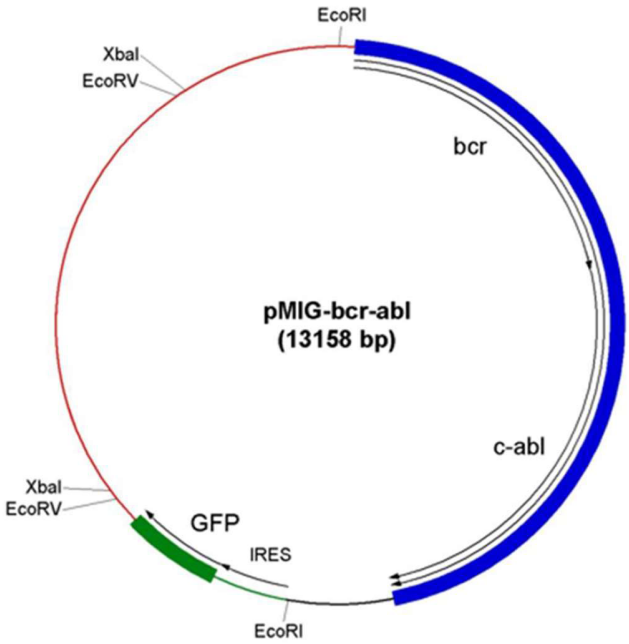

Figure 4: *Vector map of the pMIG-p185 BCR-ABL plasmid.*

The MIG vector is derived from the murine stem cell virus (MSCV). It contains genes encoding the green fluorescent protein (GFP), internal ribosomal entry side (IRES) and the fusion oncogene p185 BCR-ABL (Miething et al., 2003b). In addition to the gene elements shown, the vector also includes the ampicilline resistance gene (AMP). MIG empty vector control contains GFP, AMP and IRES.

3.3. Methods

3.3.1. Producing the BCR-ABL and MIG Vectors

Plasmid DNA was created by transforming competent ampicillin-sensible (Amp) *Escherichia coli bacteria* (*E. coli DH5α*) (Thermo Fischer Scientific, Germany) via heat shock with MIG empty-vector control as well as MIG-p185 e1a2 BCR-ABL. Those include the Amp resistance which is necessary for the selection. Bacteria was plated on an Amp supplemented agar overnight at 37°C. One Amp resistant colony was chosen to perform a MiniPrep following the manufacturer's instructions, culturing the bacteria 16h in liquid LB-medium containing 100µg/ml Ampicillin. Plasmid isolation was performed via MaxiPrep following the manufacturer's instructions (Qiagen Inc, Hilden, Germany).

3.3.2. Isolation of Fetal Liver

For the isolation of FL B6 mice were used. Breeding pairs were set up with one female and one male mouse each. Twice a day (7am and 5pm) the vestibulum vaginae of the female mice was checked for a white substance, which traps the sperm into the uterus, the so-called vaginal plug. As the plug vanishes after about seven hours after copulation this method gives a relatively exact time point for the moment of fertilization. On day 14.5 post coitum, the female mice were checked again for pregnancy via palpation and observation. At this stage of embryogenesis, the concentration of hematopoietic stem cells and the proliferation rate reach their maximum. In general, there were six to nine embryos per pregnant mouse.

First the mother was put into a closed bottle filled with isoflurane gas. After about 45 seconds the animal was under narcosis and was then sacrificed by cervical dislocation. As quickly as possible, the abdomen was opened by cutting along the linea alba with surgical scissors. After shifting the uterus out of the abdomen, it was

removed by cutting in the area of the cervix uteri and was transferred into a bowl filled with DPBS buffer to be cleaned. After the cleaning the organ was put into a petri dish wetted with HF2 buffer. One by one the embryos were separated using a scalpel, and they remained separately in their amniotic sac. The amniotic sac was then opened carefully by using two micro-surgical forceps. After opening the allantois sac the embryo was sacrificed by decapitation with the scalpel. The FL could clearly be identified as a 2x4mm brown object right beneath the surface of the fetal skin in the abdominal area. By twitching the skin with the forceps above the liver the FL was freed and easily pulled out of the embryo. The FL was put immediately into HF2 buffer. After isolating the FLs of all embryos, the FLs were pooled and squeezed through a 70 µm cell strainer and then re-suspended in HF2 buffer before being centrifuged at 500rpm. The last step was to remove the supernatant in order to freeze the cell material with FCS containing 10% DMSO. Cells were stored 24h by -80°C and afterwards in liquid nitrogen.

3.3.3. Culturing of Phoenix™ Helper-Free Retrovirus Producer Cells

T293 is a human embryonic kidney line, transformed with adenovirus E1a and carrying a temperature-sensitive T-antigen co-selected with neomycin. This is the basis for the highly transfectable *Phoenix™ E* cell line, which is created by placing constructs into these T293 cells to help produce gag-pol and envelope (env) proteins for ecotropic viruses (***Figure 5***) *(G. Nolan, Stanford, USA)*.

Before transfection, *Phoenix cells* were cultured (0.5-1x10^6 cells per ml) in a Phoenix medium (DMEM+10%FCS) and selected. The selection was performed with a special selective medium containing DMEM + 10%, FCS + 0.3 mg/ml hygromycin and 2µg/ml diphtheria toxin. Hygromycin is used for the gag-pol selection, whereas the diphtheria toxin causes the selection of the envelope

proteins. To exclude dead cells, the supernatant needs to be removed daily and is exchanged for fresh selective medium. After five to six days selection is completed when no more cells die because of the gag-pol env deficiency (passage zero (p0)). Further, *Phoenix cells* are rescued with Phoenix medium (DMEM + 10% FCS) and expanded to the p5 to p10, as these passages are appropriate for transfection.

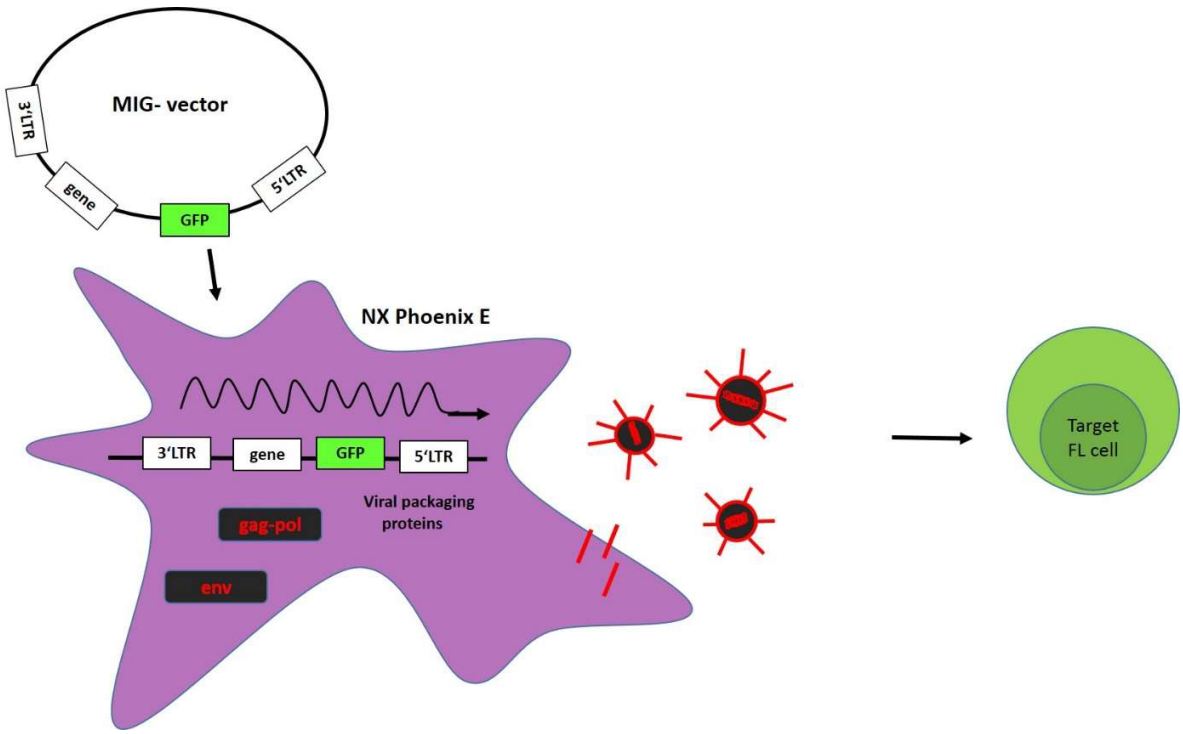

Figure 5: *Infection of BM and FL through retroviral transfected Phoenix E cells.*

Scheme of a Phoenix E cell (G. Nolan, Stanford, US) being transfected with the MIG vector. It contains gag-pol and env protein for production of retroviruses and GFP, which is expressed permanently by the vector. The target cell gets infected by the produced virus.

3.3.4. Transfection of Phoenix Producer Cells and Infection of FL Cells with MIG-p185 BCR-ABL and MIG-Empty-Vector Control

To insert the GFP protein as a marker into the FL cells, the MIG vector system is used. *Phoenix E* cells were transfected with MIG-p185 BCR-ABL or MIG empty-vector control. Prof. Dr. Nikolas von Bubnoff and his workgroup kindly provided these vectors.

A high-quality transfection can only be performed by isolating a large amount of plasmid DNA. For that, competent *Escherichia coli* bacteria are transformed by heat shock with MIG-p185 BCR-ABL vector and MIG-empty-vector control. They also contain an ampicillin resistance gene (Amp) as well as the gene-encoding GFP as already more precisely described in chapter 3.3.1.

Phoenix E ecotropic helper-free retroviral producer cells were transiently transfected with MIG-p185 BCR-ABL and MIG-empty-vector control using Lipofectamine 2000 and OptiMEM, as recommended by the manufacturer.

Thawed or freshly isolated fetal liver cells were re-suspended in BBMM (***Table 10***) containing the mIL-3 10ng/ml, mIL-6 10ng/ml and mSCF 50ng/ml growth factors and apportioned into four 12-well plates, where they were pre-stimulated for 24h at 33°C. Afterward, FL cells were infected with viral supernatants by spin-infection, which was performed four times every 12 hours at 32°C with 2,400 rpm for 90 minutes. Twelve hours after the last spin-infection, cells were harvested, washed with PBS and re-suspended in HF2 buffer. GFP expression of MIG empty vector- and MIG-p185 BCR-ABL vector-infected cells was analyzed via fluorescence-activated cell sorting (FACS) analysis. Then, the appropriate amount of GFP+ cells was calculated and cells were prepared for transplantation.

3.3.5. Transplantation of FL in Neonates

To accomplish the transplantation of FL in 129xB6 newborns (NB) the mothers were separated from their litter for radiation. As it was shown in previous experiments of our workgroup, it is necessary for the dams' acceptance to keep the newborns in their original cages during irradiation, so that their body odor does not change. Pups were irradiated with 4.5Gy and were transplanted immediately. This dose was defined on the basis of Silke Gitzelmann's and Christina Schreck's work, as a radiation of 5.0Gy neonates showed dysfunctions in their development. Silke Gitzelmann used 3.5Gy for B6 mice, but as hybrids of 129xB6 are more robust I decided empirically that 4.5Gy is the optimum for a lethal radiation in hybrid neonates to have a maximum donor cell engraftment (Gitzelmann, 2011; Schreck C. et al., 2014). The injection of infected FL was performed by using a special syringe for small volumes (Hamilton Syringe 100µL). The cell material (400,000 GFP+ FL cells re-suspended in a volume of 20-25µl) was injected into the liver of the mice. This cell number was also the result of the combination of earlier findings in our laboratory and my empirical work. As already known 100,000 GFP+ BM cells engraft a recipient. In our laboratory there were tests that showed it needs at least 4 times more FL cells than BM cells for transplantation to engraft successfully in a recipient. Due to the transparence of the pups' skin, visualizing the liver was very easy. Mice were injected with either MIG empty-vector control FL cells or MIG-p185 BCR-ABL FL cells. To easily separate the two groups afterward, one group was tattooed on the forehand and the other received no tattoo. Dam mice were treated with antibiotics for three weeks with 3% Borgal® (1ml per 250ml of drinking water) to prevent infections of the pups via breast milk during immune recovery.

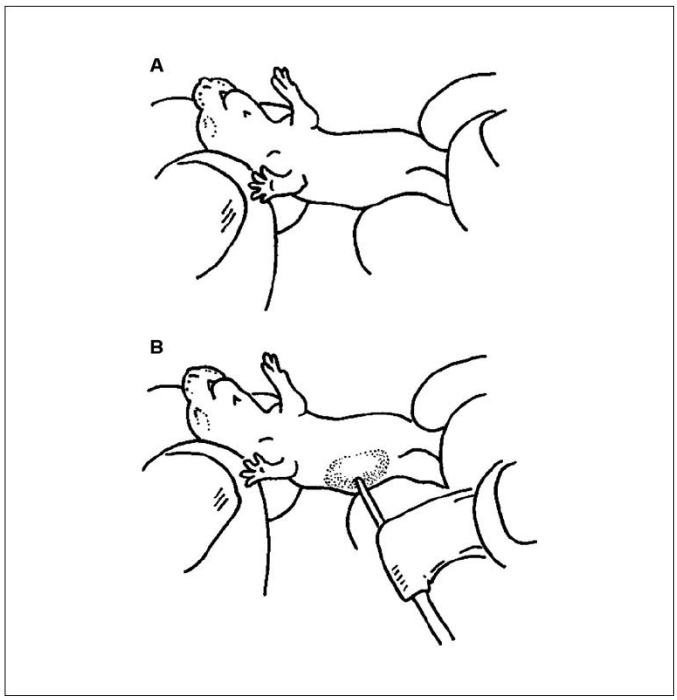

***Figure 6**: Intrahepatic transplantation.*

***(A)** Fixation for intrahepatic injection of p1-4 pup. **(B)** Scheme for placement for intrahepatic injection into a newborn pup (Pearson et al., 2008).*

3.3.6. Transplantation of FL in Young Adults (YA)

Transplantation of FL in adult mice was performed with eight-to-10-week-old 129xB6 mice. Mice were lethally irradiated with 8.5Gy and transplanted afterwards. For that purpose two commercial ashtrays were used, one to put the mouse on, and one positioned on top of the other. Through the ashtrays' notch, the tail could be isolated and then fixed with the left hand. After warming the veins with an infrared lamp and using alcohol for better definition, 1ml syringes were used containing 400,000 GFP+ FL cells. Either MIG-p185 BCR-ABL or MIG empty-vector control FL cells were injected into the lateral tail vein. The two groups, "MIG" and "BCR-ABL," were marked with ear holes. Antibiotic treatment followed for three

weeks through drinking water with 3% Borgal® (1ml per 250ml of drinking water). To ensure the intake, the supplemented drinking water was also poured over the mice's nutrition.

3.3.7. Preparation of Murine Tissues

To analyze cells of the hematopoietic hierarchy, I focused on peripheral blood, bone marrow and the spleen. All mice were sacrificed (as recommended by FELASA) under Isofluran narcosis trough cervical dislocation.

3.3.7.1. Isolation of Bone Marrow

For the isolation of bone marrow (BM) first the fur and the skin of the hind legs were removed with scissors and forceps. Afterward, the articulatio genuae was cut through and proximal to the articulatio tarsi the bone was cracked in order to free the tibia and femur easily from the muscles by only pulling the tibia distal end of the bone outside in a proximal direction. The bone was put immediately into an HF2 buffer to preserve the vitality of the cells. Afterward, the os femoris was freed from the muscles using scissors and then was dislocated from the articulatio coxae. The bone marrow was flushed with HF2 buffer by putting a small syringe into the cavum medullae of the bone. The bone marrow was collected in a tube of HF2 and re-suspended with blunt needles. After centrifuging (500 rpm, 5min, RT) bone marrow cells were prepared for FACS analysis.

3.3.7.2. Isolation of the Spleen

The spleen was isolated by opening the abdomen with a scratch right beneath the sternum. Two relief cuts, one to the right and one to the left, were performed. After illustrating the spleen, which is located on the left abdominal side of the animal, the organ was bluntly dissected with forceps. The spleen was mashed through a 100µm filter and collected in a HF2 buffer. After re-suspension, cells were again filtered through a 30µm filter and then centrifuged. The supernatant was removed and cells were prepared for FACS analysis.

3.3.7.3. Peripheral Blood Preparation

By punctuating the facial vein during the experiments or through direct collection from the heart after death, peripheral blood was obtained for analysis. It was collected in EDTA-coated vials and was analyzed with an animal blood counter. To analyze erythrocytes, blood samples were incubated with 5ml of ACK buffer per sample on ice for 15 min. All samples were centrifuged and the pellets were re-suspended with HF2 buffer.

3.3.8. Secondary Transplantation

To proof the repopulation ability and leukemic potential of the stem cell after primary transplantation (chapter 3.3.5), secondary transplantation was performed. Spleen cells from primary transplanted mice were re-injected in lethally irradiated recipients (Morita et al., 2010; Renstrom et al., 2010). For this experiment eight-week-old B6 mice were used. Three mice were transplanted secondarily, one with $1x10^6$, one with $1.5x10^6$ and one with $2x10^6$ GFP+ SP cells of a primary leukemic NB mouse (chapter 3.3.5). Additionally, because of the lower stability of B6 mice compared with the hybrids from 129xB6, $1x10^5$ BM and $5x10^5$ SP cells were injected. These

helper cells ensure a supply with immunocompetent cells during rehabilitation from the radiation. For the secondary transplantation the same method was used as for the transplantation of adult mice (see chapter 3.3.6) via injection into the tail vein after irradiation with 8.5Gy.

3.3.9. Flow Cytometry Staining

For flow cytometric analysis, cells were stained within a 100µl FACS buffer (***Table 10***) containing the primary antibody mix and incubated 15min at 4°C. After that, samples were washed with 2ml of HF2 buffer and centrifuged. Cells were stained with secondary antibody as necessary, and washed again as described above. Prior to FACS analysis, cells were re-suspended in 500µl of FACS buffer (***Table 10***) containing Propidium Iodide (PI) to distinguish alive and dead cells. Antibodies were used as listed in ***Table 7*** and ***Table 8.*** In general, for the entire BM and SP analysis $2x10^6$ cells were stained for mature hematopoietic populations and $6x10^6$ cells were stained for stem cell staining. Cells were analyzed by a CyAn ADP LxP8 flow cytometer.

3.4. Statistics

As my results showed a non-parametric distribution in the SPSS analysis program, statistical analysis was done with a Mann-Whitney-U (MWU) test for independent samples.

4. Results

Prior to this thesis, experiments with whole bone marrow were performed in young adult (YA) and newborn (NB) mice. My thesis interest was a) the development of a mouse model in NBs using fetal liver and b) how leukemia development in NB recipients would differ from leukemia development in YA recipients. By analyzing a YA population as well, I hoped to find out more about the development of leukemia under two different niche conditions: the NB niche and YA niche. The design of my experiment is shown in ***Figure 7***.

As described in chapter 3.3.1, I used the MIG-p185 BCR-ABL and MIG-empty-vector control to infect *Phoenix Eco* producer cells to form retroviral particles used to transfect E14.5 embryonic FL cells from B6 parings. These steps had to be done several times to obtain a sufficient amount of material for transplantation. During these preparations, 129 mice and B6 were paired for gaining p1 to p4 (129xB6)F1 pups as well as eight-to-10-week-old YA (129xB6)F1 hybrids for the transplantations.

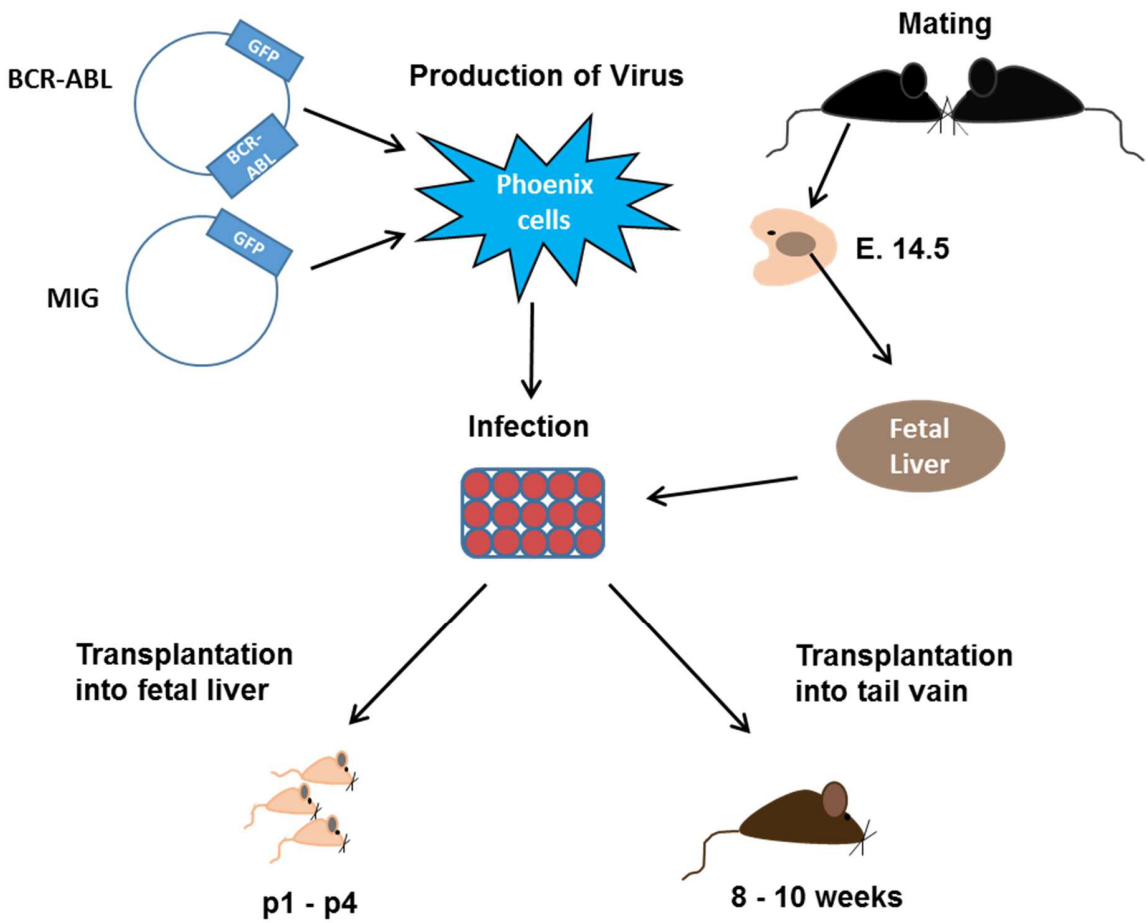

Figure 7: *Scheme of experimental design.*

Scheme of experimental design from creating the vectors until transplantation of NB and YA mice with infected FL cells. Black mice display B6, brown mice (129xB6) F1 and the nude pups result as well from the F1 hybrid of 129 and B6.

4.1. Technical Details

From every single mouse, the peripheral blood (PB), bone marrow (BM) and spleen (SP) were examined using FACS analysis. The hematopoietic tissues were analyzed according to the characteristic surface antigens for mature hematopoietic cells: CD45R (B220) for B-cells, CD4 (helper T-cells) CD8a (cytotoxic T-cells) for

T-cells as well as Ly-6G (Gr-1) und CD11b (Mac-1) for granulocytes and monocytes. To analyze stem and progenitor cells, I gated on lineage negative cells (Lin-). Lin-, Ly-6A/A (Sca-1)+ and c-Kit+ (CD117) expression to define the LSK stem cells, (Ikuta et al., 1992) while myeloid progenitors (MPs) are characterized by the expression of c-Kit but not Sca-1. With IL7R (CD127), I analyzed the emergence of common lymphoid progenitors (CLPs) (Akashi et al., 2000). To recognize the transplanted donor cells I used the MIG-vector marker GFP. FACS plots show a representative percentage of cell populations and their gating. To track the transplanted donor cells, I used the GFP, which is expressed in the MIG backbone vector. FACS plots show a representative percentage of cell populations and their gating.

4.1.1. Survival Analysis

To understand the severity of leukemia development, transplanted mice were observed until their behavioral score (***Table 3***) made immolation necessary. Since every individual mouse succumbs on a different day, analysis of the day of immolation can be analyzed as survival and plotted in survival curves. These curves generate first hints for differences in leukemia development between NB and YA mice.

The different populations can be described in the following manner. NB at p1 – p4 after birth, as well as YA at the age of eight to 10 weeks, were transplanted with an equal number of $4x10^5$ GFP+ cells from p185 BCR-ABL-infected FL cells or MIG-empty-vector-control-infected cells. After transplantation, every mouse had an individual survival time, which is characteristic for a certain leukemic expression. Recipients of MIG empty-vector control transduced cells (called here NB MIG recipients) were analyzed at the same time that the last remaining recipient of p185 BCR-ABL transduced cells-transplanted mice (called here NB BCR-ABL recipients) of the same transplantation day was sacrificed.

For every transplantation experiment being evaluated separately, all NB MIG recipients (pink line ***Figure 8A)*** survived the entire observation period (two 29 days, six 45 days and three NB mice 103 days, n=11), depending on the duration of the individual experiment and the last NB BCR-ABL recipient being sacrificed. All but one of the NB BCR-ABL recipients (blue line ***Figure 8A***) developed leukemia and were sacrificed when the health status necessitated it (according to the criteria described in chapter 3.1.2). The average survival of NB BCR-ABL recipients was 47.4 days (***Figure 8B***). An orange line visualizes the survival of the group of YA mice transplanted with MIG empty-vector-control transduced cells (named YA MIG); the YA recipients transplanted with p185 BCR-ABL transduced FL cells (named YA BCR-ABL) are shown as a green line (***Figure 8A***). The average survival period in the YA BCR-ABL group is 24.3 days (***Figure 8B***). The dot plot in ***Figure 8B*** allows me to retrace the survival time of every single recipient transplanted in the experiments. The increased survival between my new model of NB recipients and the already well-established adult model is highly significant.

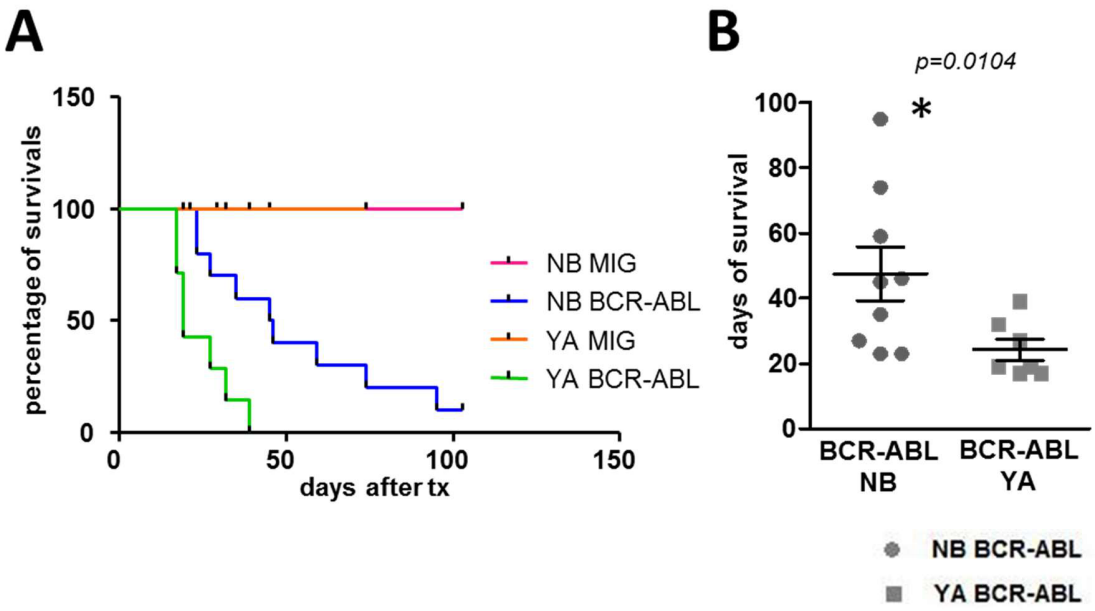

Figure 8: *Survival of NB and YA recipient mice.*

(A) *The Kaplan-Meier Survival curve shows the four different categories of recipients and the length of their survival in days after transplantation. The graph includes 11 NB MIG mice, 10 NB BCR-ABL mice, 11 YA MIG mice and seven YA BCR-ABL mice.* ***(B)*** *Dot plot describing the survival time of every single NB and YA recipient. Because one NB BCR-ABL mouse did not develop leukemia, one spot is missing in the NB column. Grey-filled circles: NB BCR-ABL, grey-filled squares: YA BCR-ABL. Statistics: MWU-test: p= 0.0104, mean= 47.44, SEM= 7.36 (NB BCR-ABL), mean=24.29, SEM= 2.54 (YA BCR-ABL).*

4.2. Total Cell Numbers of Organic Tissues

Besides the analysis of the survival time the most important focus was on the analysis of three tissues: the PB, the SP and BM. Before testing these tissues in more detail, I first compared the total cell number being counted before the preparation of the tissues for the FACS analysis to see if there were any discrepancies or aberrations. Cells were counted in a Neubauer-improved cell

counting chamber. The vitality after defrosting the cells, as well as hyperproliferations of the tissues, could be detected by this.

In PB ***Figure 9A*** shows that NB BCR-ABL recipients have a significantly higher total amount of cells being measured than the NB MIG recipients. In SP and BM, on the other hand, it stays unchanged.

In YA BCR-ABL recipients there is an increase of SP cells compared to the YA MIG recipients. Here PB and BM remain unchanged (***Figure 9B***).

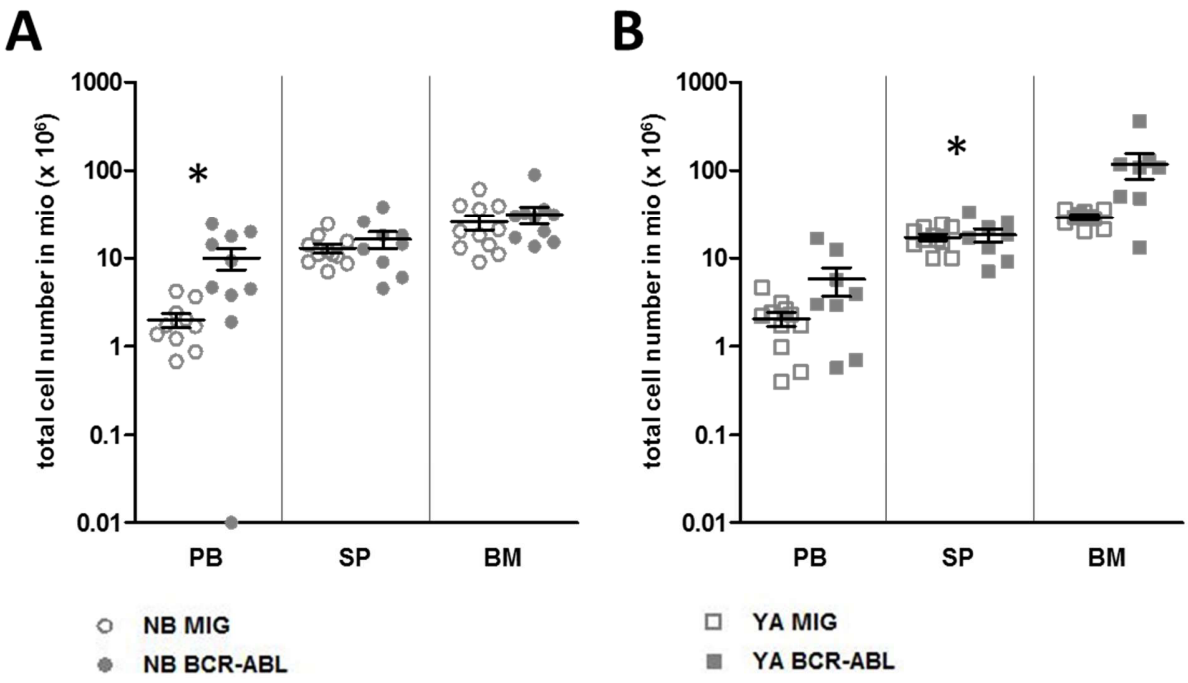

Figure 9: *Total cell numbers of the tissues used for analysis.*

(A) *Dot plot of total cell numbers in analyzed organs of NB recipients. Grey open circles: NB MIG (PB, BM, SP n= 11), grey filled circles: NB BCR-ABL (PB, SP n= 10, BM n=9).* ***(B)*** *Dot plot of total cell numbers in analyzed organs of YAs. Grey open squares: YA MIG (PB, BM n= 11, SP n=10), grey filled squares: YA BCR-ABL (PB, BM, SP n=8). MEAN±SEM, MWU-test: *p≤0.05.*

4.3. FACS Data Analysis

4.3.1. Transduction of GFP+ Cells in NB and YA Recipients

To follow the engraftment of transplanted cells in the recipients, I determined the engraftment of GFP+ cells both *in vivo* (first venipuncture four weeks after transplantation if no visual signs of leukemia had occurred so far) and after devotement via FACS analysis. As shown in ***Figure 10*** the engraftment of MIG transplanted mice after the 120 days observation period was, in general, significantly less than in BCR-ABL transplanted mice at devotement. The results show a mean engraftment of 27.94% in the blood circulation of NB BCR-ABL recipients and even 55.59% in the YA BCR-ABL population. In the BM of YA BCR-ABL recipients the GFP+ cells were able to repopulate up to a value of 69.53% (MWU-test: $p=19x10^{-5}$) and NB BCR-ABL came to a mean of 26,40% (MWU-test: $p=6.4x10^{-5}$), both of which are highly significant. The GFP engraftment in the spleen also showed high significance in both groups; in NB BCR-ABL recipients GFP+ cells averaged in general 22.85% ($p=0.0011$), and those of the YA BCR-ABL recipients 31.56% ($p=0.0032$).

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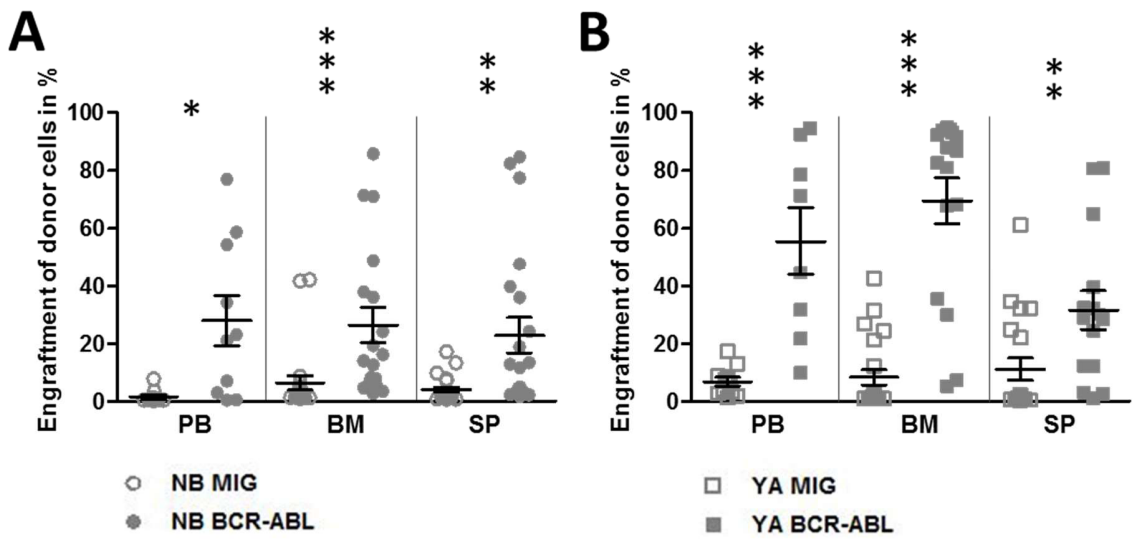

Figure 10: *Engraftment of donor cells.*

(A) *Dot plot of engraftment GFP+ cells in tissues of NB recipients. Grey open circles: MIG NB (PB n= 11, BM n= 22, SP n=22), grey filled circles: BCR-ABL NB (PB n= 10, BM n=18, SP n=21).* ***(B)*** *Dot plot of engraftment of GFP+ cells in tissues of YA recipients. Grey open squares: MIG YA (PB n= 11, BM n=22, SP n=20), grey filled squares: YA BCR-ABL (PB n=8, BM n=16, SP n=15). All results are shown in percentage MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005, ***p≤0.0005.*

4.3.2. Analysis of the Peripheral Blood

Peripheral blood of BCR-ABL recipients and MIG recipients was analyzed with an animal blood counter. I focused on the parameters determined by this equipment: white blood cells (WBC), lymphocytes (Lympho), monocytes (Mono), granulocytes (Gran), red blood cells (RBC) as well as platelets (PLT). The four experimental groups were analyzed in this manner *(**Table 14***). While the MIG population in both groups (NB and YA) was very similar throughout, differences can be seen as well in the NB BCR-ABL recipients and the YA BCR-ABL recipients. NB BCR-ABL recipients showed a highly significant alteration in four cell populations (***Figure***

11A) WBC, lymphocytes, granulocytes and RBC while in the YA BCR-ABL recipients only in lymphocytes, monocytes and RBCs show a comparably slight but significant difference compared with YA MIG recipients (***Figure 11B***).

	NB MIG	NB BCR-ABL	YA MIG	YA BCR-ABL
WBC (10^3/µl)	1.97 ± 0.33	10.99 ± 2.43	2.05 ± 0.36	3.85 ± 1.36
Lymph(10^3/µl)	1.32 ± 0.29	6.80 ± 1.54	1.27 ± 0.28	3.45 ± 1.06
Mono (10^3/µl)	0.05 ± 0.02	0.67 ± 0.19	0.06 ± 0.02	0.20 ± 0.05
Gran (10^3/µl)	0.45 ± 0.09	3.51 ± 1.01	0.54 ± 0.14	1.32 ± 0.50
RBC (10^6/µl)	5.63 ± 0.99	3.44 ± 0.78	5.72 ± 0.92	2.52 ± 0.42
PLT (10^3/µl)	489.73 ± 86.07	504.10 ± 175.30	446.55 ± 113.20	117.00 ± 30.63

Table 14: *Blood counter data.*

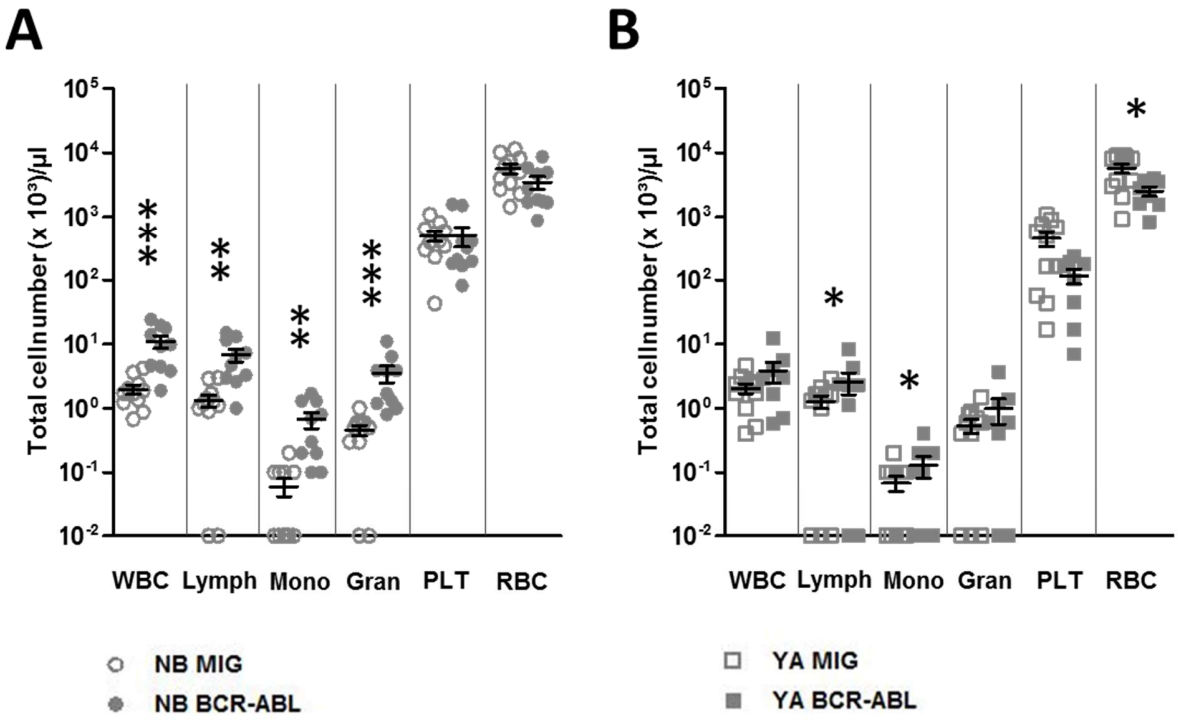

***Figure 11**: Dot plot of data obtained with an animal blood counter*

***(A)** Total cell number of white blood cells (WBC), lymphocytes (Lymph), monocytes (Mono), granulocytes (Gran), platelets (PLT) and red blood cells (RBC) in NB MIG (grey open circles; n=11) and NB BCR-ABL (grey filled circles; n=10) mice. **(B)** Total cell number of white blood cells (WBC), lymphocytes (Lymph), monocytes (Mono), granulocytes (Gran), platelets (PLT) and Red blood cells (RBC) in YA MIG (grey open squares; n=12) and YA BCR-ABL (grey filled squares; n=9) recipients. MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005, ***p≤0.0005.*

To find out more about leukemic development, and the possible mature lineages altered by transfection with p185 BCR-ABL, I also focused on the engraftment of GFP+ cells on cell differentiation such as B-cells (B220+), T-cells (CD4+/CD8a+) and, in the non-lymphoid fraction, the granulocytes (Gr1-hi CD11b-hi) and monocytes (Gr1-med, CD11b-hi). In the FACS analysis of the blood cells shown in ***Figure 12A*** one can clearly see that the NB BCR-ABL recipients developed an increase in lymphoid cells. The figure expresses the generally high increase of

these cell types, B220+ as well as CD4/8a+. As the gating in ***Figure 12A*** and the dot plot in ***Figure 12B*** show, a significant alteration of Gr1-med CD11b-hi can also be detected in NB BCR-ABL recipients.

In the experiments with YA MIG and BCR-ABL recipients (***Figure 12C and D***), cells engrafted very well but significant alterations in the FACS analysis of the PB besides the highly increased GFP+ cells ins YA BCR-ABL can only be seen in B220+ cells, as they show a significant increase as well.

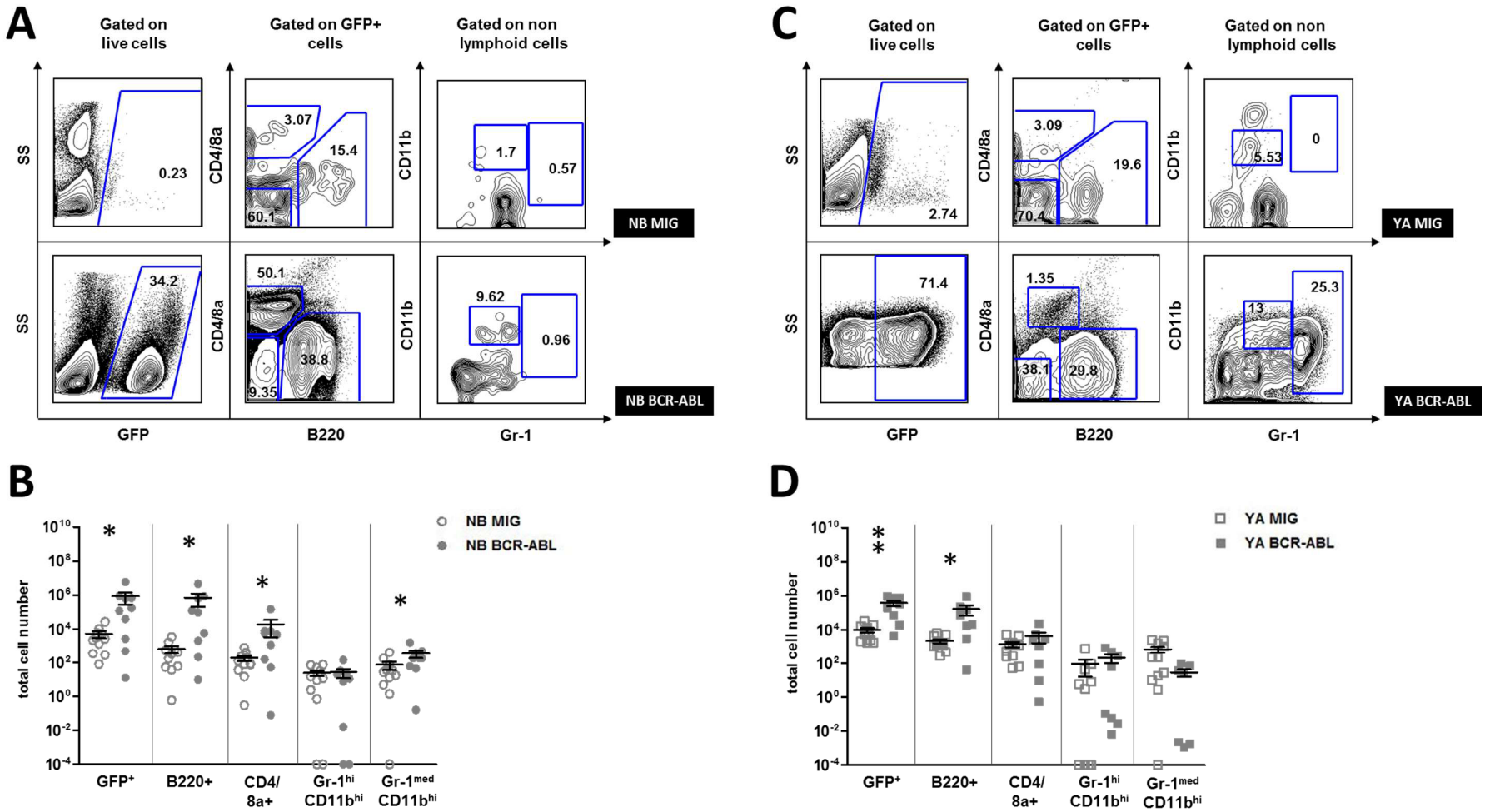

Figure 12: *Gating strategy and analysis of mature blood cells*

(A) *Gating strategy with representative FACS plots, percentage values. First row = NB MIG, second row = NB BCR-ABL* ***(B)*** *Total cell number of GFP+, B220+, CD4+/CD8a+cells, Gr1-hi CD11b+hi and Gr1-med CD11b+hi cells. Grey open circles: NB MIG (n=11), grey filled circles: NB BCR-ABL (n=10).* ***(C)*** *Gating strategy with representative FACS plots, percentage values. First row = YA MIG, second row = YA BCR-ABL.* ***(D)*** *Total cell number of GFP+, B220+, CD4+/CD8a+cells, Gr1-hi CD11b-hi and Gr1-med CD11b-hi cells. Grey open squares: YA MIG (n=11), grey filled squares: YA BCR-ABL recipients (n=8). MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005.*

4.3.3. Analysis of the Spleen

4.3.3.1. Anatomic-Pathological Observation

The spleen is a hematopoietic organ, and it is involved in the extra medullary leukopoiesis observed in many patients with leukemia, including BCR-ABL+ chronic myeloid leukemia. Thus, quantifying extra medullary hematopoiesis gave me a number of possibilities for analysis and the study of leukemia development. First, the spleen size and weights of each animal were documented at immolation. As shown in ***Figure 13A*** the spleen weight shows obvious differences, depending on the experimental group. ***Figure 13B*** emphasizes clearly the splenomegaly of NB and YA BCR-ABL recipients. While the NB and YA MIG recipients have measurements of 1.35 cm each, YA BCR-ABL spleens measure 2.35 cm and NB BCR-ABL spleens have an even larger size, with a mean of 2.5 cm.

Equally interesting is the result of weighing these organs. Both NB BCR-ABL and YA BCR-ABL recipients show highly significantly increased spleen weights in comparison with the MIG vector-control populations. In NB BCR-ABL recipients the mean weight of the tissue was with 0.299g, about 3.6-fold higher than for NB MIG recipients, which had a mean of 0.082g. In YA BCR-ABL recipients, the increase of weight was with a mean of 0.294g, about 2.8-fold higher than the spleen weights of the YA MIG recipients with a mean of 0.107g.

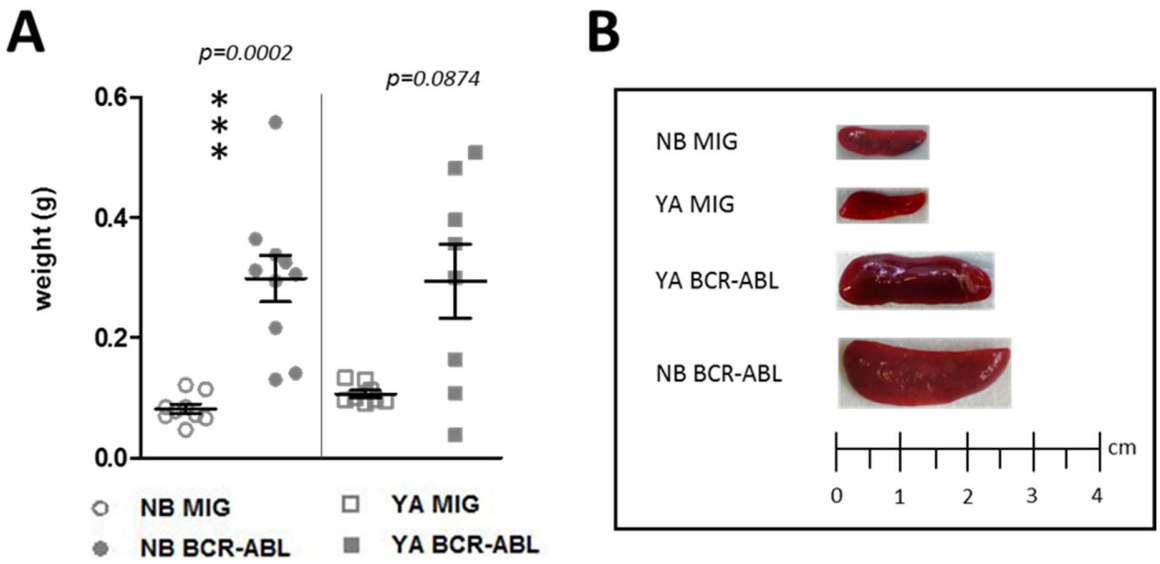

***Figure 13**: Comparison of SP in NB and YA mice.*

***(A)** Total spleen weight (g); grey open circles = NB MIG (n= 9), grey filled circles = NB BCR-ABL (n= 10), grey open squares = YA MIG (n= 8) and grey filled squares = YA BCR-ABL (n=7). MEAN±SEM, MWU-test ***p≤0.0005. **(B)** Representative pictures of spleens.*

4.3.3.2. FACS Analysis of SP Mature Cells

After the size and weight measurements were taken, the spleens were prepared for FACS analysis.

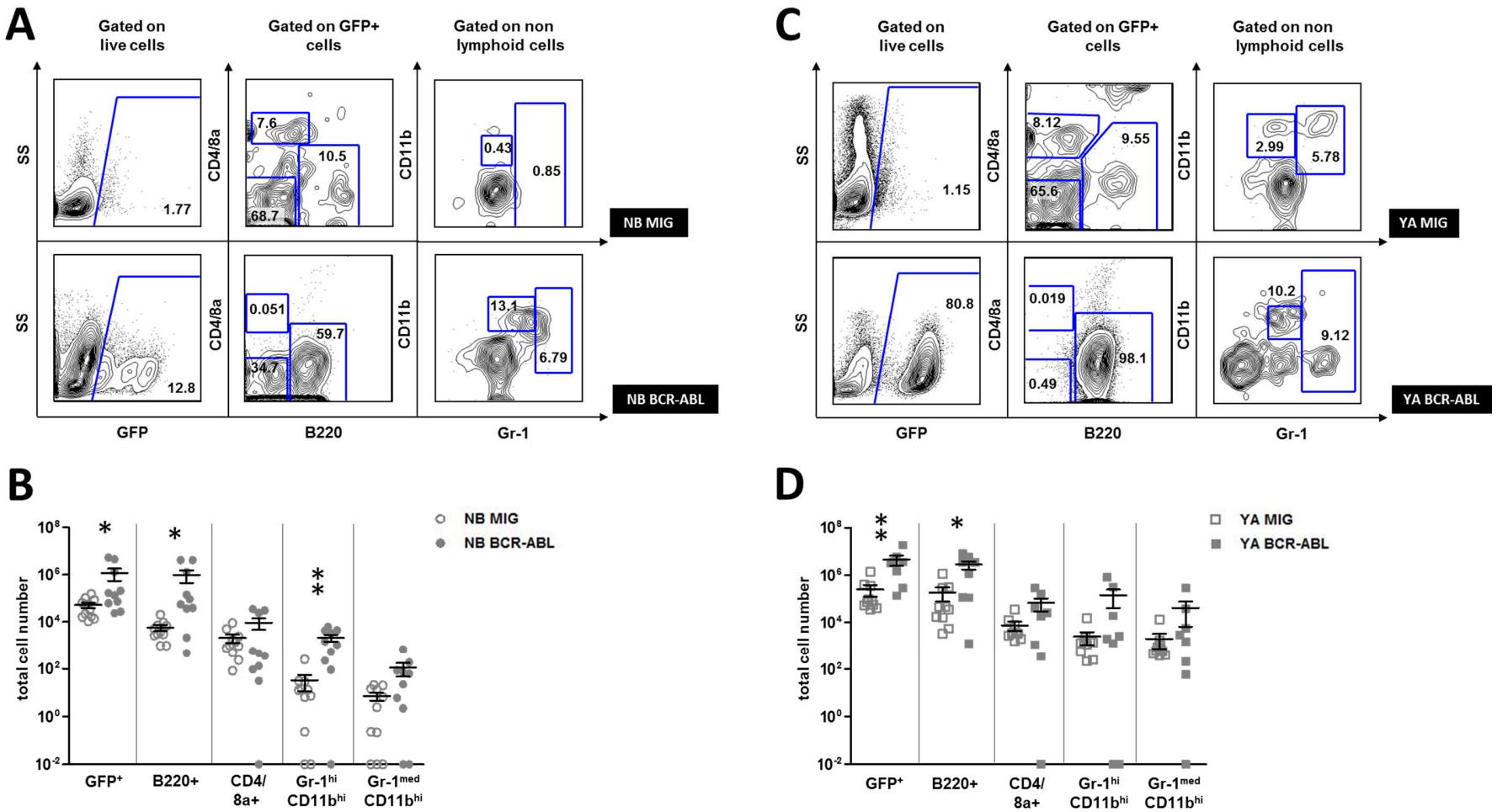

Figure 14: *Gating strategy and analysis of mature spleen cells.*

(A) *Gating strategy with representative FACS dot plots, percentage values. First row = NB MIG, second row= NB BCR-ABL* ***(B)*** *Total cell number of GFP+, B220+, CD4/8a+, Gr-1-hi CD11b-hi and Gr-1-med CD11b-hi cells calculated from spleen cell number and percentages of live cells from FACS analysis. Grey open circles = NB MIG (n=11), grey filled circles = NB BCR-ABL mice (n=10).* ***(C)*** *Gating strategy with representative FACS dot plots, percentage values. First row = YA MIG, second row= YA BCR-ABL.* ***(D)*** *Total cell number of GFP+, B220+, CD4/8a+, Gr-1-hi CD11b-hi and Gr-1-med CD11b-hi cells. Grey open squares = YA MIG (n=10), grey filled squares = YA BCR-ABL mice (n=8). Comparisons of MIG and BCR-ABL mice: MEAN±SEM, MWU-test:*p≤0.05, **p≤0.005.*

So that further insights could be gained about the development of leukemia in the recipients of the four experimental groups, FACS analysis was performed. The gating strategy can be seen in ***Figure 14A*** and ***C.*** In the NB recipients, it can be seen that engraftment of GFP+ cells is significantly higher in NB BCR-ABL recipients than in the NB MIG recipients. Whereas another increase in B220+ cells is visible, CD4/8a+ cells stay unchanged. Interestingly, the amount of Gr1-hi/CD11b-hi cells rises significantly in SP, whereas in PB their cell numbers did not change compared with NB MIG recipients. The Gr1-med/CD11b-hi granulocytic cell fraction is not affected by the expression of BCR-ABL (***Figure 14B***). This result also differs from the result of the PB analysis (***Figure 12B***).

In the experimental group including YA MIG and YA BCR-ABL recipients, the high engraftment of GFP+ cells is clear (***Figure 14D***). Of the engrafted cells, the only cell population that significantly increased is the B220+ cell population. Despite the increase in spleen size and weight, the calculated total numbers of the CD4/8a+, Gr1-med/ CD11b-hi, and Gr1-hi/CD11b-hi populations are similar with respect to the YA MIG recipients that, in essence, correspond to the results of the PB analysis.

4.3.3.3. FACS Analysis of SP Stem Cells

BCR-ABL+ leukemia is transmitted by so-called leukemic stem cells (LSCs), with the lineage- Sca-1+ Kit+ surface phenotype (Schemionek et al., 2010). To determine the presence of such cells in the extra-medullar spleen, in this part of my thesis work I focused on stem and progenitor cells (***Figure 15***). HSCs do not express lineage markers such as CD3, B220, Gr-1 and CD11b (Lin-). LSK cells are defined as Lin- but express the surface antigens Sca-1+ and c-Kit+. In the hematopoietic hierarchy, these LSKs can develop into MPs (myeloid progenitor cells), which have lost the expression of Sca-1. In addition to these two cell populations, I also focused on the IL7R+ and Lin- CLP cells (common lymphoid

progenitor cells). Together, the MP and CLP populations may give information about the myeloid or lymphoid nature of the BCR-ABL+ leukemic cells.

The strategy showing the different populations is shown in ***Figure 15A***. In the population of NB MIG and NB BCR-ABL recipients (***Figure 15B***), as also shown in the analyses above, a rise of GFP+ cells can be detected in the NB BCR-ABL recipients. Lin- cells stayed inconspicuous in the NB BCR-ABL recipients compared with the NB MIG recipients, as did the LSK population, which includes the stem cells. Interestingly, both the CLP and the MP populations show a highly significant increase compared with the corresponding MIG control recipients (***Figure 15B***).

Figure 15C shows the gating strategy for the stem cell analysis of spleen cells for YA recipients in FACS analysis. The dot plot in ***Figure 15D*** reveals that no significant changes in the development of the cell populations in YA BCR-ABL recipients can be observed, compared with the YA MIG recipients. In general, only a few GFP+ LSK cells either were detected both in YA MIG and in YA BCR-ABL recipients.

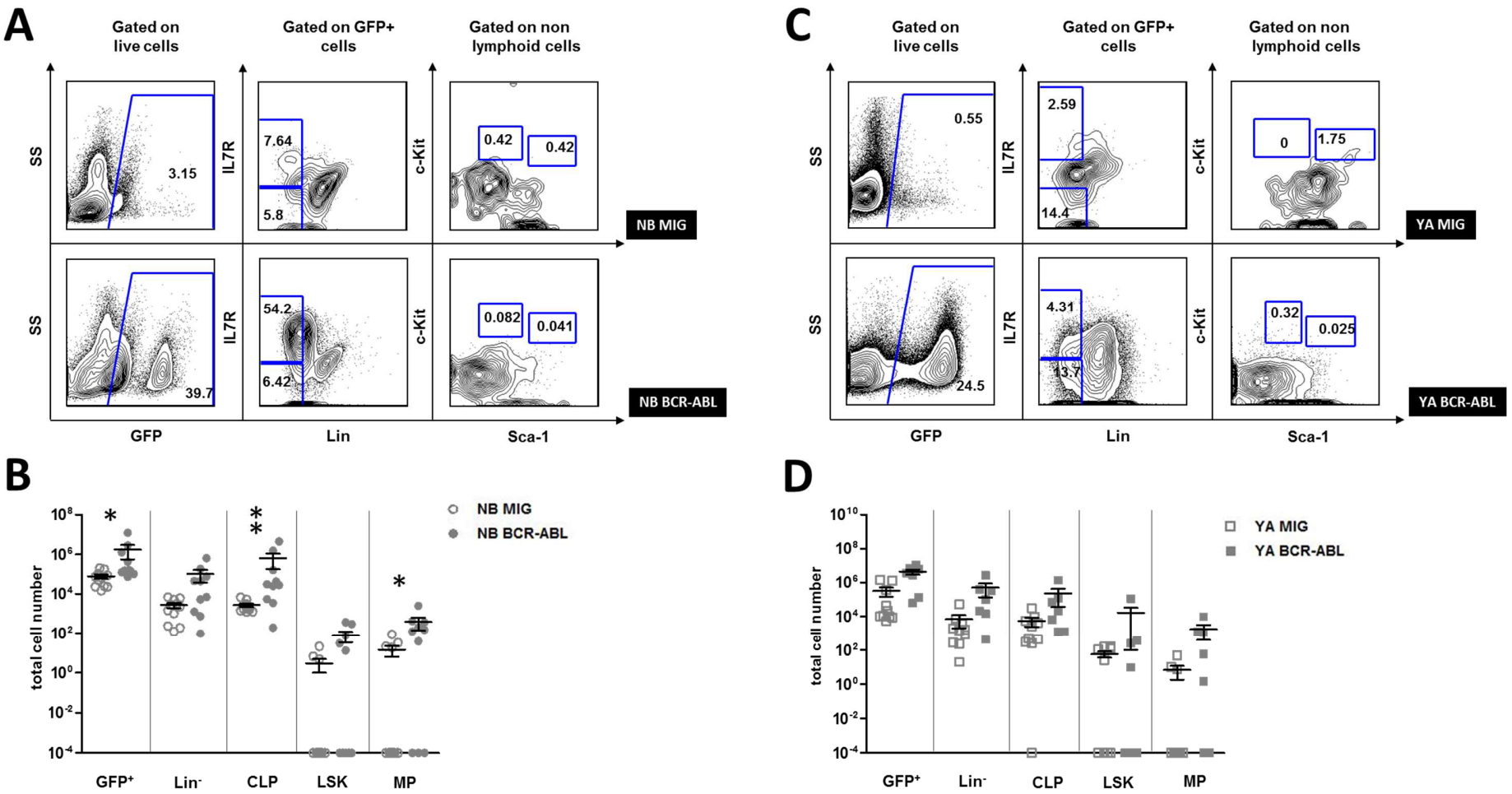

Figure 15: *Gating strategy and analysis of spleen stem cells.*

(A) *Gating strategy of HSCs and progenitor cells in FACS analysis and representative FACS plots, percentage values. First row = NB MIG, second row = NB BCR-ABL.* ***(B)*** *Dot plots of total cell numbers of NB GFP+ cells, Lin-neg, CLP, LSK and MP cells. Grey open circles = NB MIG (n=11), grey filled circles NB BCR-ABL (n=10). MEAN±SEM, MWU-test: *p≤0.05, **p≤0.05.* ***(C)*** *Gating strategy of HSCs and progenitor cells in FACS analysis and representative FACS plots, percentage values. First row = YA MIG, second row = YA BCR-ABL.* ***(D)*** *Dot plots of total cell numbers of YA GFP+ cells, Lin-neg, CLP, LSK and MP cells. Grey open squares = YA MIG (n=10), grey filled squares = YA BCR-ABL mice (n=7) MEAN±SEM.*

4.3.4. Analysis of the Bone Marrow

4.3.4.1. FACS Analysis of BM Mature Cells.

To complete the FACS analysis of different hematopoietic tissues, bone marrow samples were also studied. Representative dot plots with a gating strategy are shown in ***Figure 16A***. In line with my results in the PB and SP analysis, GFP+ cells have again significantly increased in the BM of NB and YA BCR-ABL recipients. Additionally, the B220+ cell fraction is increased significantly in NB BCR-ABL recipients as well, up to over 600 times as high as the mean of NB MIG recipients. I asserted the other lymphoid compartment, the CD4/8a+ cells rising to a multiplicator of 368 compared with the NB MIG recipients. On the contrary, no changes can be seen in Gr1-hi/CD11b-hi and Gr1-med/CD11b-hi cells. These results can be seen in ***Figure 16B***.

The logarithmic scale in ***Figure 16D*** shifts in YA BM mature cells between the YA MIG recipients and the YA BCR-ABL recipients. As is also visible in ***Figure 10***, the engraftment of GFP+ in YA BCR-ABL recipients is clearly higher. Interestingly, in the YA experimental groups, no changes in the lymphoid compartment, B220+ and CD4/8a+ cells in between the YA BCR-ABL and the YA MIG recipients could be detected, but there was a significant alteration in Gr1-hi/CD11b-hi cells. Here, YA BCR-ABL recipients show a population 37 times as high as in YA MIG recipients. However, no changes are seen for the Gr1-med/CD11b-hi monocyte population. The gating strategy for FACS analysis in YA mature BM cells is shown in ***Figure 16C***.

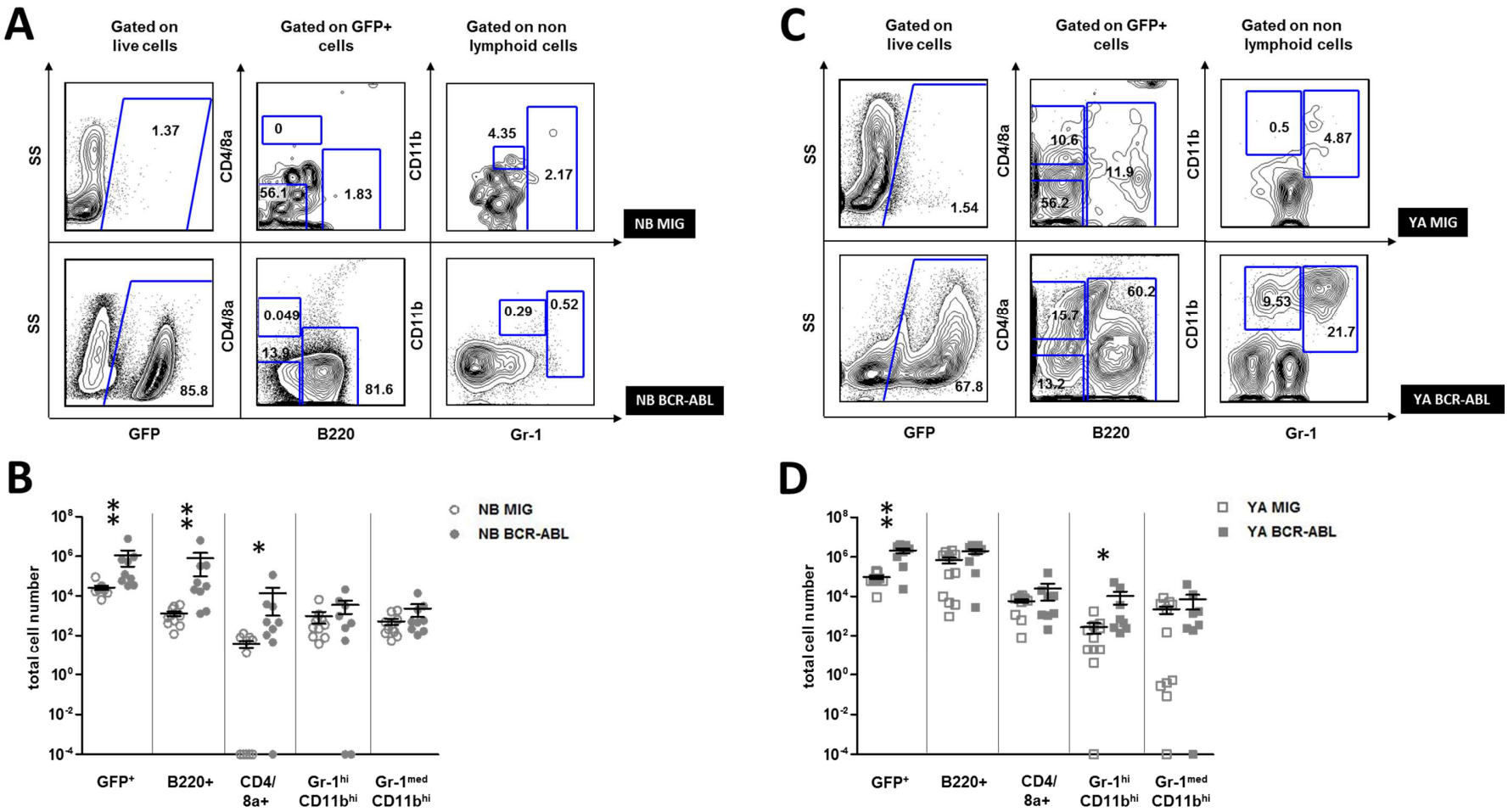

Figure 16: *Gating strategy and analysis of mature bone marrow cells.*

(A) *Gating strategy in FACS analysis and representative FACS plots, percentage value. First row = NB MIG, second row= NB BCR-ABL* ***(B)*** *Total cell number of GFP+, B220+, CD4/8a+, Gr1-hi/CD11b-hi and Gr1-med/CD11b-hi cells. Grey open circles = NB MIG (n=11), grey filled circles = NB BCR-ABL (n=9). MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005.* ***(C)*** *Gating strategy in FACS analysis and representative FACS plots, percentage values. First row = YA MIG, second row= YA BCR-ABL.* ***(D)*** *Total cell number of GFP+, B220+, CD4/8a+, Gr1-hi/CD11b-hi and Gr1-med/CD11b-hi cells. Grey open squares = YA MIG (n=11), grey filled squares = YA BCR-ABL (n=8). MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005.*

4.3.4.2. FACS Analysis of BM Stem Cells.

As I studied SP cells to determine whether I could detect LSC populations, I also analyzed LSKs and the progenitor populations in BM. ***Figure 17A*** shows the gating strategy for stem cells in flow cytometry in the NB experimental group, whereas ***Figure 17B*** displays the dot plots of the total cell number results. A mean of 70.6% of GFP+ cells shows the significantly higher engraftment in this tissue for NB BCR-ABL compared with 1.5% GFP+ cell engraftment in the NB MIG recipients. In line with the results from the SP (***Figure 15A and B***), I also found an increase of GFP+ Lin- IL7R+ CLP cells. The CLP increased from a mean of 6% of the GFP+ cells in NB MIG recipients to a mean of over 25% of GFP+ cells in NB BCR-ABL recipients. In the other HSC and progenitor cell compartments such as LSKs, Lin-neg cells and MPs, I did not detect large diversions from the number of cells in NB MIG recipients.

A closer look at these results (***Figure 17C***) reveals a remarkable increase of Lin-neg cells in the YA BCR-ABL recipients, perhaps reflecting a decreased differentiation towards fully mature cell types. The Lin- cell population rises to a mean of 8.2% of GFP+ cells in the YA BCR-ABL recipients, whereas in the YA MIG BM only 0.61% of GFP+ Lin- cells can be detected. However, CLPs and LSKs do not show marked alterations in cell numbers. In the MP population, however, the FACS analysis, as shown in ***Figure 17D***, shows an interesting decrease compared with the YA MIG recipients. This result was unexpected, considering the increase of cell number in spleens of YA BCR-ABL recipients (***Figure 15D***).

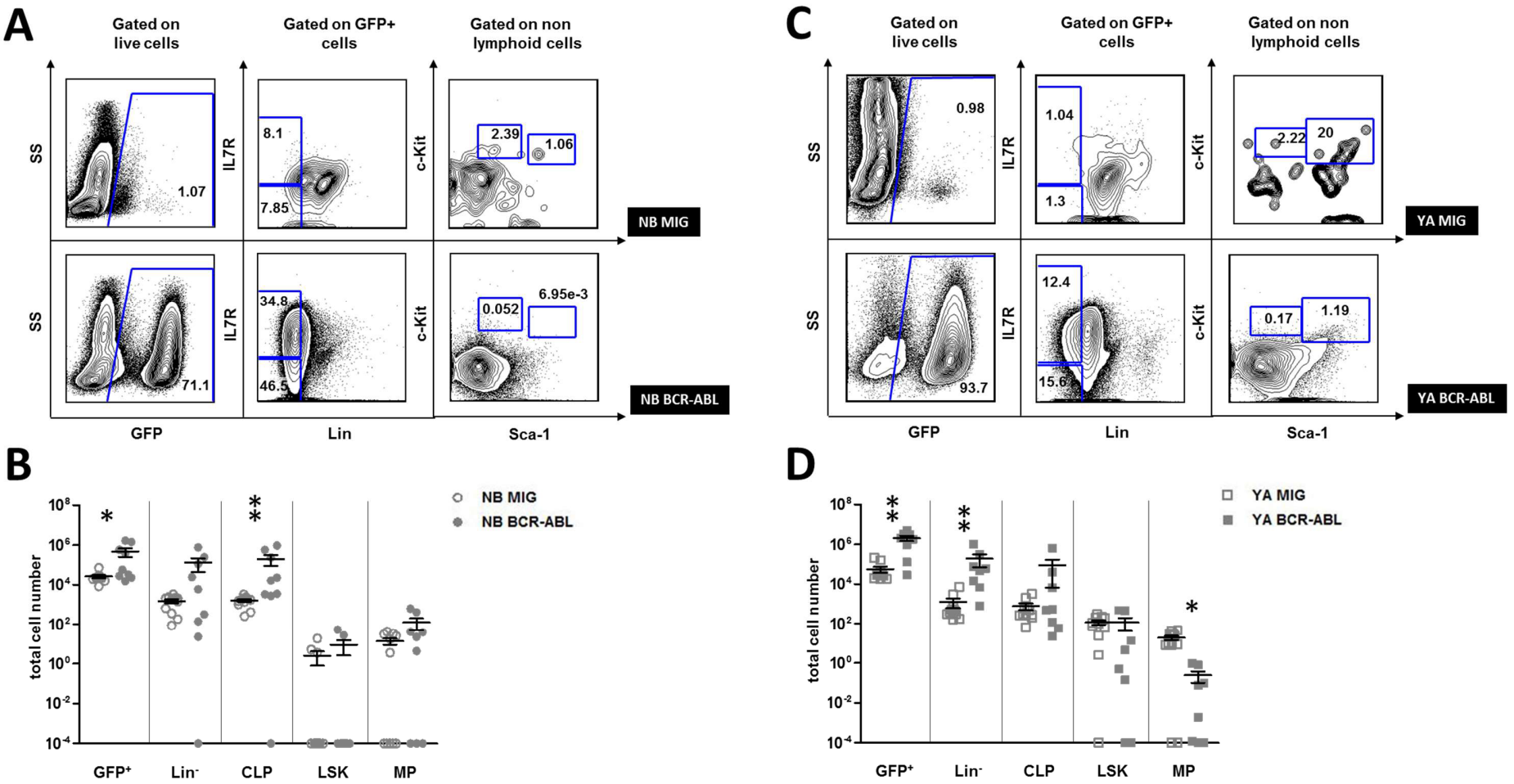

Figure 17: *Gating strategy and analysis of BM stem cells.*

(A) *Gating strategy of HSCs and progenitor cells in FACS analysis and representative FACS plots, percentage values. First row = NB MIG, second row= NB BCR-ABL.* ***(B)*** *Dot plots of total cell numbers of NB GFP+ cells, Lin-neg, CLP, LSK and MP cells. Grey open circles = NB MIG (n=11), grey filled circles NB BCR-ABL (n=9). MEAN±SEM, MWU-test: *p≤0.05, **p≤0.05.* ***(C)*** *Gating strategy of HSCs and progenitor cells in FACS analysis and representative FACS plots, percentage values. First row = YA MIG, second row = YA BCR-ABL.* ***(D)*** *Dot plots of total cell numbers of YA GFP+ cells, Lin-neg, CLP, LSK and MP cells. Grey open squares = YA MIG (n=11), grey filled squares = YA BCR-ABL (n=8) MEAN±SEM, MWU-test: *p≤0.05, **p≤0.005.*

4.3.5. Secondary Transplantation

One of the distinguishing characteristics of stem cells is their ability to self-renew, which is to produce more stem cells after cell division. As the engraftment of transplanted cells in primary recipient mice does not give definitive information about self-renewal but only a form of lympho-myeloproliferative disorder (LMPD), it is necessary to determine the presence of repopulating cells in secondary recipients. In the BCR-ABL model, LMPD cells from primary recipients were isolated after immolation and re-transplanted into lethally irradiated mice to find out whether a secondary leukemia would develop and thus show whether GFP+ CD 34- LSKs (leukemic stem cells (LSC)) were present in the transplants (Morita et al., 2010).

Firstly, I established a pilot experiment for secondary transplantation to determine the amount of GFP+ cells that would successfully engraft and perhaps develop a secondary leukemia. In YA BCR-ABL recipients it had been described that $0{,}8x10^6$ GFP+ SP cells ($3x10^6$ GFP+ cells into four mice) should suffice for the development of secondary leukemia (Miething et al., 2006). Thus, using this value as a starting point, I transplanted three eight-week-old B6 secondary recipients with escalating GFP+ cell numbers ($1x10^6$, $1.5\ x10^6$, and $2x10^6$cells). Additionally, every animal was injected with $5x10^5$ spleen helper cells for radioprotection after the 9.0 Gy lethal radiation. After 20 days, all three secondary recipients showed exactly the same symptoms (arched back, slightly swollen face, slow motion and slight signs of anemia) (criteria described in ***Table 2***) as signs of a rapidly developing leukemia.

The secondary recipients were sacrificed and analyzed as the primary NB recipients. Interestingly, in spite of the overt leukemia in the primary recipient mice, the autopsy of the secondary transplanted recipients did not show signs of splenomegaly but still had very similar weights to those of the NB BCR-ABL transplanted mice. Whereas the mean of NB transplanted BCR-ABL mice is a weight of 0.299g (***Figure 13A***), all three spleens from the secondary recipients

were very similar and showed a mean weight of 0.282g. To obtain a more detailed impression of the developing secondary leukemia, a detailed examination was performed using flow cytometry (***Figure 18*** and ***Figure 19***).

In ***Figure 18A***, the analysis of SP from NB BCR-ABL #1 (Tx 17.12.12), which served as the donor, is shown. Upon examination of the BM of the secondary recipients, there was evidence of a mean engraftment of 73.2% GFP+ cells. In mouse models, two types of malignant diseases can be distinguished: lympho-myeloproliferative disorders (LMPD), which do not form secondary leukemia, and true leukemia, which can be transplanted into secondary recipients. As different fusion oncogenes give rise to either LMPD or leukemia, an important part of disease classification is to find out whether the primary transplantation gives rise to LMPD or leukemia. It is generally believed that secondary leukemia is initiated by LSC with self-renewal properties. Since all three mice succumbed to overt B-cell leukemia, the evidence suggests that LSC were present in the transplanted primary SP cells.

Comparison of the donor cells with the secondary leukemic cells shows (***Figure 18B***) that the donor NB BCR-ABL recipient contained nearly 70% of B-cells and 27% non-lymphoid cells and almost no (0.43%) CD4/8a+ T-cells. Indeed, the secondary leukemia was also dominated by 85.5 – 98.7% of B220+ cells and only 0.13 – 0.21 % of CD4/8a+ cells. One major difference was that Gr1-hi/CD11b-hi and Gr1-med/CD11b-hi cells were very low (***Figure 18B***), suggesting that myeloid and lymphoid disease were perhaps initiated by different cells, of which only the lymphoid cells showed self-renewal.

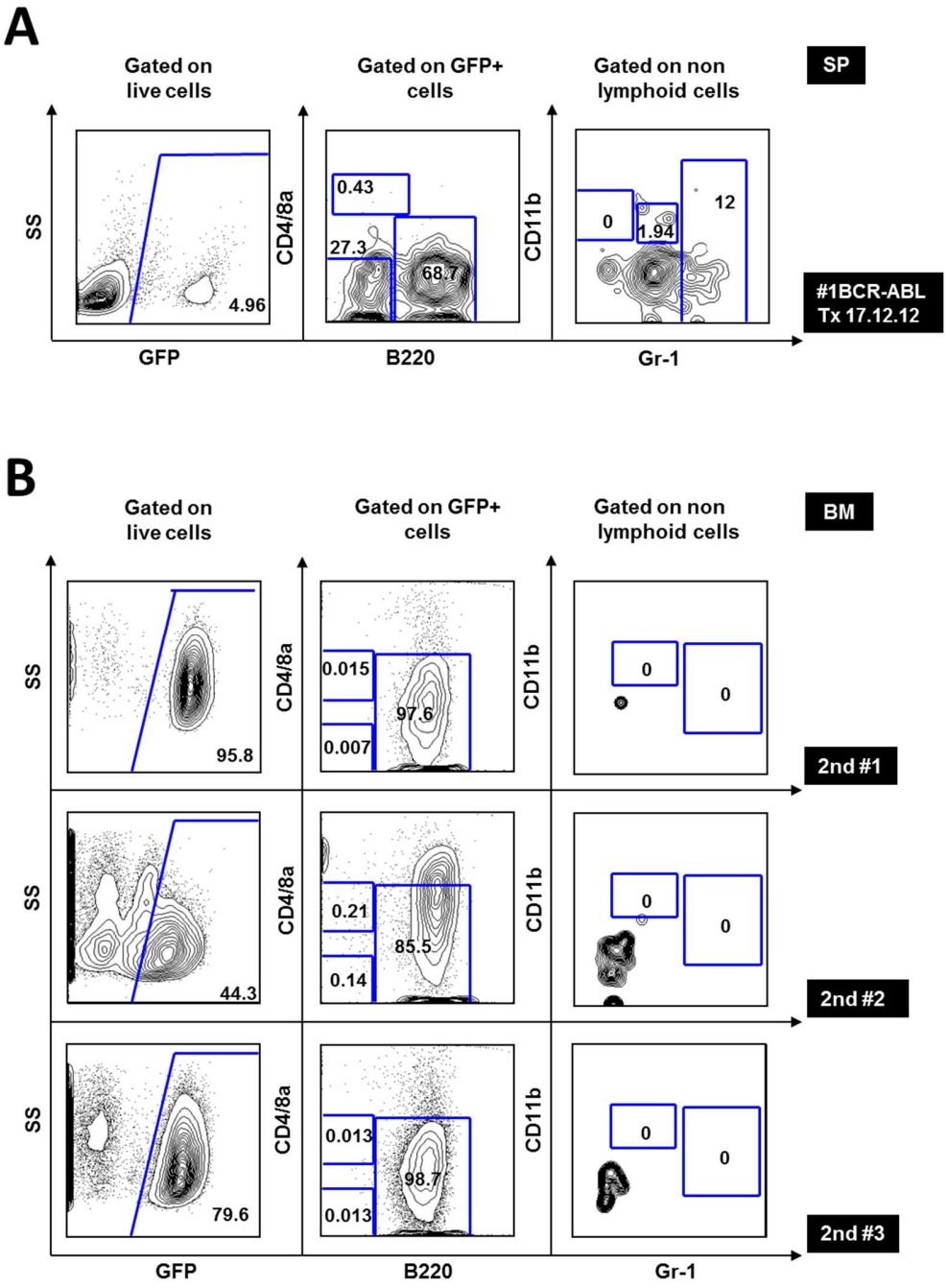

Figure 18: *SP analysis of donor and mature cell analysis of BM in secondary transplanted mice.*

(A) *Gating of spleen donor cells for secondary transplantation usage.* ***(B)*** *Gating of BM of secondary transplanted mice in FACS analysis and representative FACS plots, percentage values. First row = mouse #1, middle row = mouse #2, lower row = mouse #3.*

Furthermore, a closer examination of the outcomes of the BM FACS analysis allows me a first step in medical diagnostics of the type of developed leukemia in NB and YA recipients. Another piece of that information comes from closer examination of the peripheral blood. In ***Figure 19***, an increase of GFP+ cells up to a mean of 87.8% of the PB is visible, confirming engraftment of the donor cells in the blood system. The prominence of the B220+ cells can also clearly be seen, both in the FACS plots and the summarizing dot plot of the data (***Figure 19A and B***). Despite the lack of involved myeloid cells in the GFP+ compartment in the BM, both Gr1-med/CD11b-hi and Gr1-hi/CD11b-hi GFP+ cells are readily detectable in the blood system of the secondary transplanted recipients.

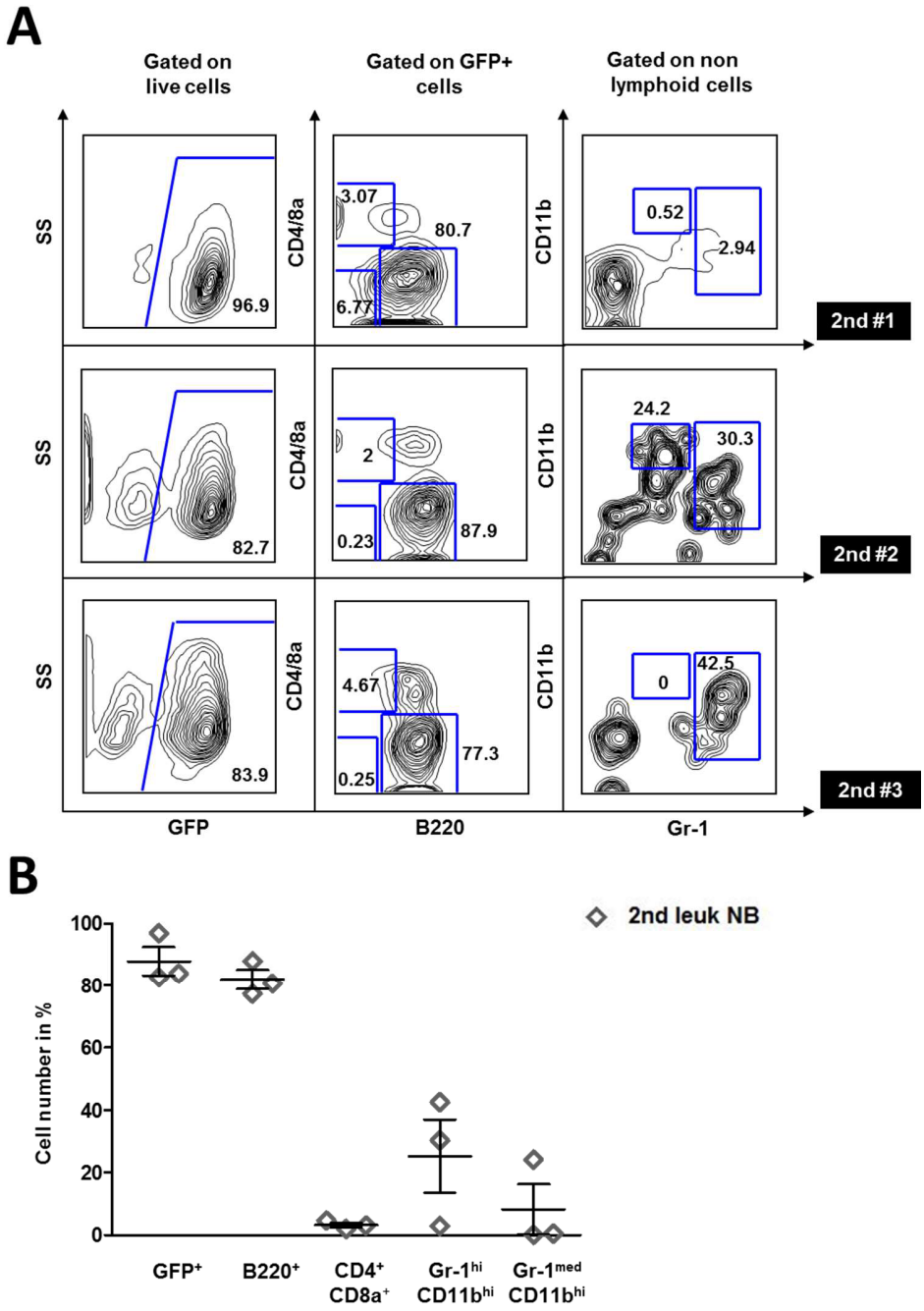

Figure 19: *Analysis of mature blood cells in secondary transplanted mice.*

(A) *Gating strategy of PB of secondary transplanted mice and FACS plots.* ***(B)*** *Dot plot of percentages in different cell populations in blood of secondary transplanted mice n = 3. MEAN±SEM.*

4.4. Classification of Recipients into Different Types of Leukemia

4.4.1. Classification of the Recipients

In the work described above, I developed a new model of BCR-ABL+ leukemia that uses BCR-ABL-transfected FL cells as donor cells and neonates as recipients. Although p185 BCR-ABL is mainly found in ALL, a myeloid disease can also possibly develop. In the case of p210 and p230 BCR-ABL oncogenes the mutation mainly gave rise to myeloid leukemia. To classify which type of leukemia develops from the transplanted GFP+ FL cells, each BCR-ABL recipient was evaluated separately for the balance between lymphoid and myeloid cells in the BM, PB and SP (***Table 15, 16, 17***). The types of leukemia that I focused on are described as "lymphoid type," "myeloid type" or "mixed type." As there are no standardized methods for identifying these types, I defined the different types as follows: When more than 70% of the GFP+ cells either express B220+ or CD4/8a+; with low cell numbers (below 30%) of Gr1-med/CD11b-hi and Gr1-hi/CD11b-hi cells, I defined as "lymphoid leukemia." Mice with over 70% of Gr1-med/CD11b-hi and Gr1-hi/CD11b-hi cell populations with less than 30% of B220+ and CD4/8a combined are defined as "myeloid leukemia," and all other proportions are defined as "mixed leukemia". This definition is related to the work of Schreck (Schreck C., 2015).

In ***Table 15*** all three organs in the NB BCR-ABL mice were analyzed. Here, all NB recipients showed a high tendency to develop lymphoid leukemia with a mean value of 95.13% lymphoid cells of all GFP+ cells. Only one recipient, designated as number #2w, could be defined as having mixed leukemia with 66.19% lymphoid cells and 33.81% myeloid cells. The same result was also found when summarizing the FACS results of the BM. The same mouse, #2w, showed a mixed lymphoid/myeloid population, whereas all the others clearly showed a dominant lymphoid leukemia with 91.18% B220+ and CD4/8a+ cells. In the SP, however, all

of the recipients showed mainly (>70%) GFP+ lymphoid cells, including number #2w.

The evaluation of the YA BCR-ABL recipients is shown in ***Table 16***. Here the PB results differ from the ones from NB BCR-ABL recipients of ***Table 15****.* According to the criteria above, 12.5% of the YA BCR-ABL recipients showed mixed leukemia and 25% showed myeloid leukemia. The remaining 62.5% of the YA BCR-ABL recipients developed a lymphoid leukemia. Interestingly, the two mice that displayed myeloid leukemia in the PB showed no signs of a myeloid disease in the BM and SP, where an almost pure lymphoid leukemia can be seen. However, the YA BCR-ABL recipient with mixed leukemia in the PB also showed a mixed-type of leukemia in the SP.

To move on, the same definitions were used for the secondary transplanted mice (***Table 17***). The donor mouse can be classified as a lymphoid type of leukemia, and so all three of the secondary transplanted recipients can be classified as lymphoid as well. Here, both analyzed tissues, PB and BM, showed no signs of a myeloid involvement. All three secondary recipients are close to 100% of a B220+ lymphoid type.

NB BCR-ABL														
Date of TX		6.11.12	6.11.12	6.11.12	17.12.12	17.12.12	22.3.13	22.3.13	22.3.13	22.3.13	22.3.13	% of BCR-ABL		
organ	ratio of	10	20	30	u1	10	1	2	3	6	2w	transplanted mice	mean	sem
PB	lymphoid and myeloid cells to GFP+ cells 100%	97.17	95.11	77.31	16.18	73.84	23.33	83.53	0	79.51	20.83			
PB	lymphoid cells to GFP+ cells	99.98	99.97	99.96	92.30	99.59	99.95	99.95		98.27	66.19	88% lymphoid	95.13	13.02
PB	myeloid cells to GFP+ cells	0.02	0.03	0.04	7.70	0.41	0.05	0.05		1.73	33.81	0% myeloid	4.87	3.71
												12% mixed		
BM	lymphoid and myeloid cells to GFP+ cells 100%	28.96	25.64	37.63	0	48.89	36.12	81.76	2.00	62.40	9.20			
BM	lymphoid cells to GFP+ cells	99.38	98.55	99.79		98.44	82.40	99.86	85.15	95.41	61.63	88% lymphoid	91.18	8.96
BM	myeloid cells to GFP+ cells	0.62	1.45	0.21		1.56	17.60	0.14	14.85	4.59	38.37	0% myeloid	8.82	4.30
												12% mixed		
SP	lymphoid and myeloid cells to GFP+ cells 100%	76.46	84.75	96.05	73.08	91.60	41.25	78.53	2	66.65	10.86			
SP	lymphoid cells to GFP+ cells	99.84	99.44	99.68	94.73	99.78	86.55	99.80	100	89.65	85.09	100% lymphoid	95.45	11.55
SP	myeloid cells to GFP+ cells	0.16	0.56	0.32	5.27	0.22	13.45	0.20	0	10.35	14.91	0% myeloid	4.55	1.92
												0% mixed		

Table 15: *Classification of NB BCR-ABL recipients.*

The table shows ratios of lymphoid and myeloid cells to GFP+ cells in all transplanted NB BCR-ABL recipients. Numbers are raw data percentages of cell populations. PB= peripheral blood, BM= bone marrow, SP= spleen. Yellow = majority in lymphoid cells, red = majority of myeloid cells, green = mixed cell population (lymphoid and myeloid). MEAN ± SEM

YA BCR-ABL												
Date of TX		25.1.13	25.1.13	25.1.13	25.1.13	25.1.13	8.11.13	8.11.13	8.11.13	% of BCR-ABL		
organ	ratio of	1	2	3	60	5	1	3	4	transplanted mice	mean	sem
PB	lymphoid and myeloid cells to GFP+ cells 100%	95.51	53.69	16.79	45.74	96.19	61.35	66.17	5.37			
PB	lymphoid cells to GFP+ cells	99.96	83.38	13.11	68.10	99.91	13.25	90.83	86.11	62.5% lymphoid	69.33	11.58
PB	myeloid cells to GFP+ cells	0.04	16.62	86.89	31.90	0.09	86.75	9.17	13.89	25% myeloid	30.67	12.76
										12.5% mixed		
BM	lymphoid and myeloid cells to GFP+ cells 100%	98.86	98.76	17.27	96.08	98.31	96.50	80.02	52.76			
BM	lymphoid cells to GFP+ cells	99.98	99.95	96.66	97.10	99.96	99.86	94.85	99.62	100% lymphoid	98.50	10.56
BM	myeloid cells to GFP+ cells	0.02	0.05	3.34	2.90	0.04	0.14	5.15	0.38	0% myeloid	1.50	0.71
										0% mixed		
SP	lymphoid and myeloid cells to GFP+ cells 100%	97.34	98.21	85.50	77.02	96.29	46.24	62.66	0.08			
SP	lymphoid cells to GFP+ cells	99.96	99.90	99.95	57.20	99.90	95.95	86.02	100	87.5% lymphoid	92.36	11.97
SP	myeloid cells to GFP+ cells	0.04	0.10	0.05	42.80	0.10	4.05	13.98	0	0% myeloid	7.64	5.31
										12.5% mixed		

Table 16: *Classification of YA BCR-ABL recipients.*

The table shows ratios of lymphoid and myeloid cells to GFP+ cells in all transplanted YA BCR-ABL recipients. Numbers are raw data percentages of cell populations. PB= peripheral blood, BM= bone marrow, SP= spleen. Yellow = majority in lymphoid cells, red = majority of myeloid cells, green = mixed cell population (lymphoid and myeloid). MEAN ± SEM

organ	2nd from NB ratio of	2nd 1	2nd 2	2nd 3	% of secondary transplanted mice	mean	sem
PB	lymphoid and myeloid cells to GFP+ cells 100%	84.00	90.03	82.08			
PB	lymphoid cells to GFP+ cells	99.72	99.86	99.87	100% lymphoid	99.82	0.05
PB	myeloid cells to GFP+ cells	0.28	0.14	0.13	0% myeloid	0.18	0.05
					0% mixed		
BM	lymphoid and myeloid cells to GFP+ cells 100%	97.62	85.71	98.71			
BM	lymphoid cells to GFP+ cells	100	100	100	100% lymphoid	100	0
BM	myeloid cells to GFP+ cells	0	0	0	0% myeloid	0	0
					0% mixed		

***Table 17**: Classification of secondary transplanted mice*

The table shows ratios of lymphoid and myeloid cells to GFP+ cells in all secondary transplanted mice. Numbers are raw data percentages of cell populations. PB= peripheral blood, BM= bone marrow. Yellow = majority in lymphoid cells, red = majority of myeloid cells, green = mixed cell population (lymphoid and myeloid). MEAN ± SEM

4.4.2. Survival in Correlation to the Type of Leukemia

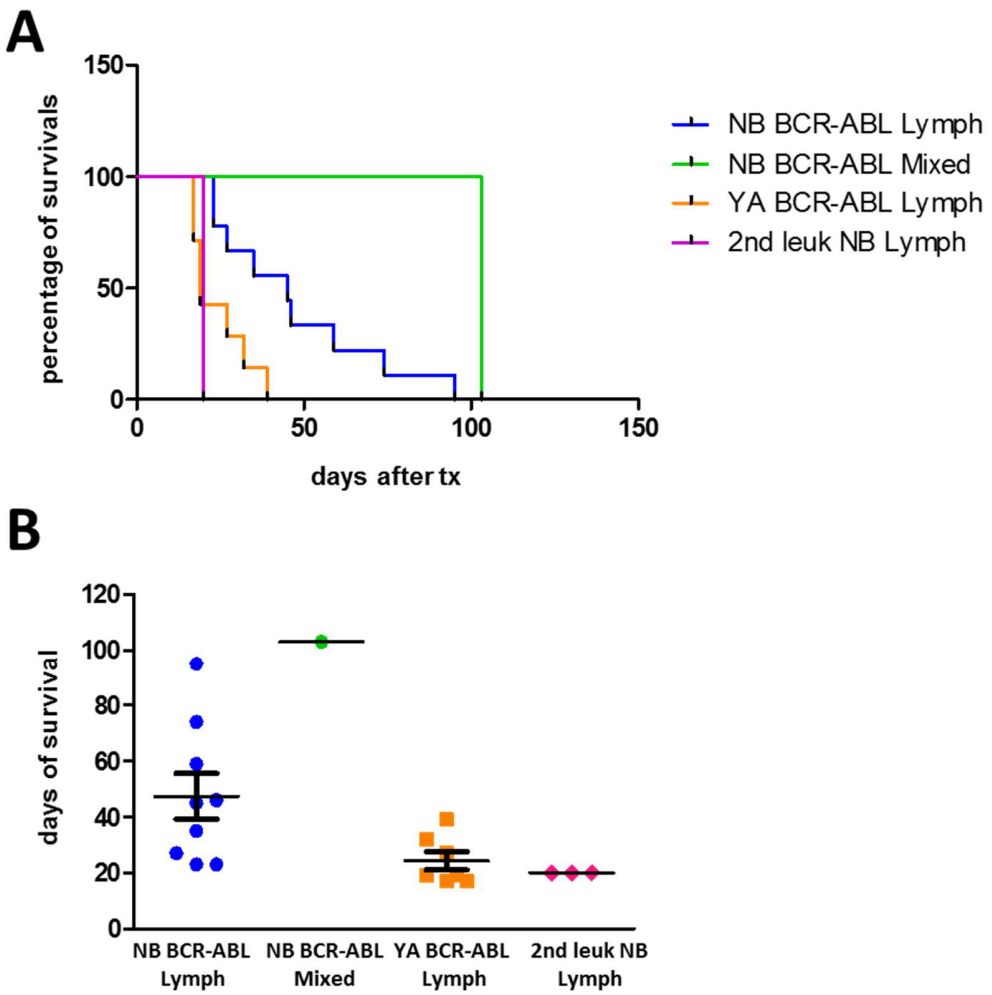

Figure 20: *Survival analysis depending on type of leukemia.*

(A) *Blue line = NB BCR-ABL with lymphoid leukemia type (n=9), green line = NB BCR-ABL with mixed leukemia type (n=1), orange line= YA BCR-ABL with lymphoid leukemia type (n=7), pink line = secondary transplanted recipients (NB BCR-ABL donor) with lymphoid leukemia type (n=3).* ***(B)*** *Dot plot of days of survival classified by the type of leukemia. MEAN ± SEM.*

In the literature, it has been shown that BCR-ABL and expression of other (fusion) oncogenes can induce different types of leukemia, with different latency periods

(Miething et al., 2003a). To find out whether mice with lymphoid, myeloid or mixed leukemia show different latencies, I constructed a survival curve depending on the type of leukemia to see whether any statements of a dependency can be made between the type of leukemia and the survival duration (***Figure 20A***). My results show that mice with lymphoid leukemia (pink, orange and blue lines) have a shorter survival than mice with a mixed type. The secondary transplanted mice with only lymphoid cell populations had to be sacrificed after 20 days. YA BCR-ABL mice showed lymphoid dominance, as seen in ***Table 16***, but still develop a low percentage of myeloid cells. Here, the mean survival time lies around 24 days. The recipients of the NB BCR-ABL group show an even longer survival, with 47 days, which expresses a lymphoid leukemic type. The longest survival in my experiments could be observed in the single mixed leukemia mice (***Figure 20B***). These results show an absolute emphasis on lymphoid types after transplantation with the p185 BCR-ABL oncogene, using my new transplantation model as well as the gold standard. Further conclusions follow in the discussion.

5. Discussion

HSCs are characterized by their ability to regenerate all blood cell lineages. *In vivo* models are the only way to gain information about the proper development of long-term repopulating HSCs. In addition, *in vivo* models are essential for research on the development of malignant leukemia and lymphomas and the study of leukemic stem cells (LSCs). Based on the early work of Till and McCulloch, who first proposed that spleen colonies in irradiated transplanted adult mice derive from HSCs (Becker et al., 1963) a diversity of transplantation models have evolved that allow distinction of host and donor HSCs on the basis of male/female or transgenic markers (PCR analysis), enzymatic activity (glucose-6-phosphate isomerase; Gpi1a/b), and surface markers (Thy1.1/2 and Ly5.1/2), and prove the ability of donor stem cells to repopulate a whole hematopoietic system after lethal radiation (Akashi et al., 2000; Harrison et al., 1993; Reya et al., 2001). Indeed, the hematopoietic transplantation models are, so far, the only experimental system in which transplantation of single HSCs have been shown to regenerate a complete tissue, the blood cell system (Dykstra et al., 2007).

Transplantation models were also instrumental in showing that fetal HSCs are actively cycling, whereas adult HSCs are not (Bowie et al., 2007). Furthermore, it was shown that early fetal HSCs do not engraft adult recipients. However, in contrast, they do engraft newborn recipients (Yoder et al., 1997b), suggesting that the newborn environment supports fetal HSCs better than the adult environment.

This observation also suggests that leukemia developing in either a newborn or adult environment may not be the same, and that the newborns support pediatric leukemia better than adults.

To study this hypothesis, I decided to study leukemogenesis induced by the p185 BCR-ABL1 fusion oncogene (Philadelphia chromosome, Ph+), which is known to be expressed in 5-10% of childhood leukemia presenting as ALL (Suryanarayan et

al., 1991) whereas it is found in about 76% of adult lymphoid leukemia patients and in only 0.3% of Ph+ myeloid leukemia that predominantly express the p210 e13/e14/a2 BCR-ABL fusion (Jones et al., 2008). Several genetically engineered mouse models of Ph+ leukemia exist. Nevertheless, juvenile models are still rare (Hauer et al., 2014).

Transplantation models of donor HSCs into neonate recipients have been described. In these methods, the donor cells are either transplanted intravenously (i.v) (Yoder et al., 1996), subcutaneously (s.c.) (Sands et al., 1999) or intrahepatically (i.h.) (Traggiai et al., 2004). As in adult recipients, the newborn recipients require conditioning to promote engraftment of the donor HSCs. For this purpose, I used irradiation, a method that was previously developed in the laboratory of Dr. S. Gitzelmann (Gitzelmann, 2011). I here developed a possible model for Ph+ childhood leukemia. For this model, I hypothesized that transplantation of leukemia-initiating cells (LIC) from fetal livers in neonatal recipients would be a closer model for childhood leukemia than using Ph+ LIC from adult donors, as the combination of the developmental stage of the donor cells and the recipient niche is critical for the success of donor HSC engraftment (Arora et al., 2014).

5.1. Feasibility of Intrahepatic Transplantation of the BCR-ABL Vector in NB Compared with the Already-Established i.v. Leukemia Model of YA

To understand microenvironmental events and the source of their effect on leukemia, *in vivo* models are indispensable. The recognition of animal care and the guidelines for replacement, reduction and refinement developed by FELASA and

the EU (EU directive 2010/63/EU) demand effective methods combined with minimal stress and burden in research. To this aim, I studied a model of p185 e1a2 BCR-ABL leukemia in newborn pups as a more efficient and less-stressful *in vivo* model for acute lymphoblastic leukemia. In addition, I studied whether this model is a more appropriate model for Ph+ childhood ALL than the more commonly used Ph+ transplantation in adult recipients.

5.1.1. The Transplantation Procedure

For the model of i.h. transplantation in neonates the handling and execution of transplantation is rapid and easy. First, the newborn pups are easy to handle, as they are not as aggressive as adult mice of some strains. Thus, the risk of an accidental self-infection or an injury to the scientist is very low. Additionally, no superior surgical skills are necessary for the i.h. transplantation, since the liver of the newborn mice is clearly visible through their transparent skin. This means a reduction of stress for the animals, since the transplantation procedure is rapid and simple to perform.

Another issue of possible stress is the conditioning using irradiation. While adult mice need to be radiated lethally with at least 8.5Gy to ensure optimal engraftment of the infected cells, previous work in the laboratory established that neonatal recipients need a sub-lethal radiation dose of only 4.5Gy. An important feature of the irradiation is that the newborns are neither touched nor removed from their nest during the procedure. The mother of the mice is taken out of the cage during the procedure and shows signs of light stress because of the absence of her pups. The combination of preservation of the nest during the irradiation and the gentle dose of radiation led to a survival rate of 100% of the neonates, as well as a good and reproducible engraftment. Previous work has shown that the engraftment overall is slightly lower than after i.v. transplantation in adults. However, where up to 20% (depending on the mouse strain) of the adult recipients may succumb to radiation

damage due to insufficient take of the transplanted cells, survival is 100% in the sub-lethally irradiated newborn recipients.

5.1.2. BCR-ABL Mouse Model for Leukemia in Neonatal Recipients

This Ph.D. thesis is the first description of a successful establishment of a p185 BCR-ABL leukemia model using i.h. transplantation and FL cells in neonatal recipients. As ***Figure 8*** shows, all of the primary recipients succumb to disease with a mean time to lethal disease of 47 days. This is significantly later than the adult recipients, which succumb at half that time, after 24 days. One hallmark of a mouse model of leukemia is that the lympho-myeloproliferative disease from the primary recipients is transferable to secondary recipients. Indeed, my experiments show that all secondary transplanted mice develop an acute B-cell leukemia (***Table 17***). Importantly, this result additionally demonstrates that p185 BCR-ABL transduced FL cells cause leukemia in newborn recipients, thus validating the model as a leukemia model.

Our results of the control experiments in adult recipients closely resemble the original description of p185-BCR-ABL-transduced 5-FU-treated bone marrow cells transplanted into adult recipients, which mostly develop a very aggressive form of ALL (Kelliher et al., 1991).

Since the donor cells I have used derive from the embryo (fetal liver cells), and the neonatal recipients succumb to disease before reaching an adult age, we hypothesize that the model I studied represents a model for childhood leukemia.

5.2. Biological and Medical Effects in BCR-ABL Leukemic Vector-Transplanted NBs

5.2.1. Statistically Longer Survival Rate of BCR-ABL Transplanted NBs

For a proper repopulation by stem cells, the cells need sufficient time to rebuild the hematopoietic system after radiation and transplantation. Repopulation is a process in which both proliferation and differentiation take place, and it takes at least three to four weeks to produce sufficient mature blood cells for the blood system to function (Otsuka et al., 2010). The overall interest of the Oostendorp Lab is the study of how the stem cell microenvironment (also called: the niche) influences the development of leukemia. In many known leukemia models in adult recipients, the disease develops rapidly and mice may succumb to leukemia between 2 and 4 weeks after transplantation. In order for the microenvironment to influence the transformations of the p185 BCR-ABL transduced FL cells, a longer exposure time would be desirable. As ***Figure 8A*** shows, there are differences in the time line concerning the detection of the disease and, for that, as well in the end of the experiment in the different experimental groups. Whereas YA BCR-ABL recipients have a mean survival time of 24 days, the NB BCR-ABL model shows an average of 47 days. These differences in survival could also be a reason for the development of different appearances of leukemic diseases, as it is known that LSCs alter due to their microenvironment (Wu et al., 2007). One reason for diversity between NB and YA microenvironments could be the less-intensive engraftment of the transplanted leukemic cells in NBs than in the YA (***Figure 10***). In general, I found that donor engraftment in the sub-lethally irradiated NB recipients is around 25%, whereas donor engraftment in YA is 52%. The different levels of engraftment can have several underlying causes. First, NB and YA recipients are conditioned using different doses of radiation; the common radiation dose for adult mice is 8.5 to 9Gy, which is lethal. In contrast, the NB pups are only

radiated with 4.5 Gy. Thus, in NB recipients there is internal competition of engrafting HSCs, since most HSCs will survive the radiation procedure, whereas in YA recipients endogenous competition will be below 10% of the total blood system after reconstitution. Another cause of reduced engraftment may be that the NB recipients are injected intrahepatically, meaning that the HSCs and LSCs need to egress from the liver and only then can circulate and enter the bone marrow. In YA recipients, the i.v. injection makes the egress phase unnecessary, perhaps making the migration to the bone marrow more efficient. However, it has been shown that i.v.-transplanted adult HSCs home strongly to the liver of NB recipients (Arora et al., 2014). This is probably due to a propensity of NB recipients to support engraftment of cycling HSCs.

5.3. PB Analyses in the Assessment of Recipients of BCR-ABL+ FL Cells

In human oncology analysis of a BM sample is the prime material for identification of leukemia. Testing of peripheral blood is usually the first diagnostic indication for further study of a BM sample. In my analyses of NB and YA recipients of p185-transduced FL cells, I first analyzed blood samples for the presence of the GFP marker in mature cell subsets. These analyses revealed a wide range of alterations in NB BCR-ABL recipients compared with the NB recipients transplanted with MIG control-transduced FL cells. The first step in the diagnostic analysis was the measurement of different parameters in a blood counter (BC), a procedure which is also performed in the diagnosis of human blood cell samples. These analyses showed that in both NB and YA recipients a similar increase of the white blood cell (WBC) and lymphocyte count is notable (***Figure 11***). In addition, this analysis also shows a clear increase in monocytes and granulocytes. The blood counter measures the different subpopulations in cell samples using physical parameters, such as size and granularity. Thus, the results of the BC are similar, but not the

same as those from flow cytometry, which has the additional expression of surface antigens. Thus, the flow cytometric assessment of B- and T-lymphocytes confirms the increase in lymphocytes in recipients of BCR-ABL+ cells. However, the flow cytometry (***Figure 12A***) does not reflect the BC results, as the Gr1-hi/CD11b-hi population remain unchanged. Thus, in the analysis of leukemic mice, as in the diagnosis of human subjects, BC analysis is only a first step in the examination of the PB; detailed analysis of lymphocyte and myeloid subpopulations requires further dissection by flow cytometry and bone marrow analysis (see below).

5.4. Analyses of the Spleen (SP) in Leukemia

The spleen is the largest lymphatic organ in the mammalian body that can support hematopoiesis during embryonic and neonatal development. In adult subjects, the spleen can serve as a reservoir of extra medullary hematopoiesis in situations of stress, such as in the case of leukemia (Kim, 2010; Swirski et al., 2009). Many hematopoietic malignancies are characterized with splenomegaly. Indeed, the same is true for experimental leukemia models, as I have presented in ***Figure 13***. Other than detecting splenomegaly, the diagnostic value of the spleen is limited and does not extend to invasive procedures such as spleen biopsies. This is different in experimental models, as the recipients are sacrificed when moribund, and the different tissues can be harvested and thoroughly analyzed by different techniques. My results from the experiments shown in ***Figure 14*** and ***Figure 15*** suggest that the SP of NB recipients is more permissive for the engraftment of LSCs than adult SP. In NB recipients, immature CLP and MP and corresponding mature B-cells as well as Gr1-hi/CD11b-hi cells are greatly increased compared with the NB MIG recipients. In contrast, in YA BCR-ABL recipients, I observed a significant increase of B-cells, but not of monocytes, CLPs or MPs. Thus, NB spleens support a more diverse collection of leukemic cells than YA spleens. This finding also supports the statement that engraftment efficiency in different tissues

depends on the development of the niche and therefore on the recipients' age and developmental stage (Arora et al., 2014).

5.5. Analyses of the Bone Marrow (BM) in Leukemia

When the peripheral blood analyses shows indications of a possible leukemic disease in clinical situations, a BM puncture is the next step in the diagnostic process. In the experimental setting, whole BM is harvested and analyzed using different techniques. I analyzed cell numbers and cellular composition of the BM using flow cytometry. In the NB model, I found over 30% of the GFP+ cells were of the B-cell lineage, whereas CD4 and/or CD8+ T-cells only show a mean of 2.5% of the BCR-ABL+ GFP+ cells. An important difference of the BM compared with other tissues was that in PB and SP there is a hint of BCR-ABL+ cells circulating or residing in the SP. I detected no clear increase in leukemic Gr1-hi/CD11b-hi or Gr1-med/CD11b-hi cells in the BM. Besides the increase of B- and T-cells in the BM of NB BCR-ABL recipients, the rise in the number of CLPs is notable. This observation supports findings described in another report, which also showed an increase of CLPs in p185 BCR-ABL-induced leukemia (Hauer et al., 2014).

In contrast with NB recipients, in YA BCR-ABL recipients' BM tissue I found a significant increase in the GFP+ Gr1-hi/CD11b-hi population. This finding suggests that BCR-ABL-induced leukemia in YA recipients has a larger myeloid component than in NB recipients. Even though it is not significant, an increase of MPs can be observed in the YA BCR-ABL recipients as well. The reason for that could be that the different routes of transplantation used, i.h. in NB and i.v. in YA recipients, preferentially promote BM engraftment of myelogenous progenitor cells. What is more likely perhaps is that, since the donor population is the same in both NB and YA recipients, the aging microenvironment supports different types of stem cells,

as the NB environment would promote lymphoid-biased stem cells more than YA or old recipients, in which progressively more myeloid cells will develop (Benz et al., 2012). Another question is: Is an increase of Gr1-hi/CD11b-hi cells compared with the YA MIG control-transplanted cells the underlying cause of a myelogenous leukemia?

5.6. The BCR-ABL i.h. Transplantation in NB as a Model for BCR-ABL induced ALL

Mostly for practical reasons, the gold standard in experimental mouse leukemia models is i.v. transplantation of oncogene-expressing BM cells into lethally irradiated adult mice (Arora et al., 2014). Instead of BM, FL-derived donor cells are increasingly being used for leukemic research on wild type (WT) mouse models (Hemann, 2015). These experiments suggest that distinct factors play a role in the type of leukemia that develops. Such factors can be e.g., the age of the donor (embryonic to adult), the age of the recipient and the tissue used for the donor cells, e.g., BM, FL, SP (Hauer et al., 2014). This also implies that the age of the niche of the recipient may influence the development and ultimately also the outcome of various experiments with leukemia mouse models.

In this thesis, the different outcomes of transplantation of p185 BCR-ABL transfected FL in two different types of recipients: YA and NB mice, was compared. As ***Table 15*** and ***Table 16*** show, in NB a very high percentage of 90% lymphoid GFP+ cells in the various tissues can be observed. In the work of Rafiei, p185 BCR-ABL is described as a main driver for ALL (Rafiei et al., 2015). Thus, my results show that the i.h. transplantation model of p185 BCR-ABL FL donor cells into neonatal mice can be regarded as a model for ALL. A closer look at the results in ***Table 15*** reveals that the GFP+ population in the spleen are 100% lymphoid cells, with only one single mouse (#2w) in which a mixed population of myeloid and

lymphoid cells developed that could be observed in the BM as well as in PB. Unfortunately, due to time constraints and the number of mice transplanted, I could not distinguish whether the frequency of one in 10 NB recipient mice will always develop this mixed-type leukemia, or whether this one recipient represents a spurious experimental artifact. To examine this issue in more detail, more NB recipient mice should be studied.

Interestingly, when we combine these results of almost complete lymphoid transformation with the results of the mean disease-free survival time, the lymphoid leukemia show a mean disease-free survival of 47 days (***Figure 20B***). The single mixed type leukemia mouse shows an even longer survival time with 103 days. To make clear statements about this fact, it would be interesting to do more research and to see whether this observation can be reproduced.

In comparison, YA BCR-ABL recipients (***Figure 20B***) not only show a much shorter mean survival time of 24 days, but also the analysis of the GFP+ cells shows (***Table 16*** #3 dark green, #1 light green) a clear population of myeloid cells in the PB analysis of two mice. In addition, the recipient with a mixed leukemic cell population in the PB also shows a mixed population in the spleen. Surprisingly, however, the GFP+ myeloid populations are not found in the BM, which shows an almost complete lymphoid population of GFP+ cells in all YA recipients. Again, the statistical power of these observations is unclear, and further experiments in this direction should be done to determine the frequency of YA recipients with a stronger myeloid component to assess whether this frequency is, in fact, different from NB recipients.

In conclusion, in this thesis a new model for p185 BCR-ABL+ leukemia has been established in which retro-virally transduced FL cells are transplanted into NB recipients. This model has been compared with the more conventional transplantation model into YA recipients. The NB model has several advantages over the YA model, such as easier handling of the transplantation in NB recipients, a longer disease-free survival and development of a more homogeneous B-

lymphoblastic leukemia. Since the model will be studied further to determine the influence of alterations in signaling pathways in niche cells on leukemia development, the fact that disease-free survival is prolonged is an important advantage of the NB model over the YA model. Indeed, this model has already been used to determine the relevance of Wnt5a from the environment in the development of leukemia (Schreck C.; et al., Submitted 2015). One other important issue I started to address is whether the NB model more closely resembles BCR-ABL+ pediatric ALL, compared with the YA recipient model. Since I used an embryonic source of donor cells (E14.5 fetal liver cells), which serve as a model for cells oncogenically transformed in-utero, and I used neonatal recipients which succumb to leukemia at a juvenile age, the NB model is closer to childhood ALL than the usage of adult recipients would be (Plasschaert et al., 2004). Considering the differential biology and treatment outcomes of pediatric and adult ALL allows the study of why adult ALL usually has a worse prognosis than pediatric ALL and development of more effective therapies for adult ALL.

In addition, because of the prolonged disease-free survival of NB recipients, the mechanism of leukemia development can be worked out in more detail. Although BCR-ABL is known as a dominant and strong oncogene, it does require additional mutations for full leukemia development. Known collaborative mutations are: deletion of the *Ikzf1* gene and mutations in the *Tp53*, *Asxl1*, *Dnmt1*, *Runx1*, *Tet2* and *Btg1* genes (Schmidt et al., 2014; Xie et al., 2014). The significance of these is unclear at present. For the study of additional mutations that accelerate leukemia development and their possible relevance in the therapy of recurring disease, the NB leukemia model would be particularly useful.

These outcomes go hand in hand with the results of Pulte et al., who described that ALL disease exists in both age groups, infants and adults, but differs in time of survival, as for adults the disease develops more acutely than for children (Pulte et al., 2014).

6. Perspectives

In the past decades, research in the field of stem cells and leukemia has created a variety of mouse models to be able to examine *in vivo* processes. However, we still have a long way to go to improve our methods and the outcomes of animal experiments.

This new model can contribute to these problems, as it is simple in handling with a very high rate of successful transplanted recipients and a high survival rate. These factors can help in continuative research, as leukemia can be produced with a high guarantee in the recipients. The next step after the development of this model should be the examination of certain proteins said to be responsible for the development of childhood leukemia. One of those could be *Ikfz1* as it is a transcription factor for lymphocyte development and regulates the differentiation and proliferation of B- and T- cells (Westman et al., 2002). Plus, the deletion of *Ikfz1* was found to cause even worse prognosis in children with B-cell ALL (Mullighan et al., 2009; Yang et al., 2011).

Another outlook is that this new model can be the basis for other leukemic vector models, such as MLL-AF9 or MLL-AF4 that appear also very often in conjunction with childhood leukemia (He J. et al., 2005; Pui et al., 2015). As the transplantation method is now established other vectors can be tested and their influence on the niche and their reaction to certain treatments could be tested.

Moreover, another direction to follow is to examine whether the difference in leukemic types in this model really changes the duration of survival, as my work shows evidence for a longer survival of mixed-type leukemia patients. For that, several more animals would be necessary, but additionally the reason why the same vector can produce in one individual a lymphocytic leukemia, whereas the other produces myelogenous leukemia, could be detected.

These findings can help in future to better categorize leukemic diseases in patients and for that optimize the treatment concerning the chance and durance of survival.

7. Summary

The Significance of the Microenvironment for the Development of p185 BCR-ABL Triggered Leukemia: A Comparison of Mouse Models in Neonatal and Adult Recipients

Scientists are still trying to understand the origins and catalysts of childhood leukemia in order to develop new healing approaches. However, no standardized model for leukemia, particularly in juvenile stadium has been developed so far.

This thesis fills the gap by developing a new model for the study of leukemia in a juvenile organism with the oncogene p185 BCR-ABL. Furthermore, the model was compared with the gold standard of adult leukemic models and tested for its practicability.

For the first time p185 BCR-ABL-transduced FL cells were introduced into wild-type (WT) recipients via intrahepatic transplantation, which constitute with their hybrid background of 129/B6 a similar microenvironment to the human one. This transplantation model turned out to be less stressful for the recipients and easy to perform. The fact that 10 out of 11 mice engrafted, constitutes a highly satisfying result. The NB recipients' time of survival was with a mean of 47 days significantly longer than those of the YA recipients. This suggests that the microenvironment has more time to adapt to the new circumstances. Hence, the analysis of the niche is more revealing. The finding that the only mixed leukemic mouse was the one who survived the longest is an interesting direction for further research.

In the microbiological analysis of NB BCR-ABL recipients 88% developed a lymphatic leukemia. This is also described in other mouse models with the oncogene p185 BCR-ABL (Hauer et al., 2014). In turn, only one out of nine recipients developed more T-cells than B-cells; the other eight recipients showed a

nearly pure B-cell leukemia. Thus, this newly developed mouse model is highly suited for further study of the microenvironment in B-cell leukemia in juveniles.

The comparison with adult animals which were transplanted i.v. with p185 BCR-ABL transduced FL cells shows: 1.the handling of adult animals is more demanding, 2. the transplanted animals' time of survival is significantly shorter. Therefore, one could assume that adult recipients will accumulate less alterations in the microenvironment in the short time leukemia is developing. For that reason this model is less suitable for analyzing leukemia in the range of the niche. Despite the varying life spans of the two groups NB and YA, the results hypothesize that B-cell emphasized lymphoid leukemia is provoked in both.

To sum up: The thesis at hand establishes a new mouse model for leukemia in a juvenile organism in which B-cell leukemia is provoked through i.h. transplantation of p185 BCR-ABL transduced FL cells. In comparison to the widespread adult model, it is not only more practicable in terms of handling and less stressful for the recipients but also more meaningful in the field of niche research in childhood leukemia, thanks to the longer exposure to the microenvironment.

8. Zusammenfassung

Die Signifikanz der Mikroumgebung für die Entwicklung einer durch p185 BCR-ABL ausgelösten Leukämie: Ein Vergleich von Mausmodellen neugeborener und adulter Empfängertiere

Die Forschung versucht nach wie vor die Ursachen und Auslöser der kindlichen Leukämie besser zu verstehen, um neue Heilungsansätze zu entwickeln. Bisher existiert jedoch kein standardisiertes, auf die Leukämie im Juvenilstadium speziell ausgelegtes Modell.

Diese Arbeit füllt diese Lücke, indem sie ein neues Modell für die Untersuchung der Leukämie in einem kindlichen Organismus mit dem Onkogen p185-BCR-ABL entwickelt. Zudem wurde es mit dem Goldstandard der adulten Leukämie-Modelle verglichen und die Praktikabilität getestet.

Zum ersten Mal wurden p185-BCR-ABL-transduzierte-fötale-Leber-Zellen per intrahepatischer Transplantation in Wildtyp-Empfängertiere eingebracht, die mit ihrem hybriden Hintergrund aus 129xB6 eine dem Menschen ähnliche Umgebung darstellen. Es zeigte sich, dass dieses Transplantationsmodell weniger stressig für die Empfängertiere und einfach in der Handhabung war. Die Tatsache, dass 10 aus 11 Mäusen engrafteten, ist ein hoch zufriedenstellendes Ergebnis. Die Überlebensdauer der Neugeborenen (NB) Empfängertiere von durchschnittlich 47 Tagen war signifikant länger als die der jungen Adulten (YA) Empfänger. Das lässt vermuten, dass die Mikroumgebung mehr Zeit hat sich an die neuen Umstände anzupassen. Dies macht die Untersuchung der Nische aufschlussreicher. Dass die einzige gemischt-leukämische Maus am längsten überlebte, bietet einen interessanten Ansatz für weitere Forschungsprojekte.

Bei der mikrobiologischen Untersuchung bildeten 88% der NB BCR-ABL-Empfängertiere eine lymphatische Leukämieerkrankung aus. Dies wird auch in anderen Mausmodellen mit dem Onkogen p185 BCR-ABL beschrieben (Hauer et al., 2014). Von diesen erkrankten Mäusen bildete nur eine aus neun mehr T- als B-Zellen aus. Die acht anderen Mäuse zeigten eine nahezu reine B-Zell-Leukämie. Somit ist dieses neu entwickelte Mausmodell hervorragend dazu geeignet die Mikroumgebung einer B-Zell-Leukämie in Kindern weiter zu untersuchen.

Der Vergleich mit den adulten Tieren denen i.v. p185-BCR-ABL-transduzierte-fötale-Leber-Zellen transplantiert wurden ergab folgende Ergebnisse: 1. Das Handling von erwachsenen Tieren ist anspruchsvoller, 2., Die Überlebensdauer der transplantierten Tiere ist signifikant kürzer. Daraus kann man folgern, dass adulte Empfängertiere in der kurzen Zeit in der sich die Leukämie entwickeln kann, weniger sichtbare Veränderungen in der Mikroumgebung hervorrufen werden. Aus diesem Grund eignet sich dieses Modell weniger zur Untersuchung der Leukämie mit Augenmerk auf die Nische. Trotz der unterschiedlichen Überlebensspannen der beiden Gruppen NB und junge Adulte (YA) lässt sich aufgrund der Ergebnisse vermuten, dass in beiden Empfängertieren eine lymphoide B-Zell-betonte Leukämie ausgelöst wird.

Zusammenfassend lässt sich folgendes sagen: In der vorliegenden Arbeit wurde ein neues Mausmodell für Leukämie im kindlichen Organismus entwickelt, in dem eine B-Zell Leukämie durch i.h. Transplantation fötaler, p185-BCR-ABL-transduzierter-Leberzellen, ausgelöst wurde. Im Vergleich zum adulten – bisher weit verbreiteten – Modell ist es nicht nur praktikabler in der Umsetzung und stressfreier für die Empfänger, sondern durch die bessere Entfaltung der Mikroumgebung auch aussagekräftiger im Bereich der Nischenforschung in der kindlichen Leukämie.

9. Index of Figures

10. Index of Tables

11. Index of Abbreviations

Abbreviation	Description
Abl	Abelson Musine Leukemia Viral Oncogene Homolog 1
AGM	Aorta-gonad-mesonephros area
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
Amp	Ampicillin resistance gene
B6	C57BL6J ola
Bcr	Breakpoint cluster region
Bcr-Abl	*Bcr-Abl* gene
BCR-ABL	BCR-ABL protein
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony-forming-unit
CLL	Chronic lymphocytic leukemia

Abbreviation	Description
CML	Chronic myelogenous leukemia
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitors
DAPI	4`,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimetylsulfoxide
DNA	Desoxyribunucleic acid
E	Embryonic
EE	Environmental enrichment
env	Envelope
F1	First filial generation
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum

Abbreviation	Description
FELASA	Federation of European Laboratory Animal Science Associations
FITC	Fluoresceinisothio-cyanate
FL	Fetal liver
GF	Growth factors
GFP	Green fluorescent protein
Gpi1a/b	Glucose-6-phosphate isomerase1a /b
Gr /Gran	Granulocytes
HBSS	Hanks's buffered salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hi	High
HSC	Hematopoietic stem cells
IL	Interleukin
kd	Kilodalton
LIC	Leukemia-initiating cells
Lin	Lineage
LMPD	Lympho-myeloproliferative disorder
LSC	Leukemic stem cell
LSK	Lin- SCA-1+ KIT+
LT-HSC	Long-term repopulating hematopoietic stem cell
Lympho	Lymphocytes
MDS	Myelodysplastic syndrome
MIG	Murine stem cell virus (**M**SCV), internal ribosomal entry side (**I**RES), green fluorescent protein (**G**FP)
Mono	Monocytes
MP	Myeloid progenitor
MPP	Multipotent progenitor
MWU	Mann-Whitney-U
NB	Newborn
neg	Negative
NK	Natural killer cell

Abbreviation	Description
Nnt	Nicotinamide nucleotide transhydrogenase
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ph	Philadelphia chromosome
PI	Propidium iodite
PLT	Platelets
pos	Positive
RBC	Red blood cells
RNA	Ribonucleic acid
RT	Room temperature
SE	Social environment
SEM	Standard error of mean
SP	Spleen
SPF	Specific-pathogen-free
ST-HSC	Short-term repopulating hematopoietic stem cell
Tx	Transplantation
WBC	White blood cells
WT	Wild type
YA	Young adult
ZPF	Zentrum für präklinische Forschung
129	129S2/svHsd

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13. Publications

During this work, the following publications were generated:

- Schreck, C., Bock, F., **Grziwok, S.**, Oostendorp, R. A., & Istvanffy, R. (**2014**). Regulation of hematopoiesis by activators and inhibitors of Wnt signaling from the niche. *Ann N Y Acad Sci*, 1310, 32-43. doi: 10.1111/nyas.12384

- Rouzanna Istvánffy, Baiba Vilne, Christina Schreck, Franziska Ruf, Charlotta Pagel, **Sandra Grziwok**, Lynette Henkel, Olivia Prazeres da Costa, Johannes Berndt, Volker Stümpflen, Katharina S. Götze, Matthias Schiemann, Christian Peschel, Hans-Werner Mewes and Robert A.J. Oostendorp. (**2015**) Stroma-Derived Connective Tissue Growth Factor Maintains Cell Cycle Progression and Repopulation Activity of Hematopoietic Stem Cells In Vitro. *Stem Cell Reports*.

- Schreck, C., Istvanffy, R., Ziegenhain, C., Ruf, F., Taubenberger, A., Mende, N., Gärtner, F, Vieth, B., Pagel, C., Henkel, L., **Grziwok, S.**, Götze, K., Guck, J., Massberg, S., Waskow, C., Schiemann, M., Peschel, C., Enard, W., Oostendorp, R. . (**Submitted 2015**). Niche WNT5A is required to regenerate functional repopulating hematopoietic stem cells through the actin regulatory pathway. *Journal of Experimental Medicine*.

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