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CALM/AF10 Leukämie: Ein gewebespezifisches knock-in Mausmodell und die Analyse von BMI1 als Kollaborateur in der Leukämogenese

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For my mother Smt Shibani Datta and my father Sri Dipak Datta

Table of Contents

1. Introduction **1**
1.1 The genetic basis of leukemia: **1**
1.2 Chromosomal translocations and leukemia **1**
1.3 The causes of chromosomal translocations **2**
1.4 Possible consequences of chromosomal translocations **3**
1.5 Importance of studying chromosomal translocations **3**
1.6 The CALM/AF10 fusion **3**
1.7 Clathrin Assembly Lymphoid Myeloid gene (*CALM* or *PICALM*) **5**
1.8 AF10 (MLLT10) **6**
1.9 Mechanism of CALM/AF10 mediated leukemogenesis **7**
1.10 Mouse models of CALM/AF10 leukemia **8**
Aim 1: Analysing the requirement for tissue-specific expression of CALM/AF10 in leukemogenesis using a murine knock-in model **8**
Aim 2: Testing *BMI1* as a potential collaborator in CALM/AF10-mediated leukemogensis using a murine retroviral bone marrow transplantation model **9**

2. Materials and Methods **10**
2.1 Materials **10**
2.1.1 Chemicals 10
2.1.2 Enzymes 14
2.1.3 Primers and Probes 16
2.1.4 Antibodies and Cytokines 19
2.1.5 Buffers and Solutions: 21
.1.6 Culture Media 23
2.1.7 Antibodies: 24
2.1.8 Mammalian Cell Line: 24
2.1.9 Transgenic Mouse Lines: 24
2.1.10 Kits and Consumable Materials 25
2.1.11 Instruments 26
2.1.11 Software 27
2.2 Methods **28**
2.2.1 Methods related to animal cell culture 28
2.2.2. Microbiology Methods 38
2.2.3 Methods in molecular biology 39

3. Results **54**
3.1 Rosa26 knock-in mouse model for CALM/AF10 leukemia **54**
3.1.1 Genaration of Rosa26-loxPstoploxP-CALM/AF10 mouse line **54**
3.1.1.1 Cloning of the knock-in construct 54
3.1.1.2 Generating ES cell lines with the Rosa26 Knock-in and generation of the chimeric mouse 57
3.1.1.3 R26LSLCA mice do not express CALM/AF10 fusion without Cre protein activity60
3.1.1.4 Hematopoietic cells of CA+/Cre- mice and syngeneic wild type mice (CA-/Cre-) have a similar immunophenotype 60
3.1.2 Ubiquitous expression of CALM/AF10 using CreERT2 ..... **67**
3.1.2.1 Analyzing the efficiency of Cre recombination of the R26LSLCA allele: 70
3.1.2.2 Ubiquitous expression of CALM/AF10 after Cre-ERT2-mediated recombinatoin does not cause leukemia 72
3.1.2.3 Analysis of CA+/Cre-ER+ Tamoxifen-treated mice 72

3.1.3 Expression of the CALM/AF10 fusion gene in the entire hematopoietic compartment after Vav-Cre mediated recombination 75
3.1.3.1 Expression of the CALM/AF10 fuson gene in Vav expressing cells leads to the development of an acute myeloid leukemia..... 78
3.1.3.2 Leukocytosis and anemia in the leukemic mice: 80
3.1.3.3 Splenomegaly..... 81
3.1.3.4 Blast-like cells in the peripheral blood, bone marrow and spleen of the leukemic mice 83
3.1.3.5 Infiltration of multiple organs by the blast cells in leukemic mice..... 84
3.1.3.6 Myeloid origin of the leukemic blasts 84
3.1.3.7 The leukemic blasts in 40% of the CA+/Vav-Cre+ leukemic mice co-express myeloid and lymphoid markers..... 85
3.1.3.8 Most leukemic cells stain positive for monocytic markers..... 90
3.1.3.9 Flow cytometric analysis of the peripheral blood, bone marrow and spleen shows significant alterations in leukemic CA+/Vav-Cre+ mice..... 92
3.1.4 B cell-specific expression of CALM/AF10 using CD19-Cre and Mb1-Cre transgenic mice 96
3.1.4.1 CALM/AF10 fusion transcript is expressed in the B cells of CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice 99
3.1.4.2 The expression of the CALM/AF10 fusion gene in the B cells from the early pro-B cell stage onwards does not lead to the development of leukemia 102
3.1.4.3 Flow cytometric analysis shows normal immunophenotype of bone marrow and spleen cells in CA+/Mb1-Cre+ mice..... 102
3.1.4.4 Immunophenotypic analysis of peripheral blood, bone marrow and spleen cells from CA+/CD19-Cre+ mice shows a normal pattern..... 104
3.1.5 Comparative analysis of CA+/Vav-Cre+,CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice 107
3.1.5.1 Comparative immunophenotype of the leukemic CA+/Vav-Cre+ mice and non - leukemic CA+/Mb1-Cre+ mice and CA+/CD19-Cre+ mice 108
3.1.5.2 Comparable levels of the CALM/AF10 fusion transcript were detected in the leukemic bone marrow cells and non-leukemic cells of CA+/Mb1-Cre+ mice 111
3.1.5.3 The HoxA cluster genes and the Hox co-factor *Meis1* are overexpressed in leukemic cells from CA+/Vav-Cre+ mice..... 113
3.1.5.4..... 116
The *Hoxa* cluster and *Meis1* overexpression is only found in the leukemic cells from CA+/Vav-Cre+ mice and not in non-leukemic cells of CA+/Mb1-Cre+ mice..... 116
3.2 A murine retroviral bone marrow transplantation model to examine the role of *BMI1* in CALM/AF10-mediated leukemias 119
3.2.1 Approach 1: Retroviral expression of *BMI1* in the bone marrow cells from IgHCALM/AF10 transgenic FVB mice..... 119
3.2.1.1 The retrovirus producer cell line E86-BMI1-FLAG-YFP 120
3.2.1.2 Transplantation experiments with pMIY-BMI1 transduced IgH-CALM/AF10 bone marrow 121
3.2.1.3 Transplanted *BMI1* expressing bone marrow cells do not show any growth advantage *in vivo* 122
3.2.1.4 Eight of 12 mice from the tr-IgH-CA&BMI1 group developed leukemia 123
3.2.1.5 Engraftment of the leukemic mice from the tr-IgH-CA&BMI1 group 127
3.2.1.6 Presence of the blast cells in the peripheral blood of the leukemic mice from the tr-IgH-CA&BMI1 group 128
3.2.1.7 Flow cytometric analysis of cells from the hematopoietic organs of the leukemic mice from the tr-IgH-CA&BMI1 group 129
3.2.1.8 Immunophenotype of the two mice from the tr-IgH-CA&BMI1 group that developed T-ALL 130
3.2.1.9 Immunophenotype of tr-IgH-CA&BMI1 mice with AML..... 132
3.2.1.10 Secondary Transplantation..... 135

3.2.2. Approach 2: Co-transduction of wild type bone marrow cells with the CALM/AF10-minimal fusion gene and *BMI1* and transplantation into FVB wild type recipients... 139
3.2.2.1 The CALM/AF10 minimal fusion ... 139
3.2.2.2 Experimental procedure ... 140
3.2.2.3 Bone marrow cells transduced with pMIG-CA-MF show high engraftment potential ... 141
3.2.2.4 The mice in the CA-MF and CA-MF&BMI1 groups develop an acute myeloid leukemia after a short latency ... 142
3.2.2.5 All the leukemic mice show high WBC count and splenomegaly ... 144
3.2.2.6 All leukemic mice were highly engrafted ... 144
3.2.2.7 GFP+/YFP+ double positive leukemic cells infiltrate multiple organs in mice from the CA-MF&BMI1 group ... 146
3.2.2.8 Multiple organ infiltration with leukemic cells ... 147
3.2.2.9 The infiltrating cells have myeloid characteristics ... 147
3.2.2.10 The immunophenotypes of the leukemia from mice of the CA-MF&BMI1 and the CA-MF group are similar ... 147

4. Discussion ... 151
4.1 A knock-in mouse model with tissue-specific expression of the CALM/AF10 fusion gene ... 151
4.10 Both CA-MF together with BMI1 and CA-MF alone induce an aggressive AML in a murine bone marrow transplantation model ... 159
4.11 Evidence for collaboration of *BMI1* with CALM/AF10 in leukemogenesis ... 160
4.12 Is the BMI1 overexpression in CALM/AF10 fusion induced leukemia due to a position effect? ... 162

5. Summary ... 163

6. Zusammenfassung ... 166

7. References ... 169

8. Appendix ... 177

Acknowledgement ... 189

Curriculum Vitae ... 191

1. Introduction

1.1 The genetic basis of leukemia:

Cancer is a genetic disease. It is caused by genetic abnormalities or mutations that are commonly somatically acquired rather than inherited. Leukemia arises from the malignant transformation of hematopoietic stem or progenitor cells in the bone marrow. As a result of specific genetic abnormalities, the transformed cell exhibit unregulated growth, leading to acute or chronic leukemia. Untreated leukemia, especially acute leukemia, is invariably fatal. Approximately 29,000 adults and 2000 children are diagnosed with leukemia in United States every year (Weir et al., 2003). Leukemia, like all other cancers, is a complex disease. It is the results of a multistep process involving the acquisition of several mutations. These mutations can be of different kinds: Point mutations, i.e. single nucleotide changes such as base substitution, insertions and deletions, gross chromosomal rearrangements, which include gene deletions, gene amplification, chromosomal translocations and also epigenetic changes in the promoter and enhancer regions of genes (Lin and Aplan, 2004). Presently, there is no evidence that the mutation of a single gene can lead to a fully transformed leukemic cell. Different mutations collaborate with each other and promote abnormal cell proliferation, differentiation and survival (Gilliland and Tallman, 2002). In contrast to epithelial tumors, which often have a complex pattern of numerical and structural chromosomal aberrations, hematological neoplasms show a much simpler picture, sometimes with none or only one chromosomal abnormality such as a single balanced translocation. It is likely that fewer mutations are required to transform a hematopoietic cell than an epithelial cell. Gilliland and Tallman postulated that at least two cooperating mutations are required for leukemic transformation with one mutation causing increased proliferation and the other disrupting cellular differentiation (Gilliland and Tallman, 2002).

1.2 Chromosomal translocations and leukemia

Among all the mutations found in leukemia, recurrent chromosomal translocations have been studied most extensively. Chromosomal translocations or inversions are found in up to 65% of the acute and chronic leukemias (Raimondi, 1993; Solomon et al., 1991). Chromosomal translocations involve the exchange of chromosomal regions between non-homologous chromosomes. Translocations are classified as reciprocal or non-reciprocal. In reciprocal or balanced translocations no genetic material is lost, while non-reciprocal or unbalanced translocations lead to the net loss or gain of genetic material.

1.3 The causes of chromosomal translocations

Chromosomal translocation can originate from double strand DNA breaks. If two breaks occur simultaneously on different chromosomes, and the two breaks from non-homologous chromosomes are joined together, a chromosomal translocation results. Several molecular mechanisms have been proposed to explain recurrent translocations found in leukemia. These mechanisms include: illegitimate V(D)J recombination; short or long interspersed nuclear element (SINEs, LINEs) mediated recombination; DNA topoisomerase II subunit exchange, homologous and error prone non-homologous end joining after a DNA double strand break. Some regions in the genome also show an increased susceptibility to DNA double strand breaks, for example regions containing stretches of pyrimidine-purine repeat sequences, scaffold and matrix attachment regions, and DNA topoisomerase II cleavage sites (Aplan, 2006). However, there still remain many questions regarding the causes of recurrent translocations and their occurrence in specific types of leukemia. Recently, using next-generation sequencing techniques, very complicated genomic rearrangements have been analyzed in detail in certain cancer cells. In these rearrangements, a large number of translocations, deletions, and inversion involving a single or several chromosomes were found in a cell. It is thought that these complicated events result from the faulty repair of a single "chromosome shattering" event. The literal translation of "chromosome shattering" into Greek is "chromothripsis" (Stephens et al., 2011). One of the causes of chromothrispsis might be a chromosome segregation error during mitosis with the formation of micronuclei in which the localized shattering and re-ligation of DNA fragments take place (Holland and Cleveland, 2012; Stephens et al., 2011).

Several inherited syndromes are associated with increased genetic instability and predispose to chromosomal translocations. For example, patients with ataxia telangiectasia, which is caused by a mutation in the *ATM* gene, are prone to acquire chromosomal rearrangements involving T cell or immunoglobulin antigen-receptor loci (Rotman and Shiloh, 1998). ATM is a protein, which plays an important role in DNA double strand break recognition and repair. Nijmegen's breakage syndrome is another genetic instability disease caused by mutations in the *NBS1* gene, which encodes the DNA double strand break repair enzyme NBS1. Patients with this disease are also predisposed to chromosomal translocation and lymphoid malignancies (Digweed and Sperling, 2004).

1.4 Possible consequences of chromosomal translocations

Translocations can have either of two consequences. They can bring the coding region of one gene into close proximity of the promoter or enhancer region of another gene resulting in the overexpression or ectopic expression of the former gene. The example of such an event is translocation t(8;14)(q24;q32) found in Burkitt's lymphoma and the t(14;18)(q32;q21) found in follicular lymphoma. As a result of these translocations, the expression levels of *MYC* and *BCL2,* respectively, are greatly increased since they are juxtaposed to the immunoglobulin heavy chain gene enhancer, which are very active in B cells.

Translocations can also give rise to chimeric or fusion genes which act as oncogenes. An example of such an event is the translocation t(9,22) (Rowley, 1973), which results in the formation of the oncogenic fusion BCR/ABL (Heisterkamp et al., 1990)

1.5 Importance of studying chromosomal translocations

Chromosomal translocations have been shown to be causal in hematological malignancies and also in several solid tumors. The analysis of recurrent, non-random chromosomal translocations has been very useful in understanding the pathogenesis of hematological malignancies leading to improved diagnosis and classifications and to the development of novel targeted therapies (Rowley, 1999, 2001).

1.6 The CALM/AF10 fusion

The t(10;11)(p13;q14) translocation is a rare but recurring translocation, which results in the in-frame fusion of the the *AF10* gene on chromosome 10, band p13, with the *CALM* gene on chromosome 11, band q14, generating both a CALM/AF10 fusion transcript and a CALM/AF10 fusion protein and a reciprocal AF10/CALM fusion (Dreyling et al., 1996). The CALM/AF10 fusion transcript encodes for almost the entire CALM and AF10 proteins, whereas the reciprocal AF10/CALM fusion transcript encodes only for the first 80 amino acids from AF10 and last 4 amino acids from the CALM gene due to the asymmetric positioning of the breakpoints in the *AF10* and *CALM* genes. The CALM/AF10 fusion transcript has been detected consistently in all patients with a t(10;11)(p13;q14), whereas the reciprocal AF10/CALM transcript is not always present. This suggests that the CALM/AF10 fusion transcript and the resulting CALM/AF10 fusion protein is the important driving force of the leukemias in these patients (Bohlander et al., 2000;Carlson et al., 2000; Narita et al., 1999). Due to alternative breakpoints, both in *AF10* and in *CALM*, several different CALM/AF10 fusion transcripts have been detected in patients (Bohlander et al., 2000;Kumon et al., 1999; Silliman et al.,

1998). Four breakpoints in the *AF10* gene and at least two breakpoints in the *CALM* gene have so far been reported. There is no indication that the different CALM/AF10 fusion transcripts result in different disease phenotypes. Identical fusion transcipts have been reported in AML and in ALL patients (Bohlander et al., 2000).

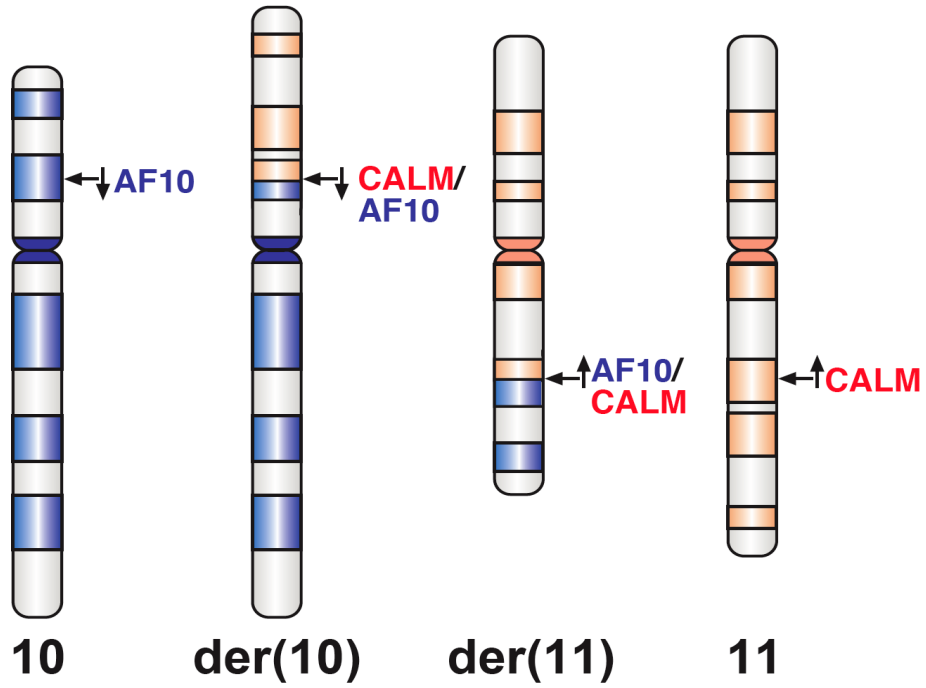


Fig 1.1: Translocation t(10,11)(p13;q14) leading to the in-frame fusion of the *CALM* gene on chromosome 11 with the *AF10* gene on chromosome 10. The arrows indicate the breakpoints and the direction of the gene. (Figure kindly provided by Stefan Bohlander)

Originally, the CALM/AF10 fusion gene was cloned from the monocytic cell line U937 (Dreyling et al., 1996). The U937 cell line was derived from a patient with diffuse histiocytic lymphoma with some monocytic features (Sundstrom and Nilsson, 1976). The CALM/AF10 fusion is found in patients with undifferentiated acute myeloid leukemia (AML) (FAB M0, M1), acute lymphoblastic leukemia (ALL), and malignant lymphoma (Dreyling et al., 1998; Kumon et al., 1999; Narita et al., 1999). The CALM/AF10 fusion has been identified in mixed-lineage leukemia with the co-expression of lymphoid (T-cell) and myeloid antigens (Kumon et al., 1999; Narita et al., 1999). Some cases of more mature AML with CALM/AF10 fusion have also been reported (FAB M4, M5, M7)(Abdou et al., 2002; Carlson et al., 2000; Jones et al., 2001; Nakamura et al., 2003; Salmon-Nguyen et al., 2000). The CALM/AF10 fusion transcript is predominantly found in children and young adult patients (Dreyling et al., 1998; Kobayashi et al., 1997; Kumon et al., 1999; Silliman et al., 1998) and is frequently associated with difficult to treat cases and a very poor prognosis (Kumon et al.,

1999; Narita et al., 1999). In T-ALL with TCR γδ rearrangement, CALM/AF10 is the most frequent chromosomal abnormality with up to 30% cases being positive for this fusion (Asnafi et al., 2003).

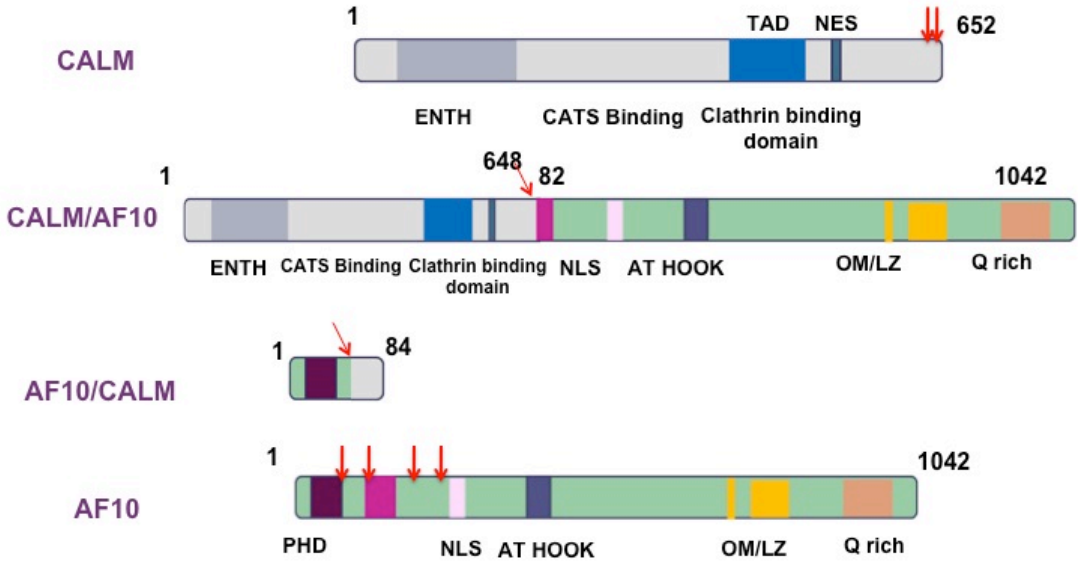


Fig 1.2: Schematic diagram of the full length CALM and AF10 gene with their domains. Red arrows indicate the different breakpoints on each gene. The CALM/AF10 fusion and the reciprocal AF10/CALM fusion, which is rarely present among patients, are also shown. ENTH-Epsin N terminal homology domain, NES-nuclear export signal; OM/LZ- octapeptide motif/leucine zipper; PHD- plant homeo domain; NLS- nuclear localization signal, TAD-transactivation domain, CATS-CALM/AF10 interacting protein expressed in thymus and spleen

1.7 Clathrin Assembly Lymphoid Myeloid gene (*CALM* or *PICALM*)

The *CALM* or *PICALM* gene encodes for a 652 amino acid long protein with extensive homologies to the neuronal-specific endocytic protein AP180 (Morris et al., 1993). It is a ubiquitously expressed protein, which is predominantly cytoplasmic but is known to shuttle between nucleus and cytoplasm (Archangelo et al., 2006; Tebar et al., 1999; Vecchi et al., 2001). CALM plays a role in the endocytic machinery, specifically in the adaptor protein 2 (AP2)-mediated formation of the clathrin-coated vesicles from cell membranes (Tebar et al., 1999). It interacts with the clathrin heavy chain via its C-terminal third and with phosphoinositides via its AP180 N terminal homology domain (ENTH) facilitating the assembly of clathrin triskelia into clathrin cages (Ford et al., 2002; Ford et al., 2001). Overexpression and downregulation of CALM have been implicated in the inhibition of receptor-mediated endocytosis and impaired endosome trafficking in the trans-golgi network (Huang et al., 2004; Tebar et al., 1999). *fit1* mutant mice carry a nonsense point mutation in the mouse ortholog of the *CALM* gene, and these mice show hematopoietic abnormalities, growth retardation and

abnormal iron metabolism and a shortened life span (Klebig et al., 2003). The human *CALM* gene has also been shown to play an important role in iron homeostasis and cell proliferation (Scotland et al., 2012). The clathrin-binding domain at the carboxy terminus of CALM is critical for CALM/AF10-mediated leukemogenesis. (Deshpande et al., 2011; Stoddart et al., 2012). Another recent study showed that the nuclear export signal (aa 544-553) within the clathrin-binding domain of CALM is important and sufficient for leukemogenesis when fused to the AF10 protein. (Conway et al., 2013)

1.8 AF10 (MLLT10)

AF10 (*MLLT10* or ALL1-fused gene from chromosome 10) is a putative transcription factor, which was identified as a fusion partner of *MLL* (Mixed Lineage Leukemia), the human homolog of the *Drosophila Trithorax* gene (Yu et al., 1998). The t(10;11)(p13;q23) MLL-AF10 fusion was initially detected in a patient diagnosed with AML-M5 (Chaplin et al., 1995). In this translocation, *AF10* and *MLL* are often involved in complex rearrangements like inversion/insertion because they are transcribed in opposite directions with respect to the centromere-telomere orientation (Beverloo et al., 1995). The *MLL* gene on 11q23 is frequently involved in translocations in leukemia and has over 50 different partner genes (Meyer et al., 2013). Interestingly, *AF10* is the only fusion partner of *MLL*, which independently rearranges with a third gene, *CALM* (Dreyling et al., 1996). Of note, the CALM/AF10 fusion is associated both with myeloid and lymphoid hematological malignancies while the MLL/AF10 fusion is exclusively found in AML (Chaplin et al., 1995).

AF10 encodes for a 1084 amino acid long protein. Like the MLL protein, AF10 also has a conserved LAP/PHD finger domain at its N terminal end (Aasland et al., 1995; Saha et al., 1995). In the C terminal region, AF10 contains an octapeptide motif followed by a leucine zipper (OM/LZ) domain. AF10 has a nuclear localization signal (NLS) (Chaplin et al., 1995; Linder et al., 2000). Due to breakpoint variability, there are different MLL/AF10 and CALM/AF10 fusion transcripts, however, the OM/LZ domain of AF10 is retained in all fusions (Bohlander et al., 2000; Narita et al., 1999). The OM/LZ domain of AF10 has been shown to interact with many interesting proteins, which play important roles in the hematopoietic development and chromatin regulation. The histone H3 lysine 79 (H3K79) methyletransferase hDOT1L interacts with AF10 via the OM/LZ domain. CALM/AF10-mediated leukemia is marked by a global hypomethylation of H3K79 but locally H3K79 hypermethylated loci like certain *HoxA* loci (Lin et al., 2009; Okada et al., 2005; Okada et al., 2006). It is proposed that the

deregulation of the global H3K79 methylation in CALM/AF10 leukemia is due to the disruption of the AF10 mediated association of hDOT1L with chromatin (Lin et al., 2009). Among the other interactors of the OM/LZ domain of AF10, the lymphoid transcriptional regulator Ikaros (IKZF1) is significant (Greif et al., 2008). Ikaros interacts with chromatin remodeling factors and is known to play a key role in the transcriptional regulation and cell cycle control during lymphoid differentiation (Georgopoulos et al., 1992; Gomez-del Arco et al., 2005; Gomez-del Arco et al., 2004). The OM/LZ domain of AF10 is required for CALM/AF10-mediated leukemogenesis (Deshpande et al., 2011). Another OM/LZ interaction partner of AF10 is GAS41; GAS41 is known to interact with INI1, the human homologue of yeast SNF2, which is a part of the SWI/SNF complex (Debernardi et al., 2002). SWI/SNF is a conserved multi-protein complex, which plays a role in chromatin remodeling in an ATP dependent manner. Among the other interactors of AF10, FLRG is interesting from the point of view of the hematopoiesis. FLRG is a secreted glycoprotein, which participates in erythrocyte commitment. AF10 interacts with FLRG via its PHD domain and this interaction enhances AF10 mediated transcription (Forissier et al., 2007; Maguer-Satta and Rimokh, 2004). In a recent publication, *AF10* was shown to play a key role in the survival of undifferentiated hematopoietic cells (Chamorro-Garcia et al., 2012).

1.9 Mechanism of CALM/AF10 mediated leukemogenesis

Very little is known about the molecular mechanism of how the CALM/AF10 fusion leads to leukemia (Caudell and Aplan, 2008). It is known that the interaction of AF10 with H3K79 methyltransferase hDOT1L is critical for the pathogenesis of MLL-AF10 mediated leukemia. Mistargeting of hDOT1L to the *Hoxa9* locus potentially plays a key role in the leukemogenesis of MLL-AF10 (Okada et al., 2005). Recently, our group reported that the expression the CALM/AF10 fusion gene results in the global reduction of H3K79 methylation in human and murine leukemic cells (Lin et al., 2009). This reduction of H3K79 methylation leads to global genomic instability (Lin et al., 2009). It has also been shown by several groups that overexpression of *Hoxa* cluster genes is a molecular signature of CALM/AF10 driven leukemias (Caudell et al., 2007; Dik et al., 2005; Okada et al., 2006). Together with the *Hoxa* cluster genes, the *Hox* cofactor *MEIS1* and the polycomb group gene *BMI1* were also found to be upregulated in CALM/AF10 positive T-ALL patients (Dik et al., 2005; Mulaw et al., 2012). Our group also reported recently, that CALM/AF10-positive leukemias show a global upregulation of genes involved in DNA repair and chromatin assembly (Mulaw et al., 2012).

1.10 Mouse models of CALM/AF10 leukemia

In a bone marrow transplantation model, overexpression of CALM/AF10 fusion resulted in the development of an aggressive AML with a median latency of 10 weeks. A proportion of the leukemic cells showed co-expression of the lymphoid marker B220 with the myeloid markers Gr1 and Mac1 (MM) (Deshpande et al., 2006). B220 is the murine homolog of human CD45R, a pan B lymphocyte specific marker, which is expressed on early and mature B cells. For the identification of the 'leukemia propagating cell', the leukemic cells were subdivided into 3 groups on the basis of their surface marker expression: B220+/MM-; B220+/MM+ and B220-/MM+. Using a limiting dilution transplantation approach, Deshpande and colleagues were able to show that the frequency of the 'leukemia propagating cell' was highest in the B220+/MM- cell population with about 1 in 34 cells (Deshpande et al., 2006). This intriguing finding led to the hypothesis that the CALM/AF10 fusion gene induces an aberrant myeloid development of B220+ early lymphoid progenitors (Deshpande et al., 2006).

However, when CALM/AF10 was expressed as a transgene in all hematpoietic cells under the control of the *Vav* promoter in mice, only 40% of the animals developed acute myeloid leukemia with a long latency of 12 months (Caudell et al., 2007). In the leukemic cells the Hoxa cluster genes were overexpressed and an impaired thymus development was noted. The results from this CALM/AF10 transgenic model are quite different from the bone marrow transplantation model where all mice developed leukemia after a very short latency.

A former member of our group, Alexander Krause, generated two transgenic CALM/AF10 mouse lines, in which the CALM/AF10 fusion is expressed from B cell specific IgH enhancer/promoter or from the proximal Lck promoter. None of the transgenic mouse lines developed leukemia even after a long observation period of up to 1 ½ years (Krause, 2006).The results from the different CALM/AF10 models strongly sugggest that it is critical for the CALM/AF10 fusion to be expressed in the correct hematopoietic cell type for leukemia development.

Aim 1: Analysing the requirement for tissue-specific expression of CALM/AF10 in leukemogenesis using a murine knock-in model

To investigate at which stage of the hematopoietic development the expression of the CALM/AF10 fusion would be leukemogenic, we generated a knock-in mouse model of the CALM/AF10 fusion. In the Rosa26 loxP-stop-loxP-CALM/AF10 (R26LSLCA) mouse line, the CALM/AF10 cDNA is preceded by a loxP flanked transcriptional stop

cassette and integrated into the Rosa26 locus. Using tissue-specific expression of the Cre recombinase or a Tamoxifen controllable version of the Cre recombinase (Cre/Estrogen receptor fusion), the stop cassette can be removed, enabling the expression of the CALM/AF10 fusion from the Rosa26 promoter. By crossing R26LSLCA mouse line with lines that express Cre in a tissue-specific manner, we were able to achieve cell type-specific expression of the CALM/AF10 fusion protein.

Aim 2: Testing *BMI1* as a potential collaborator in CALM/AF10-mediated leukemogensis using a murine retroviral bone marrow transplantation model

BMI1, a polycomb group gene, was reported to be specifically upregulated in both human and murine CALM/AF10-positive leukemias (Dik et al., 2005; Mulaw et al., 2012). *BMI1* was originally identified at a retroviral integration site that led to leukemia development in an Eμ-Myc transgenic mouse model (van Lohuizen et al., 1991). *BMI1* has been shown to be important for the self-renewal capacity of hematopoietic stem cells and to play a role in leukemogenesis (Lessard and Sauvageau, 2003; Park et al., 2003; Rizo et al., 2010).

To determine the role of *BMI1* in CALM/AF10 leukemia, we expressed *BMI1* retrovirally together with CALM/AF10. We used two approaches. In the first approach, *BMI1* was retrovirally over-epressed in IgH-CALM/AF10 transgenic bone marrow cells and the retrovirally-modified cells were transplanted into wild type mice. In the second approach, we retrovirally co-expressed both *BMI1* and CALM/AF10 in a murine bone marrow transplantation model.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Table 2.1 List of chemicals

Name	IUPAC Name	Molecular Formula	Molar mass (g/mol)	Company
Agarose	(14)-3,6-anhydro-α-L-galactopyranosyl-(13)-β-D-galactopyranan		167.6 ± 7.8 x 10^3	Carl Roth ® GmbH, Germany
Acetic Acid		$C_2H_4O_2$	60.05	Carl Roth ® GmbH, Germany
Acrylamid Rotiphorese ® Gel 30(37.5:1)	Prop-2-enamid	C_3H_5NO	71.08	Carl Roth ® GmbH, Germany
Aprotinin		$C_{284}H_{432}N_{84}O_{79}S_7$	6511.51	Sigma Aldrich ® USA
Ammonium Chloride Solution		NH_4Cl	53.49	Stemcell ™ Technologies, USA
Ammonium Persulfate		$(NH4)_2S_2O_8$	228.18	Sigma Aldrich ® USA
Bromophenol Blue	2,6-dibromo-4-[3-(3,5-dibromo-4-hydroxyphenyl)-1,1-dioxo-3-benzo[c]oxathiolyl]phenol	$C_{19}H_{10}BR_4O_5S$	669.96	NEB®, USA
Bovine Serum Albumin (BSA)				Sigma-Aldrich®,

				USA
Bradford Reagent (BioRad Protein Assay reagent)				BioRad Laboratory GmbH, Munich.Germany.
β-Mercaptoethanol	2-hydroxy-1-ethanethiol	C_2H_6SO	78.13	Sigma Aldrich ® USA
Chloroform		$CHCl_3$	119.38	Carl Roth ® GmbH, Germany
Ciprobay				Bayer Schering Pharma AG
DEPC	Diethylpyrocarbonate	$C_6H_{10}O_5$	162.14	Invitrogen
DMSO	Dimethyl Sulfoxide	C_2H_6OS	78.13	Carl Roth ® GmbH, Germany
DNazol				Invitrogen, Germany
dNTP mix (10mM mix of dATP, dGTP, dCTP, dTTP)				Invitrogen, USA
DPBS				Pan Biotech, Germany
DTT (Dithiothreitol)	2S,3S-1,4-Bis-sulfanylbutane-2,3-diol	$C_4H_{10}O_2S_2$	154.26	Invitrogen, USA
ECL™ Plus Western Blotting Detection reagent				Amersham ,Germany
EDTA (Ethylenediaminetetraacetic acid)	2[2(bis(carboxymethyl)amino)ethyl(carboxymethyl)amino] acetic acid	$C_{10}H_{16}N_2O_8$	292.25	Carl Roth ® GmbH, Germany
Ethanol	-	CH_3CH_2OH	46.068	Carl Roth ® GmbH, Germany
5-FU	5-Fluorouracil	$C_4H_3FN_2O_2$		Medac

FBS (Fetal Bovine Serum)				Biochrome AG, Berli,. Germany
Formaldehyde (37%)		CH_2O	30.03	Carl Roth ® GmbH, Germany
Giemsa Solution				Merck Darmstadt, Germany
Glycerol		$C_3H_8O_3$	92.09	Carl Roth ® GmbH, Germany
Heparin (Heparin Sodium injectable solution)				B.Braun Melsungen AG
Hydrochloric Acid		HCl	36.46	Merck, Darmstadt, Germany
Hydrogen Peroxide		H_2O_2	34.0147	Merck, Darmstadt, Germany
Isopropanol	Propan-2-ol	C_3H_8O	60.10	Merck, Darmstadt, Germany
Leupeptin	Ac-Leu-Leu-Arg-H; N-acetyl-L-leucyl-L-leucyl-D,L-argininaldehyde		426.6	Sigma Aldrich ® USA
May-Grünwald's eosine methylene blue solution				Merck, Darmstadt, Germany
Methanol		CH_4O	32.04	Carl Roth ® GmbH, Germany
Milk powder				Carl Roth ® GmbH, Germany
Phenol/Chlorform/isoamylalcohol(25:24:1)				Invitrogen.
Pefabloc	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	$C_8H_{10}FNO_2S$	203.235	Sigma-Aldrich®, USA

Penicilin/Streptomycin				Pan Biotech, Germany
Pepstatin	3-hydroxy-4-[2-[3-hydroxy-6-methyl-4-[3-methyl-2-[3-methyl-2-(3-methylbutanoylamino)butanoyl]amino-butanoyl]amino-heptanoyl]-aminopropanoylamino]-6-methyl-heptanoic acid	$C_{34}H_{63}N_5O_9$	685.89	Sigma-Aldrich® USA
Potassium Chloride		KCl	74.55	Carl Roth ® GmbH, Germany
Propidium Iodide		$C_{27}H_{34}I_2N_4$	668.39	Sigma-Aldrich®, USA
Protamine Sulfate				Sigma-Aldrich®, USA
Proteinase Inhibitor cocktail				Sigma-Aldrich®, USA
Sodium Acetate		$C_2H_3NaO_2$	82.03	Carl Roth ® GmbH, Germany
Sodium Chloride		NaCl	58.44	Carl Roth ® GmbH, Germany
SDS	Sodium dodecyl sulfate	$NaC_{12}H_{25}SO_4$	288.38	Carl Roth ® GmbH, Germany
Sodium Hydroxide		NaOH	39.99	Carl Roth ® GmbH, Germany
di-Sodium Hydrogen Phosphate		Na_2HPO_4	141.96	Carl Roth ® GmbH, Germany

Monosodium phosphate		NaH_2PO_4	119.98	Carl Roth ® GmbH, Germany
TEMED	Tetramethylthylendiamin	$C_6H_{16}N_2$	116.2	Sigma-Aldrich® USA
Tris	2.Amino-2-hydroxymethyl-propane-1,3-diol	$C_4H_{11}NO_3$	121.14	Carl Roth ® GmbH, Germany
Trizol®				Invitrogen, Germany
Trypsin EDTA				PAN Biotech
Trypan Blue				Invitrogen, Germany
Tween 20	Polyoxyethelene(20) sorbitan monolaurate	$C_{58}H_{114}O_{26}$	1227.54	Sigma-Aldrich® USA
Xylene Cyanol		$C_{25}H_{27}N_2O_6S_2Na$	538.61	Carl Roth ® GmbH, Germany

2.1.2 Enzymes

Table 2.2: List of Enzymes

Name	Function	Unit Definition	Company	Assay Condition
Benzonase	Degrades all forms of DNA and RNA. Used to remove nucleic acid from protein samples	Amount of enzyme required to digest 37μg of DNA completely under standard reaction condition	Sigma-Aldrich®, USA	Mg2+ Conc:1-2mM, pH 8.0-9.0 Temperature: 37°C β-ME 0-100mM Monovalent Cation Conc: 0-20mM
DNase I	Eliminates DNA from RNA preparations.	One unit increases the absorbance of a high molecular weight DNA solution at a rate	Invitrogen	M NaAc(pH 5.0) 5mM $MgCl_2$ 50μg/ml calf

		0.001A_{260}units/min/ml of reaction mixture at 25°C		thymus DNA
Proteinase K	Proteinase K is a non-specific, subtilisin-related serine protease with a very high specific activity. Used to remove protein from DNA preparation	One unit is defined as the amount of enzyme required to liberate folin-positive amino acids and peptides corresponding to 1 µmol of tyrosine in 1 minute at 37°C in a total reaction volume of 250 µl	NEB	30 mM Tris HCl, pH 8.0
RNaseH	Specifically degrades RNA strands in a DNA-RNA hybrid	One unit is defined as the amount of RNase H that solubilizes 1 nmol poly(A) in 20 minutes at +37°C	Invitrogen	220 mM Tris-HCl, 0.1 M KCl, 10mM MgCl2, 0.1 mM DTT, 5%(W/V) Sucrose, 0.5nmol 3H labelled Poly(A)poly(dT) Temperature: 37°C
Thermoscript Reverse Transcriptase	Synthesizes DNA from RNA template	One unit incorporates 1 nmol of dTTP into acid-precipitable material in 10 min at 37°C using poly(A)ïoligo(dT)25 as template-primer .	Invitrogen	250 mM Tris acetate(pH 8.4), 375mM potassiun acetate,40mM magnesium acetate
Taq DNA Polymerase	The enzyme has a template independent terminal transferase that adds 3' deoxyadenosine to the product end and has a 5'-3' exonuclease activity (but no 3'-5' exonuclease activity)	One unit of Taq DNA polymerase incorporates 10 nmol of deoxyribonucleotide into DNA in 30 minutes at 74°C.	Invitrogen	

2.1.3 Primers and Probes

Table 2.3 List of Primers:

Name	Sequence	Purpose
pMIG F	5'CCCTTGAACCTCCTCGTTCGACC	Amplification of the insert in the MSCV vector.
pMIG R	5' CGTCGCCGTCCAGCTCGACCA	Amplification of the insert in the MSCV vector.
C/A brkpnt F	5' GCAATCTTGGCATCGGAAAT	Amplification of the breapoint region of the CALM/AF10 fusion.
C/A brkpnt R	5'GCCTGTCGACATCCATGTTT	Amplification of the breapoint region of the CALM/AF10 fusion.
Vav Cre F	5' GACAGGCAGGCCTTCTCTGAA	Genotyping Vav cre transgenic mice.
Vav Cre R	5'CTTCTCCACACCAGCTGTGGA	Genotyping Vav cre transgenic mice.
Mb1 Cre F	5'ACCTCTGATGAAGTCAGGAAGAAC	Genotyping Mb1 Cre transgenic mice.
Mb1 Cre R	5'GGAGATGTCCTTCACTCTGATTCT	Genotyping Mb1 Cre transgenic mice.
CD19 Cre F	5'AACCAGTCAACACCCTTC	Genotyping CD19 Cre transgenic mice.
CD19 Cre R	5'CCAGACTAGATACAGACCAG	Genotyping CD19 Cre transgenic mice
Cre 7	5' TCAGCTACACCAGAGACG	Genotyping CD19 Cre transgenic mice

Cre-ER F	5' AATCGCCATCTTCCAGCAGG	Genotyping Rosa26 Cre ER mice
CRE-ER R	5' GATCGCTGCCAGGATATACG	Genotyping Rosa26 Cre ER mice
Beta act F	5' TGGGAATGGGTCAGAAGGAC	Amplification of beta actin from cDNA
Beta act R	5' CGGTCAGGATCTTCATGAGG	Amplification of beta actin from cDNA
ROSA CALM fu F	5'CGGTTGAGGACAAACTCTTCG	Detection of the recombination efficiency in Rosa26LSLCA mice
ROSA CALM fu R	5'TGCCAACTGTGGGATGTTCAC	Detection of the recombination efficiency in Rosa26LSLCA mice

Table 2.4: Primers and Probes used in Real-time PCR

Name	Sequence 5' to 3'	Purpose
CA brkpnt Probe (MGB Probe)	6-FAM-GGAGCACAGAGATGTGAA	Detection of CALM/AF10 fusion transcript along the break-point
RTCAFWD	AAACCCCTTTGGCCCTGTA	Detection of CALM/AF10 fusion transcript along the break-point
RTCAREV	AAGCTCCATCCTTATGGGGAC	Detection of CALM/AF10 fusion transcript

		along the break-point
Bmi Probe (MGB Probe)	6-FAM-TGTGATTCCAGGTTCA	Detection of mouse *Bmi1* transcript
Bmi1 FWD	TGGAGACCAGCAAGTATTGTCCTA	Detection of mouse *Bmi1* transcript
Bmi1 REV	TTATGTTCAGGAGTGGTCTGGTTTT	Detection of mouse *Bmi1* transcript
HoxA5 Probe (MGB Probe)	6-FAM AGGCTCATGATTAAAAG	Detection of mouse *HoxA5* transcript
HoxA5 FWD	ACAGGCGCTATAATGGCAATAAA	Detection of mouse *HoxA5* transcript
HoxA5 Rev	CCCTGTTCTCGTTGCCCTAA	Detection of mouse *HoxA5* transcript
HoxA7 Probe (MGB Probe)	6-FAM CCATTGGCTTCAGCC	Detection of mouse *HoxA7* transcript
HoxA7 FWD	TGTGACTTTCCCACCCTCTGT	Detection of mouse *HoxA7* transcript
HoxA7 Rev	CCAGAGGCCATTTCCAGAAC	Detection of mouse *HoxA7* transcript
HoxA9 Probe (MGB Probe)	6-FAM ATCCTGCGGTTCTGG	Detection of mouse *HoxA9* transcript
HoxA9 FWD	CGGTCCTTGTTGATTTTCTTCAT	Detection of mouse *HoxA9* transcript
HoxA9 Rev	CGAAAGGCAGGTCAAGATCTG	Detection of mouse *HoxA9* transcript

HoxA10 Probe (MGB Probe)	6-FAM CAGTAGGAGCTCTCTTC	Detection of mouse *HoxA10* transcript
HoxA10 FWD	TTGTCCGCAGCATCGTAGAG	Detection of mouse *HoxA10* transcript
HoxA10 Rev	TCGTGTTCTTTTGCGCAGAA	Detection of mouse *HoxA10* transcript
Meis1 Probe (MGB Probe)	6-FAM CATTGTTGGTGACTGCAG	Detection of mouse *Meis1* transcript
Meis1 FWD	ACTGCCTGCAACAGCTGATTAA	Detection of mouse *Meis1* transcript
Meis1 Rev	AATCGGGAAGGATGGGAAAA	Detection of mouse *Meis1* transcript

2.1.4 Antibodies and Cytokines

Table 2.5 : Antibodies

Name	Company	Dilution
PE-Rat Anti-Mouse CD19	BD Pharmingen	1:200
APC Hamster Anti-MouseCD3e	BD Pharmingen	1:200
APC Rat Anti-Mouse CD43	BD Pharmingen	
PE-Rat Anti-Mouse CD45R/B220	BD Pharmingen	1:200
PE Rat Anti-Mouse Ly-6G and Ly-6C/Gr1	BD Pharmingen	1:200
PE Rat Anti-Mouse CD11b/Mac1	BD Pharmingen	1:200
APC Rat Anti-Mouse CD11b/Mac1	BD Pharmingen	1:200
APC Rat Anti-Mouse Ly-6G and Ly-6C/Gr1	BD Pharmingen	1:200

and Ly-6C/Gr1		
PE Rat Anti-Mouse TER-119	BD Pharmingen	1:200
APC Rat Anti-Mouse CD45R/B220	BD Pharmingen	1:200
PE Rat Anti-Mouse Ly-6A/E/Sca1	BD Pharmingen	1:200
APC Rat Anti-Mouse CD117/c-Kit	BD Pharmingen	1:200
PE Rat Anti-Mouse CD4	BD Pharmingen	1:200
APC Rat Anti-Mouse CD8a	BD Pharmingen	1:200
Mouse Anti Bmi1	Abcam	1:2000
Mouse Anti FLAG	Sigma	1:5000
Goat Anti-Mouse-IgG-HRP	Santa Cruz Biotechnology	1:3000
F4/80 (BM8)-FITC	Santa Cruz Biotechnology	1 μg per $1X10^6$ cells
PE Rat anti mouse IgM	BD Pharmingen	1:200
PE Rat Anti Mouse CD24	BD Pharmingen	1:200
FITC Rat Anti-Mouse CD23	BD Pharmingen	1:500
CD 45R Micro-beads, Mouse	Miltenyibiotec	10 μl beads for 10^7 cells
CD43 Microbeads, Mouse	Miltenyibiotec	10 μl beads for 10^7 cells

Table 2.6: Murine Cytokines

Name	Company
murine Interleukin 3 (mIL3)	Tebu-bio, Offenbach, Germany
murine Interleukin 6 (mIL6)	Tebu-bio, Offenbach, Germany
murine SCF	Tebu-bio, Offenbach, Germany
mM CS	Tebu-bio, Offenbach, Germany
mGM-CSF (Lyophilized)	Tebu-bio, Offenbach, Germany

2.1.5 Buffers and Solutions:

Table: 2.7 Buffers and Solutions used in Molecular Biology

Name	Component
Lysis Buffer for mouse tails:	5 mM EDTA pH 8.0 200 mM NaCl 100 mM Tris HCl pH 8.5 100μg/ml Proteinase K (added freshly) Storage: RT
FACs Buffer:	PBS 1% FCS 1 mg/l propidium iodide Storage: Light protected, at 4°C
MACs Buffer:	PBS pH 7.2 0.5% BSA 2mM EDTA 0.09% Azide Storage: Light protected, at 4°C
Cell Freezing Buffer:	10% DMSO in FBS (V/V) Made freshly, can be stored at 4°C for a month
50X TAE Buffer	242 gm Tris base 57.1 mL Acetic Acid 100mL 0.5M EDTA Volume adjusted to 1 L, pH 8.5 (Adjusted using KOH) Storage: at 4°C
TE Buffer:	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0 Storage: at 4°C
P1 Buffer for miniprep	50 mM Tris.Cl pH 8.0 10 mM EDTA 100 μg/ml Rnase A
P2 Buffer for miniprep	200 mM NaOH 1% SDS (W/V), always prepared fresh
P3 Buffer for miniprep	3 M potassium acetate pH 5.5

Table:2.8 Buffers Used in Protein Chemistry

Name	Component
Freeze- thaw Lysis Buffer (Protein Extraction)	600 mM KCl 20 mM Tris-Cl (pH 7.8) 20% Glycerol Storage: at 4°C Added freshly: 0.4 mg/ml Pefabloc 10 μg/ml Leupeptin 10 μg/mL Pepstatin 5 μg/mL Aprotinin
Tris-HCl	1.5 M, pH 8.8 Tris powder in d_2H_2O, adjusted pH with HCL 1 M, pH 6.8 Tris powder in d_2H_2O, adjusted pH with HCL 1 M, pH 8.0 Tris powder in d_2H_2O, adjusted pH with HCL 1 M, pH 7.5 Tris powder in d_2H_2O, adjusted pH with HCL Storage at 4°C
EDTA	0.5M EDTA in d_2H_2O, pH 8 adjusted by KOH Storage at 4°C
10% SDS	Sodium Dedocyl Sulfate in d2 H2 O (W /V) Storage at 4°C
10%APS	Ammonium persulfate in d_2H_2O, (W/V) (Aliquots stored at -20°C)
2X Laemmli Sample Loading Buffer	10% β -mercaptoethanol 6% SDS 20% Glycerol 0.2 mg/ml Bromophenol blue 0.025x Laemmli stacking buffer (optional) Storage: RT, up to 2 months
1X TBS	10 mM Tris/HCl pH 8.0 150 mM NaCl Storage: RT

10x Tris-Glycine Electrophoresis Buffer	250 mM Tris 1.92 M Glycine 1% SDS Storage: RT
Gel Transfer Buffer (Western Blotting)	25 mM Tris 192 mM Glycine 20% Methanol Storage: 4°C
1X TBST	1X TBS 0.1% Tween 20 Storage RT
Blocking Solution	5% dried milk powder (W/V) in TBST
Stripping Solution	62.5 mM Tris/HCl pH 6.8 0.1 M β -mercaptoethanol 2% SDS Storage: RT

1.6 Culture Media

2.1.6.1 Media for Animal Cell Culture:

The following media were used under sterile conditions for culturing animal cells and were stored at 4°C

Name	**Company**
Dulbecco's Modified Eagle Medium (DMEM)	PAN Biotech GmbH, Germany
Roswell Park Memorial Institute Culture Medium (RPMI 1640)	PAN Biotech GmbH, Germany
Methocult 3434 (For Colony Forming Cell Assay)	Stem Cell Technologies, Vancouver, Canada.

2.1.6.2 Media for Bacterial Culture

LB media (Luria-Bertani Media)

1% Tryptone
0.5% yeast Extract

1% NaCl

pH 7.0

LB Agar

1.5% Agar in LB media

A ready to use powder of LB media and LB agar from Carl Roth GmbH was used to prepare bacterial culture media. The required amount of powder was dissolved in distilled water, autoclaved and stored at room temperature.

2.1.7 Antibodies:

Name	Concentration	Company
Penicillin-Streptomycin(100X)	10,000 unit Pen/mL 10 mg Strep/ mL	PAN Biotech GmbH, Germany
Kanamycin Sulphate (1000X)	50mg/mL	PAN Biotech GmbH, Germany

2.1.8 Mammalian Cell Line:

Name	Description	Source
GP+E86	Mouse fibroblast cell line	American Type Culture Collection (ATCC)
NIH3T3	Contact inhibited NIH swiss mouse embryonic fibroblast cell line	DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)
U937	Established from the pleural fusion of a 37 year old man with Histiocytic lymphoma	DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

2.1.9 Transgenic Mouse Lines:

Mouse line	Backgound
Rosa26-Cre-ERT2	C57BL/6
Mb1-Cre	C57Bl/6

CD19-Cre	C57Bl/6
Vav-Cre	C57Bl/6
Wild type C57Bl/6	C57Bl/6
Wild type FVB	FVB
IghCALM/AF10 Transgenic line	FVB
Rosa26-loxP-stop-loxP-CALM/AF10 knock-in line	C57Bl/6

2.1.10 Kits and Consumable Materials

Name	Company
Blotting Paper	Schleicher & Schuell
Cell strainer 40μm Nylon	BD Falcon
Cover slips	Menzel Gläser
Cryotubes	Carl Roth GmbH, Karlsruhe, Germany
Electroporation cuvettes 2 mm	EquiBio, Kent, UK
Microcentrifuge tubes	Eppendorf, Hamburg, Germany
FACS Polystrene round-bottom tubes	Becton Dickinson, Meylan, France
Freezing container (Cry0 1° C)	Nalgene TM, USA
Glassware	Schott, Jena, Germany
Hybond Nitrocellulose membrane	Amersham, Freiburg, Germany
Hypercassete™ 18x24 cm Amersham, Freiburg	Amersham, Freiburg, Germany
Hyperfilm ECL high performance	Amersham, Freiburg, Germany
LS columns	Miltenyi Biotech, Germany
Microscope slides	Menzel-Gläser®
Morter and Pestle	Sigma-Aldrich labware.
MiniMACS Seperator	Miltenyi Biotech, Germany
Nuclease free tubes , 1.5 ml	Ambion® Life technologies.
PCR tubes,0.2ml	Biozym GmbH

Pipette tips	Carl Roth, Karlsruhe, Germany
Plastic cuvettes for photometer	Carl Roth, Karlsruhe, Germany
Plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Plastic ware for cell culture	Corning, USA
Polypropylene conical tubes	Becton Dickinson, Meylan, France
QIAquick Gel extraction kit	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Syringes and needles	Braun, Melsungen, Germany
Surgical instruments	Medicon Instrument

2.1.11 Instruments

Name	**Company**
Axiophot Widefield Flourescence Microscope	Carl-Zeiss, Jena, Germany
Axiovert 135 Microscope	Carl-Zeiss, Jena, Germany
BD FACSCalibur	BD Biosciences
BioPhotometer 6131	Eppendorf Humburg Germanyc
Centrifuge 5424 R	Eppendorf Humburg Germany
Centrifuge Rotanta 46 RC	Hettich zentrifugen, Germany
Cytospin-2-Shandon	Block Scientific,Inc
Electroporator (Easyject Prima)	EquiBio Peqlab, Germany
E-Box- gel documentation system	Vilber Lourmat Deutschland GmbH
Film developing machine M35X-OMAT Processor	Kodak AG, Stuttgart, Germany
Gel electrophoresis systems	Bio-rad, Munich, Germany
Incubator (For mammalian cell culture: CO2 control)	Heraeus Instruments, Langenselbold, Germany
Innova™ 4400 Incubator Shaker	New Brunswick Scientific, Nürtingen, Germany

Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad Laboratories, Hercules, CA
NanoDrop ND 1000 spectrophotometer	Thermo scientific, Delaware, USA
TaqMan 7900HT- Real-Time PCR system	Applied Biosystems/ Life Technologies
Thermocycler: Cyclone 25	PeQlab, Erlangen, Germany
Thermomixer Compact	Eppendorf, Hamburg, Germany
Vortex T Ginie 2	Scientific Industries,Inc

2.1.11 Software

Name	**Company**	**Usage**
MS office	Microsoft Corporation, USA	For text editing, data analysis
Sigma Plot	Systat Software Inc	For generating graphs and data analysis
SDS 2.3	Applied Biosystems	For analysis of real-time PCR data
Macvector™12.0	Oxford Molecular Group	Sequence annotation, restriction site mapping.
Primer Express Ver 3	Applied Biosystems	Designing primers and probes for real-time PCR
Endnote X4	Thomson Reuters	Bibliography management
WinMDI 2.9		Flow cytometry data analysis

2.2 Methods

2.2.1 Methods related to animal cell culture

2.2.1.1 Cultivation of mammalian cells

All the mammalian cell lines used in the study were cultivated in incubators at 37°C in the presence of 95% relative air humidity and 5% CO_2. All the cell culture media used in the study were supplemented with 10% Fetal Calf Serum (FCS) and penicillin-streptomycin (final concentration: penicillin: 100 U/ml and streptomycin: 100 µg/ml).

2.2.1.2 Cultivation of adherent cells

All the adherent cell lines were grown in complete Dulbecco's Minimum Essential medium (DMEM) under above mentioned conditions. For harvesting, the confluent cultures were washed once with pre-warmed (37°C) phosphate buffered saline (PBS) and treated with trypsin-EDTA at 37°C for 3-5 minutes for the detachment of the cells from the surface of the culture flask and separation of the cells from each other. Adding complete DMEM stopped the activity of the trypsin and the cell suspension was either used for subculturing or for the preparation of frozen stocks. The cell lines were propagated and maintained by dividing the confluent cultures at a ratio of 1:3, 1:5 or 1:10 every 3-5 days depending on the growth characteristics of the cell lines.

2.2.1.3 Freezing and thawing of the cells

Mammalian cells can remain viable for years if they are stored properly in liquid nitrogen. After re-dissolving the cell pellet in FBS containing 10% vol DMSO, $1X10^7$ cells were frozen in 1.5 ml cryotubes. DMSO serves as a cryoprotective agent and is used to minimize the cellular injury induced by the freezing and thawing procedures. The cryotubes were then placed in a cryo freezing container inside a -80° C freezer to gently freeze cells at a cooling rate of about 1° C/min. While short-term preservation of cell lines at -80°C is possible, storage in liquid nitrogen (-196°C) or its vapor (-120°C) is preferred for longer preservation of cell viability.

For optimal recovery and viability of the cells, rapid thawing is essential.

Therefore, the cells were thawed quickly in a 37°C water bath and, as DMSO in the freezing medium is toxic to the cells, the cells were subsequently washed with 10-20 ml of DPBS or culture medium and centrifugation at 1000 rpm (~300 x g) for 5 min. The supernatant was discarded and the pellet resuspended in fresh culture medium.

All the thawed cell lines were cultured for 3 days before being used.

2.2.1.4 Determination of cell viability: Trypan Blue exclusion method

Determination of the number of viable cells is an essential step in cell culture before seeding the cells for culturing further or for simply determining the cell number. Trypan Blue is the most commonly used dye to determine cell viability. The dye is mixed with the cells, as essential characteristic, viable cells actively exclude the dye but dead cells stain with the dye. When seen under microscope, dead cells appear blue. On the basis of this staining, living cells can be separated and the number of viable cells can be determined.

For the determination of the number of viable cells, the cell suspension was diluted with trypan blue at a ratio of 1:2 to 1:5 (cell suspension: trypan blue) and the numbers of viable (unstained) cells in a 4 x 1 mm^2 area were counted with the help of a Neubauer haemocytometer under a phase contrast microscope. The total number of viable cells per ml of the cell suspension (non-diluted) was calculated using the following formula:

$$\text{Total number of viable cells /ml} = \left(\frac{\text{number of viable cells in 4x 1mm}^2\text{ area}}{4}\right) \text{x dilution factor x } 10^4$$

2.2.1.5 Preparation of high titre virus producing stable cell line:

To transduce murine bone marrow cells with retrovirus, high titre virus producing cell lines are required. E86 is a mouse fibroblast cell line, which was genetically engineered for stable retrovirus production. MSCV 2.1 (Murine Stem Cell virus) is a vector, which allows delivery and stable expression of the gene of interest along with a flourescent protein into dividing cells. The gene of interest is cloned into the Multiple Cloning Site (MCS), which is located upstream of the Internal Ribosome Entry Site (IRES). An enhanced Yellow Fluorescent Protein (YFP) (MSCV 2.1 with YFP: pMIY) or Green fluorescent Protein (GFP) (MSCV 2.1 with GFP: pMIG) gene is present downstream of the IRES so that the gene of interest and YFP or GFP will be co-expressed (Fig 2.1). The viral LTR promoter drives the expression of the gene of interest and the fluorescent marker gene. The vector is transfected into the E86 cells to obtain stably virus producing cell lines.

Figure 2.1: A map of the pMIY vector

The E86-BMI1-FLAG-YFP (B lymphoma Mo-MLV insertion region 1 homolog) cell line was kindly provided by Dr Keith Humphries (Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada). The E86 cell line contains a MSCV vector in which the human *BMI1* cDNA is cloned in the MCS along with a C terminal FLAG tag (Fig 2.2). A frozen vial with cells was thawed and expanded for two days in a 10 cm dish. After the cells started growing healthily, Yellow Flourescence marker (YFP) positive cells were sorted using Fluorescence Activated Cell Sorting (FACs). After three rounds of sorting, a 99% YFP positive cell line was established, which was used to transduce murine bone marrow cells.

Figure 2.2: A map of pMIY vector with *BMI1*.

Determining the viral titre of the producer cell line

To determine the viral titre produced by the packaging cell line (E86), we employd the following method:

$1x10^5$ NIH-3T3 cells were plated in each well of a 12 well adherent cell plate in three replicates and cultured for 24 hours. After 24 hours, Virus Containing Medium (VCM) was collected from a 70-80% confluent plate of virus producing E86 cell line. 500 μl VCM + 20 μl Protamin Sulfate (PS) were mixed in an Eppendorf tube and in another tube, 50 μl VCM and 450 μl DMEM medium were mixed with 20 μl PS. The medium from the NIH-3T3 cells was removed and VCM in different dilutions was carefully overlaid on the NIH-3T3 cells and incubated for 4-5 hrs at 37 °C. Cells in the third well were allowed to grow as untransduced controls. After 4-5 hours, 3 mL DMEM supplemented with 10% FBS were added to each well and incubated for 48 hours at 37 °C. After 48 hours, the NIH-3T3 cells were harvested and GFP or YFP positivity was analyzed using flow cytometry. Using the following formula, the viral titre can be calculated in transducing unit per ml (TU/ml):

$$\frac{\frac{\%\text{ of GFP positive cells}}{100} \times \text{Number of cells transduced}}{\text{Volume of virus(ml)}}$$

2.2.1.6 Syngeneic bone marrow transplantation in mice

To investigate the effect of a gene of interest in the hematopoietic system, a murine syngeneic bone marrow transplantation model can be used. In this model system, bone marrow cells from donor mice are extracted and manipulated to introduce the gene of interest and then transplanted into sub-lethally irradiated recipient syngeneic mice. To analyse the effect of *BMI1* over-expression in Igh-E/P-CALM/AF10 transgenic FVB mice, a retroviral bone marrow transplantation model was used. The experimental strategy and plan are shown in Figure 2.3.

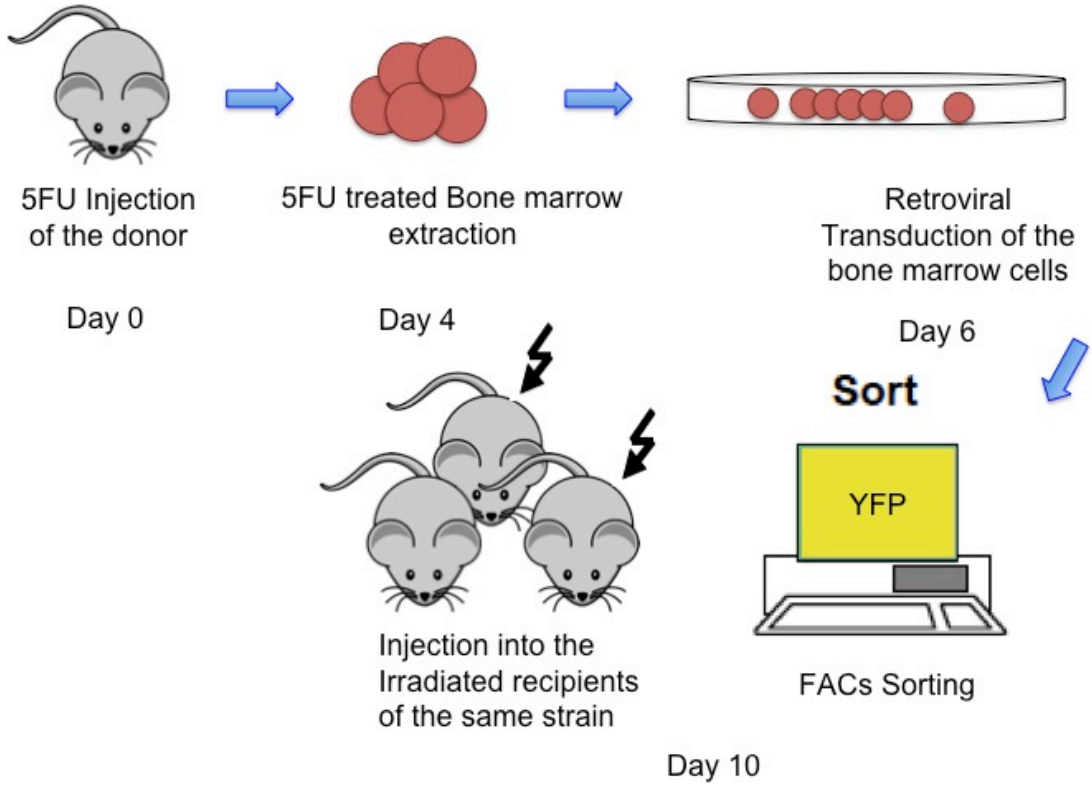


Figure 2.3: A flow chart of the retroviral bone marrow transplantation method.

5FU injection of the donor mice

To kill the cycling cells and subsequently to enrich the hematopoietic progenitor population in the bone marrow, donor mice were injected with 5-flourouracil (5FU). The dose of 5FU varies for different mouse strains. For the FVB strain, 90 mg 5FU per kg was administered via tail vein injection. 8-12 week-old animals were used as donors.

Preparation of murine bone marrow cells

Four days after the 5FU injections, the donor mice were sacrificed, and their femur, tibia and the pelvic bones were dissected with sterile surgical scissors and forceps. The muscles and tissues were carefully removed as much as possible with a sterile scalpel without breaking the bones at the joints. The bones were then crushed with the help of a mortar and pestle in 3% FBS supplemented PBS (sterile). While crushing, the bones break and the bone marrow cells are released into the PBS. The marrow cell containing PBS-FBS suspension was then carefully collected with pipettes and filtered through 45 µm sterile Millipore filters. The bone marrow cells will pass through the filter but some tissue debris will be retained. The crushed bones were repeatedly washed with PBS-FBS until the effluent PBS becomes colorless to ensure collection of as many bone marrow cells as possible. The harvested bone marrow cells were pelleted at 1070 RPM for 10 mins at 4°C. The pellet was carefully resuspended in ammonium chloride (NH_4Cl) and incubated for 10 mins on ice to lyse the RBC. After 10 mins, the cells were centrifuged at 1070 RPM for 10 mins at 4°C again. The pellet was washed with PBS to remove remaining NH_4Cl and finally the bone marrow cells were cultured in DMEM media supplemented with 15% FBS and cytokines (10 ng/mL mIL-6, 6 ng/ml mIL-3 and 100 ng/mL murine stem cell factor).

Irradiation of E86 viral producer cell lines:

E86 cells carrying the gene of interest were harvested from ≈80% confluent plates in 15% DMEM media (10 ml final volume from one confluent plate). If a double transduction was planned then the two E86 producer cell lines were mixed according to their retroviral titre. Then the cells were ready for irradiation. 10 ml of each sample was taken in a 15 ml Falcon tube and the caps were sealed with parafilm (optional). The cells were irradiated with 40 Gy. Irradiated cells were plated in new plates for adherent cells

Retroviral transduction of bone marrow cells

On day 6, two days after the bone marrow extraction, the bone marrow cells were retrovirally transduced by co-culturing with the virus producing E86 cell line carrying the gene of interest. This was done as follows:

The bone marrow cells were harvested and transferred to a Falcon tube. Before transfer, cells were filtered again using a 45 µm filter to remove clumps. Adherent cells were collected with a scraper. Bone marrow cells were centrifuged at 1070 RPM at 4°C for 10 mins. The pellet was resuspended in 15% FBS DMEM media. Then

the volume was adjusted so that for each plate to be transduced 10 ml of media would be available. Cytokine and protamine sulfate (PS) was added accordingly. The cells were suspended in media with cytokine and PS. Irradiated plates of E86 cells were handled carefully and kept in the incubator as long as possible. (The cells tend to dislodge from the monolayer at this step). The medium was carefully removed from the E86 cells, and the bone marrow cells were overlaid on the E86 cells without disturbing the E86 cell layer. Then the plates were incubated at 37°C for 25 to 36 hours. After this incubation, the bone marrow cells were carefully removed from the E86 cells, washed and replated with fresh media supplemented with cytokines.

Transplantation

Four days after the transduction (the 10th day after 5FU injection), successfully transduced bone marrow cells were sorted for YFP positivity. Non-transduced cells were also collected to aid hematopoietic reconstitution of the recipient mice.

Recipient mice were from the same inbred strain (syngeneic) as the donor (in our experiment the FVB strain) and 8-12 weeks old. Before the transplantation, recipients were lethally irradiated (Cs^{137} gamma radiation). The dosage of the irradiation depended on the mouse strain. FVB mice were irradiated with 800 cGy. After irradiation, each recipient received 0.5 million transduced and 0.5 million non-transduced bone marrow cells via tail vein injection. Transplanted animals were kept in Individually Ventilated Cages (IVC) for four weeks after transplant. Their drinking water was supplemented with ciprofloxacin and acetic acid to prevent infection.

Follow-Up

Following transplantation, mice were regularly examined and closely monitored for any signs of leukemia or other diseases. The engraftment of the bone marrow was assessed in blood collected from the tail veins. We also performed WBC and RBC counts.

When high WBC counts accompanied by ruffled fur, fatigue, heavy breathing and lethargy were observed, the animals were sacrificed, since these symptoms were usually followed by the rapid demise of the animals. Peripheral blood, spleen and bones from the pelvis and legs were collected and the rest of the body was fixed in buffered formalin. The cells from peripheral blood, spleen and bone marrow were analyzed with FACS. Smears and cytospins of the cells were also prepared and stained with Giemsa-May-Grünwald for

morphology studies. Formalin-fixed organs were processed for histology and immunohistochemistry (for details see "Post mortem analysis" in the next paragraph).

2.2.1.7 Post mortem analysis of mice

Collection of the peripheral blood and its processing

After the mouse was sacrificed by CO_2 asphyxiation, it was sterilized with 70% ethyl alcohol and the body was dissected with sterile forceps and scissors. The thoracic cage was opened, and blood from the heart was carefully collected with a sterile needle and a heparin-coated syringe.

After the collection of the peripheral blood, the following procedures were performed:

Blood smear preparation:

2-3 μl freshly collected peripheral blood was placed on a glass slide and spread across the glass slide with the help of another clean slide.

Determination of WBC count:

2 μl blood was mixed with 48 μl NH_4Cl and incubated on ice for 10 mins. WBCs were counted using a hemocytometer. Depending on the number of cells, additional dilutions were made with trypan blue.

The final formula to determine the number of WBC in the peripheral blood/mL is

(Number of total living cells counted in all 4 squares of the Neubauer chamber)/4 X 25 X dilution (if any) X 10^4 = WBCs per mL

Determination of RBC count:

1 μl of blood was mixed in 10 mL PBS and the RBCs were counted using a Neubauer hemocytometer. The number of RBCs in the peripheral blood/mL was calculated with the following formula:

(Number of total cells counted in 4 squares of the Neubauer chamber)/4 X dilution (if any) X 10^8 = RBCs per mL

Preparing peripheral blood mononuclear cells (PBMC):

Total blood was treated with NH_4Cl to lyse the RBCs on ice for 20 mins. After 20 mins the cells were spun down and was washed with PBS to obtain the PBMCs.

Preparation of splenocytes:

The spleen was dissected from the sacrificed mouse and preserved in PBS with 3% FBS. The size and weight of the spleen was documented. A small part of the organ was transferred to buffered formalin for histology.

The rest of the spleen was crushed in PBS using a plunger from a sterile syringe and passed through 0.45 µm filter. A single cell suspension was obtained by gently pipetting up and down. The cells were centrifuged at 1070 RPM, 4°C for 10 min. The pellet was treated with NH_4Cl for 10 mins on ice followed by a wash with PBS.

Flowcytometric analysis of the murine cells

To analyze the surface markers on the cells from different organs of the animal, flow cytometry was used.

After sacrificing the animals single cell suspensions from peripheral blood and different organs were prepared. The cells were immunostained with different murine antibodies. For each staining, $1X10^6$ cells were used. Antibodies in specific dilutions were added to the cells suspended in PBS and incubated on ice for 20 min. The cells were washed and resuspended in FACs buffer and analyzed using FACS Calibur.

Cytospin preparation

For studying the morphology of the cells in peripheral blood and in different hematopoietic organs, the cells were permanently fixed on glass slides using a cytospin centrifuge and stained with Giemsa and May-Grünwald.

The cell suspensions were prepared (PB cell, slides were air-dried.

May-Grünwald staining:

Blood smear and the cytospin slides were arranged in racks and immersed in fresh May-Grünwald solution (undiluted) for 3 mins, followed by a 5 min rinse in distilled water. Then the slides were immersed in 1:50 diluted Giemsa stain for 1 hour, followed by another 5 min wash by immersing the slide rack in distilled water. Finally, the stained slides were air dried overnight and then examined under the microscope.

2.2.1.8 Colony Forming Cell (CFC) assay

CFC assays were performed to evaluate the clonogenic potential of the hematopoietic progenitors. For this assay, transduced bone marrow or bone marrow cells from sacrificed animals were cultured in a semi solid agar called MethCult. For our experiments, MethoCult 3434 was used, which is supplemented with cytokines supporting the growth of myeloid progenitors. For this procedure the following protocol given by Stem Cell Technologies was used:

A MethCult 3434 bottle was thawed and the medium was dispensed in 3 mL aliquots and stored at -20°C. 500 bone marrow cells were suspended in 300 μl of DMEM media. The 3 mL aliquot of MethoCult was thawed at room temperature and the 300 μl cell suspension was added to it and vortexed thoroughly. Then the aliquot was set aside so that the bubbles could escape. 35 mm diameter culture dishes were labeled appropriately. Each experiment was performed at least in duplicate.

After 10-20 mins the bubbles had dissipated, and the MethoCult was taken up with a 16 gauge blunt end needle attached to a 3 mL sterile syringe.

To expel most of the air from the syringe, approximately 1 mL medium was drawn into the syringe. Then by gently depressing the plunger the medium was expelled completely. This process was repeated until no air space was visible in the syringe anymore. Finally, after removing all air bubbles, the MethoCult medium with the cell was taken into the syringe and 1.1 mL were dispensed per 35 mm plate. The culture dishes for the CFC assays had been tested for minimal cell adherence. Adherence of cells during the CFC assay can cause inhibition of colony growth and obscure visualization of colonies. By gently tilting and rotating each plate, the methylcellulose medium was evenly distributed. Two 35 mm plates were placed into a 100 mm petri dish. A third, open 35 mm plate containing 3 mL of sterile water was also placed in the bigger Petri dish. The use of a 100 mm petri dish and an open 35 mm plate with water helps to maintain humidity and minimizes contamination during culture and handling.

The Petri dishes with the three smaller plates were placed in an incubator maintained at 37°C, 5% CO_2 and ≥ 95% humidity. After 7-10 days, the plates were analysed for colony growth. When colonies appeared, they were counted and from the morphology of the cells the origin of the colonies was determined. After the colonies were counted, the plates were ready for re-plating. The cells were collected by dissolving the methylcellulose in warm PBS and were then used for replating and flow cytometry analysis.

2.2.1.9 B cell harvesting from mouse spleens using magnetic cell separation

To isolate B cells from mouse spleen, a magnetic cell separation technique was used. Magnetically labeled B220 antibody-beads were used for a positive selection of B cells and magnetically labeled CD43 beads were used for negative selection of B cells from spleen. We followed the protocol from Miltenyi Biotec, the suppliers of the magnetic bead separation kits. The procedure was performed as follows:

A single cell suspension was made from the spleen in PBS or MACs buffer.

Cells were counted. For 10^7 cells, 10 μl magnetically labeled antibody beads and 90μl MACs buffer was used. After counting, the cells were pelleted. The supernatant was discarded and equal amount of beads were added directly to the pellet. After 20 mins of incbation at 4°C, the tubes were filled with 10 ml of MACs buffer. The tubes were centrifuged, and the pellet was resuspended in 500 μl MACs buffer. The LS columns were placed in the MACS separator and washed once with 3 ml of MACs buffer. The matrix of the LS Columns is composed of ferromagnetic spheres, which are covered with a cell-friendly coating allowing fast and gentle separation of cells. When placed in the magnetic field of a MACS® Separator, the spheres amplify the magnetic field by 10,000-fold, thus inducing a high magnetic field gradient within the column. This is crucial for isolation of cells which are only minimally labeled with MACS® MicroBeads, leaving enough epitopes free for concurrent antibody staining. The space between the spheres is several times larger than primary and most cultured cells. This allows the cells to freely flow through the column. Magnetically labeled cells are held in suspension within the column and do not actually "bind" the column matrix. This suspension minimizes stress on the cells and allows for efficient sterile washing by avoiding cell aggregation. LS Columns can be used to separate material less than 30 μm in size. The collection tubes were placed on ice underneath the column.
The cells (500μl) were loaded to the column. The column was washed 3 times with 3ml PBS/Ba. The flow through contained unlabelled cells (CD43 negative cells, resting B cells)

To collect the labelled cells, which adhered to the column, the column was removed from the separator, placed on the collection tube and filled with 5 ml MACs buffer. Then quickly flushed with a pestle. The eluent contained the positive fraction (B220 positive B cells).

Cells were counted in a 1 in 10 dilution.

2.2.2. Microbiology Methods

2.2.2.1 Culturing bacteria

For routine bacterial cultures, frozen cells from glycerol stocks were streaked out on LB-agar plates containing appropriate antibiotics and incubated O/N (over-night) at 37°C to obtain single colonies. A single colony was then inoculated in the desired volume of LB medium containing the appropriate antibiotic and incubated O/N at 37°C in a shaking incubator at 190 rpm. To prepare competent bacterial cells, LB agar and medium without antibiotics were used.

2.2.2.2 Preparation of bacterial stocks

850 μl of an overnight bacterial culture was mixed with 150 μl of glycerol and stored at -20°C or at -80°C for long-term storage

2.2.2.3 Preparation of electrocompetent cells:

Electrocompetent cells are specially prepared bacterial cells, which can take up naked extracellular DNA upon electropermeabilization of the cellular membrane by an externally applied electrical field. This process of delivering DNA into the bacterial cells is called electroporation. For making bacterial cells competent for electropration (electrocompetent), bacterial cells are washed several times in water to remove salt from the cell solutions. This leads to a high resistance and very low conductivity of the bacterial cell suspension, which is required for the electroporation to be successful.

For making electrocompetent cells a single bacterial colony was inoculated in 10 ml LB medium and grown overnight at 37°C. On the next day, the overnight pre-culture was inoculated into 400 ml of fresh LB and grown till an OD_{600} of 0.5-0.6 was reached (around 2 hrs). The culture was cooled on ice, and the cells were pelleted in a cooling centrifuge at 4500 RPM for 5 mins. The cell pellet was washed twice using 40 ml ice-cold water. Cells were washed several times using 20 ml 10% glycerol (cold). The pellet was finally resuspended in 800 μl 10% glycerol and 50 μl aliquots were stored in microcentrifuge tubes at -80°C.

2.2.2.4 Transformation/ Electroporation

Electrocompetent cells were thawed on ice. 1-5 μl plasmid DNA (0.1-2 ng) was mixed with the competent cells. The mix was incubated exactly for 1 min on ice. Then it was transferred to a pre-cooled cuvette. The cuvette was placed in an electroporation device and electroporation was performed at 2500 V. One ml LB was added to the cuvette and mixed gently by pipetting up and down. The mix was transferred to a new microfuge tube, incubated for 1 hour at

37°C shaking at 210 rpm. Finally, the cells were spun down at 3100 rpm at room temperature. Almost all the medium, except for 70 μl was removed and discarded. The cell pellet was resuspended in the remaining 70 μl of supernatant. Cells were spread on LB agar plates with or without antibiotics. Each transformation reaction was plated on two plates, 10 μl on one plate and 60μl on a second plate. This allowed us to pick single colony of transformed bacteria in case of very efficient transformation. Both plates were incubated overnight at 37°C.

2.2.3 Methods in molecular biology

2.2.3.1. Isolation of Plasmid DNA (Mini prep)

Approximately 1.5 ml of a bacterial overnight culture was centrifuged at 11,000 rpm for approximately 1 minute. The supernatant was discarded and the pellet was resuspended in 300 µl Buffer P1. 300 µl freshly prepared P2 was immediately added and the mix was left at room temperature for 5 minutes. Following the 5-minute incubation, 300 µl P3 was added and the sample was centrifuged at 14,000 rpm for 10 minutes. 900 µl of the supernatant was taken and transferred to a fresh tube. Plasmid DNA was precipitated by adding 0.7 volume of isopropanol. Tubes were kept at -20°C for 10 minutes and were later centrifuged for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol by centrifuging at 14,000 rpm for 15 minutes. The supernatant was discarded and the pellet was dried at room temperature for 10 minutes. The pellet was resuspended in 20 µl of 1x TE buffer and kept at -20°C for future use.

2.2.3.2 Isolation of genomic DNA from mouse tails

This protocol was adopted from Laird (Laird, 1991). The tail DNA from mice was extracted for genotyping. Small pieces of tail tips were clipped from 5-8 week old mice and the following protocol was used to extract the DNA.

Lysis*:* Tail pieces were immersed in lysis buffer containing proteinase K (0.5 ml) and shaken overnight in a thermo-mixer at 55°C.

Precipitation: The next day, the tubes containing the digested samples were centrifuged at room temperature for 10 mins at 14000 RPM. The supernatant containing the DNA was transferred to fresh tubes avoiding the cellular debris. One volume of ispropanol was added to the lysate and the tubes were vortexed until the precipitation was complete.

Recovery of the DNA: After the precipitation was complete the tubes were centrifuged for 10 min at 14,000 RPM. The pellet containing the DNA was washed using 70% ethanol, air dried and finally dissolved in 30-100 µl of TE buffer depending on the size of the pellet.

2.2.3.3 DNA extraction from mouse tissue using DNAzol

For the extraction of DNA from mouse tissues (peripheral blood, bone marrow spleen, kidney etc), we used the DNAzol reagent from Life Technologies according to the manufacturer's protocol.

1 mL DNAzol can be used to isolate DNA from 25-50 mg of tissue, 0.1mL whole blood or 1-3x10^7 cells. A single cell suspension of the cells from the tissue was prepared using the homogenizer and the cell number was determined. According to the cell numbers, DNAzol was added to the cell pellet and by gently pipetting up and down, the cells were lysed homogeneously. To precipitate DNA from the homogenate, 100% ethanol was added, 100 µl for every 1 mL of DNazol. Inverting the tubes a couple of times mixed samples. The tubes were incubated for 3 mins at room temperature until a visible precipitate had formed. The DNA precipitate was taken up with a pipette tip and attached to the side of the tube, the supernatant was carefully decanted. The DNA was washed with cold 70% ethanol two to three times and air-dried briefly. The pellet was finally dissolved in 8 mM NaOH

2.2.3.4 Extraction of RNA from animal cells/tissue using TRIzol®

First, a single cell suspension was prepared from the mouse tissue. To isolate total RNA from these cells, the following method was used, which is adapted from the protocol provided with the TRIzol reagent.

The cells were lysed in TRIzol® Reagent by repetitive pipetting. 1 ml of TRIzol® reagent was used for 5-10 x10^6 of cells. Washing the cells before addition of TRIzol® was avoided as this increases the likelihood of mRNA degradation. Insoluble material was removed from the homogenate by centrifugation at 12,000 *g* for 10 minutes at 2-8°C. The resulting pellet contains cell membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. The clear supernatant was transferred to a fresh tube. The homogenized samples were incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml chloroform per 1 ml of TRIzol® reagent was added. The tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. The samples were centrifuged at no more than 12,000 *g* for 15 minutes at 2-8°C. After the centrifugation, the mixture separates into a

lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol® reagent used for homogenization. RNA was precipitated from the aqueous phase by mixing with 0.5 mL isopropanol per 1 mL of TRIzol®. Samples were incubated for 10 minutes at 15-30°C and then centrifuged at 12,000 RPM for 10 minutes at 2-8°C. The RNA forms a gel like pellet. The supernatant was discarded. Pellets were washed with 75% ethanol, 1mL for every 1ml of TRIZOL. Samples were mixed by vortexing and then centrifuged at 7500g for 10 minutes at 2-8°C. The supernatant was removed, and pellets were air-dried. Finally, the pellet was dissolved in DEPC water by pipetting up and down and incubating the sample at 60 °C for 10 minutes.

2.2.3.5 Isolation of RNA using the RNeasy Mini Kit

RNA from the fresh mouse organs was extracted using the RNeasy Mini kit. The kit was also used for additional purification of some already extracted RNA sample. The following protocol was adapted from the RNeasy Mini handbook provided by Qiagen.

A single cell suspension was prepared from the organs. To 1 ml RTL buffer 10 μl β-mercaptoethanol were added. For 0.5-1 x 10^7 cells, 600 μl RTL buffer was used. Cells were lysed in RTL buffer by pipetting up and down several times. If adherent cells grown in monolayer were used, they were directly lysed on the plate. 1 volume of 70% ethanol was added to the cell lysate, mixed thoroughly, and then the lysate was transferred to the RNeasy spin column along with the precipitate if formed. Up to 700 μl can be transferred to the spin column at one time. Then the columns were centrifuged ≥ 8000 rpm for 15-30 sec. The flow-through was collected in the 2 mL collection tubes supplied with the columns. The RNA bound to the membrane in the column. The columns were then washed with wash buffer RW1. The flow through was discarded. 500 μl RPE buffer was added to the columns and columns were centrifuged at ≥ 8000 RPM for 15-30 sec. The RPE buffer concentrate, which comes with the kit, was dissolved in ethanol before the process was started. The wash step with RPE was repeated twice. Finally the RNA was eluted with 30-50 μl nuclease free water.

2.2.3.6 Agarose gel electrophoresis:

Electrophoresis was used to separate DNA/RNA molecules on the basis of their mobility through a gel matrix in an electric field. The mobility depends on the molecular weight of the DNA or RNA molecules and their secondary structure. Non-denaturing gel

electrophoresis was used for DNA/RNA separation and for both the analysis and isolation of DNA fragments. The agarose concentration of the gels was determined according to the desired range of separation of linear DNA according to the following table:

Agarose (%)	Range of separation of linear DNA	Used for
0.8	500 bp to 15 kb	>3.5 kb
1.0	250 bp to 12 kb	
1.2	150 bp to 6 kb	1.5 kb to 3.5 kb
1.5	80 bp to 4 kb	
1.8	50 bp to 3 kb	50 bp to 1.5 Kb

For RNA samples, 2% agarose gels were used.

The gel was prepared by dissolving agarose powder in warm 0.5X TAE with 1X SYBR® Safe (Invitrogen). The gel was poured onto a horizontal gel tray. After the gel was completely cool, it was transferred into appropriate gel-running chambers. The DNA samples were loaded in the gel pockets with loading dye added to 1x final concentration. By applying an electric field of approximately 700 V/m, the DNA fragments were separated. Under these conditions, the negatively charged DNA molecules migrate through the gel matrix to the anode at speeds inversely proportional to the length of the individual DNA fragments. The gels would typically be run for 1 to 2 hours.

2.2.3.7 Gel Purification of DNA fragments:

This protocol is adapted from Qiagen® gel extraction kit QIAquick. It is designed to extract and purify DNA fragments from agarose gels in TAE or TBE buffer.

The DNA fragment was excised with a clean and sharp scalpel from the agarose gel. The gel slice was weighed, 3 volumes of buffer QG were added to 1 volume of the gel (100 mg corresponds to 100 μl). For gels with more than 2% agarose content, 6 volumes of QG buffer were added. The mix was incubated at 50°C for 10 minutes (or till the gel slice was dissolved completely). Then 1 volume of isopropanol was added to the samples. The whole mixture was transferred to a QIAquick spin column, which was placed on the 2 ml collection tube. Spin columns were centrifuged for 1 minute and the flow-through was discarded. The columns were again washed with 0.5 ml of QG buffer. 0.75 ml Buffer PE was added to the columns to wash, centrifuged for 1 minute and the flow-through was discarded. The spin columns were spun for an additional 2

minutes to remove the remaining Buffer PE. QIAquick columns were placed on clean microcentrifuge tube. The DNA was eluted using 50 μl Buffer EB (10 mM Tris.Cl, pH 8.5). Buffer EB was added at the centre of the QIAquick membrane, incubated for 4 min and finally the column was centrifuged for 1 min at top speed to collect the DNA.

2.2.3.8 Estimation of the DNA or RNA concentration

DNA and RNA concentrations were determined using a Nanodrop spectrophotometer. 1.5 μl samples were used for each measurement. The purity of the samples was determined by OD_{260}/OD_{280} ratio. SYBR Safe stained agarose gels were also used for the estimation of DNA concentration by comparing the strength of the DNA band to be assessed with standard DNA markers.

2.2.3.9 Digestion of DNA with restriction enzymes

Restriction enzymes are site-specific endonucleases purified from different bacteria. These enzymes can recognize specific sequences in double stranded DNA and hydrolyze the phosphodiester bond. These enzymes are frequently used in molecular biology to cut DNA at specific locations. These enzymes are active only at certain salt concentration, which vary from enzyme to enzyme. The correct salt concentrations are supplied with specific buffers that are provided with the enzymes. A specific amount of DNA (genomic or plasmid, usually between 100 and 1000 ng) was incubated with required quantity of enzyme and specific buffer at the optimal temperature. Multiple enzymes can also be used at the same time using the correct buffers.

A typical restriction digestion reaction is presented below. The ingredients reagents are mixed in the following order for a 20 μl reaction.

Component	Volume
Nuclease free water	16 μl
10X buffer (recommended)	2 μl
DNA	1 μl (~1 μg)
Restriction enzyme	1 μl (5-10U)

2.2.3.10 Polymerase Chain Reaction (PCR):

The PCR technique is used to amplify DNA fragments of interest in an exponential fashion. The amplification is achieved using synthetic oligonucleotides called primers that flank the sequence to be amplified. These primers, under appropriate conditions, anneal to the denatured target sequence and, in the presence of a heat stable

DNA polymerase enzyme and dNTPs, the synthesis of a complementary strand of the target sequence takes place. As template 1 µl cDNA (about 10 to 200 ng), 1 µl genomic DNA (0.1 to 1 µg) or 0.5 ng plasmid DNA was used. The PCR reactions were prepared as follows:

Reagents	**Amount (for one 25 µl reaction)**
10X PCR Buffer	2.5 µl
50 mM $MgCl_2$	0.75 µl
10 mM dNTP mix	0.5 µl
100 pmol/µl Primer F	0.5 µl
100 pmol/µl Primer R	0.5 µl
Taq Polymerase	0.2 µl
DNA (0.5ng/µl-1µg/µl)	1-5 µl
Water	to a total of 25 µl

The total reaction volume was adjusted depending on the specific requirements.

The reaction was cycled through a specific temperature profile; a standard PCR program is given below

Steps	**Temperature**	**Time**	**No of Cycles**
Initial denaturation	95°C	5-10 min	1
Subsequent denaturation	95°C	30 sec	20-35
Primer annealing	55-60°C	30 sec	
Elongation	72°C	1-3 min	
Final Elongation	72°C	5-10 min	1

The annealing temperature can vary depending on the primers; the elongation time varied depending on the size of the amplicon. For every 1000 bp, 1 min elongation time is required.

2.2.3.11 Reverse Transcriptase Polymerase Chain Reaction (RT PCR)

RT PCR is a very sensitive and reproducible method to detect specific RNA molecules. The process involves two steps:

cDNA synthesis:

cDNA synthsis is the process in which a completary DNA copy of mRNA is prepared by the reverse transcriptase enzyme.

Amplification:

Amplification of specific regions of cDNA using specific primers by PCR.

The following protocol is for cDNA synthesis using ThermoScript system:

Primer and RNA master mix (for 1 reaction)

Components	Volume
RNA (10 pg - 5 μg) –	x μl
10 mM DNTP mix	2 μl
Random primer mix (50 ng/μl)	1 μl
DEPC treated water	to 12 μl

The above master mix was heated at 65°C for 5 mins and then placed on ice.

Reaction mix (for 1 reaction):

5x cDNA synthesis buffer was vortexed for 5 secs and placed on ice just before use. The master mix was pipetted on ice and gently vortexed

Component	Volume
5X buffer	4 μl
0.1 M DTT	1 μl
Rnase OUT 40 U/μl	1 μl
DEPC treated water	1 μl
Thermoscript RT 15U/μlt	1 μl

8 μl of the master mix was aliquoted into each of the tubes containing primer and RNA.

The tubes were transferred to the thermal cycler and the following program was used:

25°C for 30 min

50°C for 50 min

85°C for 5 min

After that, 1 μl RNase H was added to each reaction and incubated at 37°C for 20 mins. The cDNA was stored at -20°C or used directly for PCR.

PCR was carried out using Platinum® *Taq* DNA Polymerase High Fidelity. 10% of the cDNA synthesis reaction (2 μl) was used for one PCR reaction. The following components were mixed in a 0.2 or 0.5 ml, thin-walled PCR tube. Below is a pipetting scheme for a 50 μl reaction:

Components	**Volume**
10X High Fidelity buffer	5 μl
50 mM $MgSO_4$	2 μl
10 mM dNTP mix	1 μl
10 μM sense primer	1 μl
10 μM antisense primer	1 μl
Platinum Taq	0.2 μl
DEPC treated water	37.8 μl
cDNA from the synthesis reaction	2 μl

The PCR reactions were cycled through the following temperature profile on a thermal cycler:

94°C for 2 min
94°C for 30 sec
55°C for 30 sec (temperature depends on primer)
68°C for 1 min/kb extension time
4°C hold
10 μl of the amplification product was analyzed on an agarose gel.

Below is a flow chart of the process adapted from Invitrogen:

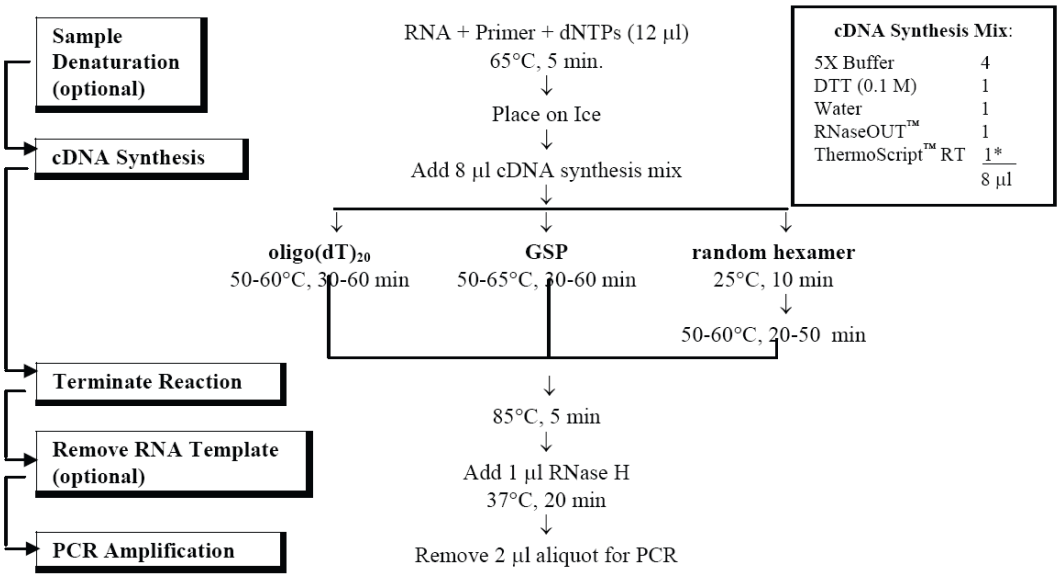


Fig 2.3: Schematic plan of cDNA RT-PCR process (adapted from Invitrogen)

2.2.3.12 Real-time PCR

Real-time PCR is a very sensitive and reliable technique to measure the products generated during the exponential phase of the PCR reaction. These measurements allow a very accurate estimate of the initial template amount. In the TaqMan real-time assay, a ''TaqMan probe'' is used which is non-extendable and homologous to the amplicon. The probe has a fluorescent reporter dye attached to its 5' end and a quencher dye at its 3' end. The probe binds to the target sequence. As long as the probe is intact, the fluorescent dye and the quencher are in close proximity so the quencher significantly decreases the fluorescence emitted by the dye through a process called FRET (Fluorescence Resonance Energy Transfer). But as soon as the primers bind the target sequence and the polymerization begins, the 5' nuclease activity of the Taq polymerase cleaves the 5' end of the probe and the quencher dye is released. Once the quencher dye is released, the close proximity between the quencher and the fluorescent dye is lost. This leads to an increase in fluorescence from the free dye. In addition, the cleavage removes the probe from the target strand and the amplification continues. With each cycle of amplification, additional reporter dye molecules are cleaved leading to an increased intensity of the fluorescence proportional to the number of specific PCR products.

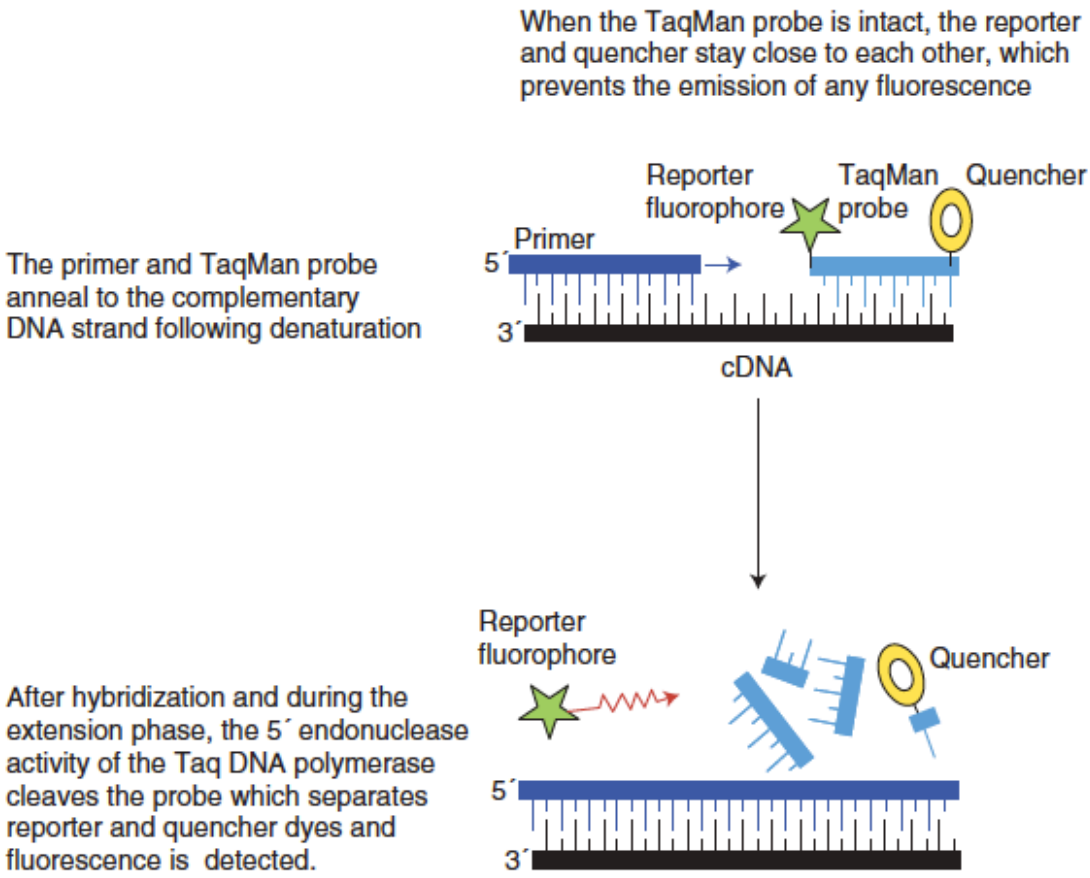


Fig 2.4:Schematic presentation of the basic principle of TaqMan assay (Adapted from Arya et.al, Expert Rev. Mol. Diagn.2005)

Below is the basic protocol for real-time PCR:

Good quality cDNA samples were prepared from total mRNA, and the concentrations of the samples were determined accurately. A master mix was prepared with the reverse and forward primers, probe and the universal PCR mix from Applied Biosystems. A standard example of real-time PCR master mix is given in the following table:

Component	Volume
cDNA	~ 100-200 ng
Probe (concentration?)	0.05 µl
Forward Primer (molarity?)	0.2 µl
Reverse Primer (molarity?)	0.2 µl
2x Taqman universal PCR mix	10 µl
Water	to make the final volume 20 µl

The master mix was then dispensed into the designated wells of the 96 well PCR plates. Known amounts of cDNA samples were appropriately added to the wells. Both technical and biological replicates were performed for a robust statistical analysis of the results. The plate was then placed into the ABI real-time thermal cycler programmed for the following cycles:

50°C, 2 min

95°C, 10 mins

40 cycles of the following steps:

95°C, 15 secs

60°C, 1 min

Following amplification, the CT values (C_T values: the cycle number at which the fluorescence exceeds a threshold value) of the target gene and internal standards were recorded and the data was analyzed using the SDS 2.1 software.

2.2.3.13 Protein extraction by the freeze-thaw lysis method

Cells were harvested using ice cold PBS and the samples were transferred to a 1.5 mL microfuge tube. The cells were pelleted in a cold centrifuge at top speed. The pellet was resuspended in FT Lysis buffer. 80 μl buffer was used per 10 cm confluent dish. After dissolving the pellet completely, the tubes were emersed in liquid nitrogen for snap-freezing. Frozen tubes were taken out of liquid nitrogen and placed on ice for thawing. After the samples were thawed, they were briefly mixed by vortexing. This process of freeze-thawing was repeated for 2 more times. Finally 250 U Benzonase was added to each sample to digest DNA in 10 min room temperature incubation. The protein concentration was measured using the Bradford method. Before proceeding for SDS-PAGE, the samples were mixed with an equal volume of 2x Laemmli buffer, heated at 95°C for denaturation and loaded on the gel.

2.2.3.14 Estimation of protein concentration

To determine the protein concentration in cell lysates, the Bradford Assay was performed (Bradford, 1976). This is a colorimetric assay. The principle of this assay is based on an absorbance shift in the Coomassie dye, when the red form of the dye changes and stabilizes into a blue form, Coomassie blue, after binding to protein.

Thus, the concentration of the total protein in the lysate can be measured by reading the absorbance at 595 nm with a spectrophotometer. The assay is performed in the following steps:

The Bradford protein assay reagent (BIO-RAD) was diluted 1:5 with water. For the standard curve, 200 μg/ml, 400 μg/ml, 600 μg/ml, 800 μg/ml and 1000 μg/ml protein standards were used. The protein standards were all made from a 10 mg/mL stock solutions. 20 μl of each dilution was mixed with 980 μl of diluted Bio-Rad protein assay reagent. The mixture was then added to a cuvette and the absorbance of these protein solutions was measured at 595 nm using a photometer. For the unknown sample, 2 μl were diluted in 18 μl water and mixed with 980 μl Bio-Rad protein Assay reagent and the absorbance was measured at 959 nm. Measurements of the unknown samples were performed two or three times as technical replicates. From the readings of the known standards, a standard curve was calculated and the concentrations of the unknown samples were determined.

2.2.3.15 Polyacrylamide gel electrophoresis (PAGE)

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight under denaturing conditions. SDS-PA gels of appropriate concentrations were prepared depending on the size of the proteins to be separated. For SDS-PAGE, the gels are prepared in two layers with different acrylamide concentration. These layers are known as stacking gels and separating or resolving gel. As the name implies, the stacking gel concentrates the proteins in a thin band before they enter the separating gel, which separates the proteins according to size. This results in sharper protein bands in the separating gel. The separating gel is poured first and then the stacking gel is poured on top of the separating gel. The polyacrylamide concentration of the stacking gel is always the same (5%), whereas the acrylamide concentration of the separating gel varies according to the size of the proteins that need to be separated. For the preparation of a 12% separating gel (as an example) and 5% stacking gel the following reagents were mixed:

For 10 ml 12% resolving gel:

Component	Volume
Water	5.3 ml
30% acrylamide mix	2.0 ml
1.5 M Tris (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% ammonium persulfate (APS)	0.1 ml

TEMED	8 µl

For 3 ml 5% stacking gel:

Component	Volume
Water	2.1 ml
30% acrylamide mix	0.5 ml
1.0 M Tris (pH 6.8)	0.38 ml
10% SDS	30 µl
10% APS	30 µl
TEMED	3 µl

TEMED and APS were always added last to the gel mixtures, just before the gel was poured. The separating gel mix was poured first into the gel cassette and was allowed to polymerize at RT. After the polymerization of the separating gel, the stacking gel mix was prepared and poured on top of the polymerized separating gel. Then the comb was positioned in the upper portion of the stacking gel to form the loading wells. Samples to be loaded onto the gel were denatured by boiling at 95°C for 5 minutes in SDS Laemmli buffer. The SDS-Laemmli buffer confers a negative charge to each protein in proportion to its mass, resulting in the movement of the protein towards the anode (+) through the gel matrix at a speed that is roughly proportional to the molecular weight of the protein in a specific weight range. The proteins were resolved by applying 60-120 V (1 kV/m) for 4-8 hours at 4°C in 1x Tris-Glycine electrophoresis buffer. 10-50 µg of each protein sample were loaded in each well. A commercial protein size marker was also loaded so that the molecular weight of the proteins could be estimated.

2.2.3.16 Western blot analysis

Western blot analysis is a widely used technique to detect the presence of a specific protein in a given sample. This technique is also used for quantitative estimation of a protein in a sample. The method involves the separation of proteins according to their molecular weight using electrophoresis through an SDS-PAGE gel, transfer of the proteins on a membrane and detection of the proteins with antibodies specific for the target protein. Below is a step-by-step description of the process:

Gel Transfer

Protein in a sample were separated on a SDS-PAGE gel according to size and then transferred to a nitrocellulose membrane using a wet transfer system (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). The transfer assembly was prepared according to the instructions of the manufacturer. For this, the gel, filter papers, sponges were all equilibrated in cold transfer buffer for 5 mins. Then the transfer sandwich was prepared in the following order:

Negative electrode, sponge, two sheets of blotting paper, gel, membrane, two sheets of blotting paper, sponge, positive electrode.

The sandwich was locked in place inside the designated cassette and placed into the transfer chamber. To keep the chamber cold, special ice packs provided with the transfer system were used. The chamber was filled with the transfer buffer. A small magnetic stir bar was placed inside the chamber. The whole assembly was placed on a magnetic stirrer in the cold room and the transfer was performed overnight at 100 V, which was ramped up to 200 V in the last 1 hour.

Blocking and Hybridization

After disassembling the transfer system, the membrane was removed carefully from the gel, and the lanes were marked with pencil. The membrane was then incubated in blocking solution and placed on a rocking platform in the cold room for overnight or at room temperature for 2 hours. The primary antibody was diluted in the blocking solution (5% milk powder in TBST), and the membrane was transferred to the antibody solution and allowed to shake on the rocking platform for 1 hour at room temperature or overnight in the cold room. The dilution of the primary antibody varied from antibody to antibody. After the incubation with the primary antidbody, the membrane was washed 3 times with TBST for 5 minutes each on the rocking platform. The secondary antibody was also diluted in the blocking solution. The membrane was incubated with the secondary antibody for 30-45 mins at room temperature. Finally, the membrane was washed 3 times with TBST as described before.

Detection

The secondary antibodies, which had the enyzme horseradish peroxidase covalently linked to them, were detected by incubating the membrane with enhanced chemiluminescence reagent for 3- 5 min at RT (ECL, Amersham Pharmacia biotech). The membranes

were then placed in an X-ray film cassette and autoradiography films were exposed for 5 sec to 10 min.

Stripping

In order to probe the same membrane with a different antibody, the immunoblots were stripped. Immunoblots were stripped by incubating in stripping solution at 56° C for 30 min, followed by two washes in TBST buffer for 10 min at RT. The membranes were then blocked again and probed with a different primary antibody.

3. Results

3.1 Rosa26 knock-in mouse model for CALM/AF10 leukemia

In order to achieve expression of the CALM/AF10 fusion in a tissue inducible manner, a knock-in mouse line was generated by Alexandre Krause, a previous member of our group, in collaboration with Dr Marlon Schneider and Prof Eckhard Wolf from the Gene Center, Munich. In this mouse line, the cDNA of the human CALM/AF10 fusion gene is knocked into the murine Rosa26 locus. In the next paragraphs, I will give an overview of how this mouse line was generated by Alexandre Krause.

In order to generate the above-mentioned line, two vectors were used pBigT and pROSA26 PA. These vectors were kindly provided by Frank Constantini from Columbia University, USA. These two vectors are commonly used to target the ubiquitously expressed Rosa26 locus in the mouse genome with a cassette that allows expression of a gene of interest from the ROSA26 locus.

3.1.1 Genaration of Rosa26-loxPstoploxP-CALM/AF10 mouse line

3.1.1.1 Cloning of the knock-in construct

First, the CALM/AF10 fusion was excised from the pIGCASV-40 construct, which is described in Alexandre Krause's thesis. Below is a schematic diagram of this construct:

Fig 3.1: Schematic representation of pIGCASV-40 construct (Taken from the doctoral thesis of Dr Alexandre Krause)

CALM/AF10 along with the SV40 splice/polyA cassette was released from this construct using *Sac*I and *Not*I and subcloned into the MCS (multiple cloning site) of the pBigT vector (Srinivas et al., 2001). In the pBigT vector, the MCS is preceded by a loxP flanked transcriptional stop cassette, which blocks expression of the transgene but can be excised via Cre-mediated recombination. This

vector allows conditional expression of the transgene. Below is the map of the pBigT vector:

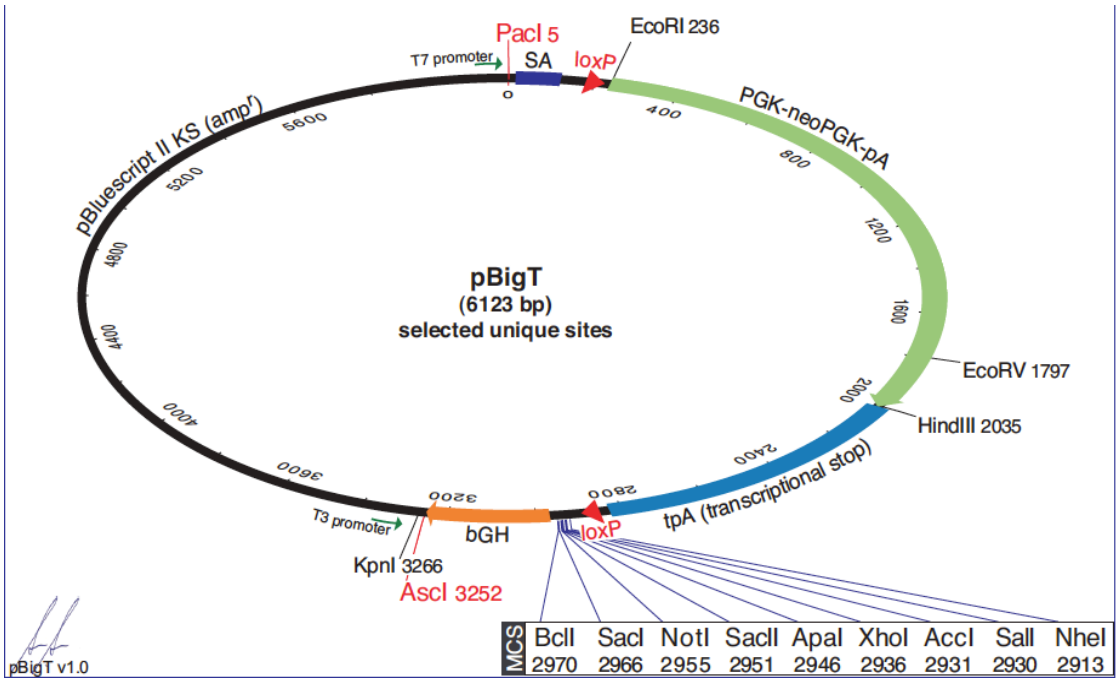


Fig 3.2: Vector map of pBigT (Source http://www.srinivas.org)

The entire inducible cassette along with the CALM/AF10 cDNA was then excised from pBigT with *Pac*I and *Asc*I and cloned into the Rosa26PA vector. This vector contains the genomic sequence to target the murine Rosa26 locus. Below is the map of pRosa26PA vector:

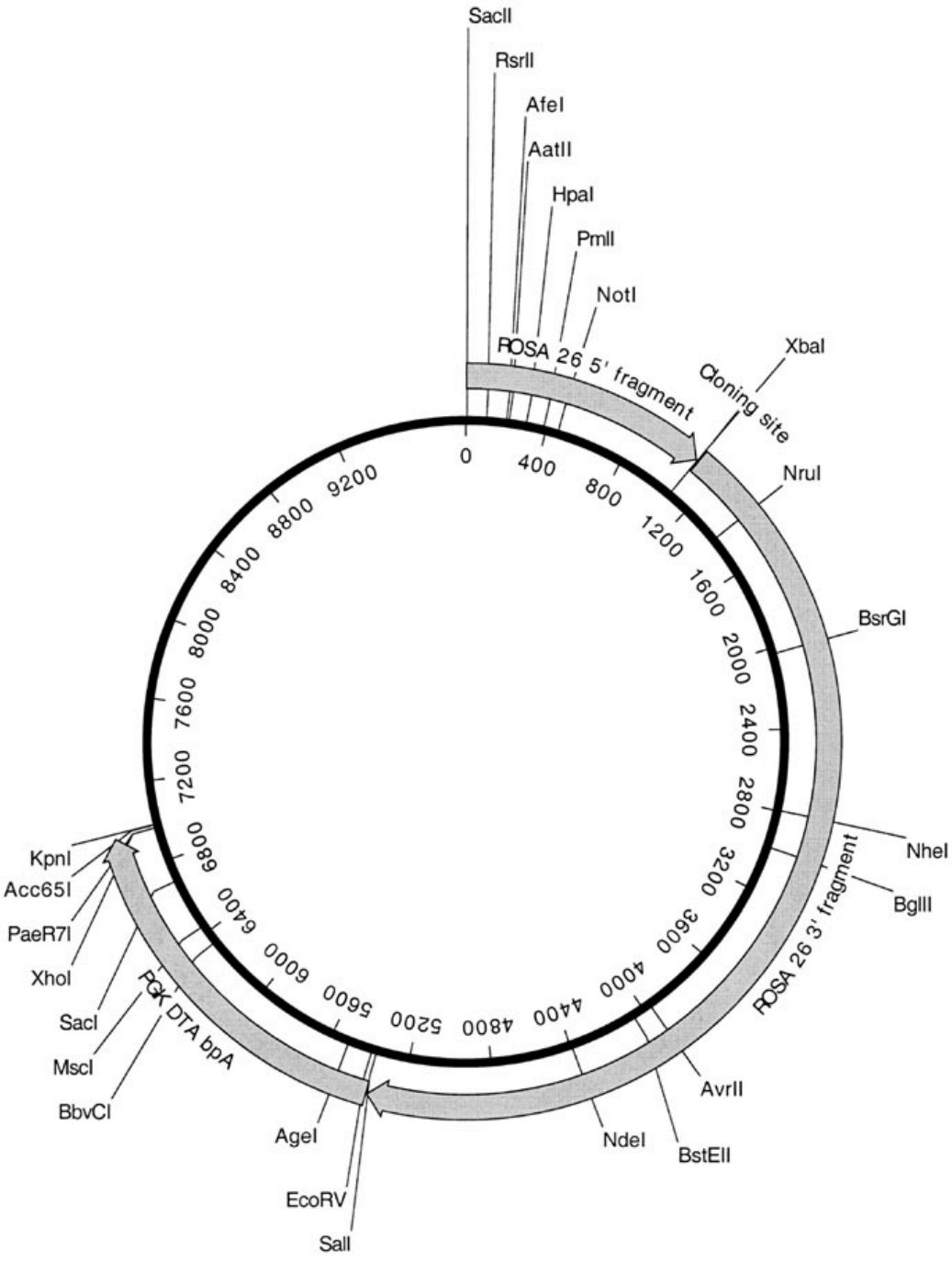


Fig 3.3 Vector map of Rosa26PA (Source: Addgene;Originally generated by Philipp M Soriano)

In the final construct, CALM/AF10 is preceded by a strong transcriptional stop sequence flanked by loxP sites. The transcriptional stop cassette prematurely terminates the transcription from the Rosa26 promoter. So the knocked-in CALM/AF10 fusion gene is not expressed in Rosa26 loxP-Stop-loxP CALM/AF10 (R26LSLCA) mice. However, when these mice are crossed with Cre-producing transgenic mice, the Cre-mediated

excision of the floxed termination sequence leads to the expression of the CALM/AF10 gene from the Rosa26 promoter. By crossing R26LSLCA mice with different strains of Cre-transgenic mice, which express the Cre recombinase in different tissues, tissue specific and conditional expression of the fusion can be achieved.

Below is the diagram of the final construct:

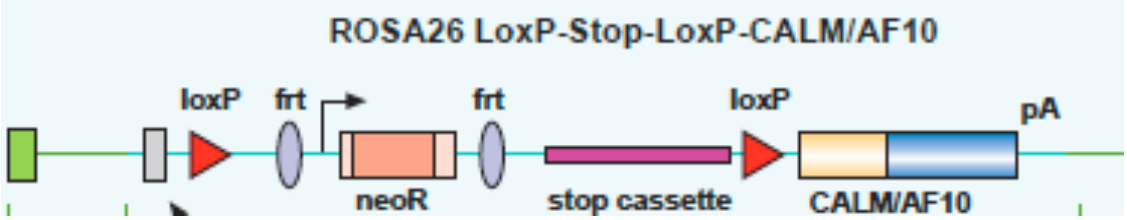


Fig 3.4: The final construct Rosa26 loxP-stop-loxP CALM/AF10 (R26LSLCA)

3.1.1.2 Generating ES cell lines with the Rosa26 Knock-in and generation of the chimeric mouse

The R26LSLCA construct was sequenced to confirm its correctness. The vector was then linearized with *Kpn*I and electroporated into mouse embryonic stem (ES) cells derived from the 129X1/SvJ strain. The ES cells were grown under Neomycin selection. Only stably transfected ES cells would grow in the selection medium. A total of 49 ES cell clones were amplified. The DNA was prepared from the clones and site-specific integration of the vector was confirmed by Southern blot analysis. A 387 bp region from the Rosa26 promoter was used as probe. The genomic DNA of ES cell clones were digested with *Eco*RV. The Rosa 26 locus specific insertion of the construct leads to the appearance of a new band of ~ 4.2 Kb size in addition to the 11 kb wild type band. The schematic presentation of the Southern strategy and the Southern blot performed by Alexandre Krause is shown below:

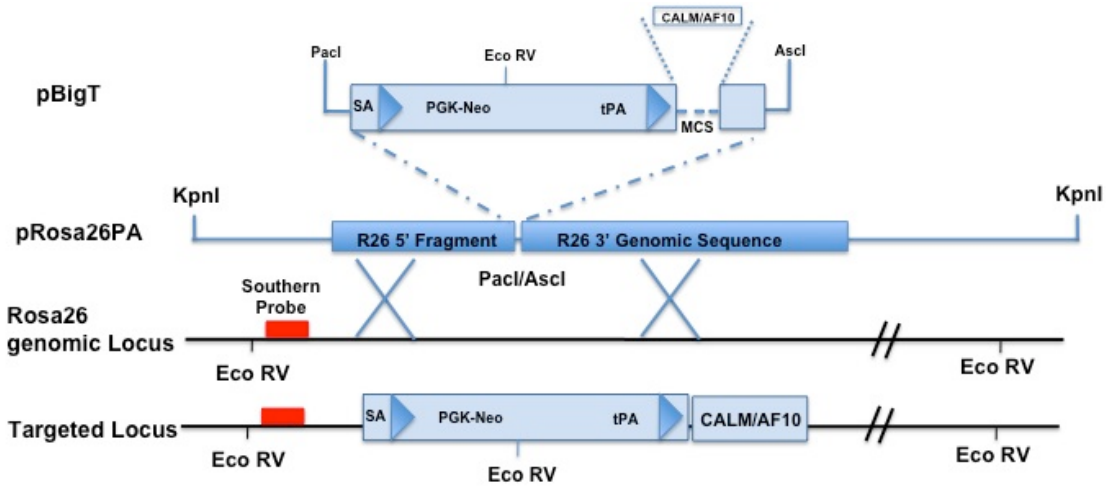


Fig 3.5: Southern strategy: The Southern probe (red) is located outside of the targeting construct in the region of the Rosa26 promoter. The genomic DNA was digested by *Eco*RV. If the Rosa 26 locus was successfully targeted, a recombinant band will appear because of the introduction of a new *Eco*RV site. (original figure is adapted and modified from Srinivas S *et al*. BMC developmental Biology 2001)

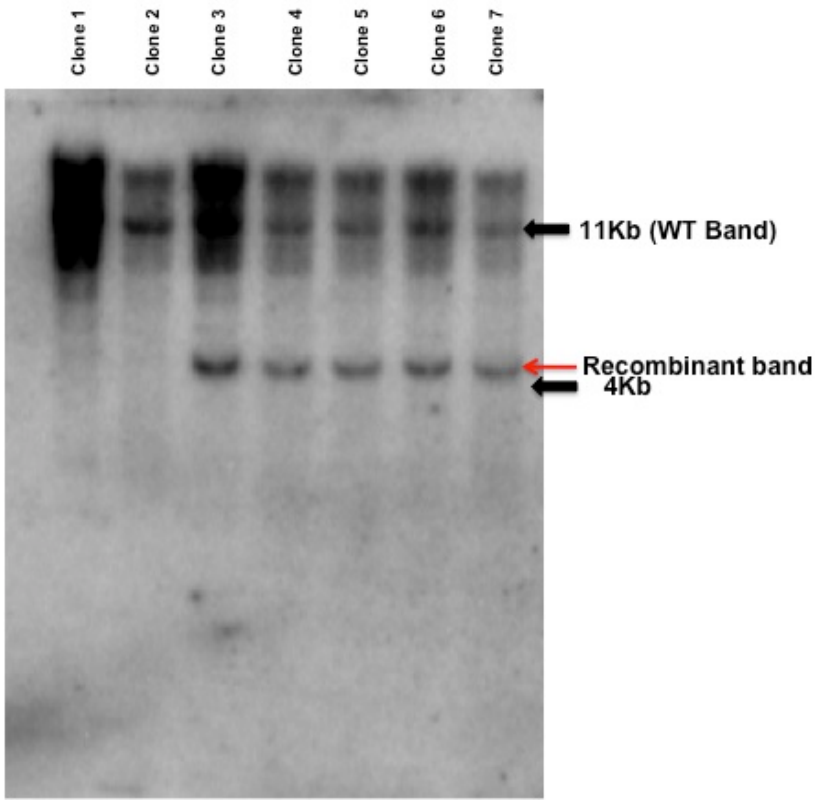


Figure 3.6: Southern blot for screening ES cell clones positive for targeted recombination. Genomic DNA of 7 ES cell clones digested with *Eco*RV and probed with the Rosa26 promoter probe. Clone 1 and 2 are wild type but clone 3, 4, 5, 6, 7 are heterozygous for the targeted allele. (Performed by Alexandre Krause)

7 positive ES clones confirmed by Southern blot were selected for further functional analysis. These clones were transiently transfected with Cre expressing vectors (experiment done by Dr PD Marlon Schneider at the Gene Center) and then cDNA was extracted from those cells. The successful excision of the transcriptional stop-cassette from the Rosa26 locus and the expression of the CALM/AF10 fusion were confirmed by RT PCR.

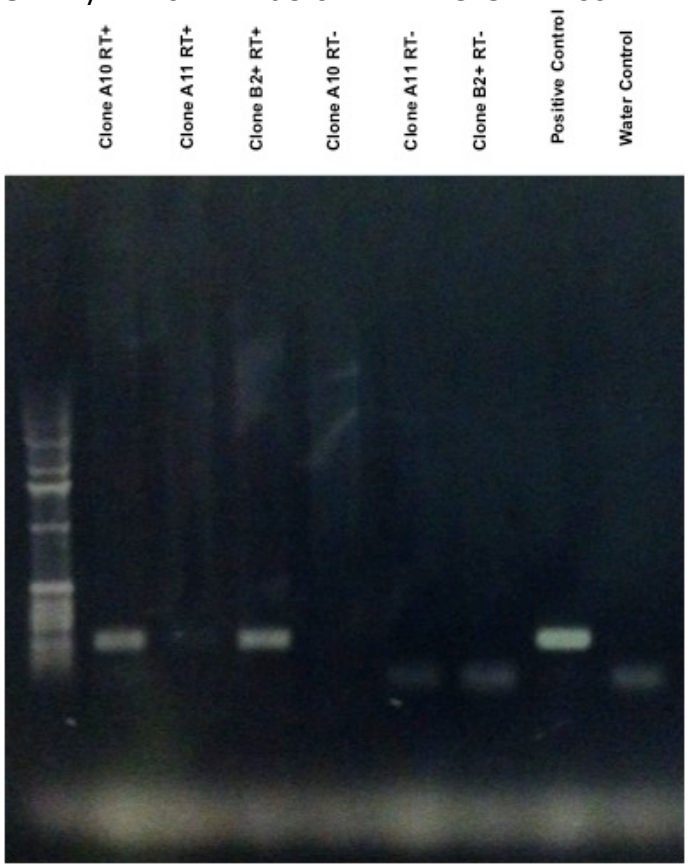


Figure 3.7: RT PCR from the cDNA of 3 recombinant ES cell clones. ES cells were transiently transfected with Cre protein expressing plasmids, RNA was extracted and a RT-PCR using CALM/AF10 specific primer pair was performed. (Lane1: Clone A10 cDNA with reverse transcriptase (RT); Lane 2: Clone A11 cDNA with RT; Lane 3: Clone B2 cDNA with RT; Lane 4: Clone A11 cDNA without RT; Lane 5: clone A11 cDNA without RT; Lane 5: B2 cDNA without RT; Lane 6: Positive control; Lane 7: water control. (Experiment done by Alexandre Krause)

In the previous experiment, the expression of the CALM/AF10 fusion is clearly visible in clone A10 and B2. Clone A10 was used for the blastocyst injection. ES cells were injected into the C57BL/6J

blastocyst and then the injected blastocyst was transferred to a surrogate mother. Chimeric mice were born and one of them showed germline transmission of the knock-in allele and the Rosa26LSL-CALM/AF10 (R26LSLCA) line was established. The work in my thesis project was performed with this mouse line.

3.1.1.3 R26LSLCA mice do not express CALM/AF10 fusion without Cre protein activity

R26LSLCA mice were genotyped by a PCR-based genotyping assay using the CA brkpnt F and CA brkpnt R primer pair. R26LSLCA mice carrying the CALM/AF10 transgene are also called "CA+/Cre-" in this thesis. In Figure 3.8 an agarose gel picture shows the PCR genotyping assay from 7 R26LSLCA mice:

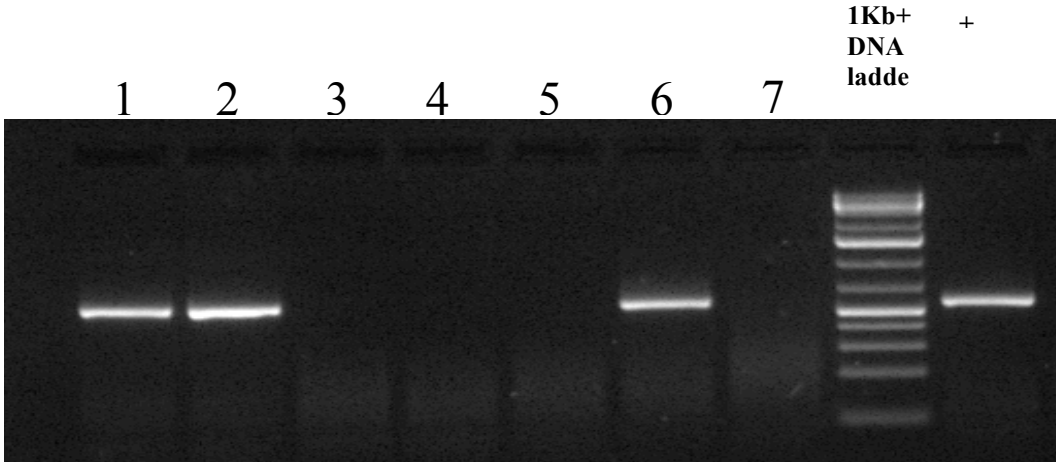


Fig 3.8: Example of the PCR genotyping assay for the R26SLSCA allele. Genomic DNA (gDNA) was extracted from the tail tips of seven 8-10 week old F1 mice from the R26LSLCA X C57BL/6 cross. The gDNA was assayed for the presence of the R26SLSCA allele by PCR. Using the C/Abrkpnt F and C/Abrkpnt R primer pair (table 2.1.3), the ~525 bp band in lanes 1, 2, and 6 indicates the presence of the R26LSLCA allele.

Bone marrow cells were isolated from 3 CA+/Cre- mice and cDNA was prepared from the bone marrow cells. Real time RT PCR was performed on those cDNA samples using realtime probe (CA brkpnt probe) and the primer pairs RTCAFWD and RTCAREV (Table 2.4) specific for the CALM/AF10 breakpoint. No detectable level of CALM/AF10 fusion transcript was identified in the bone marrow of these mice. This result indicates that in R26LSLCA mice, CALM/AF10 transcript is not produced without the activity of Cre protein.

3.1.1.4 Hematopoietic cells of CA+/Cre- mice and syngeneic wild type mice (CA-/Cre-) have a similar immunophenotype

A flow cytometry based immunophenotyping assay was performed on the peripheral blood, bone marrow and spleen cells of CA+/Cre- mice and syngenic wild type mice of similar age. These wildtype

mice are called “CA-/Cre-’’. The standard anibodies used for this assay are listed in the following table:Table 3.1: Standard panel of antibodies used for immunophenotype analysis by flow cytometry

Name of the Antibody	Specificity	Target compartment
PE-rat anti-mouse Gr1	Reacts with a common epitope Ly-6G and Ly-6C present on granulocytes and monocytes but not on erythrocytes.	Myeloid
APC rat anti-mouse CD11b or Mac1 alpha	Reacts with the 170 kDa α chain of Mac1 or CD11b which is expressed at varying levels on granulocytes, macrophages, myeloid derived dendritic cells, natural killer cells, microglia and B-1 cells	Myeloid
PE-rat anti-mouse Ly-6A/E or Sca1	Reacts with the 18kDa phosphatidylinositol-anchored protein Sca1 which is expressed on the multipotent hematopoietic stem cells in the bone marrow of mice	Stem cell, progenitor cell
APC rat anti-mouse CD117 or c-Kit	Reacts with CD117, a transmembrane tyrosine kinase receptor encoded by Kit gene. CD117 is expressed on hematopoietic progenitor cells committed to myeloid, erythroid lineages also on the precursors of B and T cells.	stem cell, progenitor cell
APC rat anti-mouse CD45R or B220	Reacts with an epitope of the extracellular domain of transmembrane CD45 glycoprotein. CD45 is expressed on B lymphocytes at all stages from pro-B through mature and activated B cells	Lymphoid
APC rat anti-mouse CD43 or Ly-48	Reacts with 115 kDa glycoform of CD43 which is expressed on pro-B cells,plasma	Lymphoid/myeloid

	cells,granulocytes, monocytes,macrophage, platelets, natural kille cells,peritoneal and splenic CD5+ B cells and most T helper cells. The resting peripheral B cells do not express this protein.	
APC hamster anti-mouse CD3e	Reacts with 25kDa ε chain of the T cell receptor-associated CD3 complex, which is expressed on thymocytes, mature T lymphocytes and NK cells.	Lymphoid
PE rat anti-mouse CD4	Reacts with the CD4 differentiation antigen expressed on most thymocytes, subpopulation of mature T cells (T helper cells) and a subset of NK-T cells.	Lymphoid
APC-rat anti-mouse CD8a	Reacts with the 38 kDa α chain of CD8 differentiation antigen expressed by cytotoxic T cells	Lymphoid
PE rat anti-mouse CD19	Reacts with CD19, a 95kDa transmembrane glycoprotein, which is a B cell lineage differentiation antigen.	Lymphoid

Aliquots of peripheral blood (PB), bone marrow (BM) and spleen cells (Spl) were stained with two antibodies at the same time conjugated either to PE (phycoerithrin) or APC (Allophycocyanin). Antibodies were used in the following combinations: Gr1-PE/Mac1-APC; Sca1-PE/cKit-APC; B220-PE/CD43-APC; CD4-PE/CD3-APC; CD4-PE/CD8-APC; CD19-PE/B220-APC. The antibody-stained cells were analyzed on a flow cytometer.

PB cells from 3 CA+/Cre- mice and BM and Spl cells from 6 CA+/Cre- mice were isolated and immunophenotyping analysis was performed. As wild type control, PB, BM and Spl cells from 5 CA-/Cre- mice were isolated and their immunophenotype was analyzed using flow cytometry. The results are summarized in the following figures:

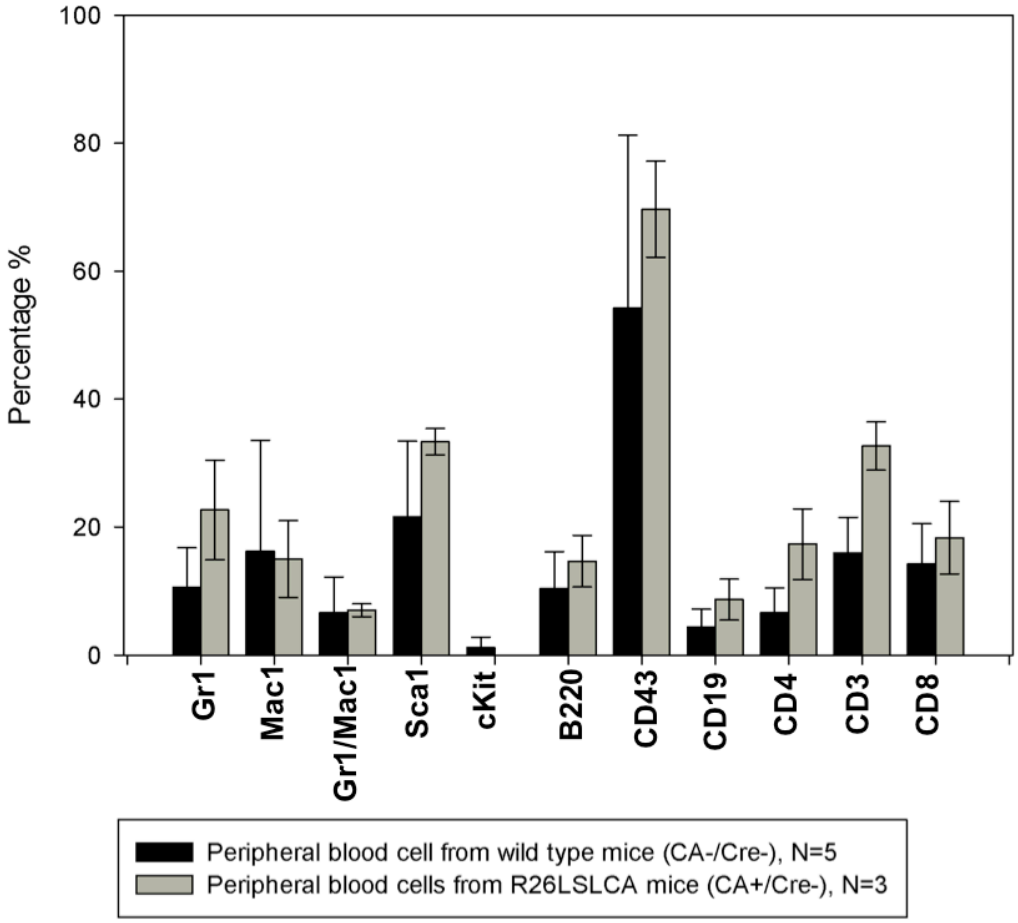


Fig 3.9: Immunophenotype of the peripheral blood cells from wild type mice (CA-/Cre-) and CA+/Cre- mice The bar graphs represent the average percentage of surface markers and their combinations. The error bars represent the standard deviation. There was no significant difference in the expression of surface markers between the two groups of animals.

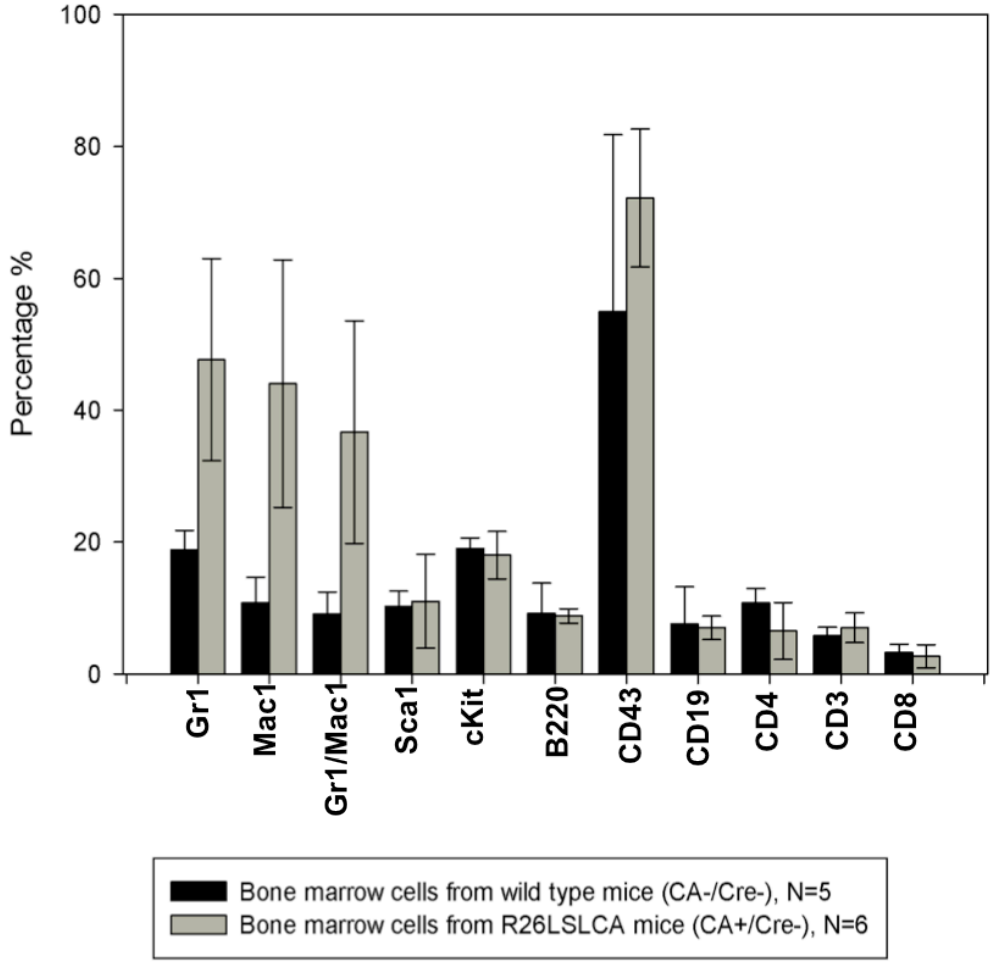


Fig 3.10: Immunophenotype of the bone marrow cells from wild type mice (CA-/Cre-) and CA+/Cre- mice The bar graphs represent the average percentage of surface markers and their combinations. The error bars represent the standard deviation. Expression of the myeloid surface markers Gr1, Mac1 was higher in the CA+/Cre- mice in comparison to the wild type mice but (p value for Gr1 is 0.002, Mac1 is 0.003 and GM++ is 0.006. All other markers show similar distributions between two groups.

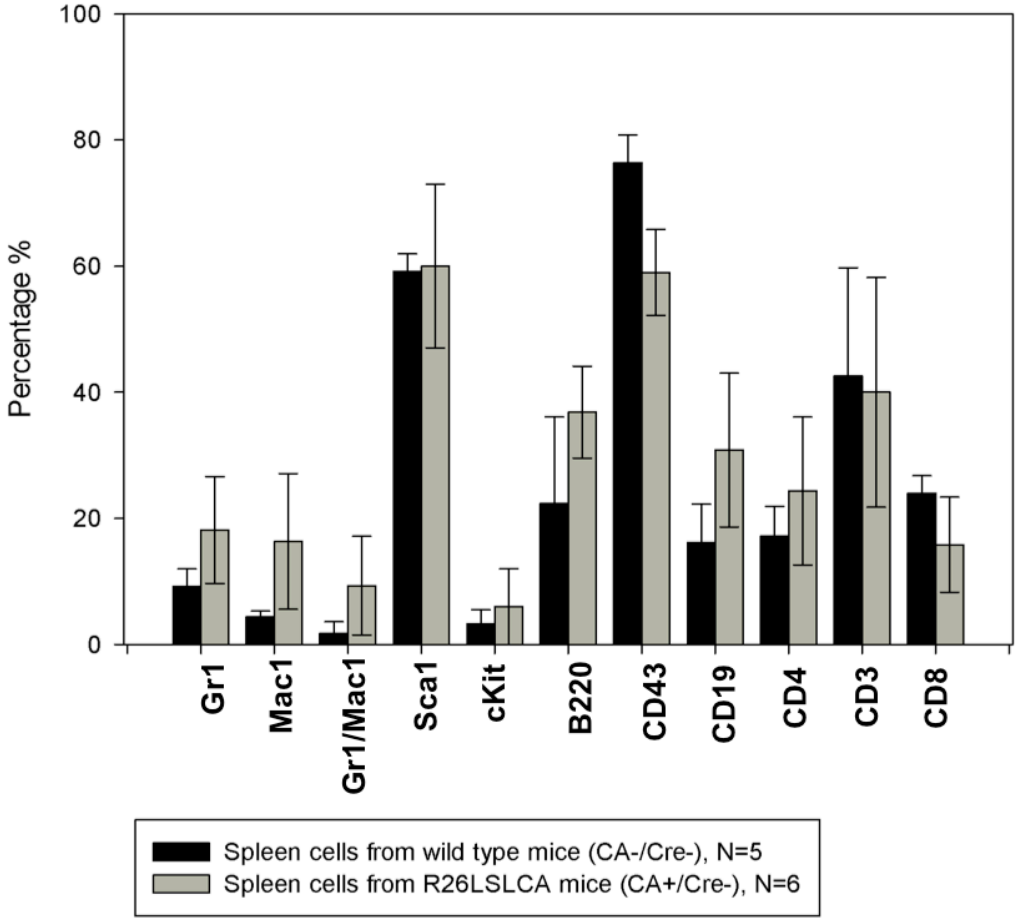


Fig 3.11: Immunophenotype of the spleen cells from wild type mice (CA-/Cre-) and CA+/Cre- mice The bar graphs represent the average percentage of surface markers and their combinations. The error bars represent the standard deviation. Mac1 positive cells were more in CA+/Cre-mice (p value 0.03) but other surface markers showed similar distribution between two groups of animals.

Hematopoietic cells from CA+/Cre- mice and the wild type CA-/Cre- mice by and large show similar immunophenotype. The variation that was observed in the expression of myeloid markers on the bone marrow and spleen cells could be considered as normal variation.

Since the hematopoietic compartments of these two groups of animals are quite similar, we combined them into one group for a larger cohort of control mice. For the comparative immunophenotypic analysis in the remainder of this thesis, we have used this combined group of mice (data from 5 CA-/Cre- and 6 CA+/Cre- animals) as controls.

3.1.2 Ubiquitous expression of CALM/AF10 using CreERT2

To achieve ubiquitous expression of the CALM/AF10 (CA) fusion, the R26LSLCA mouse line was crossed with the Rosa26 Cre-ERT2 line (kindly provided by Eckhardt Wolf's group, Gene center). These mice have a Cre recombinase/estrogen receptor fusion gene integrated in the ubiquitously expressed Rosa26 locus (Soriano, 1999). The offspring of this cross, which had both knock-in Rosa26 alleles, was called CA+/CreER+. The estrogen receptor T2 part of the fusion portein is responsible for a cytosolic location of the Cre-ERT2 fusion protein. In the presence of Tamoxifen, the Cre-ERT2 fusion protein translocates into the nucleus, where the Cre moiety of the fusion protein mediates loxP-specific recombination and removal of the transcriptional stop cassette of the R26LSLCA allele (Soriano, 1999). This results in the ubiquitous expression of the CALM/AF10 fusion protein.

The F1 generation animals were genotyped at the age of 5-8 weeks using the primer pair CA brkpnt F and R and Cre-Er F and R (Table 2.3).

The double positive (CA+/Cre-ER+) animals were injected intra-peritonially with 1 mg of Tamoxifen for 5 consecutive days. The Tamoxifen dose was not adjusted to the body weight of the individual animals, although all the injected animals were of same age (12 weeks old) and had very similar body weight. As control, R26LSLCA mice that did not have the Cre-ERT2 allele (CA+/Cre-Er-) were treated with Tamoxifen in the similar way. After 5 days of induction, one CA+/Cre-ER+ and one CA+/Cre-ER- control mouse were sacrificed and cDNA from the spleen and the bone marrow was prepared to analyze the expression of CALM/AF10 using RT-PCR. In this PCR a primer pair specific for the breakpoint in the CALM/AF10 cDNA was used.

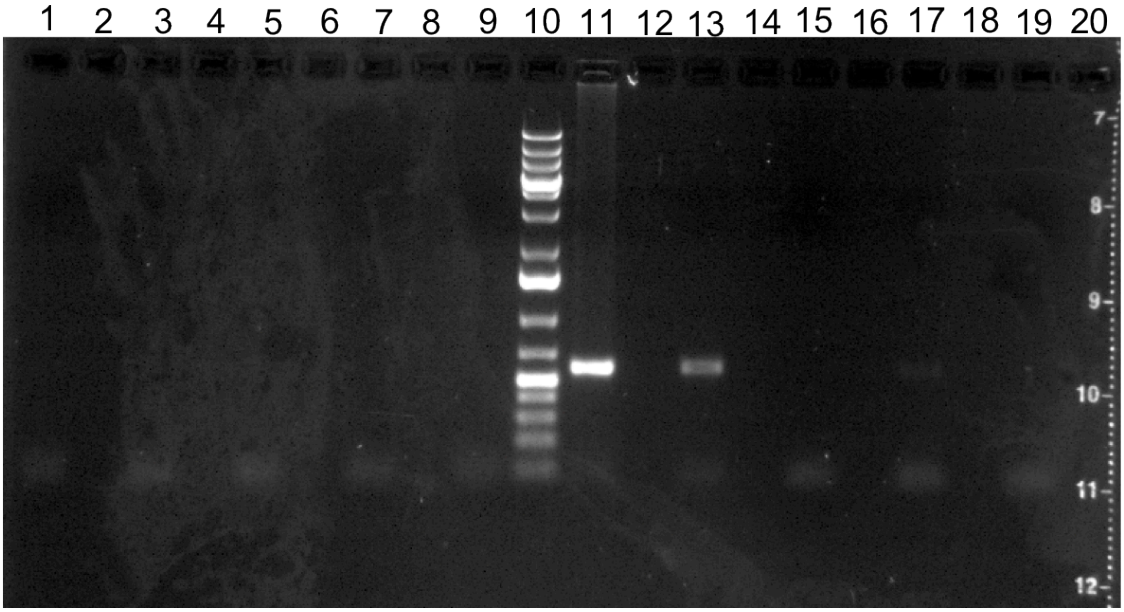


Fig 3.12: Expression of the CALM/AF10 fusion in CA+/Cre-ER+ mice after Tamoxifen treatment. Lane 1: Negative control, water; Lane 3: cDNA from CA+/Cre-ER+ bone marrow (BM) without reverse transcriptase (RT); Lane 5: cDNA from CA+/Cre-ER- BM without RT; Lane 7: cDNA from CA+/Cre-ER+ spleen (Spl) without RT; Lane 8: cDNA from CA+/Cre-ER- Spl without RT; Lane 10: Fermentas 1kb plus DNA ladder; Lane 11: Genomic DNA from CA+/Cre-ER+ as positive control for the CALM/AF10 amplicon; Lane 13: cDNA from CA+/Cre-ER+ BM with RT; Lane 15: cDNA from CA+/Cre-ER- BM with RT; Lane 17: cDNA from CA+/Cre-ER+ Spl with RT; Lane 19: cDNA from CA+/Cre-ER- Spl with RT. Primer pair specific for CALM/AF10 breakpoint (C/A brkpnt F and CA brkpnt R) was used for the assay,

In the bone marrow of the Tamoxifen treated mouse (CA+/Cre-ER+), expression of the CALM/AF10 fusion gene was detected (band size ~ 500bp; lane 13 in Fig 3.12). In the spleen from the same mouse very low levels of CALM/AF10 mRNA could also be detected (lane 17 in Fig 3.12). In the negative controls without RT no amplification was found. These controls were especially important since any small carry-over of mouse genomic DNA would also lead to a PCR product of the same size with these primers. There was also no expression of CALM/AF10 in the bone marrow and spleen of the Tamoxifen treated CA+/Cre-ER- mouse (lanes 15 and 19).

The expression of the CALM/AF10 fusion gene was also detected in a second group of Tamoxifen treated CA+/Cre-ER+ mice. 3 animals were sacrificed (12 weeks old): one Tamoxifen-treated CA+/Cre-Er+ mouse, one CA+/Cre-ER+ mouse without Tamoxifen treatment and a Tamoxifen-treated CA+/Cre-ER-. From all the mice cDNA was prepared from the bone marrow and spleen, and an RT PCR was performed using the primer pair CA brkpnt F and R (Table 2.3). To

assay the quality of the cDNA, beta-actin was used as control (Figure 3.13).

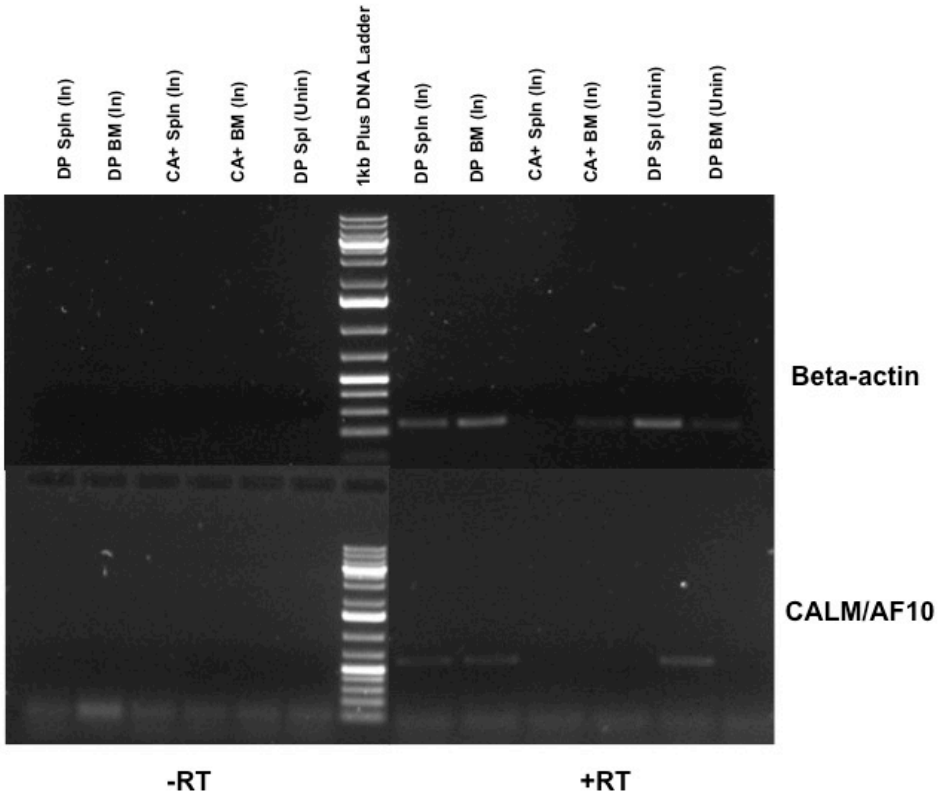


Fig 3.13: Amplification of beta-actin house-keeping gene and CALM/AF10 fusion gene from the cDNA of the mouse tissue. DP Spln (In)**:** CA+/Cre-ER+ spleen cDNA, Tamoxifen treatment; DP BM (In): CA+/Cre-ER+ bone marrow cDNA, Tamoxifen treatment; CA+Spln (In): CA+/Cre-ER- spleen cDNA, Tamoxifen treatment; CA+ BM (In): CA+/Cre-ER- bone marrow cDNA, Tamoxifen treatment; DP Spln (Unin): CA+/Cre-ER+ spleen cDNA without Tamoxifen treatment; DP BM (Unin): CA+/Cre-ER+ bone marrow cDNA without Tamoxifen treatment. Size of the beta actin amplicon ~ 250 bp, size of CALM/AF10 ~ 550 bp -RT: Control samples without reverse transcriptase; +RT: Samples with reverse transcriptase. In: induced or Tamoxifen treated; Unin: not induced, no Tamoxifen treatment

Beta-actin expression was detected in all the cDNA samples except for the cDNA from CA+/Cre-ER- bone marrow (Figure 3.13).

CALM/AF10 expression was detected in the bone marrow and spleen of the two Tamoxifen-induced CA+/Cre-ER+ mice (Figure 3.13). But CALM/AF10 expression was also detected in the spleen cDNA of the uninduced CA+/Cre-ER+ mouse. Since the -RT control did not show amplification, genomic DNA contamination can be ruled out. This result suggests Tamoxifen-independent (leaky) activity of the Cre-ERT2 recombinase.

3.1.2.1 Analyzing the efficiency of Cre recombination of the R26LSLCA allele:

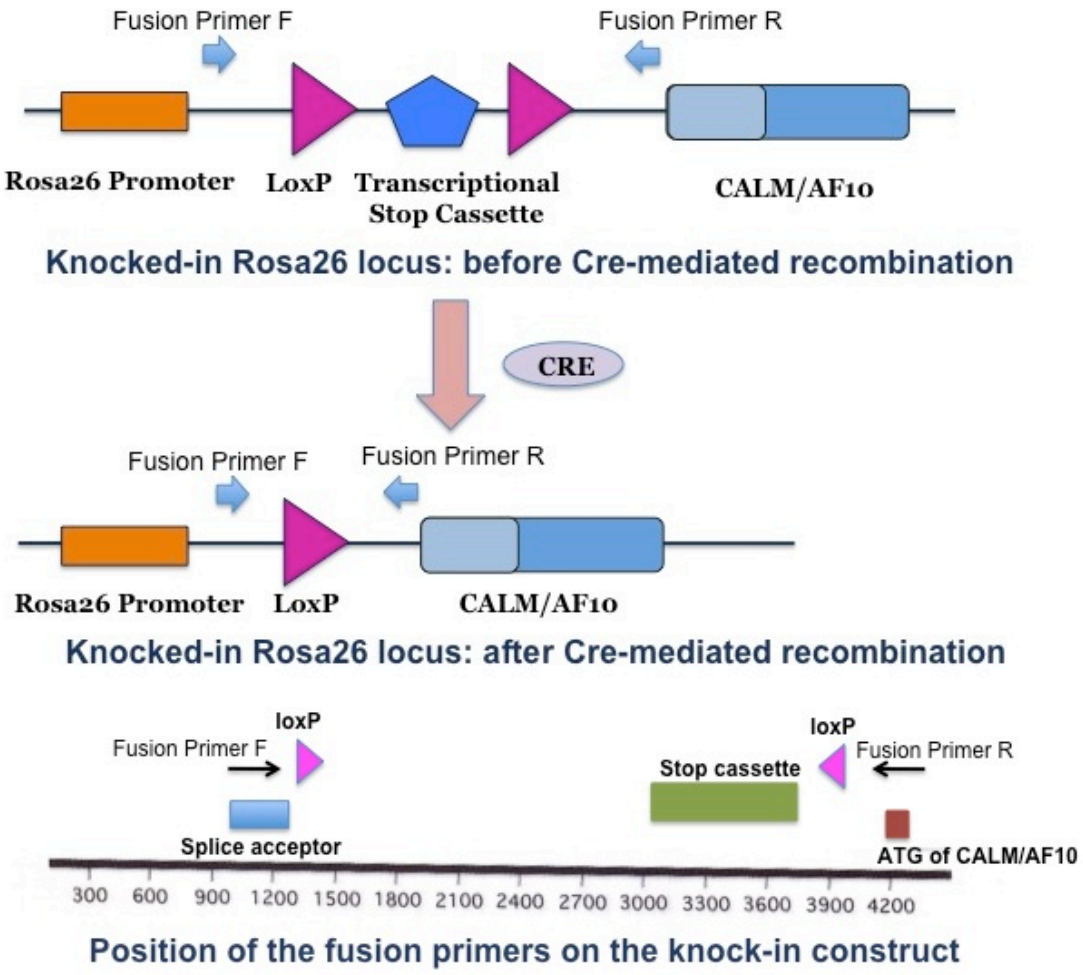


Fig 3.14: PCR based strategy for analyzing the recombination efficiency of the Cre protein. Schematic representation of R26SLSCA allele showing the position of the fusion primer F and R. These two primers amplify a 411 bp region between the 5' loxP and the start of the CALM/AF10 cDNA after Cre- mediated recombination.

To assay the recombination efficiency of the Cre recombinase at the R26SLSCA allele, a PCR based strategy was developed. In figure 3.14, the position of the forward primer is indicated as "Fusion primer F" This primer binds to the splice acceptor region, 5' of the first loxP site (bp 1167-1188). The reverse primer ("Fusion primer R") binds to the region between the second LoxP site and the start codon of the CALM/AF10 gene (bp 4258-4278). This primer pair will amplify a 411 bp region after the stop cassette has been removed by Cre-mediated recombination at the loxP sites. In the non-

recombined state, the distance between these two primers is 3.1 kb, too large to be amplified in a normal PCR experiment.

The recombination efficiency of the Cre-ERT2 protein was assayed by performing the above PCR assay on the genomic DNA of Tamoxifen treated CA+/Cre-ER+ mice. Genomic DNA from untreated and treated CA+/Cre-ER+ mice and Tamoxifen treated CA+/Cre-ER- mice were included as controls in the assay.

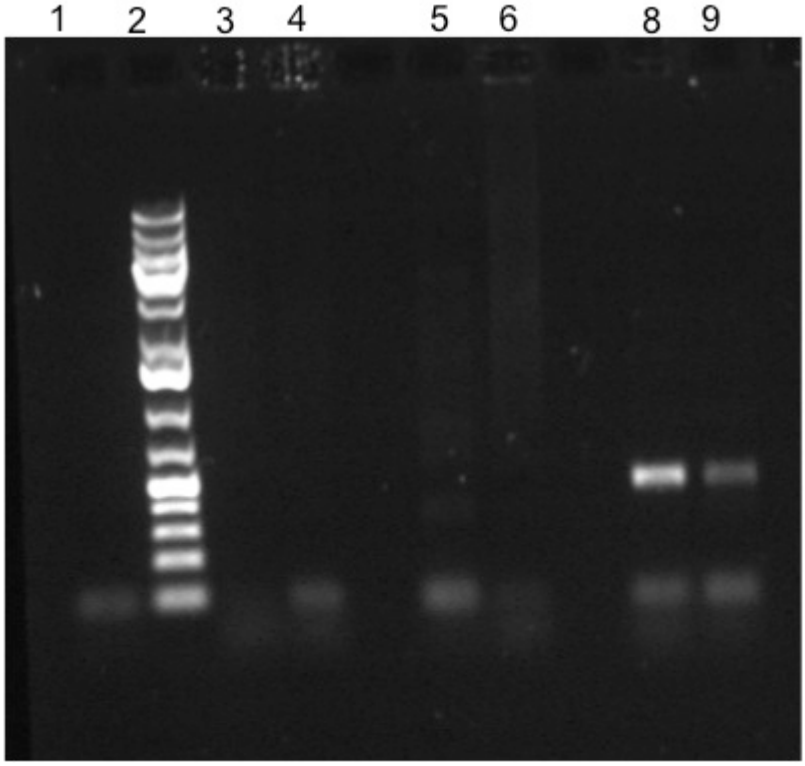


Fig 3.15: PCR from genomic DNA of bone marrow and spleen to detect CreERT2-mediated genomic recombination. Lane 1: water control; Lane 2: 1 Kb DNA ladder; Lane 3: Bone marrow DNA from a Tamoxifen treated **CA+/Cre-ER-** mouse; Lane 4: Spleen DNA from a Tamoxifen treated **CA+/Cre-ER-** mouse; Lane 5: Bone marrow DNA from a **CA+/Cre-ER+** mouse without Tamoxifen treatment; Lane 6: Spleen DNA from a **CA+/Cre-ER+** mouse without Tamoxifen treatment; Lane 8: Bone marrow DNA from a Tamoxifen-treated **CA+/Cre-ER+** mouse; Lane 9: Spleen DNA from a Tamoxifen-treated **CA+/Cre-ER+** mouse;

In the above PCR assay, the expected band of 400-500 bp, which indicated successful excision of the transcriptional stop-cassette, was only detected in the organs of Tamoxifen treated CA+/Cre-ER+ mice (Lane 8 and 9; figure 3.15). No amplification was seen from the DNA of the untreated CA+/Cre-ER+ mice or from the Tamoxifen treated CA+/Cre-ER- mice. These results clearly showed that the Cre enzyme functioned as expected in the CA+/Cre-ER+ mice, excising the loxP site-flanked transcriptional stop cassette 5' of the CA fusion gene in the RS26SLSCA allele.

3.1.2.2 Ubiquitous expression of CALM/AF10 after Cre-ERT2-mediated recombinatoin does not cause leukemia

A total of 19 CA+/Cre-ER+ mice were treated with Tamoxifen when they were 8-10 weeks old. Along with the CA+/Cre-ER+ experimental animals, 8 CA+/Cre-ER- and 8 CA-/Cre-ER+ control mice were treated with Tamoxifen in the same fashion and observed. The details of the tamoxifen treated mouse cohort are summarised in Tables A1, A2 and A3 in the appendix.

Tamoxifen-treated CA+/Cre-ER+ mice, which expressed CALM/AF10 ubiquitously, did not develop any leukemia or hematopoietic phenotype even after a long observation period of 24 months.

3.1.2.3 Analysis of CA+/Cre-ER+ Tamoxifen-treated mice

Flow cytometry

To analyze the bone marrow compartment and splenocytes, 3 clinically healthy CA+/Cre-ER+ mice that had undergone Tamoxifen treatment at 8 to 10 weeks of age were sacrificed at the age of 12 months. Their bone marrow and spleen cells were stained with antibodies from our standard antibody panel (table 3.1). The immunophenotype of the hematopoietic cells from these mice are compared with that of our control mouse group as described earlier.

The results from the experiment are shown in figure 3.16 and 3.17.

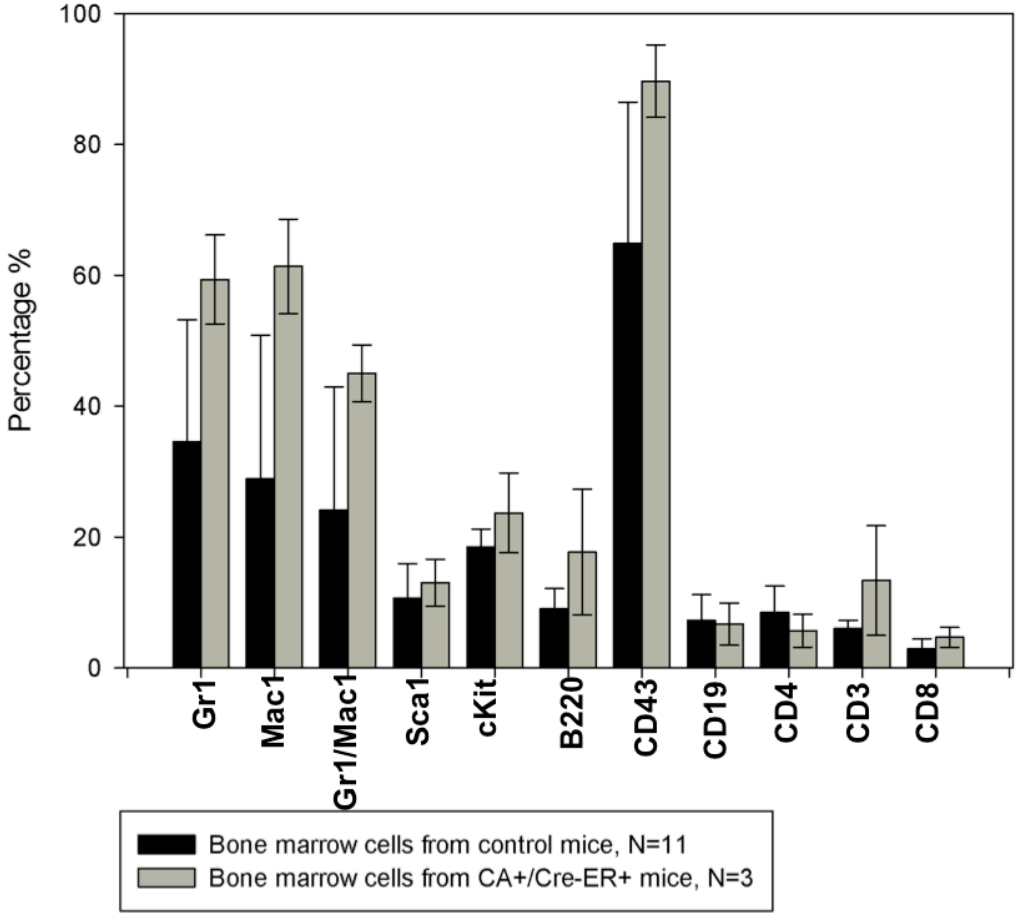


Fig 3.16: Immunophenotype of the bone marrow cells from tamoxifen treated CA+/Cre-ER+ mice (N=3) and control mice (N=11). Average percentages of epitope positive cells for each epitope are shown as bar graphs. The error bars represent the standard deviations. The number of myeloid cells were slightly higher in the bone marrow of Tamoxifen treated CA+/Cre-ER+ mice. P value for the Gr1+ cells is 0.04, for Mac1+ cells is 0.03. Other markers show more or less equal distribution between two groups of animals

The number of Mac1 positive and Gr1 positive cells was higher in Tamoxifen treated CA+/Cre-ER+ mice. Apart from that there were no significant differences in the distribution of the various surface markers on the bone marrow cells from the Tamoxifen-treated CA+/Cre-ER+ mice compared to the bone marrow cells from control mice.

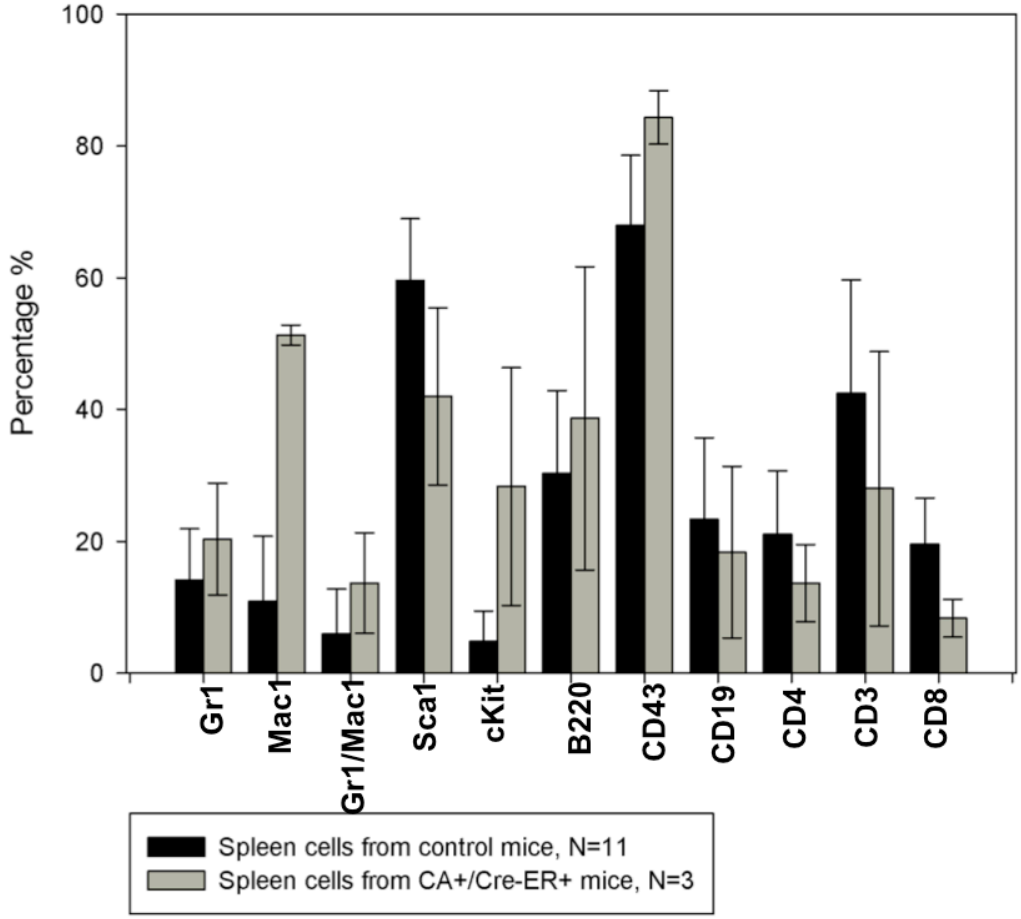


Fig 3.17: Immunophenotype of the spleen cells from Tamoxifen-treated CA+/Cre-ER+ mice (N=3) and the control mice (N=11). Average percentages of epitope positive cells for each epitope are shown as bar graphs. The error bar represents the standard deviation. Number of Mac1+ cells is very high in Tamoxifen treated CA+/Cre-ER+ mice (p value 1.69E-5).

The spleen cells from CA+/Cre-ER+ and the control mice showed a by and large similar immunophenotype. However, the percentage of Mac1 positive cells was higher in the spleen cells derived from CA+/Cre-ER+ mice than in spleen cells from control mice (p value 1.69E-5). The other markers showed a similar distribution between the two groups.

3.1.3 Expression of the CALM/AF10 fusion gene in the entire hematopoietic compartment after Vav-Cre mediated recombination

To achieve expression of the CALM/AF10 fusion gene in the entire hematopoietic compartment, Rosa26LSLCA mice were crossed with the Vav-Cre mouse line. (Kindly provided by Dr Mathias Kieslinger, Helmholtz Zentrum Munich). In this mouse line, the Cre protein is expressed under the murine *Vav* gene regulatory elements (Georgiades et al., 2002). The *Vav* gene is expressed almost exclusively in hematopoietic cells starting from early fetal liver hematopoiesis onwards (Ogilvy et al., 1999).

Rosa26LSLCA mice were crossed with Vav-Cre mice and the offsprings were genotyped using a PCR-based assay when they were 8-10 weeks old. The CALM/AF10 fusion breakpoint-specific primers (CA brkpntF and CA brkpnt R) and Vav-Cre-specific primers (Vav Cre F and Vav Cre R) were used in the assay. Double positive mice showing amplification with the CALM/AF10 fusion breakpoint primers and the Vav-Cre primers were monitored for the development of leukemia. The double positive mice are called CA+/Vav-Cre+.

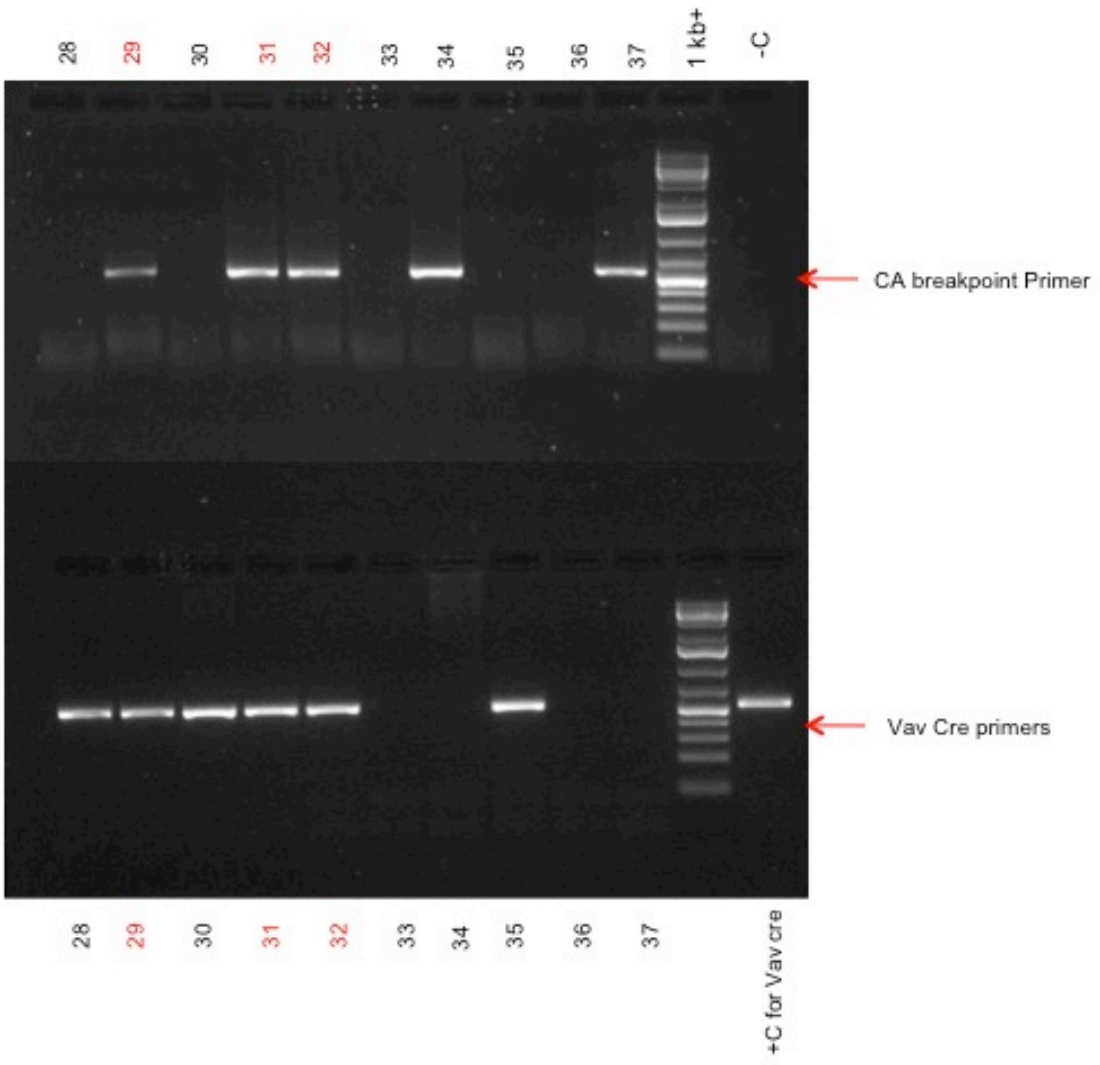


Fig 3.18: Example of the PCR-based genotyping assay for Vav-Cre. Genomic DNA extracted from the tail tips of nine 8-10 week old F1 mice from the initial R26LSLCA x Vav-Cre cross was used for the assay. The ~ 550 bp band using CALM/AF10 breakpoint specific primers (CA brkpnt F, CA brkpnt R) indicates the presence of the CALM/AF10 transgene. A ~ 500 bp band with the Vav Cre F and Vav Cre R primers shows the presence of the Vav-Cre transgene. On the above gel mice number 29, 31, and 32 are positive in both the CALM/AF10 and Vav-Cre assay.

The expression of CALM/AF10 fusion transcript was detected in the bone marrow of CA+/Vav-Cre+ mice by RT-PCR and realtime RT-PCR. cDNA was prepared from the bone marrow cells of one CA+/Vav-Cre+ mouse. As a CALM/AF10 positive control, cDNA from the U937 cell line was used. U937 is a human monocytic cell line containing CALM/AF10 translocation, from which the CALM/AF10 fusion was cloned originally. Realtime RT PCR was performed on these two cDNA samples using the CALM/AF10 breakpoint specific primer pair and probe. Amplification of the beta actin gene was used as the endogenous control. This experiment showed that the expression of the CALM/AF10 fusion transcript is slightly higher in

the bone marrow of the CA+/Vav-Cre+ mouse than in the U937 cell line. As mentioned in the introduction, the CALM/AF10 fusion was originally cloned from the U937 cell line. The results of this experiment are shown in figure 3.19

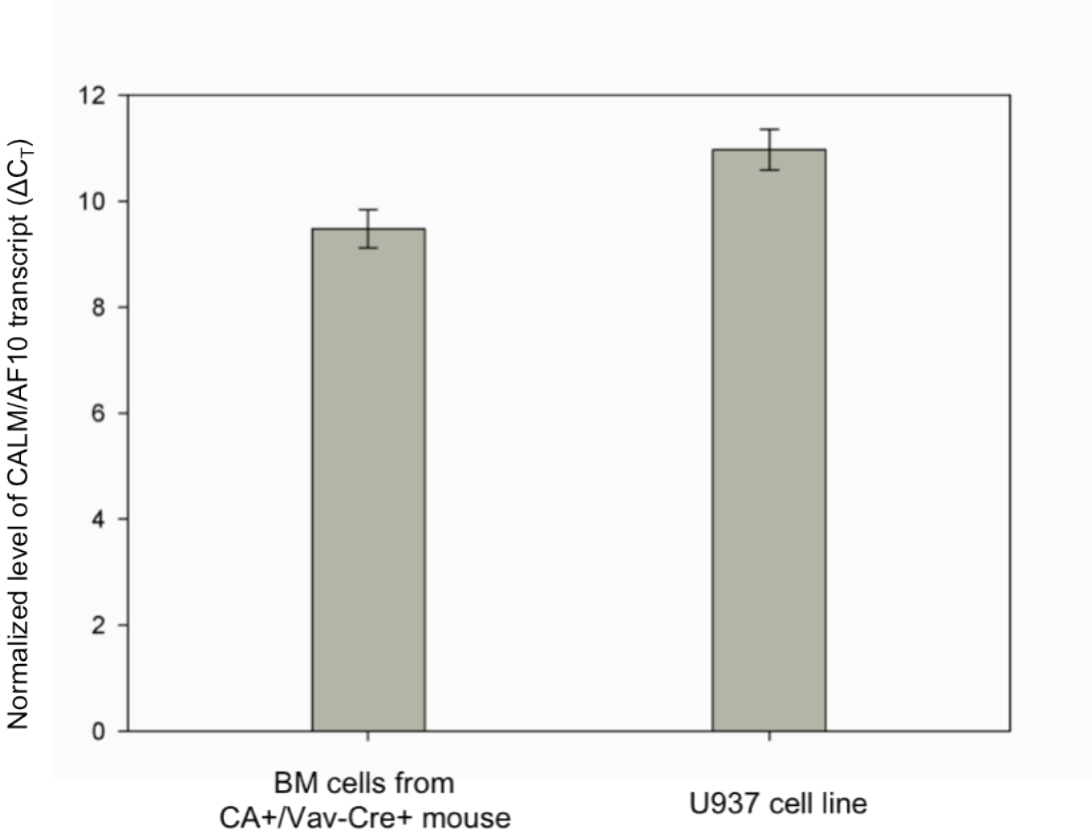


Fig 3.19: Detection of CALM/AF10 transcript in the bone marrow cells of a CA+/Vav-Cre+ mouse. cDNA was prepared from the bone marrow cells of one CA+/Vav-Cre+ mouse and U937 cells. Realtime PCR was performed using CALM/AF10 fusion breakpoint specific primer pair and probe. Amplfiication of the ACTB (beta actin) was used as endogenous control. For CALM/AF10 and beta-actin, three replicates were assayed. The bar graphs represent the normalized C_T value of the CALM/AF10 transcript. The error bars represent the standard deviation between the technical replicates. In both samples, the expression of the CALM/AF10 fusion was detected. Since the $C_T$value for CALM/AF10 transcript is slightly lower in CA+/Vav-Cre+ bone marrow cells than in U937,the level of CALM/AF10 transcript is slightly higher in the bone marrow of CA+/Vav-Cre+ mouse.

A total of 23 CA+/Vav-Cre+ mice along with 12 CA-/Vav-Cre+ controls and 15 R26LSLCA mice (CA+/Vav-Cre-) mice (common control arm for all Cre lines) were observed for leukemia development for a period of up to 600 days.

3.1.3.1 Expression of the CALM/AF10 fuson gene in Vav expressing cells leads to the development of an acute myeloid leukemia

The R26LSLCA mice expressing the CALM/AF10 fusion gene after Vav-Cre mediated recombination developed an acute myeloid leukemia with a median latency of 354 days and a 100% penetrance. A total of 23 CA+/Vav-Cre+ animals were observed. A post mortem analysis was possible in only 15 leukemic animals. 8 animals were found dead in their cages and could not be analyzed in detail. 6 out of 8 dead mice had splenomegaly, which strongly suggests that they died of leukemia. In 2 dead animals we could not do any analysis due to severe autolysis of the organs. The days of survival for each leukemic animal are listed in table A4 in the appendix.

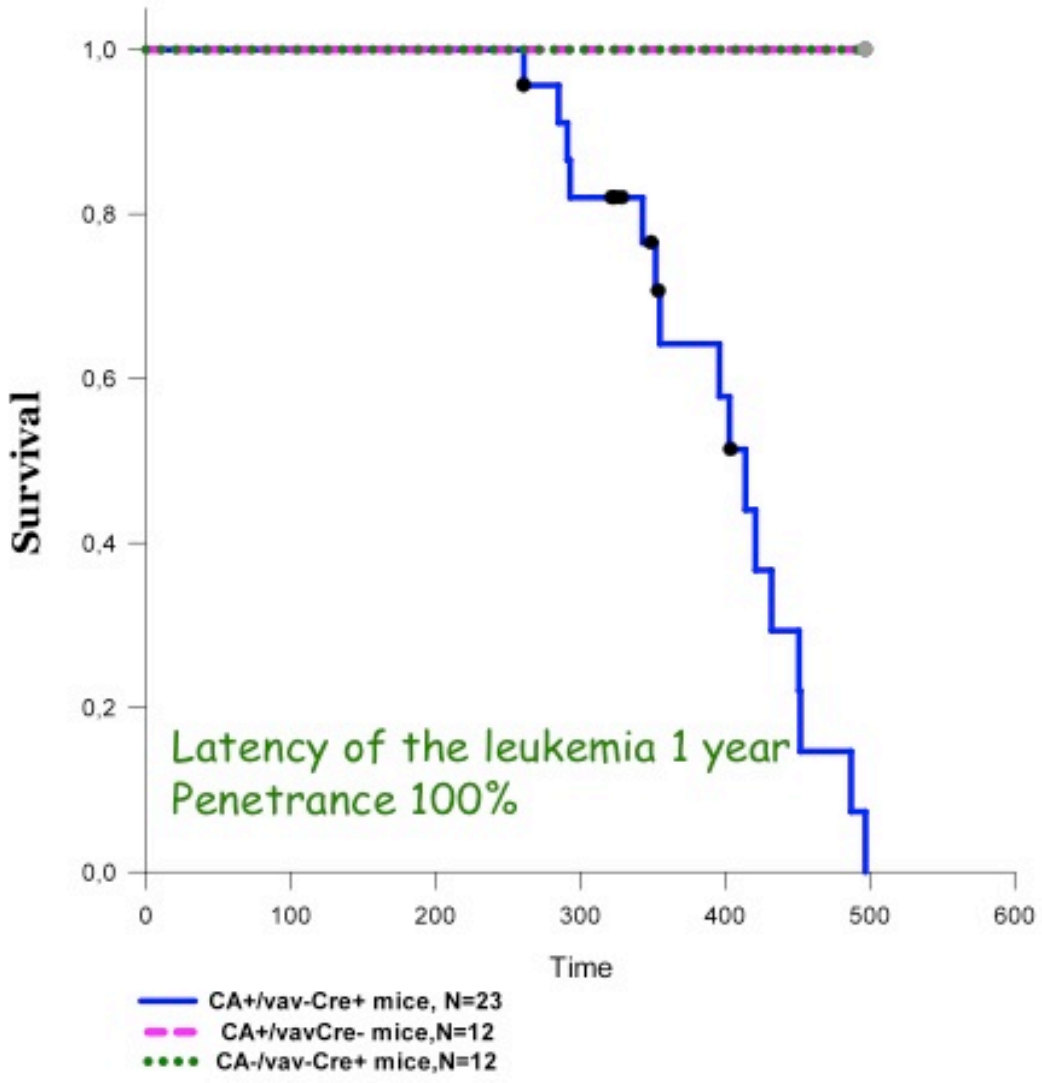


Fig 3.20: Kaplan-Meier survival plot of CA+/Vav-Cre+, CA+/Vav-Cre- and CA-/Vav-Cre+ mice. All the mice expressing the CALM/AF10 fusion gene (CA+/Vav-Cre+)(N=15) developed an acute myeloid leukemia with a median latency of 354 days (ranging from 261 to 497 days). In contrast, the mice from both control groups: CA+/Vav-Cre- (N=12) and CA-/Vav-Cre+ mice (N=12) remained healthy. Each step on the survival graph indicates one event (death of an animal). The dots indicate the censored animals, which could not be analyzed.

Shortly before their death, leukemic animals showed the typical symptoms associated with leukemia like ruffled fur, hunched back, reduced movement, weight loss and tachypnea. Animals were thoroughly monitored for the development of these signs and symptoms. Peripheral blood was regularly collected from the tail

vein of the animals under observation for routine WBC and RBC count.

3.1.3.2 Leukocytosis and anemia in the leukemic mice:

In all the analysed leukemic mice, a several fold expansion of the white blood cell (WBC) was observed. The RBC count was relatively reduced, indicating anemia.

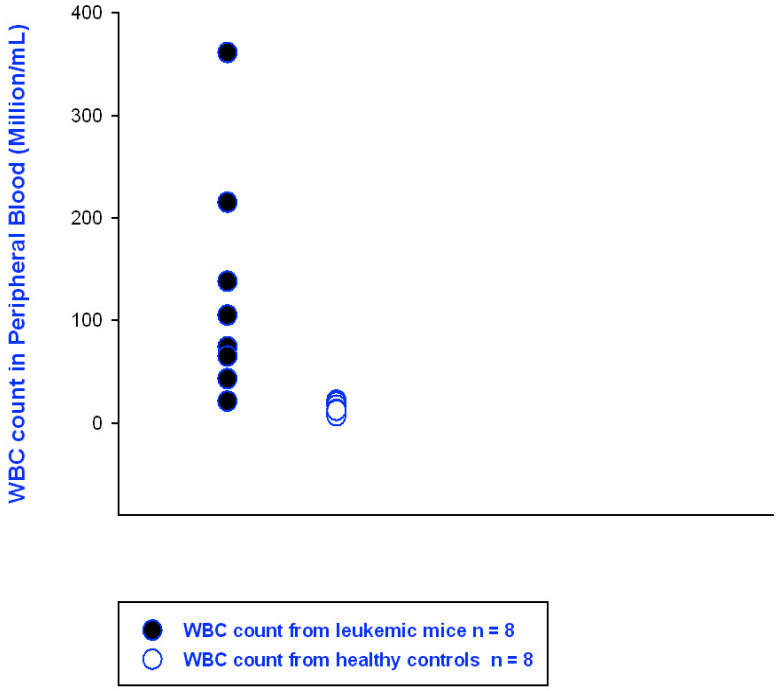


Fig 3.21: Distribution of the WBC counts in the peripheral blood of leukemic CA+/Vav-Cre+ n= 8 and healthy control mice (CA+/Cre- and CA-/Cre-), n=8. WBC counts in the leukemic mice were significantly higher compared to the healthy controls. (p value 0.026)

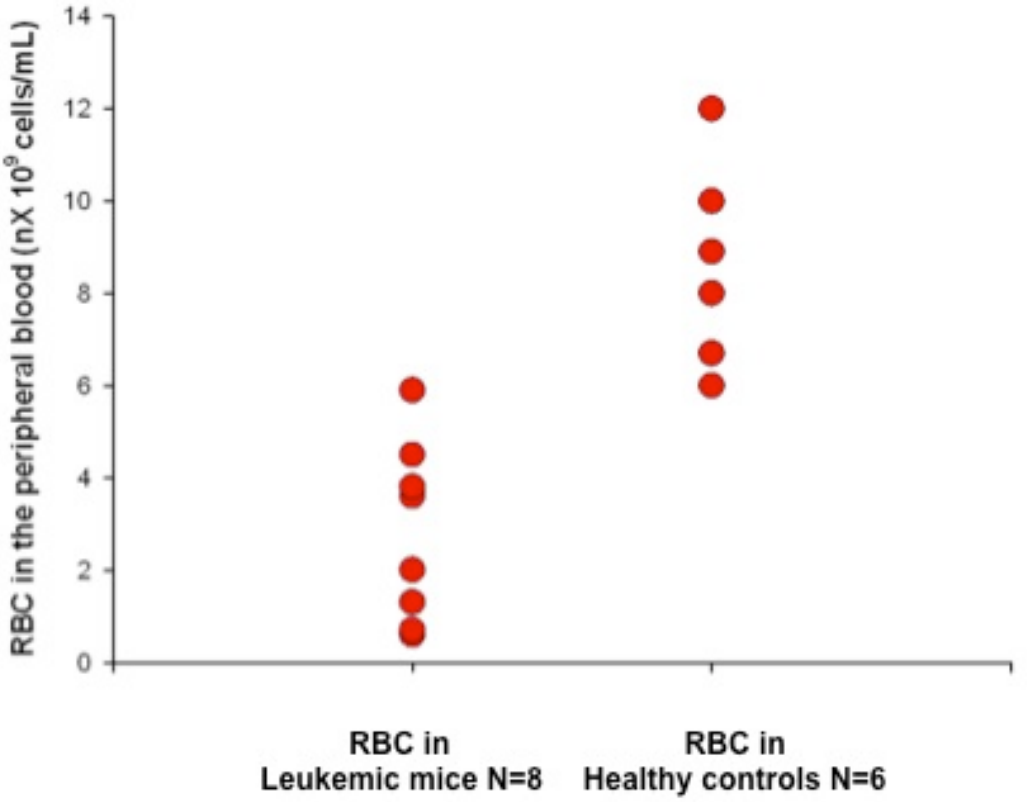


Fig 3.22: Distribution of RBC counts in the peripheral blood of leukemic CA+/Vav-Cre+ mice and the healthy controls (CA+/Cre- and CA-/Cre-). The number of RBC in the peripheral blood of leukemic mice was significantly reduced (p= 0.0001)

3.1.3.3 Splenomegaly

The spleens of the leukemic animals were enlarged. We dissected 6 out of 8 mice that were found dead in the cage and found their spleens to be enlarged.

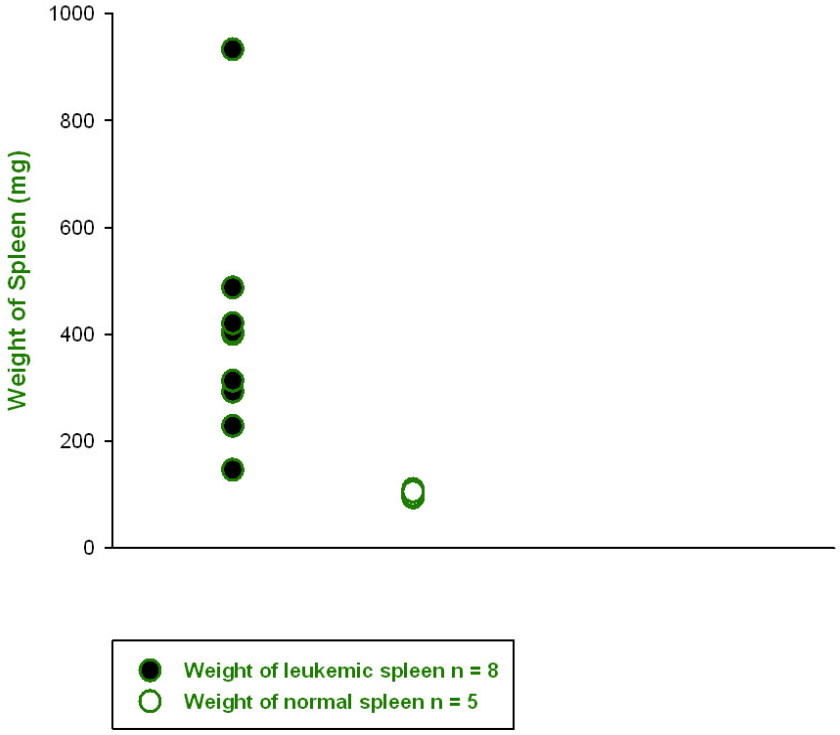
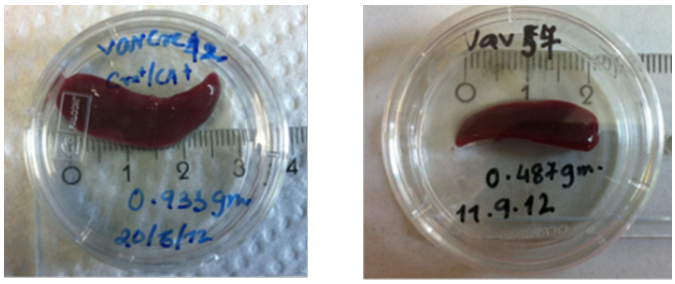


Fig 3.23: Distribution of spleen weight of leukemic CA+/Vav-Cre+ mice and healthy control mice (CA+/Cre-and CA-/Cre-). The weight of the spleen from the analyzed leukemic animals was significantly higher (p value 0.0042) than in the healthy controls.



Leukemic Spleen

Healthy Spleen

Fig 3.24: Picture of two spleens from leukemic mice (CA+/vav-Cre+) and one spleen from a non-leukemic mouse (CA+/Cre-).

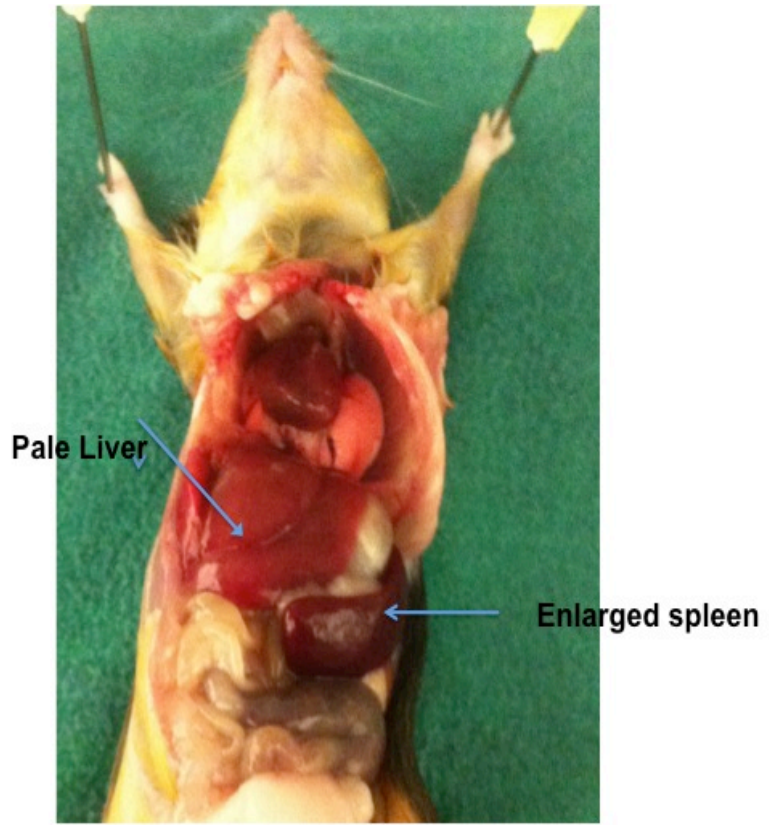


Fig 3.25: Picture of a post mortem dissection of a leukemic mouse (Id: vav 81) with splenomegaly and pale organs.

3.1.3.4 Blast-like cells in the peripheral blood, bone marrow and spleen of the leukemic mice

To study the morphology of the cells from leukemic mice, cytological slides were prepared with the peripheral blood smear and cytospin preparations with cells from bone marrow and spleen. In the PB smears, lymphocytes were almost absent. Most of the cells were of myeloid origin. Many large cells with immature appearance, prominent chromatin and bluish cytoplasm were found in the PB smears and cytospins from bone marrow and spleen from leukemic mice.

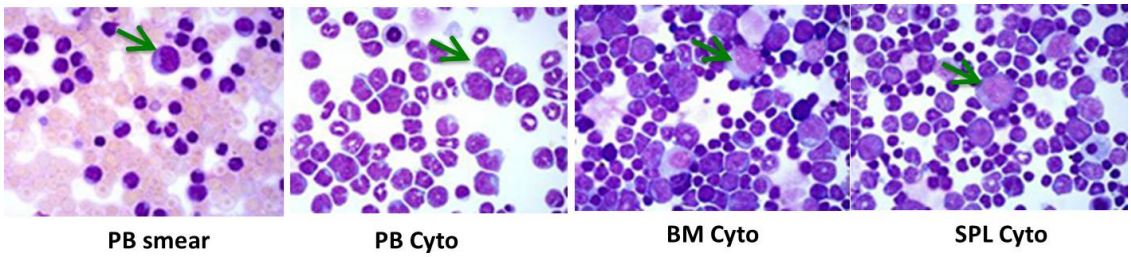


Fig 3.26: Giemsa-May-Grünwald staining of the peripheral blood (PB) smear and the cytospins from PB, bone marrow (BM) and spleen cells from a leukemic mouse (Id:Vav57). Abnormal blast like cells (arrows) was observed in all preparations. (Magnification 63X)

3.1.3.5 Infiltration of multiple organs by the blast cells in leukemic mice

Histopathological sections were prepared from formalin fixed organs of the leukemic mice. Infiltrating leukemic blasts were identified using immunohistochemistry. A diffuse infiltration of leukemic blasts in most organs examined was detected attesting to the severity and aggressiveness of the disease. Infiltrated organs included spleen, kidney, liver, lung, pancreas, salivary gland, thymus and the gastro intestinal tract.

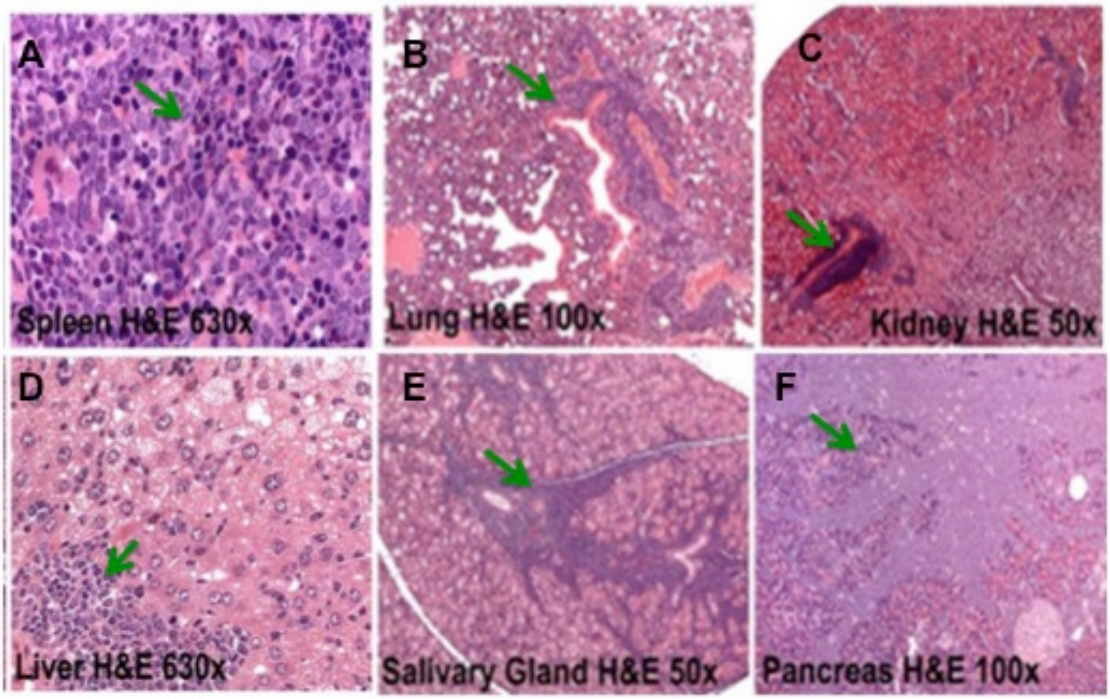


Fig 3.27: Section of organs from a leukemic CA+/Vav-Cre+ mice (Mouse Id vav 32). Different organs were infiltrated by the leukemic blast cells. The figure shows hematoxylin and eosin (H&E) stained organ sections at different magnifications. A. The infiltration in the sleepn involved predominantly the red pulp; B. infiltration in the lung; C. In the kidney section glomerular infiltration is visible; D. Extensive infiltration in the sinusoidal and the periportal space of the liver; E and F. Infiltrates were found in the salivary gland and the pancreas. Arrows indicate areas of infiltrating blast cells. (Histology was performed by Priv Doz Dr Leticia Quintanilla-Fend in the Institute for Pathology, University of Tübingen)

3.1.3.6 Myeloid origin of the leukemic blasts

To characterize the type and the differentiation stage of the infiltrating blasts in these leukemias, several immunohistochemical stainings were performed on the fixed tissue sections. Myeloperoxidase (MPO) is an enzyme present from the early granulocyte precursor stage onwards. A positive myeloperoxidase staining indicates a myeloid origin of the blasts and it distinguishes

them from immature lymphoid blasts. Chloracetate-esterase (ASDCL) is the stain used to identify cells of the granulocytic lineage from an early promyelocytic stage onwards. To identify the B and T lymphocytic blasts, B220 and CD3 stainings were performed, respectively. A positive TdT (terminal desoxynucleotide transferase) will identify cells of lymphoid origin. To confirm the nature of the infiltrating blasts, all the above-mentioned stainings were performed on the tissue sections from leukemic mice.

The infiltrating blasts were positive for MPO and ASDCL confirming their myeloid nature. Tissue sections were negative for TdT staining. CD3 and B220 staining showed a few residual lymphocytes present in the spleen of the leukemic mouse. Some mature granulocytes were also observed in the tissue sections. These analyses revealed that the CALM/AF10 fusion gene in the CA+/Vav-Cre+ mice led to the development of an acute myeloid leukemia with maturation.

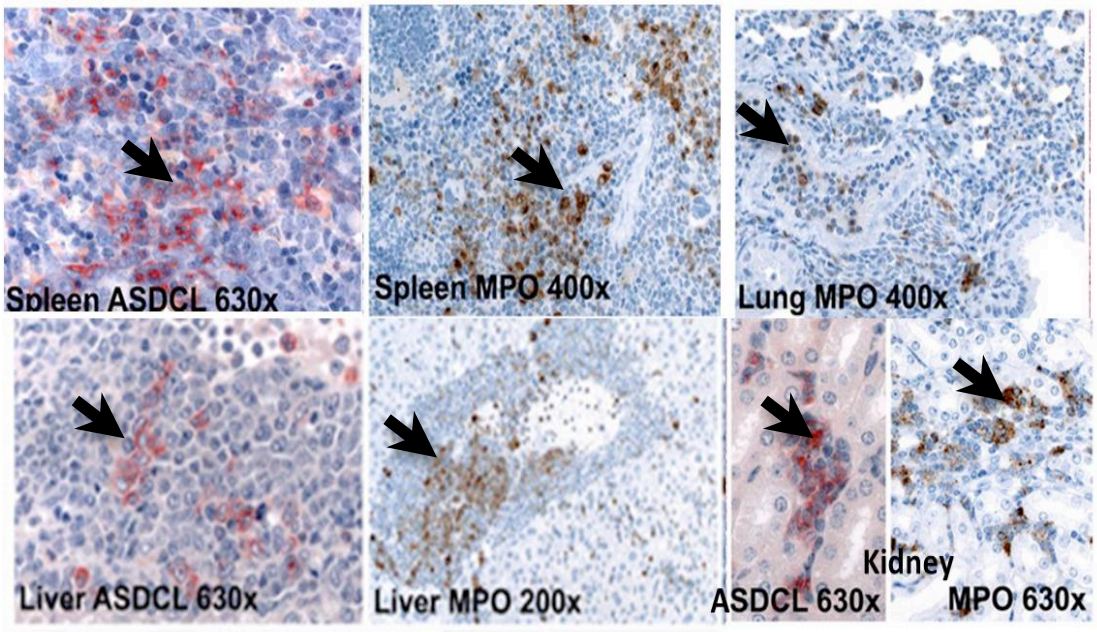


Fig 3.28: Immunohistochemistry on the tissue sections from CA+/Vav-Cre+ leukemic mice. Spleen, liver, lung and kidney sections show the presence of myeloid marker positive infiltrating blasts. Black arrows indicate areas of infiltrating blasts.

3.1.3.7 The leukemic blasts in 40% of the CA+/Vav-Cre+ leukemic mice co-express myeloid and lymphoid markers

Using flow cytometric analysis the immunophenotype of the leukemic cells from several hematopoietic organs was analyzed in detail. Single cell suspensions for flow cytometry were prepared from the peripheral blood, bone marrow and the spleen of the leukemic mice and stained with several antibodies detecting

epitopes specific for the myeloid, B and T lymphocytic compartment. Antibodies from our standard panel described in table 3.1 were used. In all the CA+/Vav-Cre+ leukemic mice, the cells were positive for the myeloid markers Mac1 and Gr1 reconfirming the myeloid nature of the leukemia. But in 6 out of 15 leukemias, the leukemic cells were also positive for lymphoid markers. As graphically represented in figure 3.29, the leukemic animals could be divided into two groups on the basis of the immunophenotype of their leukemic cells. In 6 out of 15 leukemic animals, 40±6% cells in the peripheral blood, 35±12.09% cells in the bone marrow and 44±4.58% of spleen cells were positive for both the myeloid marker Mac1 and the B lymphoid marker B220. We will call these leukemias "biphenotypic". In this group, the number of cells staining positive only for B220 was 51±15% in the peripheral blood, 45.3±17.4% in bone marrow and 57.5±6.4% in the spleen. These percentages of B220+ cells are significantly higher than in the control mice (p values for PB, BM, spleen are 2.7E-5, 1.26E-5 and 0.0001, respectively).

In contrast, in the other group of leukemias (9 mice out of total 15 animals), the number of cells staining positive for B220 was very low: 14.6±8.61% in the peripheral blood, 6.83±3.25% in the bone marrow and 23.66±12.8% in the spleen. In this group of leukemias, the majority of cells (on an average, 60% cells) stained positive for the myeloid maker Mac1. We will call the leukemias in this group "myeloid". A population of cells staining positive for both the lymphoid marker B220 and the myeloid marker Mac1 (B220+/Mac1+) was virtually absent in this myeloid group. In both groups, the number of Mac1 positive cells was very high in the blood, bone marrow and spleen. However, in the biphenotypic group the number of Gr1+ cells was not significantly different from the control mice whereas in the myeloid group there was significantly high number of Gr1+ cells present in all the hematopoietic organs. In summary, the leukemic bulk in the myeloid group was $Mac1^{high}$ $Gr1^{high}$ B220- where as in the biphenotypic group, leukemic cells were $Mac1^{high}$ $Gr1^{low}$ $B220^{high}$. The number of cells positive for the T cell markers CD3, CD4, and CD8 was greatly reduced in all cases. For the detailed values of surface markers of control and leukemic animals please refer to the appendix table A5, A6 and A7.

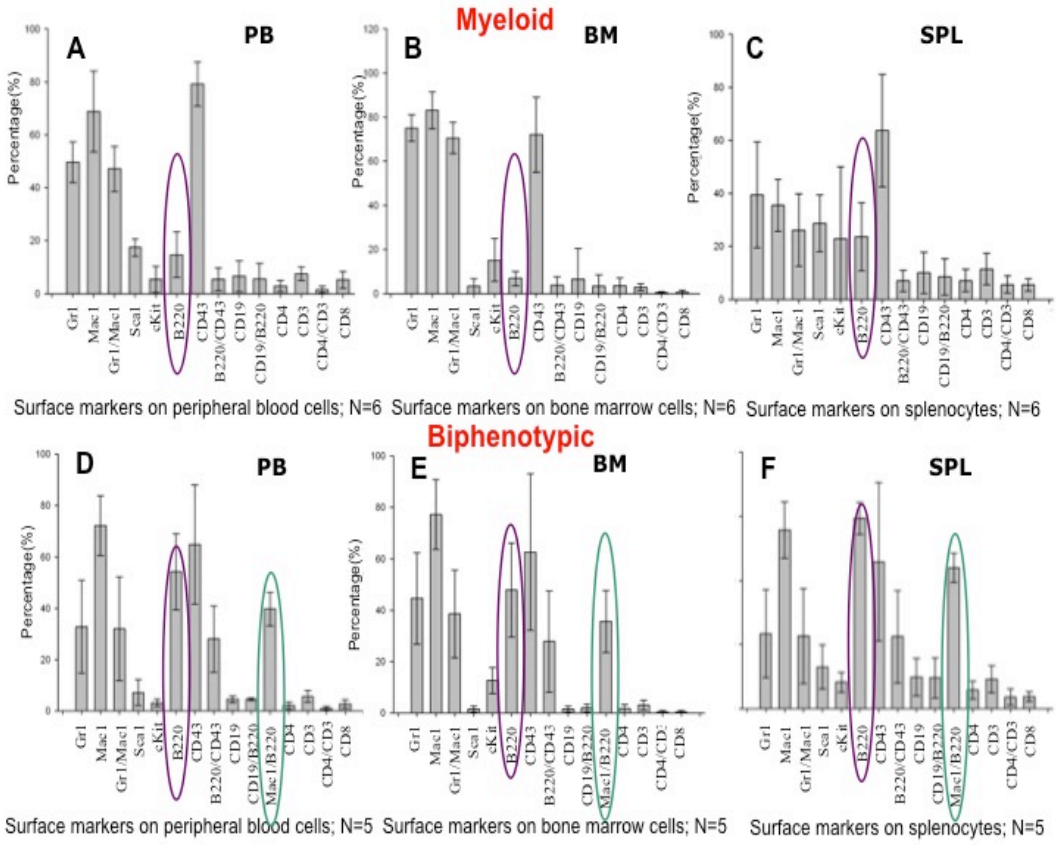


Fig 3.29: Summary of the immunophenotype of the leukemias in the CA+/Vav-Cre+ mice. Based on the phenotype of the leukemic cells, two groups of leukemias (myeloid and biphenotypic) can be distinguished. The upper panels A, B, C summarize the surface marker distribution in the peripheral blood, bone marow and on the splenocytes in the myeloid group (data from 6 of 9 animals). The majority of cells are Mac1 positive in all the organs. Only very few cells are positive for the lymphoid marker B220. The lower panels D, E, F show the surface markers on the peripheral blood, bone marrow and spleen cells from the biphenotypic group of leukemias (data from 5 of 6 animals). In this group, a high percentage of B220 positive cells are found in all three organs. And there is a very prominent population of cells co-expressing the myeloid marker Mac1 and the lymphoid marker B220 (green circles). The difference in the percentage of B220 positive cells between the two groups is significant. In peripheral blood the p value is 0.0002, in bone marrow the p value is 0.0001, in spleen the p value is 0.0001

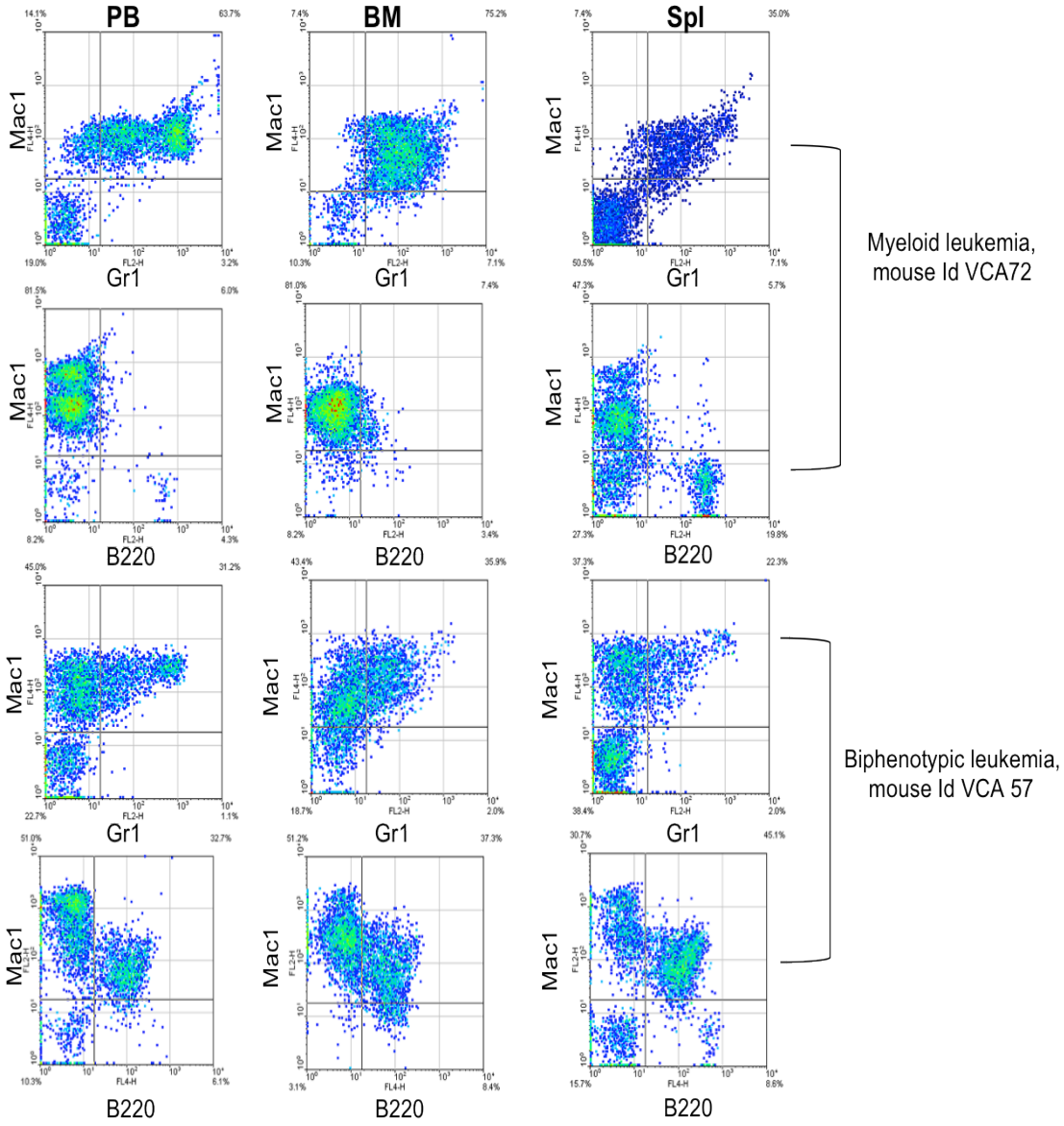


Fig 3.30: Immunostaining analysis of hematopoietic cells from the two groups of leukemia using flowcytometry. Immunostaining of the hematopoietic cells from mouse VCA 72 and VCA 57 using Gr1-PE and B220-PE in combination with Mac1-APC antibodies are shown. Mouse VCA72 developed a myeloid leukemia. Majority of the cells in the hematopoietic organs are Gr1-Mac1 positive. Immunostaining using Mac1 and B220 reveals that B220 positive cells are almost absent in all the hematopoietic organs of this mouse. In the lower panel immunostaining of the peripheral blood, bone marrow and spleen cells from the mouse VCA57, which developed biphenotypic leukemia is shown. In this case B220-PE and Mac1-APC antibody staining reveals the presence of Mac1+/B220+ biphenotypic population in all the hematopoietic organs

Immunophenotype of 2 leukemic CA+/Vav-Cre+ mice from each group of leukemia are shown in figure 3.30. The upper two rows of

the figure show hematopoietic organs from mouse VCA 72 which developed a myeloid leukemia. Gr1+/Mac1+ myeloid cells infiltrated all the organs. There were no B220 positive cells in PB and BM, very few in the spleen, the Mac1+/B220+ population is absent. The lower two rows show immunostaining of the cells of mouse VCA 57, which developed biphenotypic leukemia. High number of Mac1+ cells are present in all the organs of this mouse, number of Gr1+ cells is fewer than the VCA 72 mouse with myeloid leukemia and when we look at the B220, Mac1 staining we see the presence of biphenotypic Mac1+/B220+ cells in all the organs.

3.1.3.8 Most leukemic cells stain positive for monocytic markers

Bone marrow cells extracted from both myeloid and biphenotypic leukemias were further characterized using a few more antibodies specific for surface markers like CD23, IgM, F4/80 and CD24 antibodies. The details of the antibodies used are given in the following table:

Table 3.2 List of antibodies for characterization of the leukemic cells

Name of the antibody	Specificity
F4/80, rat monoclonal antibody, FITC conjugate	Recognizes the F4/80, which is a member of the epidermal growth factor EGF-TM7 family. This family includes class B G protein coupled receptors. Mostly present on macrophage and monocytes.
PE rat anti-mouse IgM	Recognizes specifically mouse IgM of Igh-C(a) and Igh-C(b) haplotypes.
FITC rat anti-mouse CD23	Reacts with CD23, an immunoglobulin receptor expressed on mature conventional B lymphocytes but not on T cells.
PE rat anti-mouse CD24	Reacts with CD24, a membrane associated glycoprotein present on B lymphocytes, granulocytes and monocytes.

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In figure 3.31, Mac1 positive bone marrow cells from leukemic mouse VCA 57 are co-stained with antibodies specific for CD23, CD24, F4/80 and IgM antibodies. This mouse had biphenotypic leukemia. The bone marrow cells from another mouse VCA 74 with myeloid leukemia were also stained using same antibodies and the results are summarized in figure 3.32. On an average in both groups, 47.6± 11.39 % of the bone marrow cells co-stained for Mac1 and CD24 and 48.1±9.19 % cells stained positive for Mac1 and F4/80 indicating the monocytic nature of these cells. The staining pattern of these markers was not obviously different between the two leukemic groups. Staining for CD23 and IgM markers were negative in both cases.

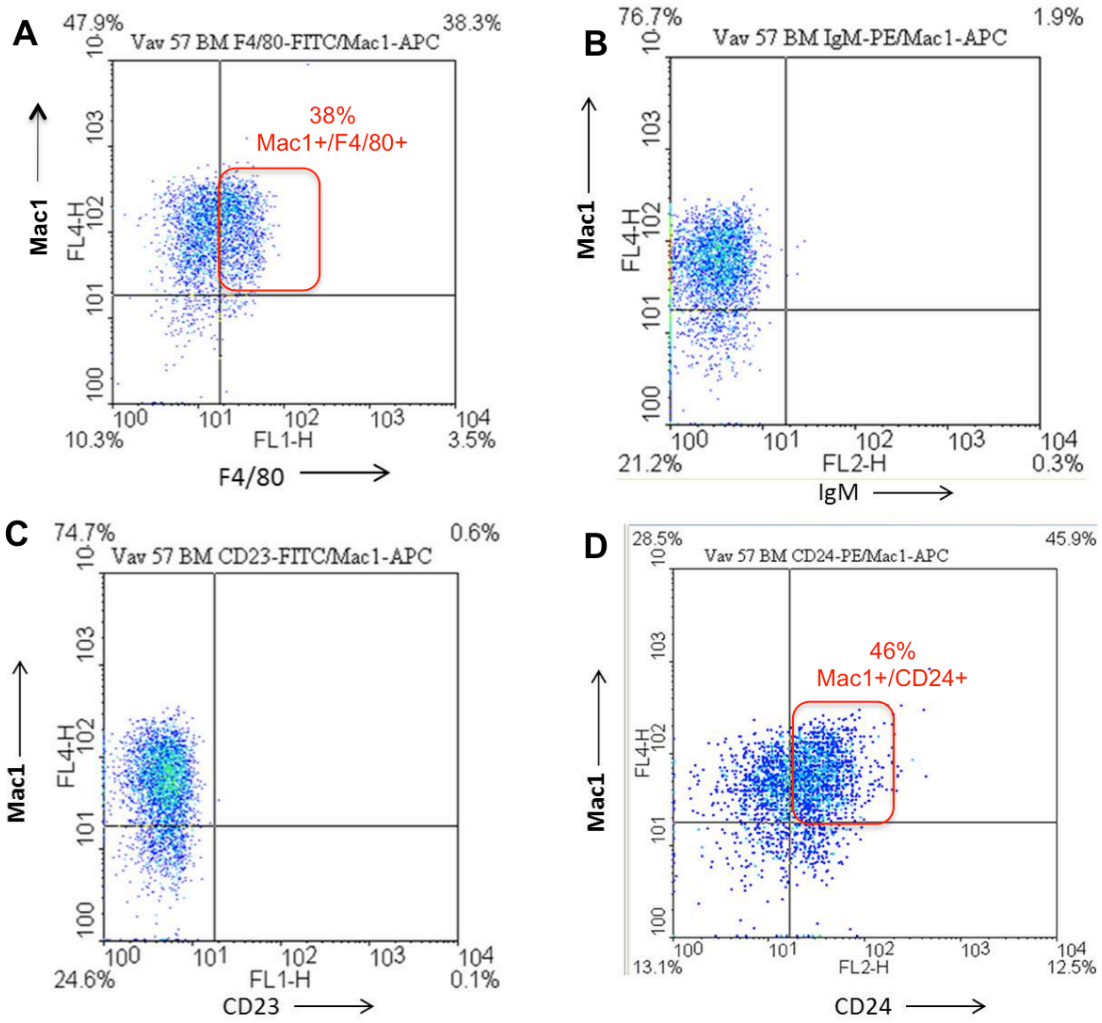

Fig 3.31: Immunostaining of the bone marrow cells from a biphenotypic leukemia sample using monocytic markers. Mac1 positive bone marrow cells from mouse VCA 57 were co-stained with Mac1 and F4/80 (A), Mac1 and IgM (B), Mac1 and CD23 (C) and Mac1 and CD24 (D). 38% cells stained positive for F4/80 marker (A) and 46% cells were positive for CD24 (D). There were no CD23 and IgM positive cells as indicated in B and C.

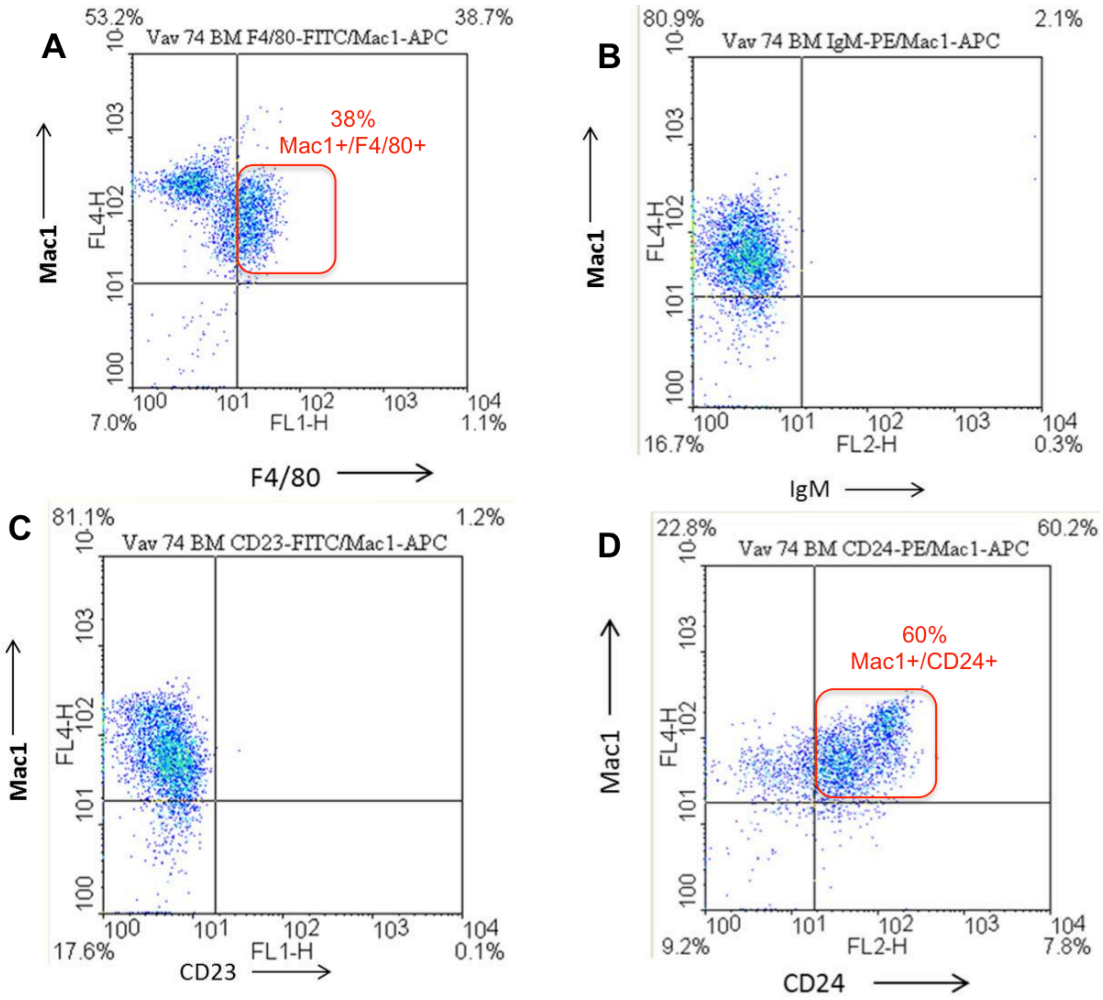

Fig 3.32: Immunostaining of the bone marrow cells from a myeloid leukemia sample using monocytic markers. Leukemic bone marrow cells from mouse VCA 74 were co-stained with Mac1 and F4/80 (A), Mac1 and IgM (B), Mac1 and CD23 (C) and Mac1 and CD24 (D). 38% cells stained positive for F4/80 marker (A) and 60% cells were positive for CD24 (D). There were no CD23 and IgM positive cells as indicated in B and C.

3.1.3.9 Flow cytometric analysis of the peripheral blood, bone marrow and spleen shows significant alterations in leukemic CA+/Vav-Cre+ mice

The immunophenotype of cells from the peripheral blood, bone marrow and spleen of leukemic CA+/Vav-Cre+ mice was compared with those of our control mouse group, as described earlier. Samples from both 7 myeloid and 6 biphenotypic leukemias are used in this analysis. In this comparison, as expected, significant differences were observed. The expansion of the myeloid compartment is visible in the blood, bone marrow and also in spleen in both groups of leukemias. The number of CD3, CD4 and CD8 positive cells (T cells) is significantly reduced in the spleen and the peripheral blood indicating a myeloid bias in hematopoiesis. The

results are summarized in the following figures (Figures 3.33, 3.34 and 3.35).

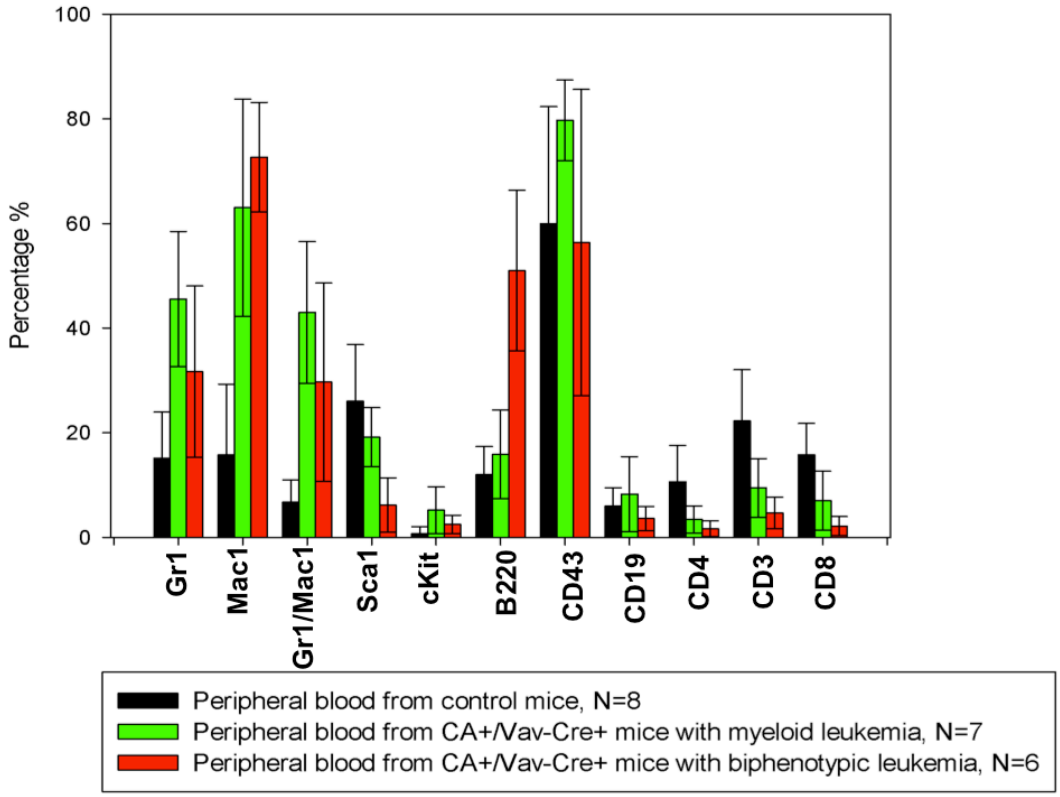

Fig 3.33: Immunophenotype of the peripheral blood cells from leukemic mice (CA+/Vav-Cre+) and the control mice. The average percentage of cells staining positive for each marker is shown as a bar graph. The error bars represent the standard deviation. A significant expansion of the myeloid compartment (Gr1+, Mac1+, Gr1+/Mac1+) in the leukemic samples from both groups is seen. In the biphenotypic leukemia number of B220 positive cells is significantly higher than the control (p value 2.07E-5) and the myeloid group of leukemia (p value 0.0002). Number of CD4, CD3, CD8 positive lymphoid cells are reduced in both groups of leukemias, in comparison to the control mice.

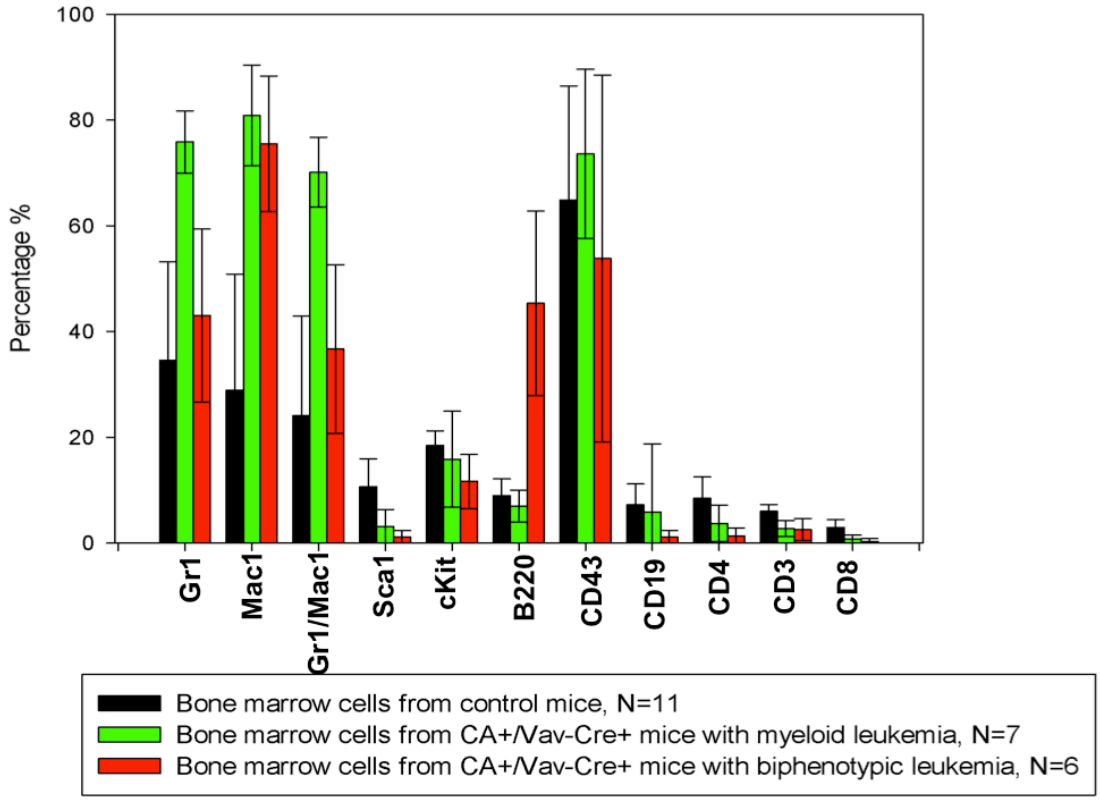

Fig 3.34: Immunophenotype of the bone marrow cells from leukemic mice (CA+/Vav-Cre+) and the control mice. The average percentage of cells staining positive for each marker is shown as a bar graph. The error bars represent the standard deviation. A significant expansion of the Mac1+ cells in the leukemic samples is seen. There is a very high proportion of Gr1+ cells in the myeloid leukemias in comparison to the controls, (p value: 3.77×10^{-5}). The biphenotypic leukemias have a lower proportion of Gr1+ cells in comparison to the myeloid leukemias (p value: 0.0001). The proportion of B220+ cells is very high in the biphenotypic leukemic samples in comparison to control (p value: 1.26×10^{-5}) and the myeloid leukemia (p value: 0.0001).

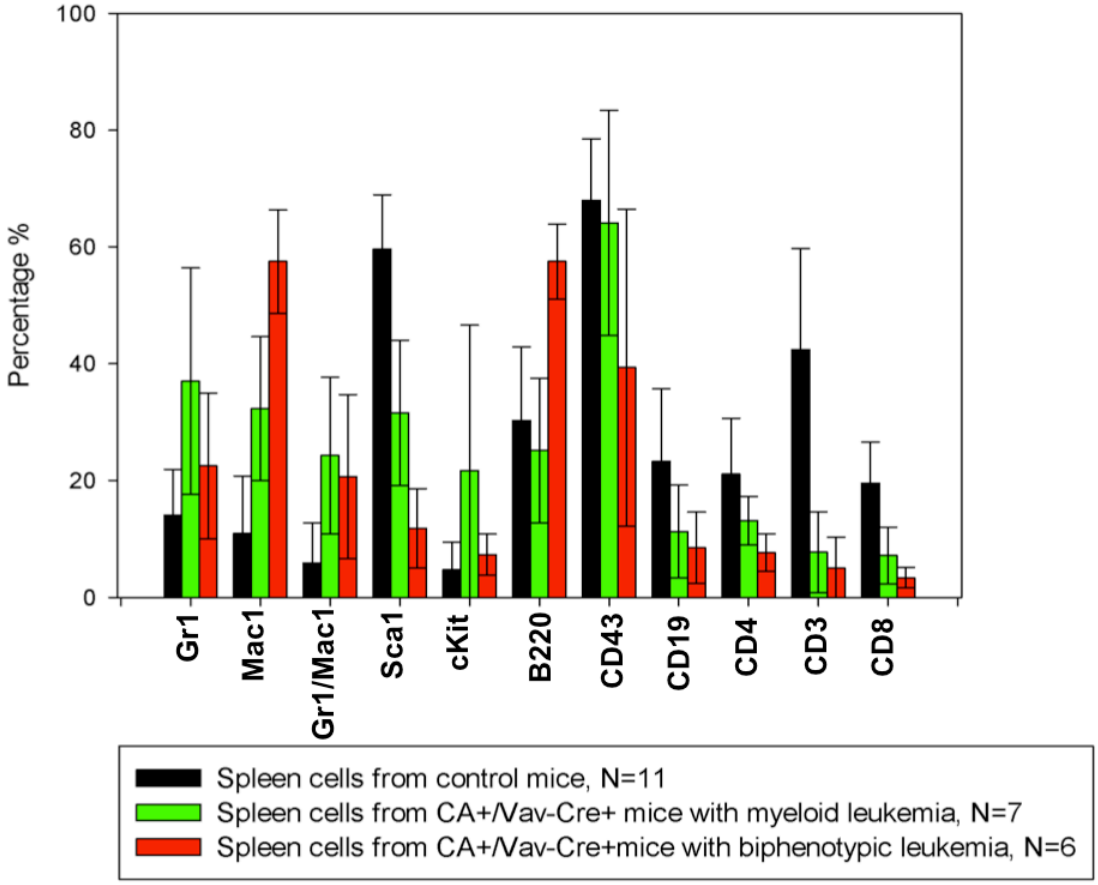

Fig 3.35: Immunophenotype of the spleen cells from leukemic mice (CA+/Vav-Cre+) and the control mice. The average percentage of cells staining positive for each marker is shown as a bar graph. The error bars represent the standard deviation. The proportion of Mac1+ cells is very high in both groups of leukemias in comparison to the controls. The percentage of Gr1+ cells is higher in the myeloid leukemias in comparison to the control (p value: 0.002). However, in the biphenotypic leukemias there are fewer Gr1+ cells and there is no significant difference from the controls (p value 0.0105). The proportion of B220 positive cells is very high in biphenotypic leukemia in comparison to the control (p value: 0.0001) or the myeloid leukemias (p value: 0.0001).

The individual values of different surface markers on the cells of the hematopoietic organs from the leukemic and the control mice are shown in tables A5, A6, A7 in the Appendix.

3.1.4 B cell-specific expression of CALM/AF10 using CD19-Cre and Mb1-Cre transgenic mice

To achieve expression of the CALM/AF10 fusion protein in the B cell compartment, the CD19-Cre and Mb1-Cre transgenic mouse lines were crossed with R26LSLCA mice. Dr Ursula Zimber-Strobl's group kindly provided both Cre lines.

Robert C. Rickert et al generated the CD19-Cre transgenic mice (Rickert et al., 1997). In this mouse line, the Cre gene is under the transcriptional control of the *CD19* locus. The *CD19* gene is expressed specifically in B cells from an early stage throughout B cell development and differentiation. In the CD19-Cre mice, a Cre expression cassette was inserted into the second exon of the *CD19* gene by homologous recombination. Due to this insertion, the coding region of *CD19* gene is disrupted but all the regulatory elements of the *CD19* locus are left intact. Mice with one CD19-Cre allele have a normal phenotype and can be used for B cell-specific deletion of loxP flanked (floxed) target regions.

The Mb1-Cre mice were generated by Hobeika and colleagues (Hobeika et al., 2006). In this mouse line, the Cre expression cassette is integrated into the *Mb1* locus. The *Mb1* gene encodes the Igα signalling subunit of the B cell antigen receptor. *Mb1* is highly expressed in B cells from the very early pro-B cell stage in the bone marrow and also in the later stages of B cell development but not in plasma cells. Different stages of B cell development are outlined in the following figure, stages expressing the CD19 and Mb1 genes are indicated.

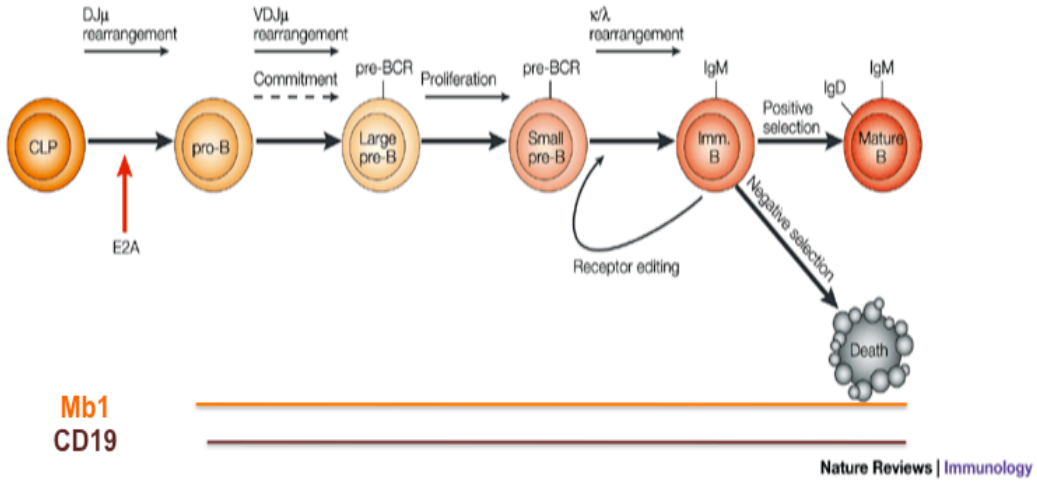

Fig 3.36: An outline of the B cell development showing the stages where Mb1 and CD19 markers express. (Original figure adapted from Nature Reviews Inmmunity, December 2001 and modified)

R26LSLCA mice were crossed with both the Mb1-Cre and CD19-Cre lines. Tail DNA from 8-10 week old F1 offspring was used for a PCR-based genotyping assay. Animals positive for both CALM/AF10 and Cre should express CALM/AF10 in the B cells where CD19 or Mb1 expression occurs. These double positive mice were called CA+/CD19-Cre+ and CA+/Mb1-Cre+, respectively.

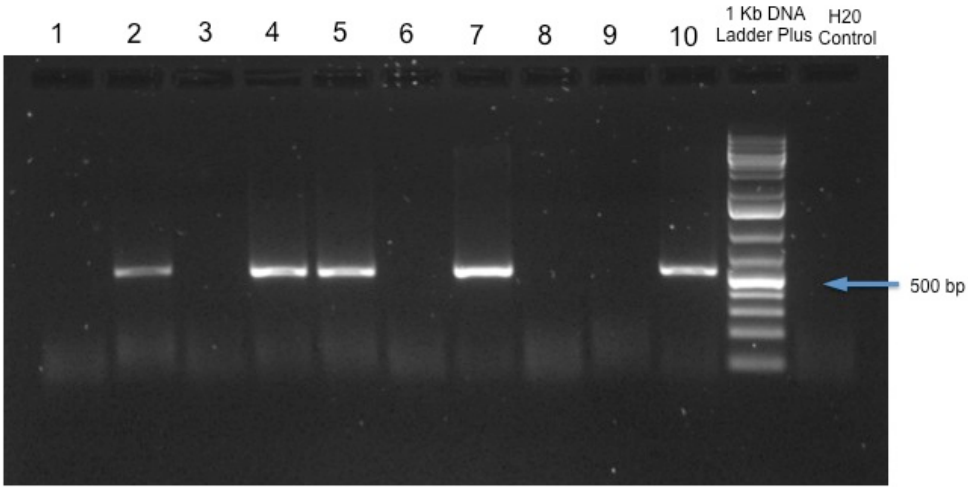

Fig 3.37: Example of the PCR genotyping assay for the CALM/AF10 breakpoint. Genomic DNA extracted from the tail tips of ten 8-10 week old F1 mice from the R26LSLCA X Mb1-Cre cross was assayed for the presence of the R26SLSCA allele. Using the C/Abrkpnt F and C/Abrkpnt R primer pair (table 2.1.3), the ~525 bp band in lanes 2, 4, 5, 7, and 10 indicates the presence of the R26LSLCA allele.

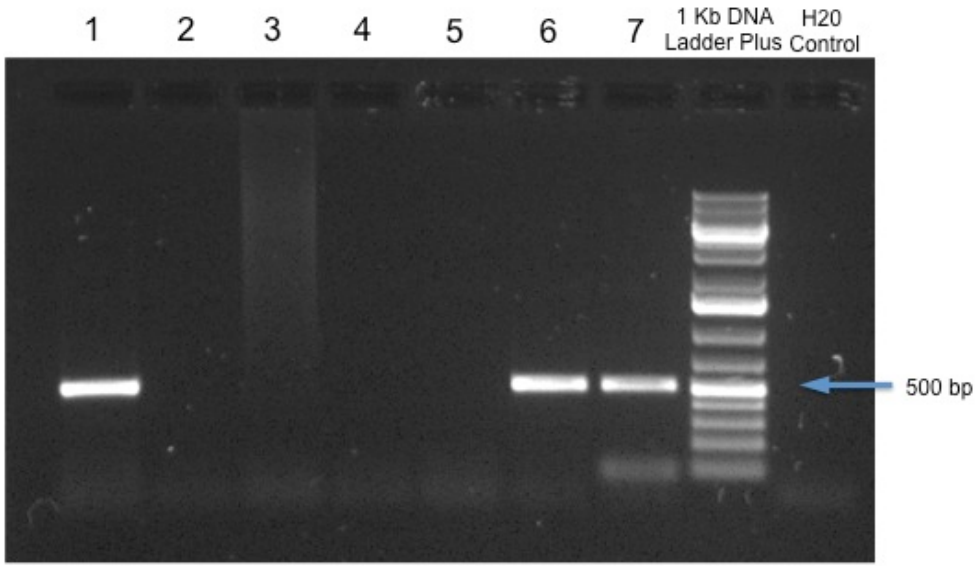

Fig 3.38: Example of the PCR genotyping assay for the Mb1-Cre allele. Genomic DNA extracted from the tail tips of seven 8-10 week old F1 mice from the R26LSLCA X Mb1-Cre cross was used for the assay. Using the Mb1 Cre F and Mb1 Cre R primer pair (Table 2.3.1**)** the appearance of a band of about 510 bp in lanes 1, 6 and 7 indicates the presence of the Mb1-Cre allele.

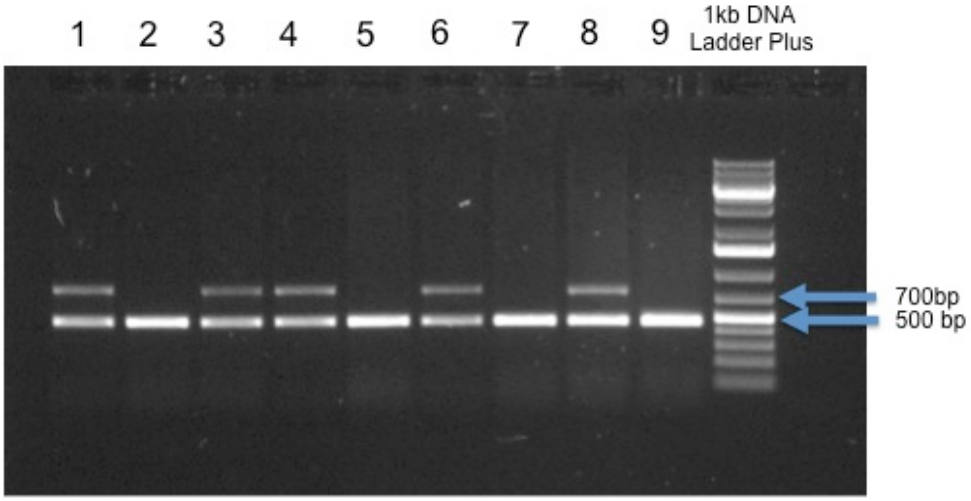

Fig 3.39: Example of the PCR genotyping assay for the CD19-Cre allele. Genomic DNA extracted from the tail tips of nine 8-10 week old F1 mice from the R26LSLCA X CD19-Cre cross was used for the assay. The ~750 bp band seen in lanes 1, 3, 4, 6 and 8 originates from the disrupted *CD19* locus where the Cre coding cassette has been integrated; the 500 bp band corresponds to the wild type *CD19* locus. The CD19 Cre F and CD19 Cre R primer pair Cre (Table 2.3.1) was used for the genotyping.

3.1.4.1 CALM/AF10 fusion transcript is expressed in the B cells of CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice

To analyze the expression of the CALM/AF10 fusion gene in the B cells of CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice, CD43 negative resting B cells were isolated from the spleens of one CA+/Mb1-Cre+ mouse and one CA+/CD19-Cre+ mouse by magnetic cell isolation (MACs), cDNA was prepared from these cells and RT PCR was performed.

CD43 is a marker for granulocyte, monocyte, macrophage, CD5+ splenic B cells, T lymphocytes. Only peripheral resting B cells are negative for CD43. Single cell suspension of spleen cells were prepared and incubated with magnetically labeled CD43 antibody. Then the cells were passed through a column placed in a strong magnetic field. CD43+ Cells were labeled with the magnetic antibodies and hence were retained in the column because of the magnetic field. Unlabelled CD43- cells were collected in the flow through. cDNA was isolated from the B cells of these two mice and RT PCR and realtime RT PCR was performed. The expression of the CALM/AF10 fusion transcript was detected in the B cells from both the CA+/Mb1-Cre+ and the CA+/CD19-Cre+ mouse.

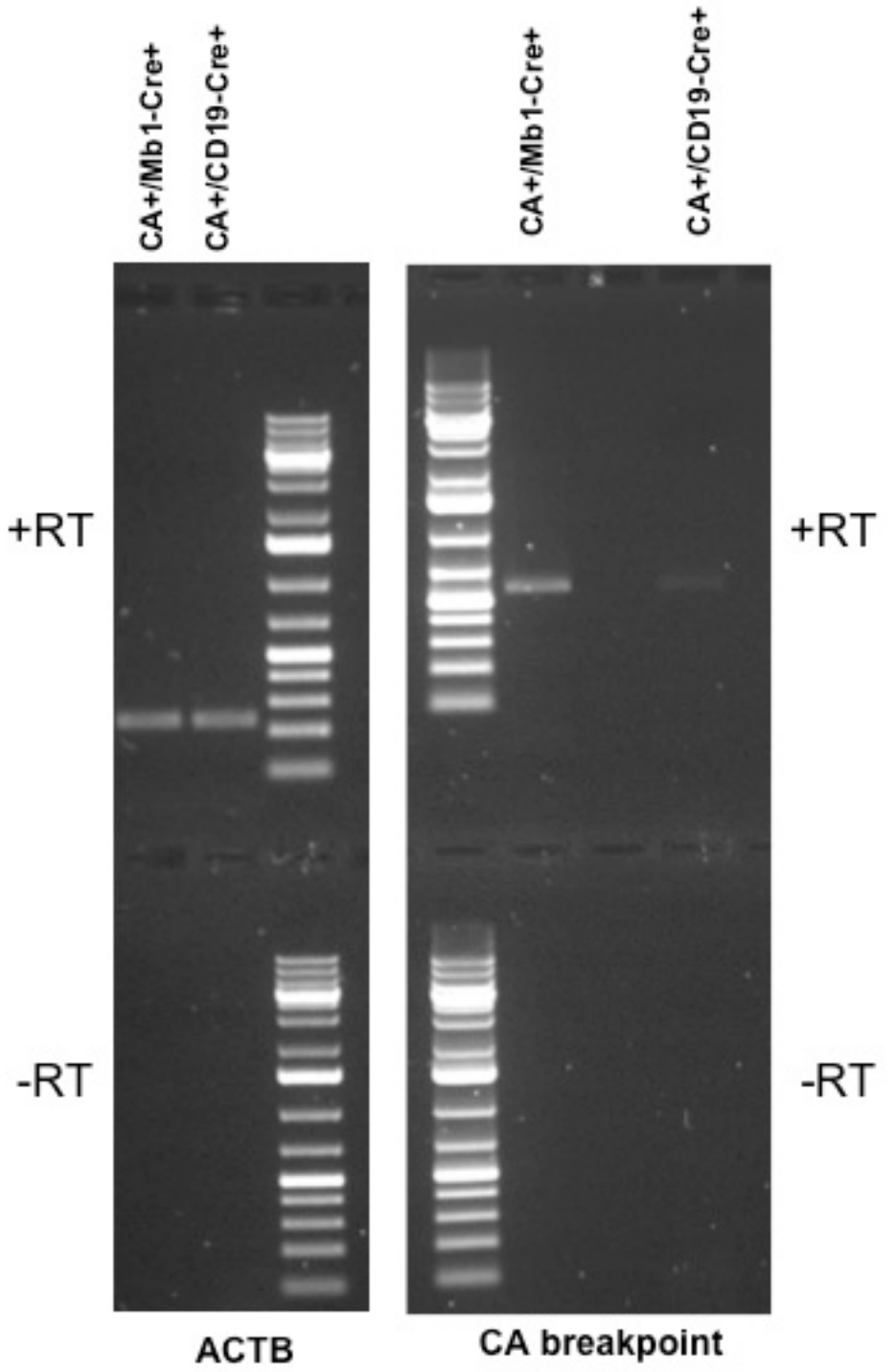

Fig 3.40: Agarose gel of the RT-PCR assay performed on the cDNA of B cells isolated from a CA+/Mb1-Cre+ mouse and a CA+/CD19-Cre+ mouse. Amplification of the internal control gene beta-actin (ACTB) from the B cell cDNA of both mice are shown in the upper-left panel. In the lower left panel is the negative control reaction (without reverse transcriptase) to detect the presence of any carry over genomic DNA. The upper right panel shows the expression of CALM/AF10 fusion in both cDNA samples. The lower right hand panel has the corresponding control without reverse transcriptase (RT).

The expression of the CALM/AF10 fusion gene was detected in the cDNA sample from the B cells of the CA+/Mb1-Cre+ mouse as well as from the CA+/CD19-Cre+ mouse. The expression level of the fusion gene was higher in CA+/Mb1-Cre+ mouse.

Realtime RT PCR was performed with the same cDNA samples from murine B cells to analyze the expression of the CALM/AF10 fusion transcript in a quantitative way. CALM/AF10 breakpoint specific probes and primers were used for the assay. cDNA from U937 cell line was used as a positive control for CALM/AF10 in the realtime RT PCR assay and to compare expression levels. U937 is a human cell line, which harbors the CALM/AF10 fusion and was originally used for the cloning of this fusion. We could detect the expression of CALM/AF10 fusion transcript in all samples. As previously seen in the RT-PCR results, we saw higher expression levels of CALM/AF10 in the B cells from CA+/Mb1-Cre+ mice. It should be noted that the expression levels both in the B cells from the CA+/Mb1-Cre+ and the CA+/CD19-Cre+ mouse are higher than in the U937 cell line. The results are summarized in the following figure.

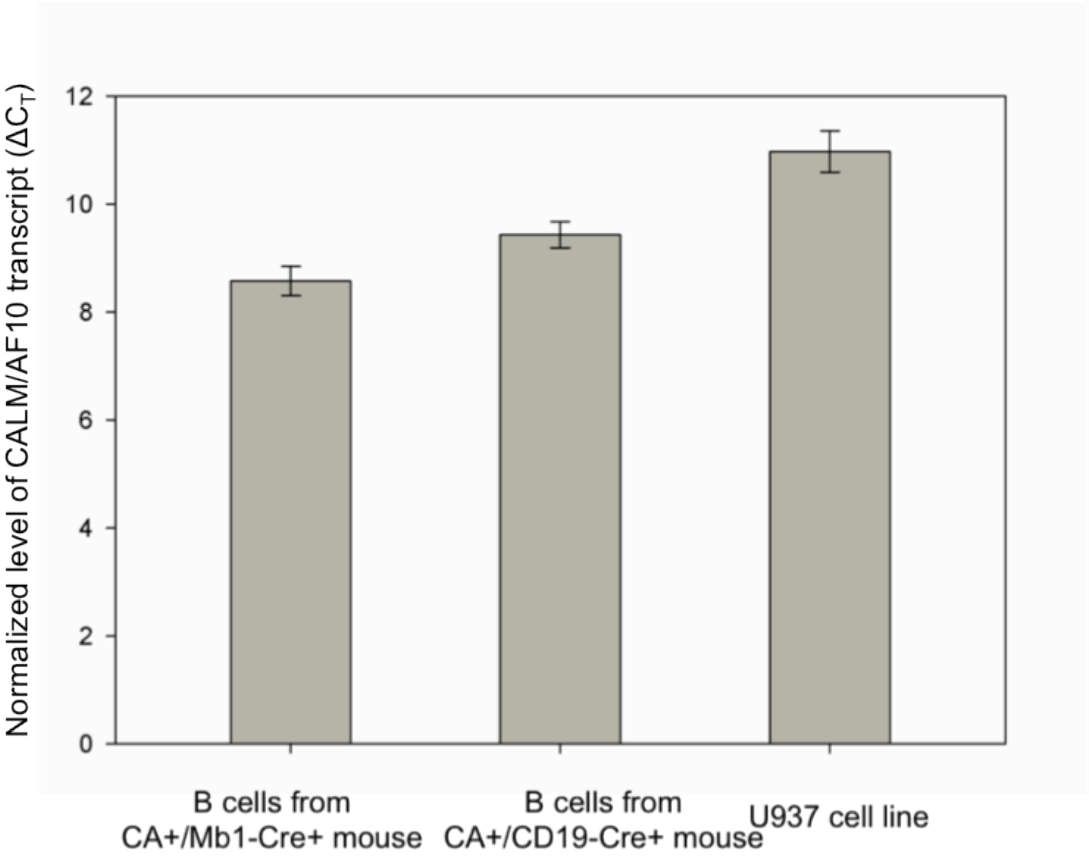

Fig 3.41: Detection of the CALM/AF10 fusion transcript in the B cells from CA+/Mb1-Cre+ mice and CA+/CD19-Cre+ mice using realtime RT- PCR. Realtime RT PCR was performed on the cDNA samples of B cells from one CA+/Mb1-Cre+ mouse and one CA+/CD19-Cre+ mouse using CALM/AF10 fusion breakpoint specific probe and primer pair (Table 2.4). cDNA of U937 cells was used as CALM/AF10

positive control. For each reaction 3 technical replicates were performed. The error bars represent the standard deviation of the technical replicates. For all the samples, beta-actin gene was used as the endogenous control for cDNA quality and amount. The C_T values of the CALM/AF10 transcripts were normalized using the endogenous control beta actin. The bar graphs represent the normalized $C_T$value of CALM/AF10 transcript in each sample. The C_T value of a transcript in realtime PCR correlates inversely with its expression. Higher C_T values of a transcript indicate lower expression levels.

3.1.4.2 The expression of the CALM/AF10 fusion gene in the B cells from the early pro-B cell stage onwards does not lead to the development of leukemia

A total of 20 CA+/CD19-Cre+ mice were observed for 12-18 months for the development of leukemia. As a control 15 CA-/CD19-Cre+ mice were also observed. These mice were all healthy during the period of observation and did not show any phenotypic abnormality**.**

25 CA+/Mb1-Cre+ and 16 CA-/Mb1-Cre+ mice were observed for 12-18 months. These mice also remained all clinically healthy and did not develop any signs of leukemia during the observation period.

3.1.4.3 Flow cytometric analysis shows normal immunophenotype of bone marrow and spleen cells in CA+/Mb1-Cre+ mice.

Four clinically healthy CA+/Mb1-Cre+ mice were sacrificed at the age of 13 months and immunophenotyping of the bone marrow and splenocytes was performed with our standard antibody panel (Table 3.1) and the data were compared with the immunophenotype of mice from the control group as described before.

The distribution of surface markers on the hematopoietic cells showed a similar pattern between the CA+/Mb1-Cre+ mice and the control mice. However, slightly higher number of Mac1+ cells was found in CA+/Mb1-Cre+ spleens and bone marrows. But the other myeloid markers, like Gr1, CD43 did not show any significant difference between the two groups. The expression of B lymphocytic markers, like B220, CD19 and T lymphocytic markers like CD4, CD3, CD8 was also quite similar between the groups. The results are summarized in figure 3.42 and 3.43.

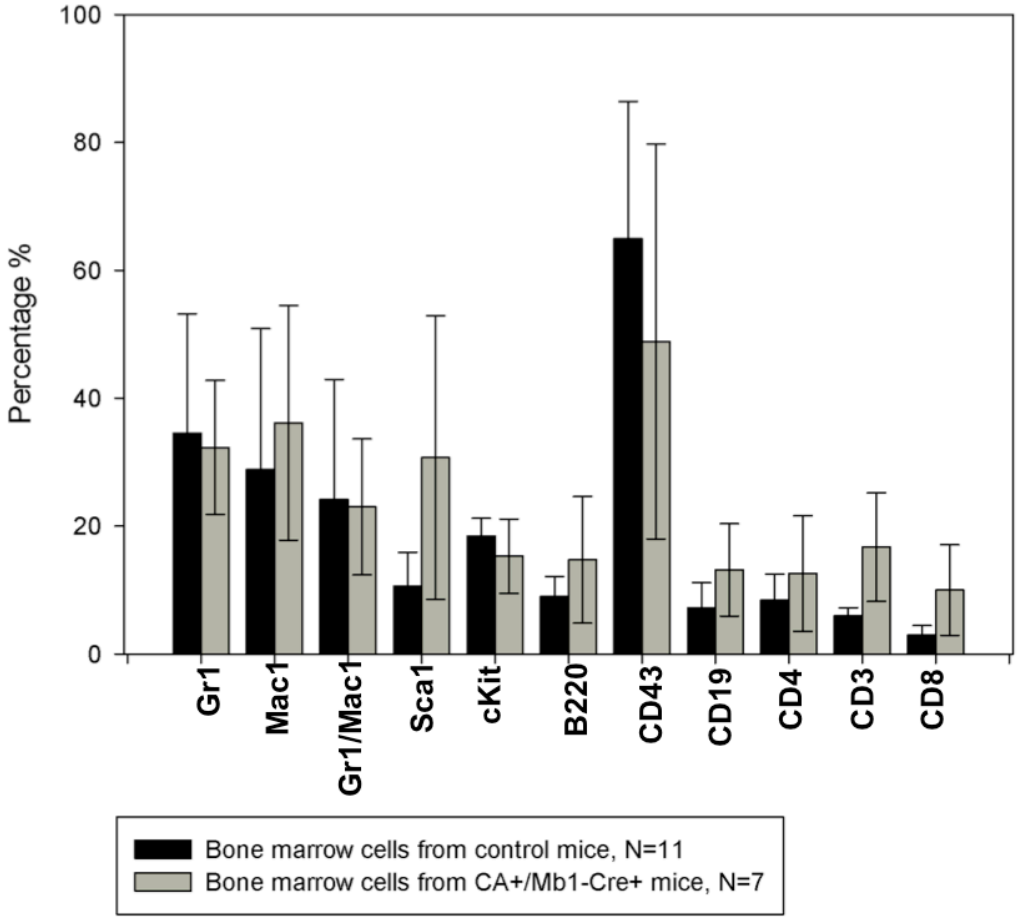

Fig 3.42: Immunophenotype of the bone marrow cells from mice expressing CALM/AF10 in the B cells by Mb1-Cre mediated recombination (CA+/Mb1-Cre+) and bone marrow cells from the controls. The average percentage of individual surface markers and their combinations as described before is represented as bar graphs. The error bars represent the standard deviations. Different surface markers show similar distribution between the two groups except for the Mac1 expression, which is slighty higher in the CA+/Mb1-Cre+ mice but difference is not statistically significant.

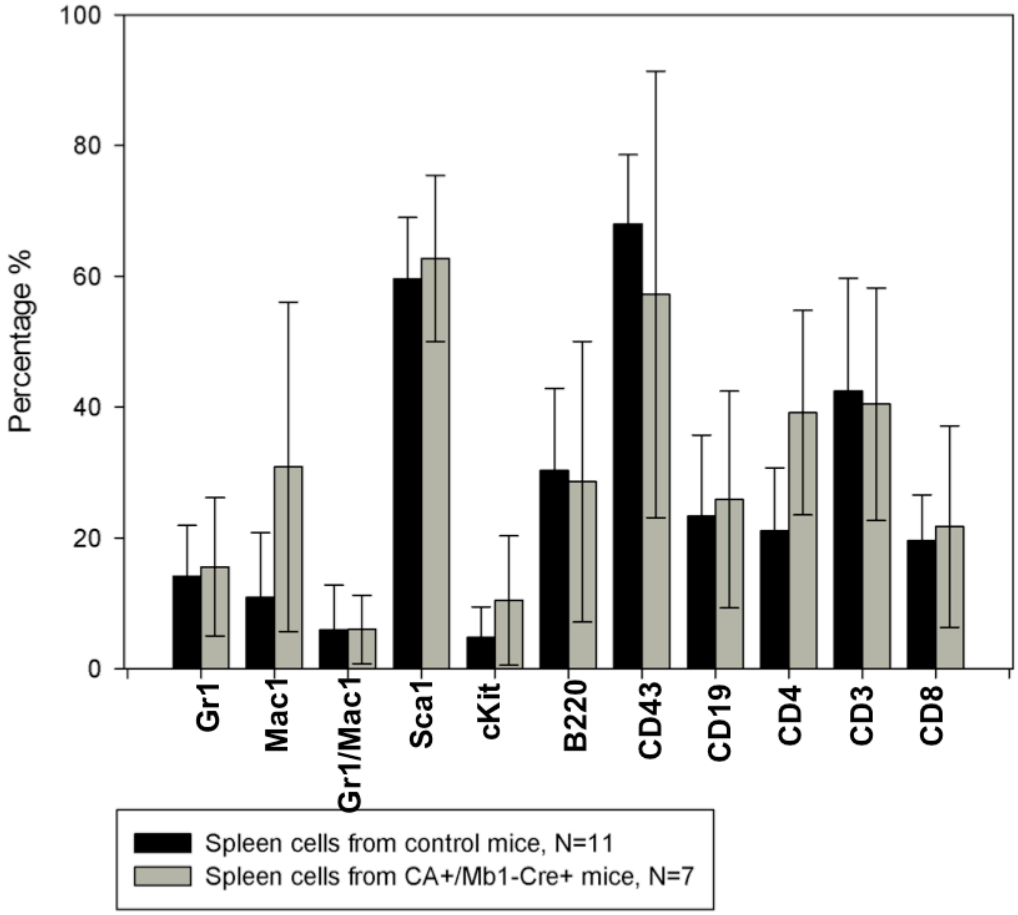

Fig 3.43: Immunophenotype of the splenocytes from CA+/Mb1-Cre+ mice and the control mice. The average percentage of individual surface markers and their combinations, as described before, is represented as bar graphs. The error bars represent the standard deviation. The number of Mac1+ cells is significantly higher in the CA+/Mb1-Cre+ mice in comparison to the control group (p value 0.08) but the other markers show a similar distribution between the two groups.

3.1.4.4 Immunophenotypic analysis of peripheral blood, bone marrow and spleen cells from CA+/CD19-Cre+ mice shows a normal pattern

6 healthy CA+/CD19-Cre+ mice were sacrificed at the age of 16 months, and their peripheral blood, bone marrow and spleen cells were extracted for immunophenotype analysis. These mice express the CALM/AF10 fusion gene in the B-lymphocytes from the early pre-B to mature B cell stage after Cre-mediated recombination. For the comparison data from our standard control group was used.

The results are summarised in figure 3.44, 3.45 and 3.46.

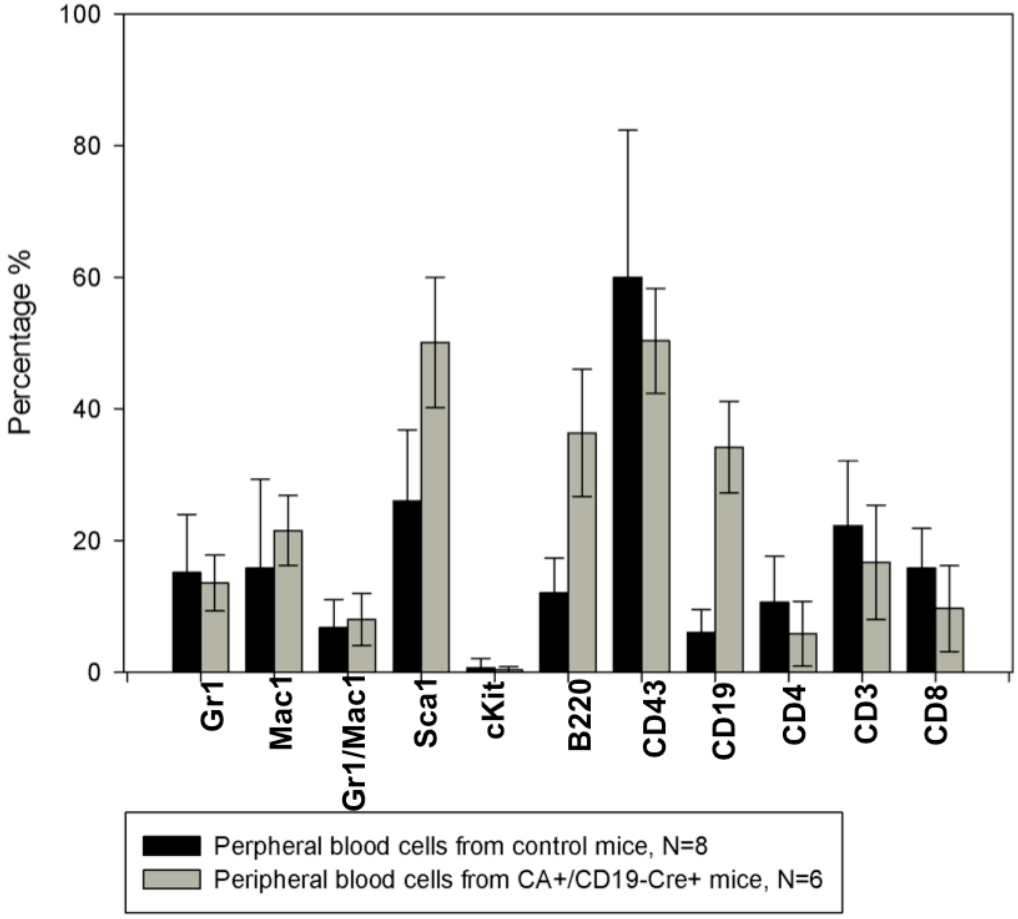

Fig 3.44: Immunophenotype of peripheral blood cells extracted from CA+/CD19-Cre+ mice and the controls. The average percentage of individual surface markers and their combinations is represented as columns. Error bars represent +/- standard deviation. The number of Sca1+ cells was significantly higher in CA+/CD19-Cre+ mice (p value 0.0009) the number of B220+ and CD19+ B cells was also significantly higher in CA+/CD19-Cre+ mice (p value for B220: 1.56E-6 and for CD19: 2.43E-6).

In the peripheral blood of CA+/CD19-Cre+ mice, the number of Sca1+ cells was significantly higher than in the control mice. The number B220+ and CD19+ cells were also significantly higher in the CA+/CD19-Cre+ mice. This difference was not so marked on the cells from the bone marrow and spleen. All the other markers showed no significantly different distribution between the two groups in PB, BM and spleen.

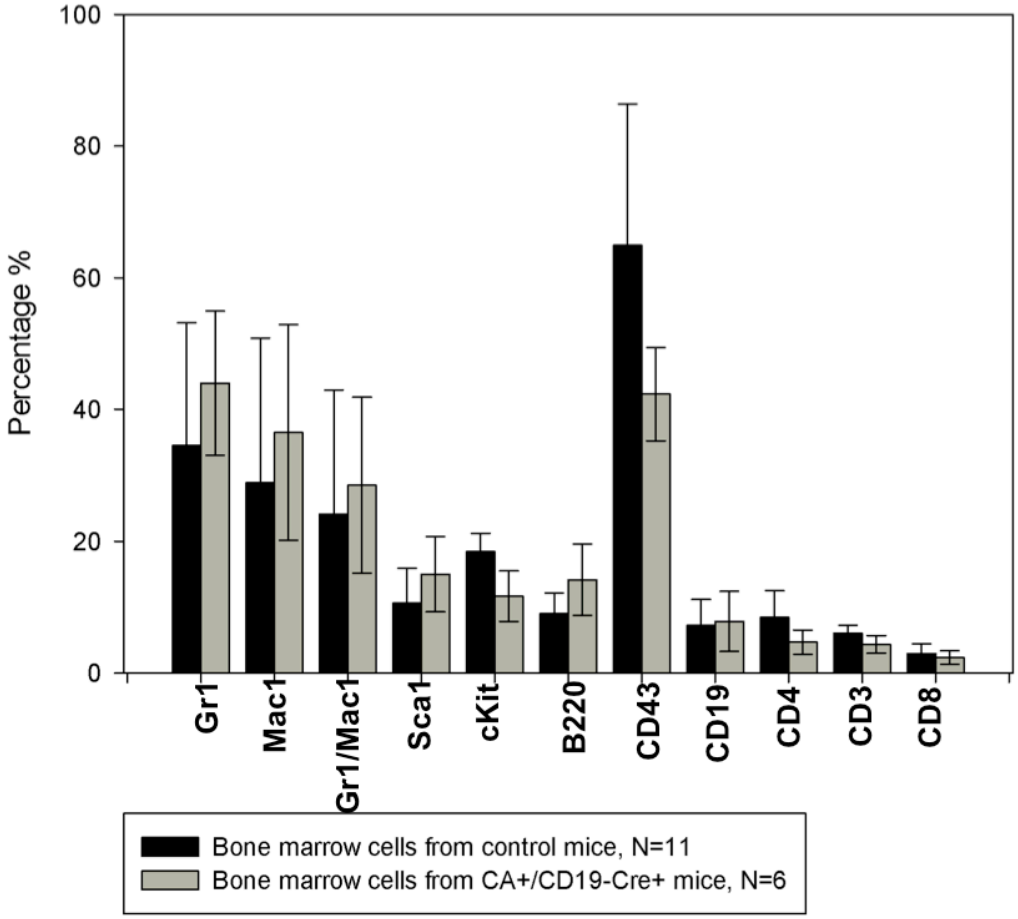

Fig 3.45: Immunophenotype of bone marrow cells extracted from CA+/CD19-Cre+ mice and the control mice. The average percentage of individual surface markers and their combinations is represented as columns. The error bars represent the standard deviation. Bone marrow cells from both groups show a similar phenotype.

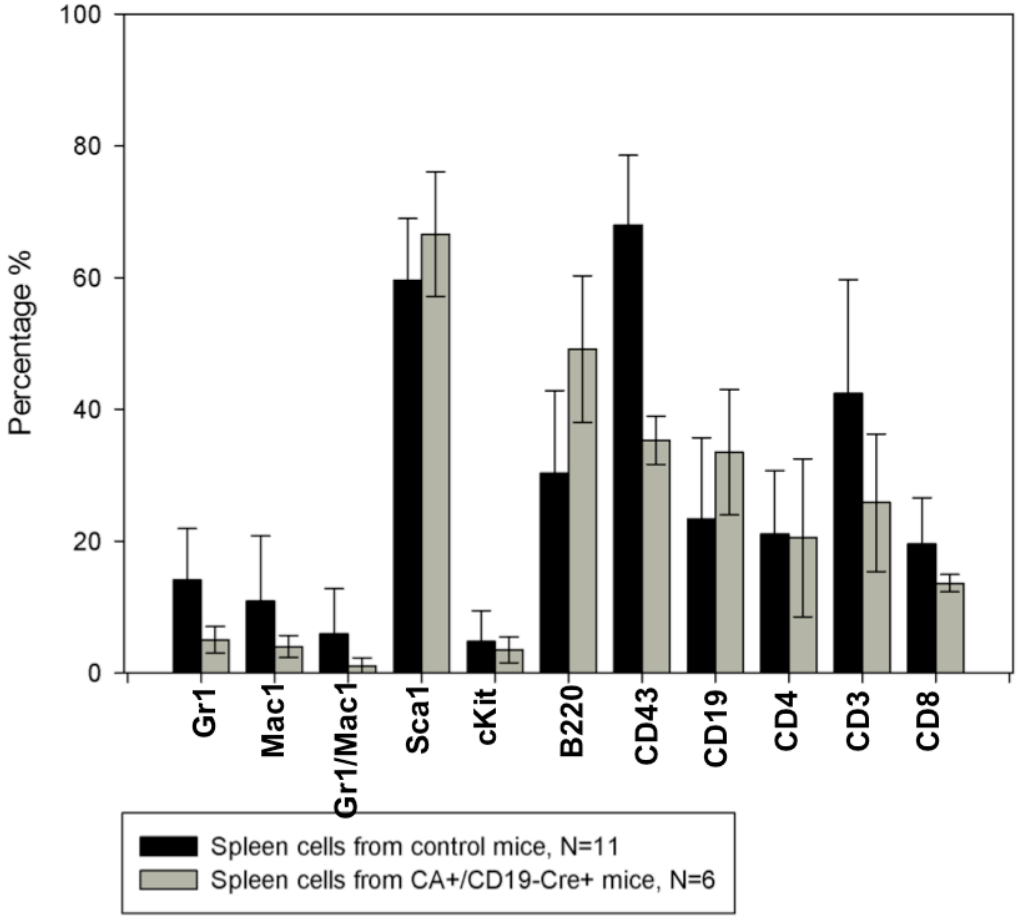

Fig 3.46: Immunophenotype of spleen cells extracted from CA+/CD19-Cre+ mice and the control mice. The average percentage of individual surface markers and their combinations is represented as columns. The error bars represent the standard deviation. Bone marrow cells from both groups show a similar phenotype. The number of B220 positive B cells is higher in the spleen from CA+/CD19-Cre+ mice (p value 0.001). There is no other significant difference in the distribution of surface markers between two groups.

3.1.5 Comparative analysis of CA+/Vav-Cre+,CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice

The expression of CALM/AF10 in cells expressing the Cre recombinase under the control of the Vav locus leads to the development of myeloid leukemia in all the mice but when CALM/AF10 is expressed in the B-lymphoid compartment in the CD19-Cre and Mb1-Cre crosses, all the mice remain healthy. For an overall picture, we are going to compare the leukemic and non-leukemic mice that express CALM/AF10 in different hematopoietic compartments. In the following figure, I shall give the survival plot

of the number of mice we have observed from each cross of the R26SLSCA mice with the three Cre-transgenic lines.

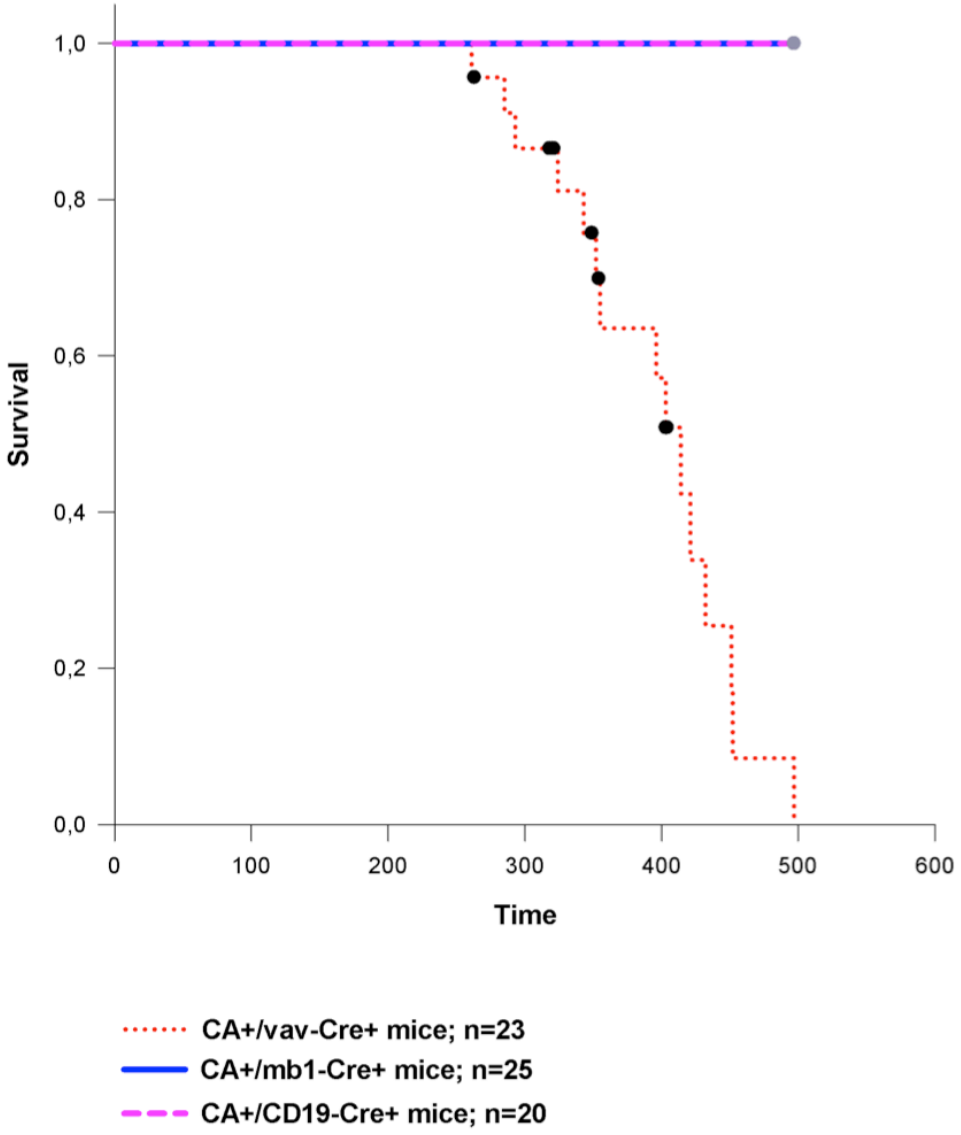

Fig 3.47: Kaplan-Meier survival plot of CA+/vav-Cre+ mice (n=23), CA+/mb1-Cre+ mice (n=25) and CA+/CD19-Cre+ mice (n=20). All CA+/vav-Cre+ mice developed leukemia with a median latency of 352 days. Post mortem analysis was possible for 15 mice. The dots indicate 8 censored mice that could not be analyzed. None of the CA+/Mb1-Cre+ or CA+/CD19-Cre+ mice developed leukemia.

3.1.5.1 Comparative immunophenotype of the leukemic CA+/Vav-Cre+ mice and non -leukemic CA+/Mb1-Cre+ mice and CA+/CD19-Cre+ mice

The immunophenotype of the leukemic CA+/Vav-Cre+ mice and non-leukemic CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice has been

compared with our control group of mice (CA+/Cre- and CA-/Cre-) previously and discussed in detail. The leukemic mice, as expected, showed significant alterations in their hematopoietic compartment. The hematopoietic cells from the CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice that expressed CALM/AF10 in B cells and did not develop leukemia showed quite a normal immunophenotype with very few exceptions.

For an overall picture, we compared the hematopoietic compartments of leukemic and non-leukemic mice expressing CALM/AF10. In the following figures, we show the immunophenotype of the hematopoietic cells from the leukemic CA+/Vav-Cre+ mice and the non-leukemic CA+/Mb1-Cre+, CA+/CD19-Cre+ mice together with our control group.

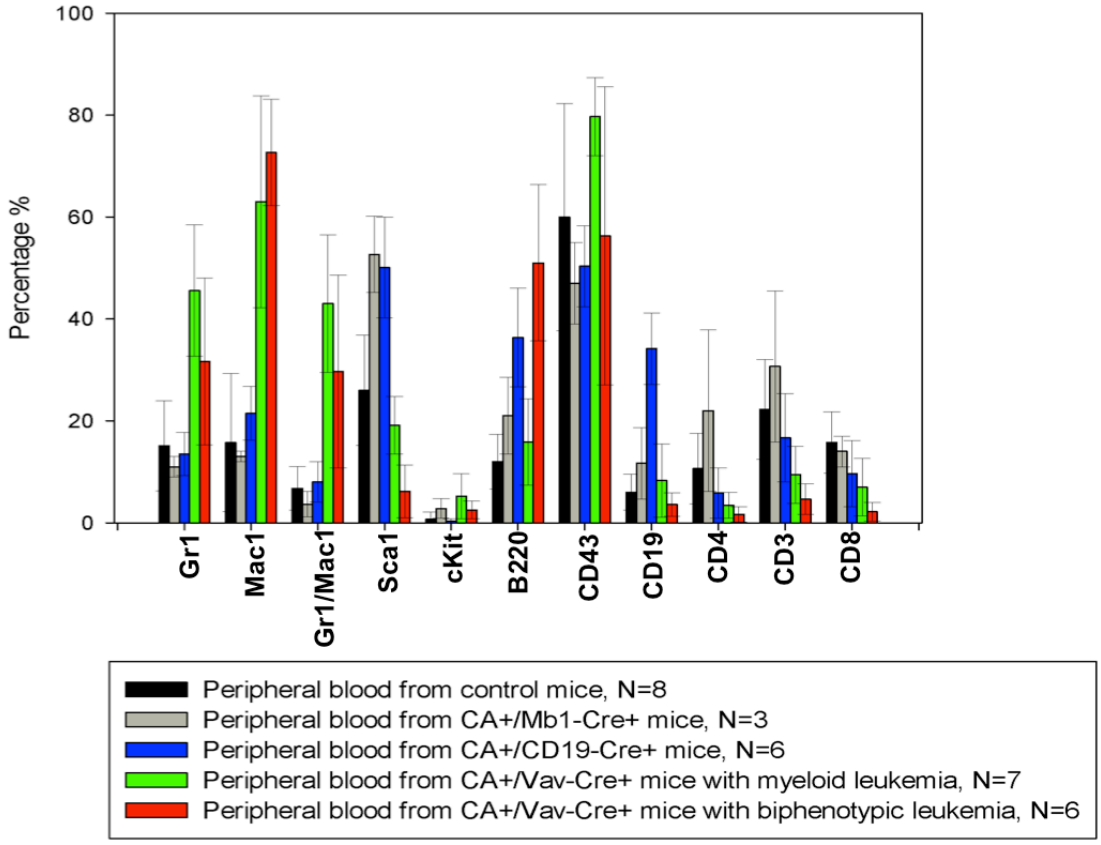

Fig 3.48: Comparative immunophenotype of the peripheral blood cells from the control mice, CA+/Mb1-Cre+ mice, CA+/CD19-Cre+ mice and the leukemic CA+/Vav-Cre+ mice. The bar graphs represent the average number of positive cells for each surface marker or their combination. Error bars represent the standard deviation. In the leukemic mice there is a massive expansion of the myeloid compartment shown here by high number of Gr1+, Mac1+ and Gr1+/Mac1+ cells. In the peripheral blood of CA+/CD19-Cre+ mice, the number of B220+ and CD19+ B cells is very high. But in these non-leukemic mice the number of myeloid cells and T lymphocytes appear normal according to our assay.

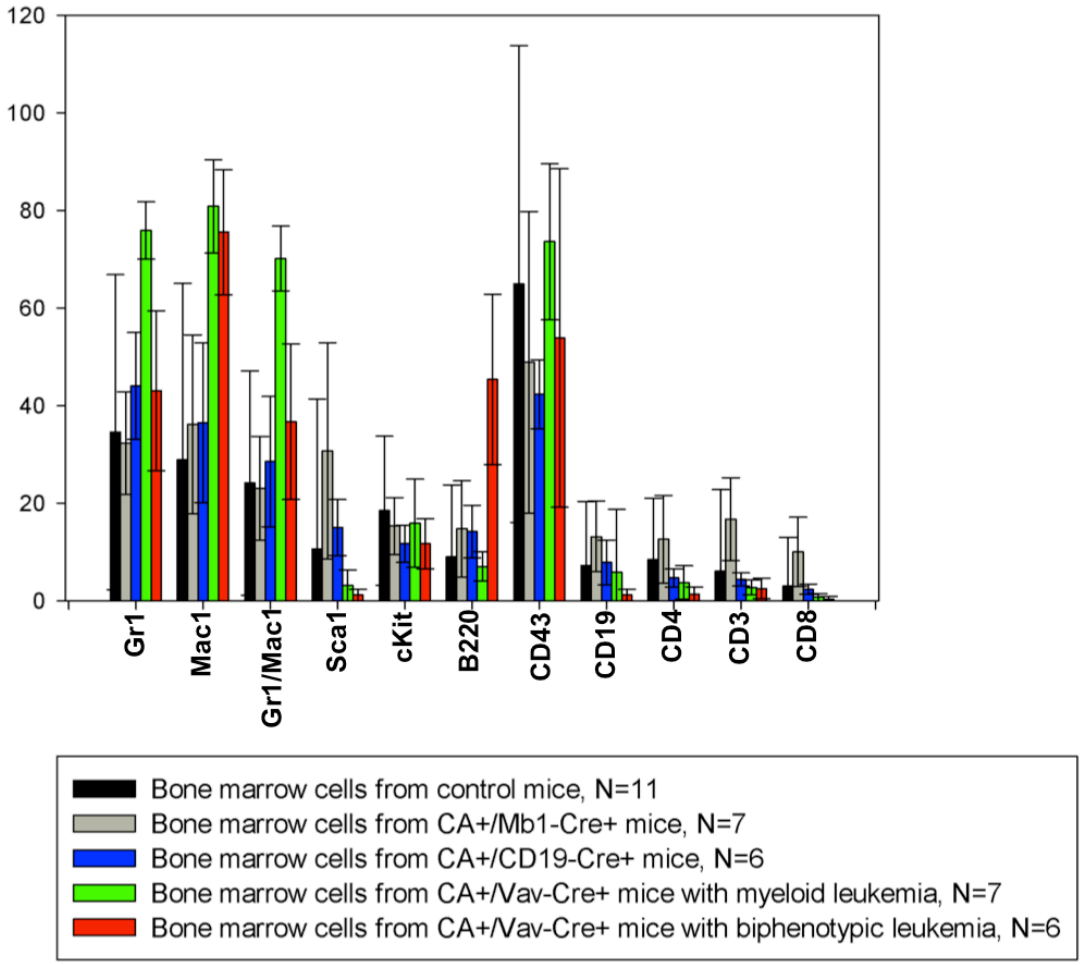

Fig 3.49: Immunophenotypes of the bone marrow cells from the control mice, CA+/Mb1-Cre+ mice, CA+/CD19-Cre+ mice and the leukemic CA+/Vav-Cre+ mice. The bar graphs represent the average number of cells positive for each surface marker or their combination. Error bars represent the standard deviation. In the bone marrow of the leukemic mice there is an expansion of the myeloid compartment with high numbers of Gr1+, Mac1+, Gr1+/Mac1+ cells and CD43+ cells.

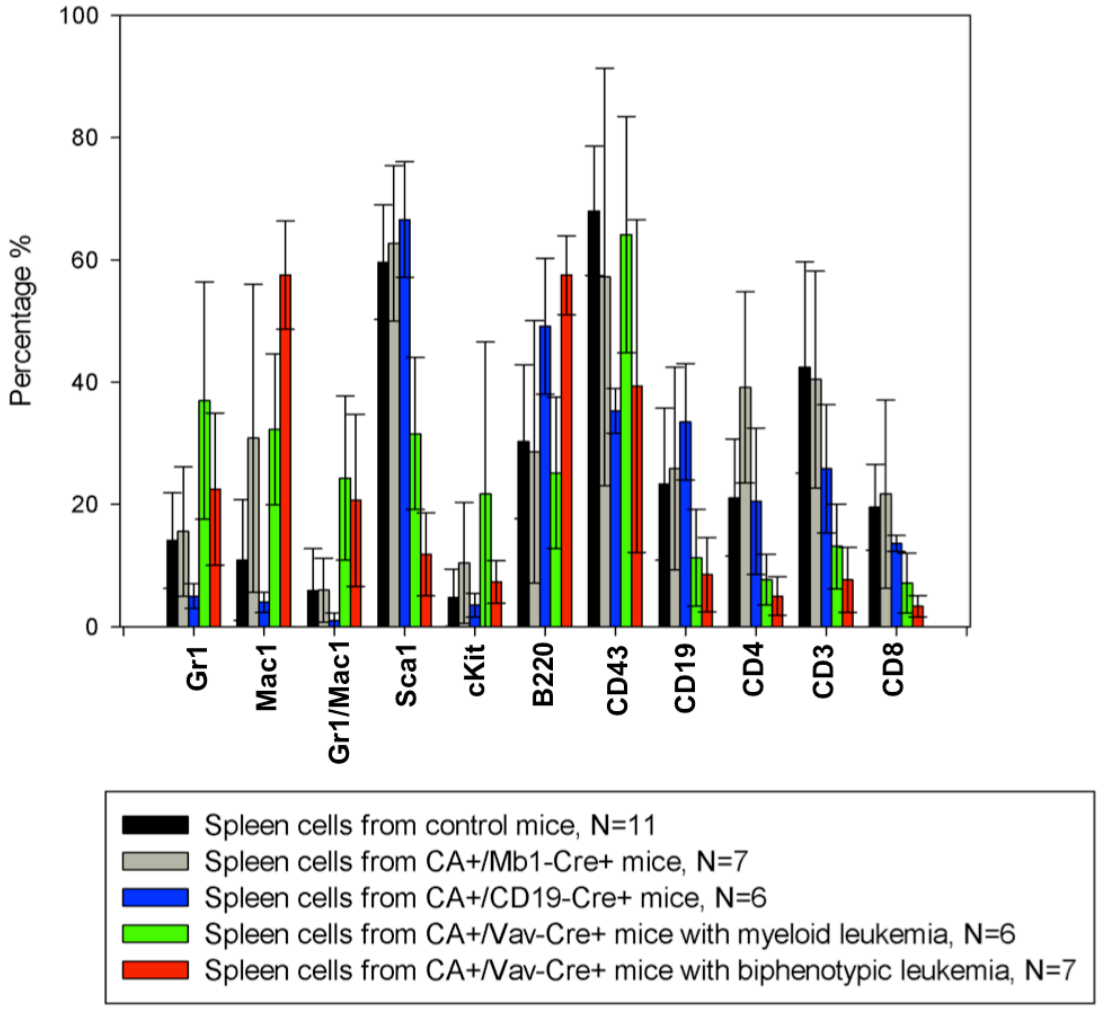

Fig 3.50 Comparative immunophenotype of the spleen cells from the control mice, CA+/Mb1-Cre+ mice, CA+/CD19-Cre+ mice and the leukemic CA+/Vav-Cre+ mice. The bar graphs represent the average number of cells positive for each surface marker or their combination. Error bars represent the standard deviation. Leukemic spleen cells show very high expression of myeloid markers like Gr1, Mac1 as expected. Surprisingly, the number of Mac1 positive cells is very high in the spleen of CA+/Mb1-Cre+, even comparable to the spleen cells from the CA+/Vav-Cre+ mice with leukemia. We can also see low number of Sca1+ cells in the leukemic spleen. The number of CD4+, CD3+ and CD8+ T cells is also reduced. The spleen cells of the other groups do not share these features. Spleen cells from CA+/CD19-Cre+ mice show a normal immunophenotype.

3.1.5.2 Comparable levels of the CALM/AF10 fusion transcript were detected in the leukemic bone marrow cells and non-leukemic cells of CA+/Mb1-Cre+ mice

To determine the level of the CALM/AF10 fusion transcript in the bone marrow and spleen of the leukemic CA+/Vav-Cre+ mice and in the bone marrow and the B cells of non-leukemic CA+/Mb1-Cre+ mice, we performed a real time RT-PCR assay.

cDNA was isolated from the unsorted bone marrow cells of 3 leukemic CA+/Vav-Cre+ mice, the unsorted spleen cells of 4 leukemic CA+/Vav-Cre+ mice, unsorted bone marrow cells of 3 non-leukemic CA+/Mb1-Cre+ mice and B220 marker positive MACs-sorted B cells of 6 CA+/Mb1-Cre+ mice (age 12 months) and 3 CA+/CD19-Cre+ mice (age 18 months). cDNA from the U937 cell line was used as a positive control for the CALM/AF10 transcript. As mentioned earlier, the CALM/AF10 fusion was originally cloned from the U937 cell line. Realtime RT PCR was performed to analyze the expression of CALM/AF10 transcript using primers detecting the CALM/AF10 breakpoint. Both the 18s rRNA gene and the beta actin gene transcripts were used as controls for cDNA quantity and integrity in this assay.

The CALM/AF10 transcript had the highest expression in the leukemic spleen and was expressed at comparable levels in the leukemic bone marrow of CA+/Vav-Cre+ mice and in the B cells of CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice. The CALM/AF10 transcript was detected at slightly lower levels in the bone marrow of the CA+/Mb1-Cre+ mice. This observation is probably due to the fact that the expression of CALM/AF10 is restricted to B cells in CA+/Mb1-Cre+ mice and that the B cells constitute only a smaller percentage of the bone marrow cells. The C_T values of the CALM/AF10 transcript in the different tissues, normalized to the beta-actin expression are shown in figure 3.51.

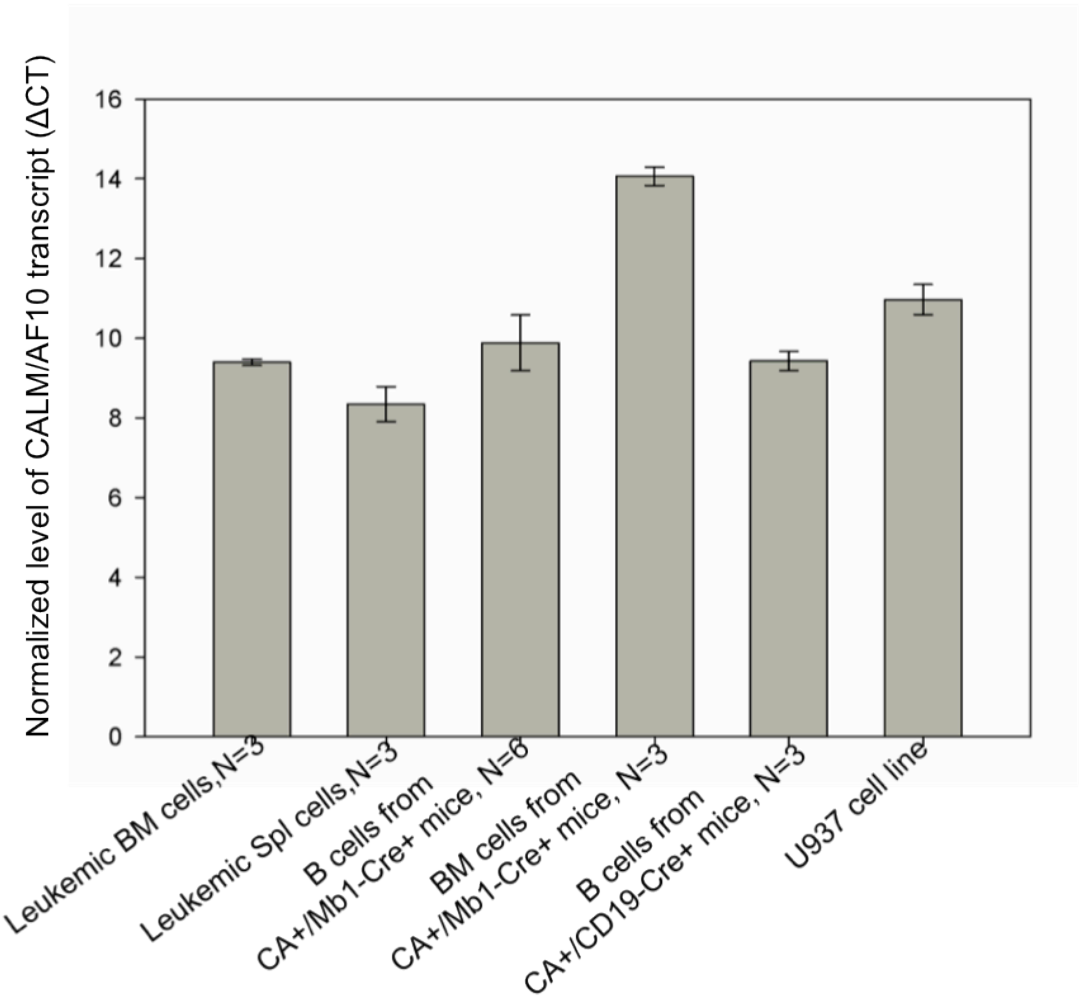

Fig 3.51: Detection of the CALM/AF10 transcript in the bone marrow and spleen cells of the leukemic CA+/Vav-Cre+ mice and in the B cells of non-leukemic CA+/Mb1-Cre+, CA+/CD19-Cre+ mice and U937 cell line.. Bar graphs represent the $C_T$value of CALM/AF10 transcript normalized to the expression of the endogenous control beta-actin. Bone marrow cells from 3 leukemic mice, spleen cells from 4 leukemic mice, B220+ B cells from 6 CA+/Mb1-Cre+ mice and 3 CA+/CD19-Cre+ mice are assayed. The error bars represent the standard deviations among the biological replicates. As a positive control, the U937 cell line was used. Three technical replicates were assayed for all samples. For U937, error bars represent the standard deviation between the technical replicates.

3.1.5.3 The HoxA cluster genes and the Hox co-factor *Meis1* are overexpressed in leukemic cells from CA+/Vav-Cre+ mice

Hox genes play important roles in hematopoietic differentiation and changes in their expression levels are found in several hematological malignancies. Several *HoxA* cluster genes are overexpressed in CALM/AF10 positive T-ALL patients (Dik et al., 2005). The expression of *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxb4*, *Bmi1* and the Hox co-factor *Meis1* was assayed using real time RT-PCR in leukemic and normal bone marrow and spleen cells from CA+/Vav-Cre+ and wild type control mice (CA-/Cre-).

cDNA was isolated from the unsorted bone marrow cells of 4 leukemic CA+/Vav-Cre+ mice. In 3 cases there were more than 80% blasts cells present in the bone marrow. One of the samples had 50% blast in the bone marrow. Among these mice two mice had myeloid leukemia and two mice had the biphenotypic type of AML. As controls, we have used unsorted bone marrow cells from 3 wildtype mice (CA-/Cre-). From the immunophenotype we know that the bone marrow of the leukemic mice contains very different cell populations compared to the wild type bone marrow. We used the control bone marrow cells just to set a normal base line for the expression of the genes we tested. As internal control both the 18s rRNA gene and beta actin were used.

All the tested genes from the *Hoxa* cluster and *Meis1* showed higher expression levels in the leukemic bone marrow. The expression level of the tested genes did not vary among the leukemic samples. The sample with 50% blasts also had very high levels of Hoxa cluster gene and *Meis1* expression.

Bmi1, a polycomb group gene, was also overexpressed in the CALM/AF10 positive T-ALL patient samples reported by Dik and colleagues (Dik et al., 2005). The *Bmi1* transcript showed just slightly higher (2 to 3 fold) expression levels in the leukemic bone marrow. Hoxb4 was taken as a control outside the HoxA cluster and as a Hox gene that is not usually over-expressed in leukemic cells. The results are summarized in figure 3.52 A and B.

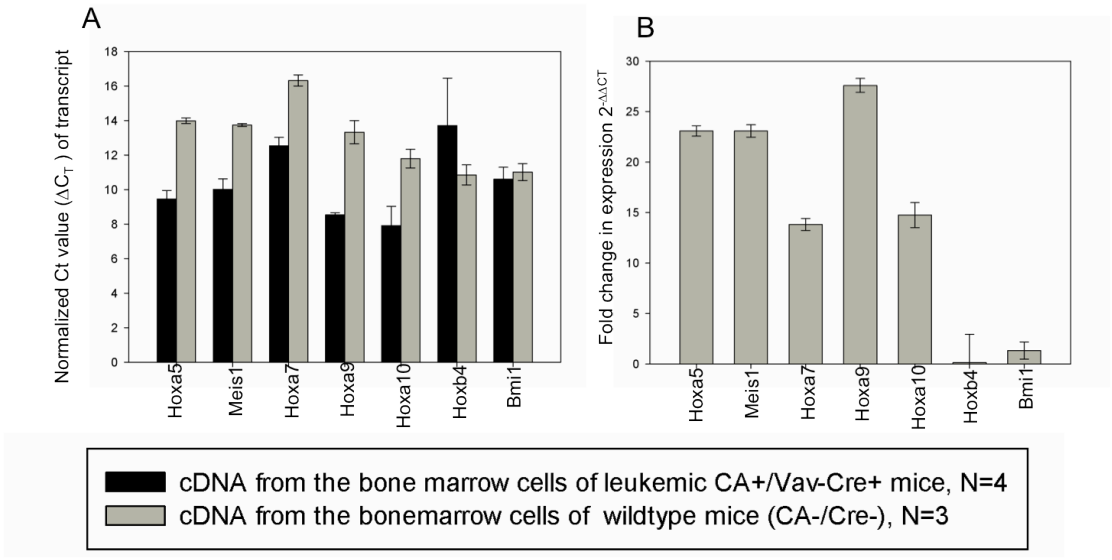

Fig 3.52: Quantification of the *Hoxa* gene and *Meis1* transcript levels in the leukemic bone marrow of CA+/Vav-Cre+ mice (N=4) relative to transcript levels in bone marrow from wild type mice (N=3). RNA transript analysis for *Hoxa5, Hoxa7, Hoxa9, Hoxa10, Meis1* was performed using real time RT-PCR from bone marrow samples from 4 leukemic CA+/Vav-Cre+ and 3 wild type mice. For each sample, 3-6 technical replications were assayed. As endogenous control, both beta-actin and 18srRNA were used. In the chart, the ΔC_T values were calculated using beta actin. The expression of *Hoxa5, Hoxa7, Hoxa9, Hoxa10, Meis1, Bmi1* and *Hoxb4* was normalized to beta actin. Chart A shows the normalized expression level of each transcript in leukemic and wild type bone marrow. The error bars show standard deviation among biological replicates. All the *Hoxa* cluster genes have lower C_T value in leukemic samples. Chart B shows the fold change in the expression level of each transcript compared to wild type bone marrow. The *Hoxb4* gene is from the *HoxB* cluster and does not show higher expression levels in the leukemic cells. The *Bmi1* transcript is expressed at slightly higher levels in the leukemic cells (1.33 fold) and all the *HoxA* genes are expressed from 13 to 27 fold higher in the leukemic bone marrow.

cDNA was isolated from the unsorted spleen cells of 4 leukemic CA+/Vav-Cre+ mice. Among the 4 chosen leukemias, all of them had more than 80% blasts in the bone marrow. Out of the 4 leukemias, 2 were myeloid and the other two biphenotypic. As controls we used cDNA from spleens of 3 wildtype mice. We assayed the level of *Hoxa5, Hoxa7, Hoxa9, Meis1* and *Hoxb4* transcripts in the leukemic spleen and compared it with their expression levels in the spleen cells from wild type mice (CA-/Cre-). 18s rRNA gene and beta-actin gene were used as endogenous controls. The results are summarized in figure 3.53 A and B. We could not detect *Hoxa7* and *Hoxa9* in wild type spleen cells but in leukemic samples these transcripts were present at a reasonably high level. *Hox*a5 and Hox co-factor *Meis1* showed very high levels

of expression in the leukemic spleen. Hoxb4 expression was unchanged. The expression of *Hoxa10* transcript was detectable neither in wild type nor in leukemic spleen cells. We did not perform qRT-PCR for *Bmi1* transcript on spleen samples.

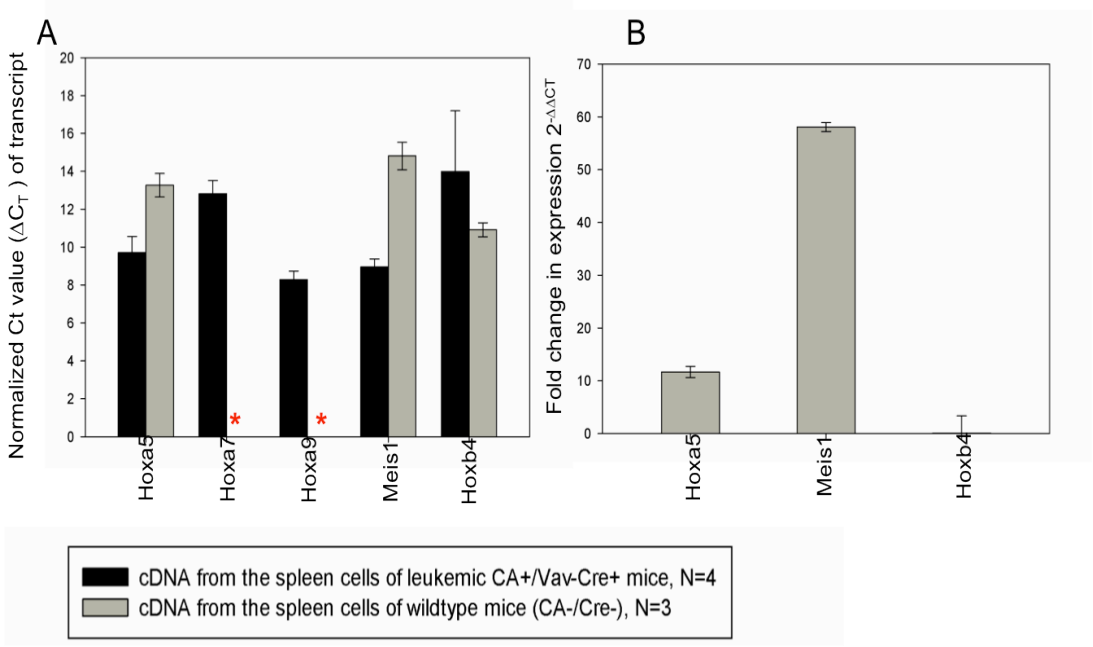

Fig 3.53: Relative quantification of the *Hoxa* gene and *Meis1* transcripts in the leukemic spleen of CA+/Vav-Cre+ mice compared to transcript levels in normal spleen. RNA transcript analysis for *Hoxa5, Hoxa7, Hoxa9, Meis1* and *Hoxb4* was performed using realtime RT-PCR on 4 leukemic and 3 wildtype spleen samples. For each sample, 3-6 technical replications were performed. Chart A shows the normalized expression level of each transcript (ΔC_T) in leukemic and wildtype spleen. The ΔC_T calculations in this chart were preformed using beta actin as endogenous control. We could not detect Hoxa7 and Hoxa9 transcripts in wildtype spleen cells, which are marked with red stars. The chart B shows the fold expression changes of *Hoxa5, Hoxb4* and *Meis1* transcripts, compared to wild type spleen cells. The *Hoxb4* gene is from the *HoxB* cluster and does not show higher expression levels in the leukemic cells. *Meis1* shows 58 fold higher expression in the spleen cells from the leukemic CA+/Vav-Cre+ mice compared to spleen cells from wild type mice. We could not calculate the fold change for *Hoxa7* and *Hoxa9* as they were not detectable in wild type spleen cells.

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3.1.5.4 The *Hoxa* cluster and *Meis1* overexpression is only found in the leukemic cells from CA+/Vav-Cre+ mice and not in non-leukemic cells of CA+/Mb1-Cre+ mice

Real time RT-PCR was performed to measure the expression of the *HoxA* cluster genes and *Meis1* transcripts in the B cells from 3 healthy CA+/Mb1-Cre+ and 4 wildtype (CA-/Cre-) mice. B220+ cells were isolated from the spleen cells of three 1 year old CA+/Mb1-Cre+ mice using MACs seperation. In these B cells, the CALM/AF10 fusion gene is expressed after Mb1-Cre-mediated

recombination. We already showed the expression of the CALM/AF10 fusion transcript in these cells (Fig 3.51). As controls, cDNA from isolated B220+ B cells from the spleen of 4 wild type (CA-/Cre-) mice was used. We measured the expression of *Hoxa5, Hoxa7, Hoxa9, Meis1, Bmi1* and *Hoxb4* transcripts. 18s rRNA and beta actin were used as endogenous controls. We wanted to compare the expression level of these tested genes between CALM/AF10 expressing B cells and the wild type B cells. We were not able to detect *Hoxa7, Hoxa9* and *Hoxa10* transcripts in any of the samples.

Hoxa5 gene or *Meis1* expression levels were not significantly different between B cells from CA+/Mb1-Cre+ and wild type mice. The results are summarized in figure 3.54

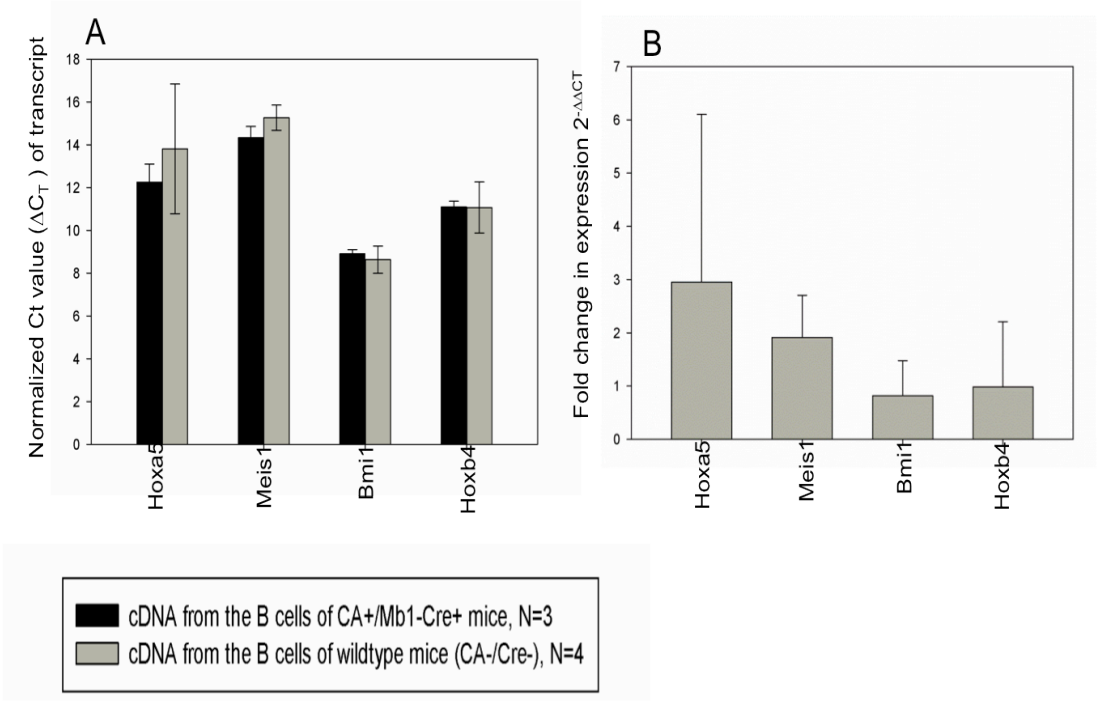

Fig 3.54: Relative quantification of the *Hoxa* genes and *Meis1* transcript levels in B cells from CA+/Mb1-Cre+ and wild type mice. Real time PCR assays for *Hoxa5, Hoxa7, Hoxa9, Meis1, Bmi1* and *Hoxb4* were performed on RNA from CA+/Mb1-Cre+ and wild type mice. B cells from 3 CA+/Mb1 Cre+ mice and from 4 wild type mice were used for this assay. For each samples 3-6 technical replicates were performed. We could not detect *Hoxa7, Hoxa9 or Hoxa10* in any of the samples. The chart A shows the normalized levels of transcripts (ΔC_T) in the B cells from wild type and CA+/Mb1-Cre+mice. For the normalization and calculation of ΔC_T, beta actin was used as endogenous control. Note that all the detected transcripts show similar ΔC_T value in both samples. In chart B, the fold change in expression is shown. *Hoxa5* and *Meis1* transcripts show 2.9 fold and 1.9 fold higher expression in CA+/Mb1-Cre+ B cells, respectively, but the error rate is very high and the difference is not statistically significant. The P value for *Hoxa5* is 0.43 and for *Meis1, it is* 0.08.

Summary:

To summarise our work with the tissue-specific, inducible knock-in mouse model of CALM/AF10 (R26SLSCA mice), we could show that the CALM/AF10 fusion is only leukemogenic when expressed in cells that have Vav promoter activity. Thus CALM/AF10 needs to be expressed in the right cell at the right stage during hematopoietic development. When CALM/AF10 is expressed in the whole hematopoietic compartment after Vav-Cre-mediated recombination an aggressive AML develops with 100% penetrance and a median latency of about 1 year. In contrast, a more B cell compartment restricted expression of CALM/AF10 using CD19-Cre and Mb1-Cre-mediated recombination, does not lead to the development of leukemia. Hematopoietic cells from the leukemic mice showed overexpression of *HoxA* cluster genes and the Hox co-factor *Meis1*. We could not detect overexpression of *HoxA* cluster genes or *Meis1* in the hematopoietic cells of non-leukemic mice that expressed CALM/AF10 (the CA+/Mb1-Cre+ mice).

3.2 A murine retroviral bone marrow transplantation model to examine the role of *BMI1* in CALM/AF10-mediated leukemias

A murine retroviral bone marrow transplantation model was developed to investigate the role of *BMI1* in CALM/AF10-induced leukemia. *BMI1* (B lymphoma Mo-MLV insertion region 1 homolog) is a polycomb group gene, and its transcript is consistently upregulated in CALM/AF10-positive T ALL and AML patients (Dik et al., 2005; Mulaw et al., 2012) and in a murine CALM/AF10 bone marrow transplantation leukemia model (Mulaw et al., 2012)

To investigate the role of *BMI1* in collaborating with CALM/AF10 in leukemogenesis, we co-expressed *BMI1* with the CALM/AF10 fusion in murine bone marrow cells. To achieve this goal, we used two approaches. In the first approach, *BMI1* was retrovirally expressed in the bone marrow cells from the transgenic mouse line IgH-CALM/AF10. Alexandre Krause generated this mouse line, on the FVB background, in our lab. These animals express the CALM/AF10 fusion under the control of the immunoglobulin heavy chain enhancer/promoter in mature B cells but do not develop leukemia (Krause, 2006). Bone marrow cells from the IgH-CALM/AF10 mice were retrovirally transduced with a *BMI1* expressing retrovirus. Successfully transduced cells were transplanted via tail vein injection into syngeneic lethally irradiated wild type FVB recipient mice.

In the second approach, bone marrow cells from FVB wild type mice were co-transduced with two retroviruses. One virus contained the CALM/AF10 open-reading frame (pMIG-CALM/AF10) and the other virus contained the *BMI1* gene (pMIY-BMI1). Successfully cotransduced bone marrow cells would co-express both the green and the yellow fluorescent protein. The double transduced cells were flow-sorted and then injected into lethally irradiated wild type FVB recipients.

3.2.1 Approach 1: Retroviral expression of *BMI1* in the bone marrow cells from IgHCALM/AF10 transgenic FVB mice

IgH(E/P)CALM/AF10 is a transgenic FVB mouse line in which the expression of the CALM/AF10 fusion gene is under the control of the B cell specific IgH promoter and enhancer. Expression of the CALM/AF10 fusion in these mice is found in mature B cells. These mice do not develop leukemia, even at old age (18 months). For the first transplantation model these mice were used as the bone marrow donors. In the bone marrow from these mice, human *BMI1* was overexpressed using retroviral transduction. Below is a schematic of the experimental design.

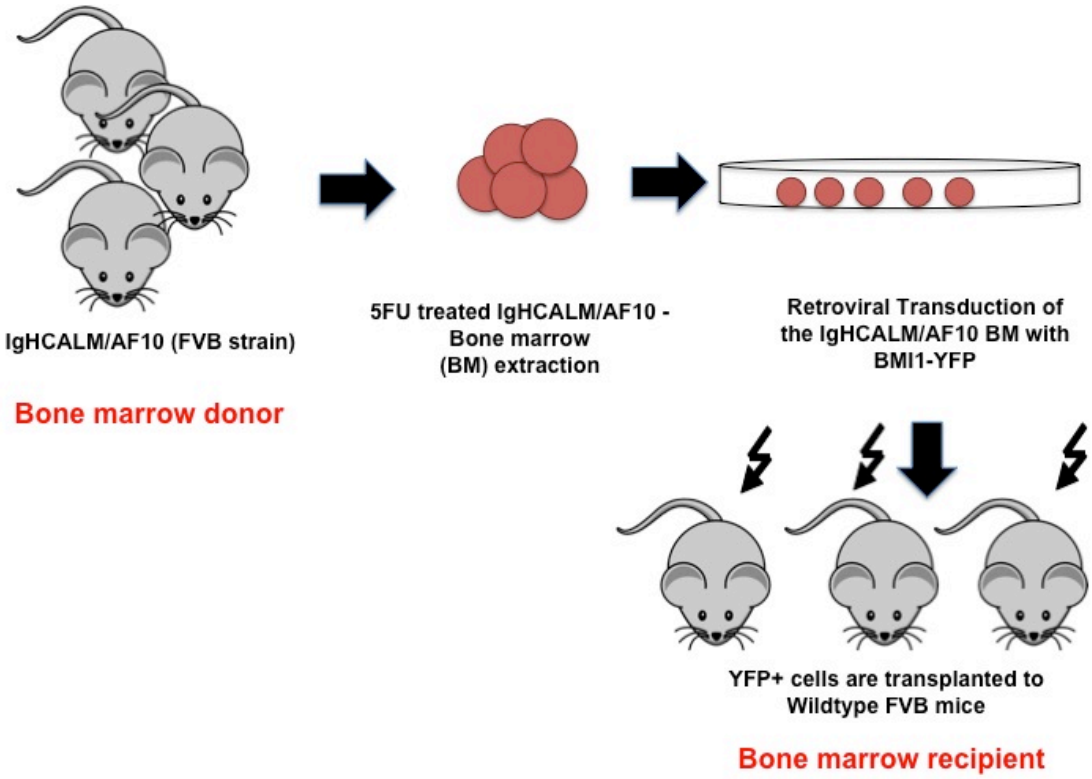

Fig 3.55: Flow chart of the first transplantation model. The bone marrow donors were the IgHCALM/AF10 transgenic FVB mice and the recipients were wild type FVB mice. 5FU treated bone marrow cells were transduced with the pMIY-BMI1 retrovirus, and successfully transduced cells were transplanted into the wildtype recipients.

3.2.1.1 The retrovirus producer cell line E86-BMI1-FLAG-YFP

The E86-BMI1-FLAG-YFP cell line was kindly provided by Dr Keith Humphries (Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver British Columbia, Canada). This E86-BMI1-FLAG-YFP cell line contains a MSCV vector with a C-terminally FLAG-tagged BMI1 cDNA. The *BMI1* cDNA is followed by Internal Ribosome Entry Site (IRES) and by the YFP gene (Figure 3.56).

Figure 3.56: Map of the pMIY-BMI1 construct

The E86-BMI1-FLAG-YFP cell line is a high titer pMIY-BMI1 retrovirus producing cell line. We performed Western blot analysis to assay the production of the BMI1-FLAG protein. As can be seen in figure 3.57, the FLAG-tagged human BMI1 protein could be detected in extracts from the E86-BMI1-FLAG-YFP retrovirus

producer cell line and in retrovirally transduced bone marrow cells. The BMI1 protein could not be detected in E86 cells transduced with the empty pMIG vector or in non-transduced bone marrow cells.

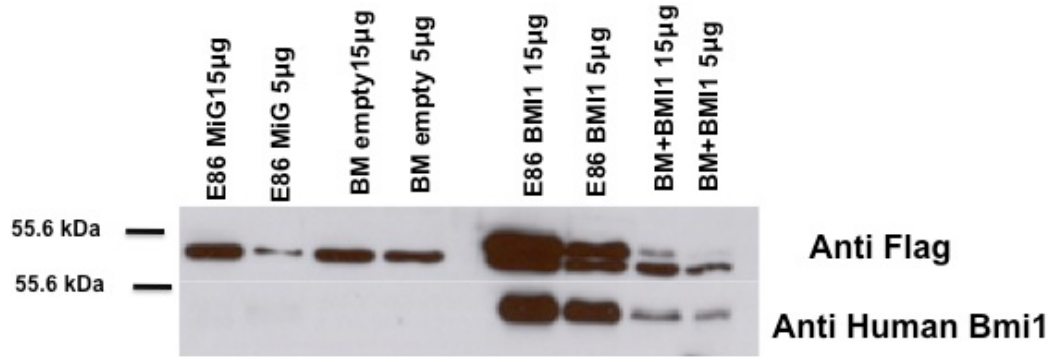

Fig 3.57: Western blot using anti-FLAG and anti hu-BMI1 antibodies. The first two lanes (1, 2) from each blot contain the protein extract from control E86-MIG cells, (E86 transduced with the empty vector pMIG), lanes 3 and 4 contain the extract from non-transduced murine bone marrow cells; lanes 5 and 6 contain the extract from the E86-BMI1-FLAG-YFP cell line and the last two lanes contain protein extracts from pMIY-BMI1 transduced murine bone marrow cells. In the upper panel the anti FLAG antibody was used. In the expected lanes (lane 5, lane 6, lane 7, lane 8), the BMI1-specific band is visible at around 50 kDa. In the lower panel, the anti human BMI1 antibody detects a specific band BMI1 band at around 50 kDA molecular weight..

3.2.1.2 Transplantation experiments with pMIY-BMI1 transduced IgH-CALM/AF10 bone marrow

In order to express the CALM/AF10 fusion and BMI1 together in murine bone marrow, bone marrow cells from 5FU-treated IgH-CALM/AF10 transgenic donor mice were retrovirally transduced using the E86-BMI1-FLAG-YFP cell line. YFP positive bone marrow cells were sorted using fluorescence activated cell sorting (FACS). $0.5x10^6$ YFP positive cells together with $0.5x10^6$ non-transduced bone marrow cells were transplanted into the lethally irradiated FVB mice via tail vein injection.

As controls, bone marrow cells from wild FVB mice transduced with pMIY-BMI1 or the empty vector (pMIY) were used. $0.5x10^6$ YFP positive cells and $0.5x10^6$ non-transduced cells were injected into the tail vein of FVB wild type mice. As a third control group, bone marrow cells from the transgenic IgH-CALM/AF10 mice were transduced with pMIY and transplanted together with non-transduced cells into FVB wild type recipients. The number of transplanted animals in the different experimental groups is shown in table 3.3:

3.3 Summary of the transplantation experiments: The number of mice transplanted with different constructs is summarized in this table. The first column includes the different experimental groups; in the second column the constructs used for each group of experiment are listed. In the third and fourth columns the strain/genetic background of the donor and recipient mice are listed. The last column contains the number of transplanted animals for each group.

Experimental group	Construct	Bone marrow donor	Bone marrow recipient	Number of recipients
tr-IgH-CA&BMI1	pMIY-BMI1	FVB IgH-CALM/AF10 transgenic mice	FVB wild type	12
WT-FVB&BMI1	pMIY-BMI1	FVB wild type	FVB wild type	12
tr-IgH-CA&MIY	pMIY	FVB IgH-CALM/AF10 transgenic mice	FVB wild type	7
WT-FVB&MIY	pMIY	FVB wild type	FVB wild type	9

3.2.1.3 Transplanted *BMI1* expressing bone marrow cells do not show any growth advantage *in vivo*

To assay the engraftment of the transduced bone marrow cells, we collected peripheral blood 4 - 6 weeks after the transplantation and measured the percentage of YFP-positive cells using flow cytometry. The transplanted animals from all the experimental groups showed a wide range in the proportion of YFP-positive cells from a very low percentage in some mice to more than 70% YFP-positivity in others. The median YFP-positivity was 44%. In all cases we had transplanted the same number of YFP-positive, retrovirally transduced and non-transduced bone marrow cells. There was no significant growth advantage of the transduced cells over the non-transduced cells in any of the experimental groups. 2 mice out of the total 12 transplanted mice in tr-IgHCA&BMI1 experimental group were not engrafted

The percentage of YFP-positive cells in the peripheral blood from the mice of the experimental groups, 4 weeks after transplantation is show in figure 3.58.

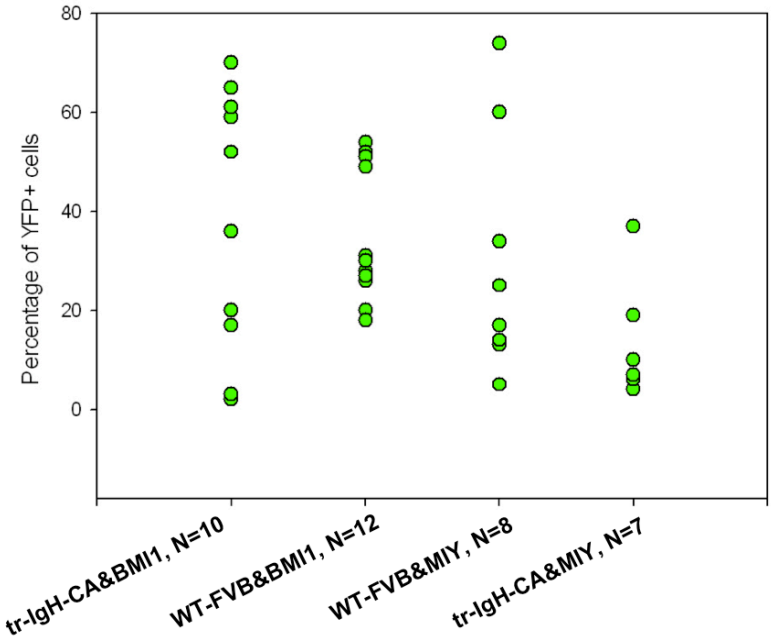

Fig 3.58: Percentage of YFP-positive cells in the peripheral blood of transplanted mice from different experimental groups. The analysis was performed between 4 and 6 weeks after the transplantation.

3.2.1.4 Eight of 12 mice from the tr-IgH-CA&BMI1 group developed leukemia

Eight of the 12 mice from the tr-IgH-CA&BMI1 group became sick and had to be sacrificed.

Of the 8 sick animals, 5 mice developed acute myeloid leukemia in 101 days (5625A#1), 197 days (5625A#2), 246 days (5619A#2), 314 days (5619B#1) and 461 days (5619B#2) post transplantation. The post mortem analysis revealed in all the sick mice signs and symptoms of leukemia like splenomegaly leukocytosis, and the presence of immature blastic cells in the peripheral blood. Only in one of the 5 mice (5619A#2), the number of WBCs in the peripheral blood was in the normal range (6 million/ml) and the spleen size was also normal (124 mg). But this mouse had abnormal numbers of myeloid marker positive cells and cKit positive immature cells in the peripheral blood bone marrow and spleen.

2 out of 8 sick animals developed T-ALL in 101 days (5625B#2) and 219 days (5619A#1) post transplantation. Both the mice had a very

high number of leukocytes in their peripheral blood. The FACs analysis revealed the presence an abnormal CD4/CD8 double positive immature T lymphocyte population in the peripheral blood, bone marrow and spleen cells of these two mice. These abnormal double positive cells were surprisingly YFP-negative. Cytospin preparations from the peripheral blood bone marrow and spleen cells of these two mice revealed the presence of immature lymphoblasts (~ 80%).

The remaining one mouse (5625B#1) out of the 8 sick animals was sacrificed 123 days post transplantation. Before it was sacrificed it showed signs of difficulties while eating moving and breathing. But the post-mortem analysis on this mouse did not lead us to a conclusive diagnosis. The mouse had a spleen slightly bigger than normal (175 mg) and WBC count in the peripheral blood was also higher than normal (12 million/ml). In the peripheral blood we did not detect any YFP positive cell. In the bone marrow and spleen 3% and 25%, respectively, of the cells were YFP positive. Flow cytometric analysis revealed that the mouse had a normal percentage of myeloid and lymphoid cells. However, Sca1 positive cells were abnormally high in blood (41%) and bone marrow (91%). We could detect immature blast cells of myeloid lineage in the peripheral blood smear and bone marrow cytospin of this mouse.

Four out of the 12 mice from the tr-IgH-CA&BMI1 group remained healthy during the observation period. Of these 4 healthy mice, 2 mice had very low percentages of YFP-positive cells (2-3%) in the peripheral blood 4 weeks after transplantation. The other 2 mice had no YFP-positive cells in their peripheral blood 6 weeks after transplantation.

The leukemia phenotype, engraftment at the time they were sacrificed and diagnosis of the mice from the tr-IgH_CA&BMI1 group is summarised in table 3.4.

3.4 Summary of the characteristics of the 8 leukemic mice of the tr-IgH-CA&BMI1 group

Mouse ID	**Latency** (Days post transplantation)	**Clinical features**	**YFP+ cells** (Measured when the mouse was sacrificed)	**Diagnosis**	**Histology**
5625A#1	101	Splenomegaly, weight 650mg Leukocytosis	PB-21% BM-36% Spl-44%	Myeloid Leukemia	Immature cells in PB, BM, Spl cytospins
5625A#2	197	Splenomegaly, weight 330 mg Leukocytosis	PB-30% BM-86% Spl-52%	Myeloid leukemia	Presence of immature cells in PB, BM, Spl cytospins
5625B#1	123	Slightly bigger spleen, weight 175 mg Leukocytosis	PB- 0% BM-3% Spl-23%	not diagnosed	few immature blastic cells in the PB smear
5625B#2	101	Small spleen, weight 90 mg, Leukocytosis	PB- 0% BM-3% Spl-3%	T-ALL	Many blasts in the peripheral blood
5619A#1	219	Splenomegaly, spleen weight 515 mg, Leukocytosis	PB-1% BM-18% Spl-14%	T-ALL	Presence of lymphoblasts, in peripheral blood all the cells were CD4 and CD8 double positive.
5619A#2	246	Normal spleen, weight 124 mg WBC count normal	PB-9% BM-4% Spl-17%	Myeloid leukemia	A few immature blastic cells in the peripheral blood.
5619B#1	341	Splenomegaly, weight 771 mg Leukocytosis	PB-42% BM-43% Spl-23%	Myeloid leukemia	Not determined
5619B#2	461	Slightly bigger spleen, weight 168 mg, Leukocytosis	PB-32% BM-12% Spl-28%	Myeloid leukemia	Immature myeloid cells in the peripheral blood smear and bone

					marrow cytospin

Leukocytosis

Post mortem analysis revealed that 7 out of 8 leukemic mice of the tr-IgH-CA&BMI1 group had a high number of WBC in the peripheral blood when they were sacrificed. One mouse had a WBC count of 6 million/mL, which is in the normal range. For the comparison we have used the WBC count of 5 wild type FVB mice as control.

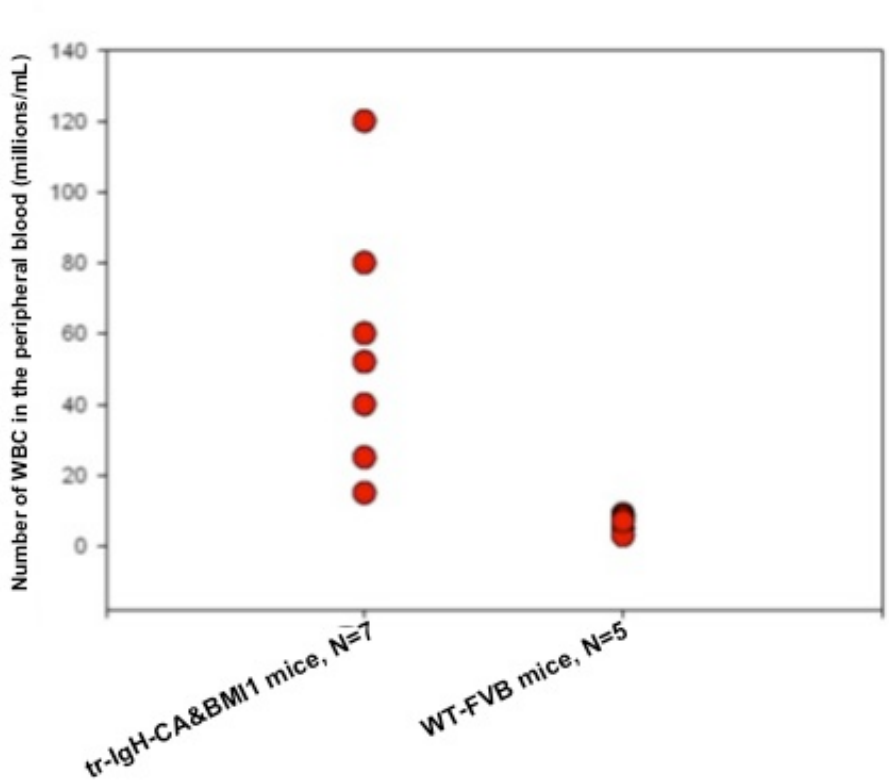

Fig 3.59: Distribution of the WBC counts in the 7 leukemic mice from the tr-IgH-CA&BMI1 group at the time of diagnosis and wild type FVB controls

Splenomegaly:

The average weight of a normal sized spleen from wild type FVB mouse is 120 mg. 4 out of 8 leukemic mice from the tr-IgH-CA&BMI1 group had very large spleens weighing from 330 mg to 770 mg. Among the other 4 leukemic mice, spleen was slightly enlarged (168 mg, 175 mg) in 2 cases. One leukemic mouse had a normal-sized spleen weighing 124 mg. Another mouse with T-ALL had a very small spleen of 90 mg.

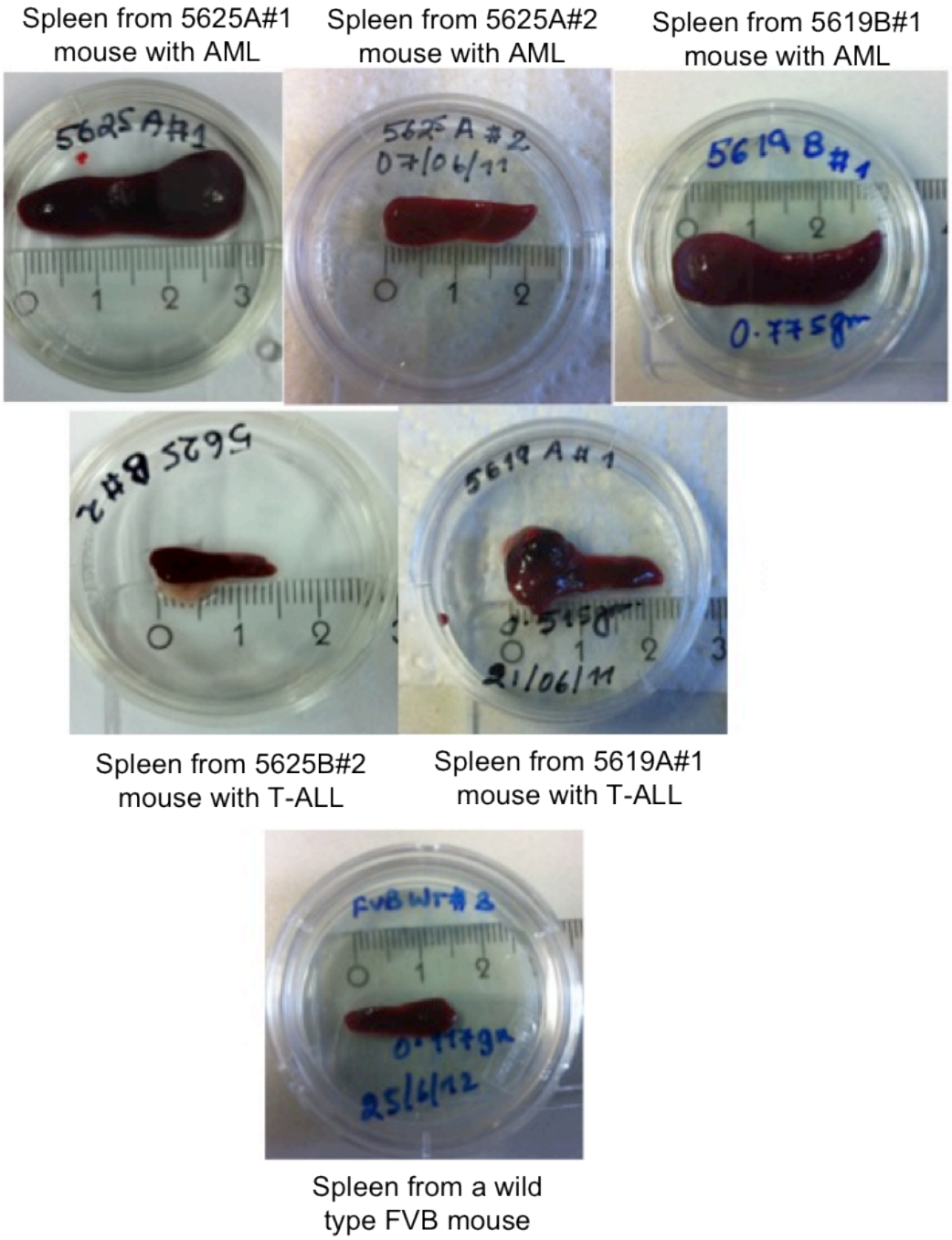

Fig 3.60: Spleens from leukemic mice from the tr-IgH-CA&BMI1 group and healthy mice.

3.2.1.5 Engraftment of the leukemic mice from the tr-IgH-CA&BMI1 group

After sacrificing the leukemic animals, cells from the peripheral blood, bone marrow and spleen were extracted and the percentage of YFP-positive cells was determined. The percentage of YFP-

positive cells in the PB, BM and spleen of the leukemic mice is shown in figure 3.61.

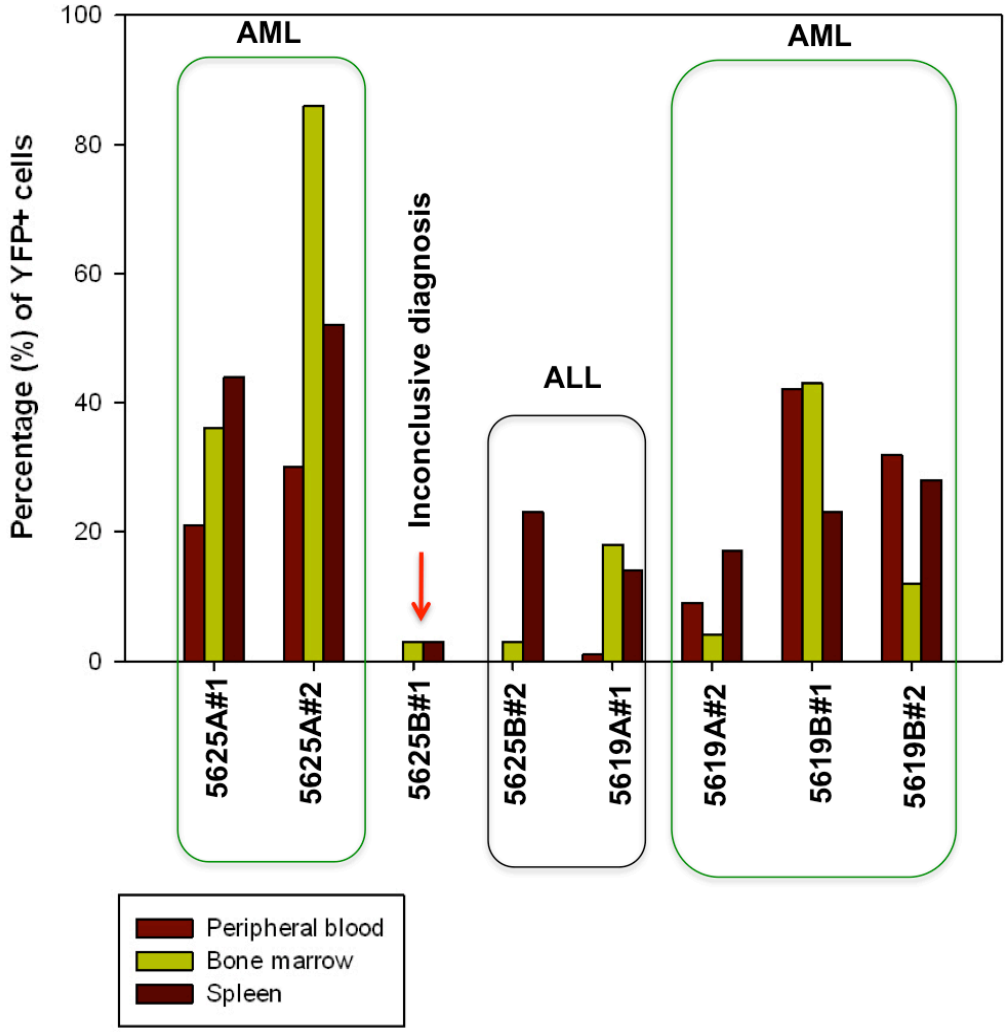

Fig 3.61: The percentage of YFP-posistive cells in the peripheral blood, bone marrow and spleen of the leukemic mice from the tr-IgH-CA&BMI1 group. The animals are marked with the type of leukemia they developed. In mouse 5625B#1 and 5625B#2 there were no YFP-positive cells in the peripheral blood.

3.2.1.6 Presence of the blast cells in the peripheral blood of the leukemic mice from the tr-IgH-CA&BMI1 group

We observed immature cells with big nuclei and bluish cytoplasm in the peripheral blood smear from the leukemic mice.

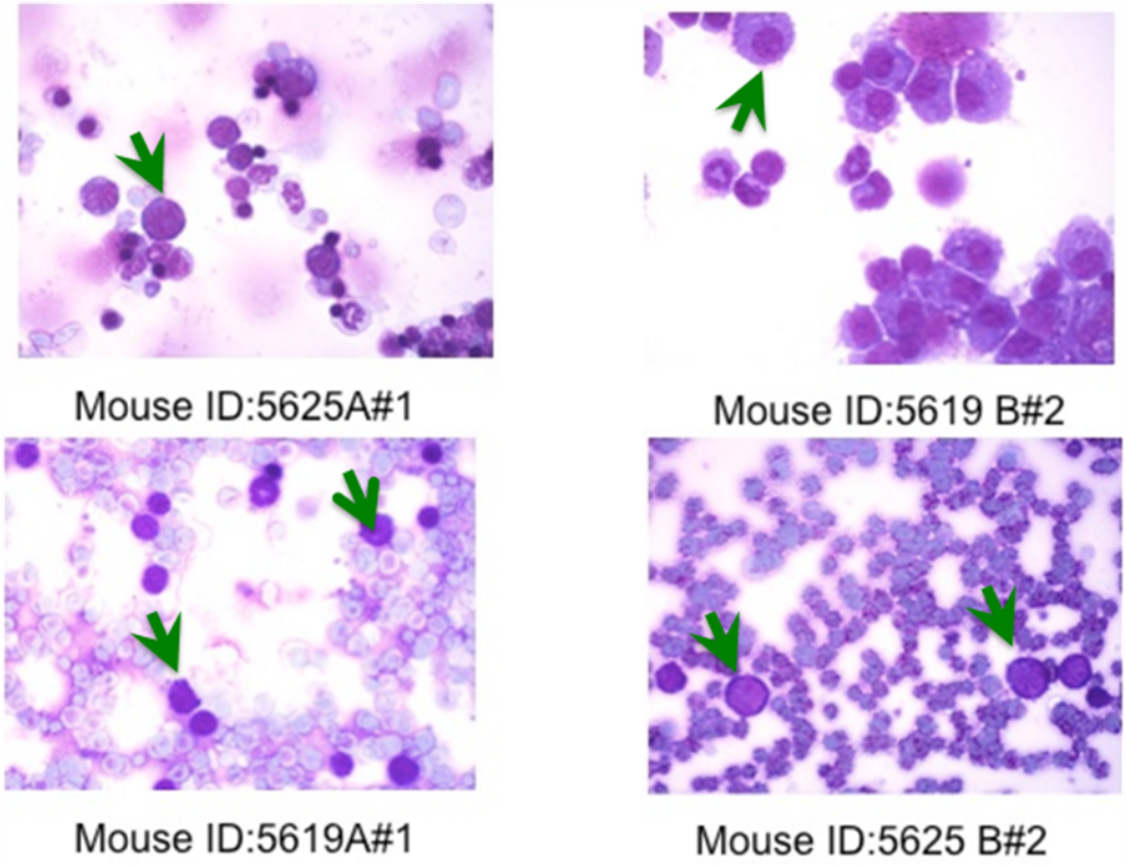

Fig 3.62: Giemsa-May-Grünwald stained peripheral blood smear of leukemic mice from the tr-IgH CA&BMI1 group. Peripheral blood smears from 4 leukemic mice are shown in the figure **(**Magnification 63x). The cells marked with green arrows are blasts. In mouse 5619B#2, many myeloblasts are visible, whereas in mouse 5625B#2, immature lymphoblasts can be seen.

3.2.1.7 Flow cytometric analysis of cells from the hematopoietic organs of the leukemic mice from the tr-IgH-CA&BMI1 group

The peripheral blood, bone marrow and spleen cells from the leukemic mice were analysed by flow cytometry. On the basis of the immunophenotype the mice were divided into two groups: one group consisting of two mice showing signs of acute T cell leukemia. In the other group consisting of 6 mice, 5 animals showed marked splenomegaly, high white blood cell count and a disturbed haematopoietic differentiation with a myeloid bias compatible with the diagnosis of AML. 1 mouse had a spleen slightly bigger than normal and many immature cells in the peripheral blood smear and bone marrow cytospins.

In the following figures, the immunophenotype of the hematopoietic tissues of the leukemic animals from the two groups are compared

with the wild type controls. Cells from the peripheral blood, bone marrow and spleen from the leukemic and wild type control mice were analyzed by flow cytometry using the following antibody combinations: Gr1-PE/Mac1-APC; Sca1-PE/cKit-APC; B220-PE/Ter119-APC; CD4-PE/CD8-APC (PE: phycoerithrin; APC: Allophycocyanin). The percentage of cells positive for each epitope from the individual leukemic animals is summarized in appendix table A8

3.2.1.8 Immunophenotype of the two mice from the tr-IgH-CA&BMI1 group that developed T-ALL

Two mice from the tr-IgH-CA&BMI1 group (5619A#1 and 5625B#2) developed signs and symptoms of T-ALL. The immunophenotype of the cells from blood, bone marrow and spleen are summarized and are graphically represented in the following figures. For comparison we show the immunophenotype of the hematopoietic cells from wild type FVB mice.

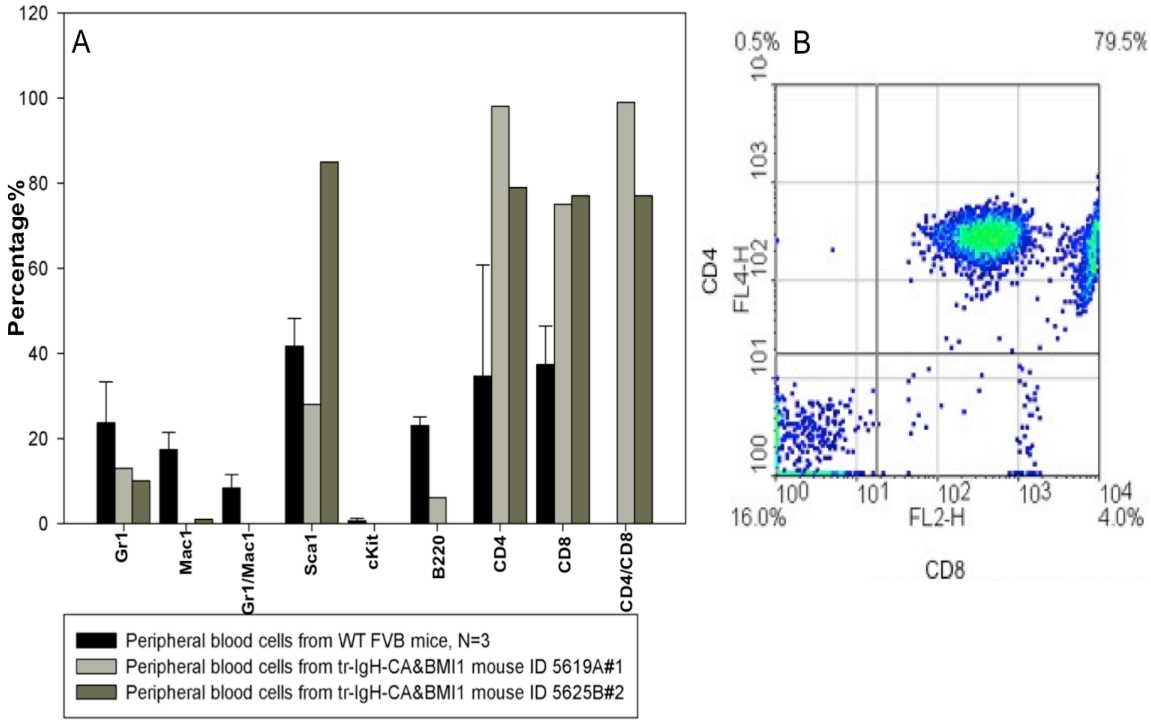

Fig 3.63: Immunophenotype of the peripheral blood cells from two mice of the tr-IgH-CA&BMI1 group that developed T-ALL and the wild type controls. A. Summary of the cell surface markers from leukemic blood and control blood; B. Flow cytometry of PB cells using CD4 and CD8 antibodies. In figure A the expansion of the lymphoid compartment (CD4+/CD8+ cells) is clearly visible. Most of the cells belong to an abnormal population of CD4/CD8 double positive cells. The myeloid compartment is considerably reduced. In figure B the scatter plot from the peripheral blood cells from mouse 5619A#1 shows that almost 80% of the PB cells are CD4 and CD8 double positive.

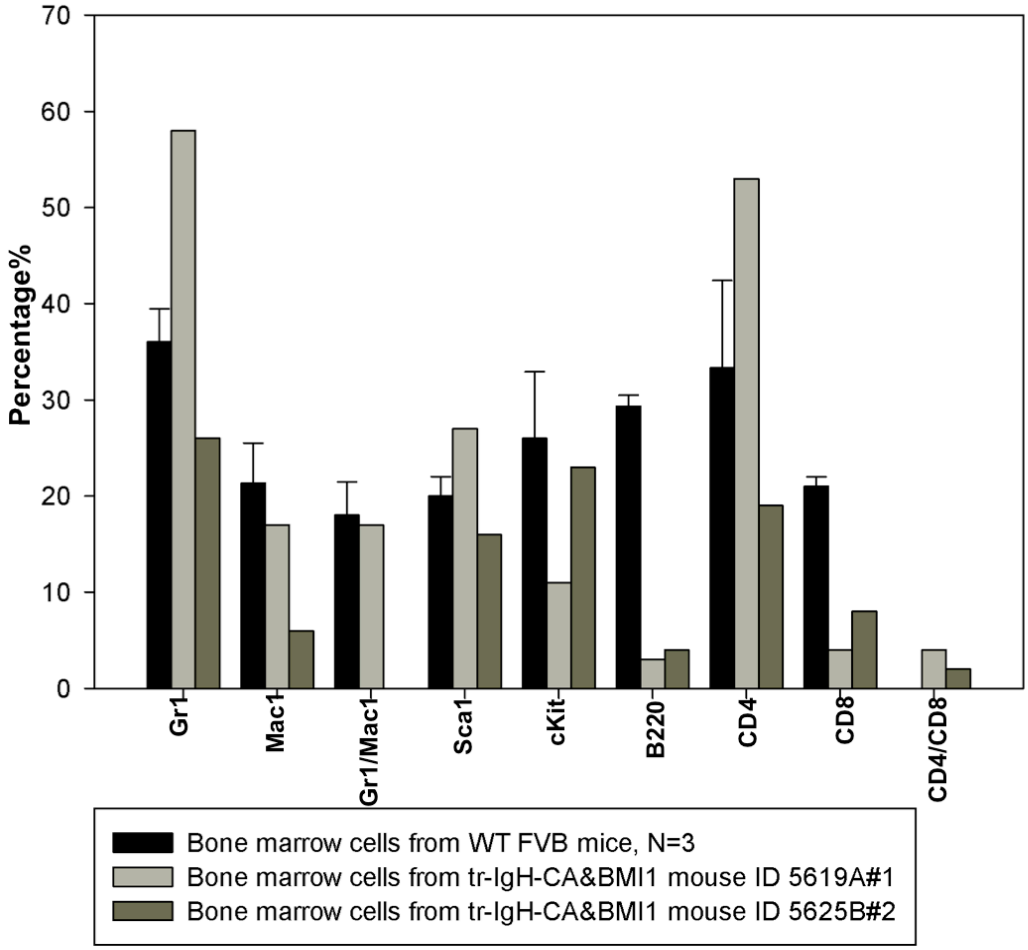

Fig 3.64: Immunophenotype of the bone marrow cells from the two mice from the tr-IgH-CA&BMI1 group with T-ALL. In the bone marrow a few CD4/CD8 double positive cells are detected. In mouse 5619A#1 number of Gr1+ cells and CD4+ cells are very high. In mouse 5625B#2 we could not detect any myeloid marker Mac1 positive cell. Number of B220 positive cells is significantly reduced in both leukemic mice.

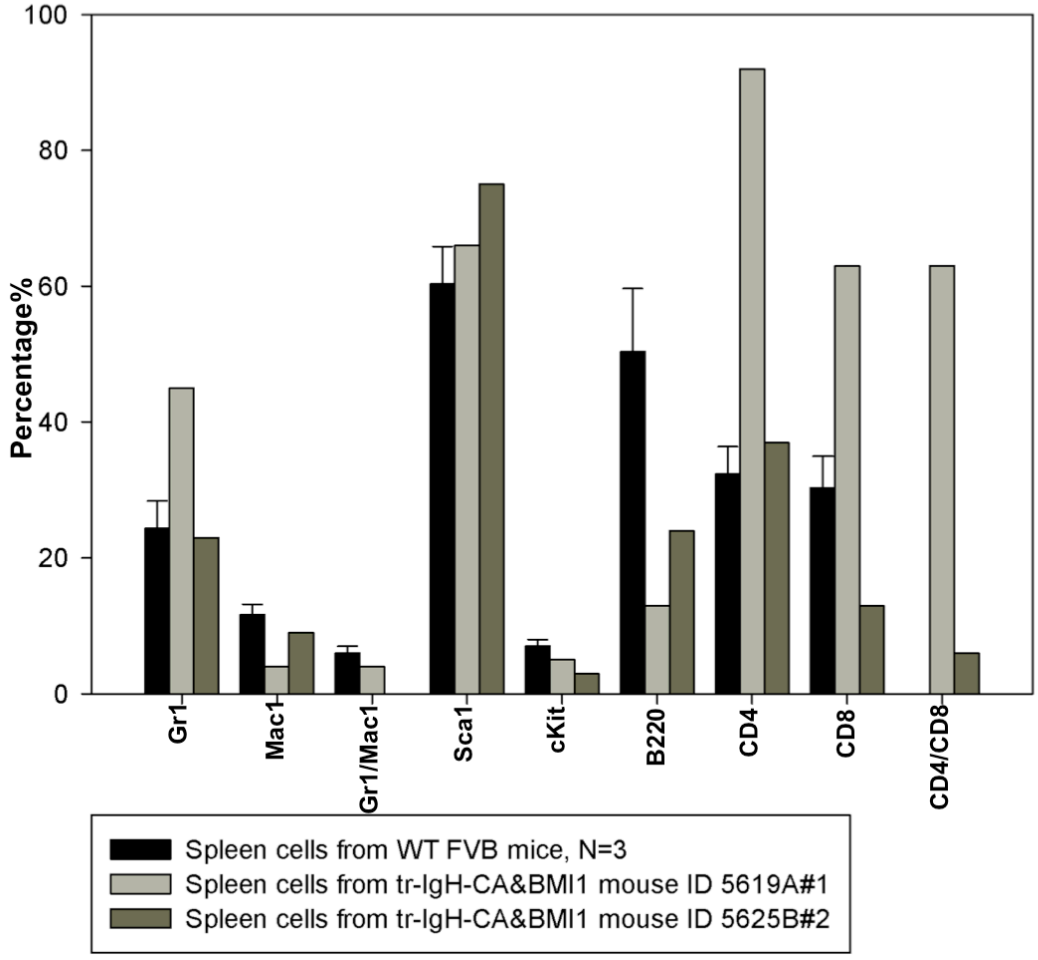

Fig 3.65: Immunophenotype of the spleen cells from the two mice from the tr-IgH-CA&BMI1 group with T-ALL. In both leukemic mice, the number of B220+ B cells is reduced significantly. The number of CD4+ and CD8+ T cells is very high in mouse 5619A#1. The abnormal population of immature T cells staining both for CD4 and CD8 is also present in both leukemic mice.

3.2.1.9 Immunophenotype of tr-IgH-CA&BMI1 mice with AML

6 mice from tr-IgH-CA&BMI1 group developed AML. The immunophenotype of the hematopietic cells are summarized in the following figures. The immunophenotype of the hematopoietic cells from 3 wild type FVB mice are used as controls. In the following figures 3.66, 3.67 and 3.68, the immunophenotype of the tr-IgH-CA&BMI1 mice with AML are summarized along with our standard controls.

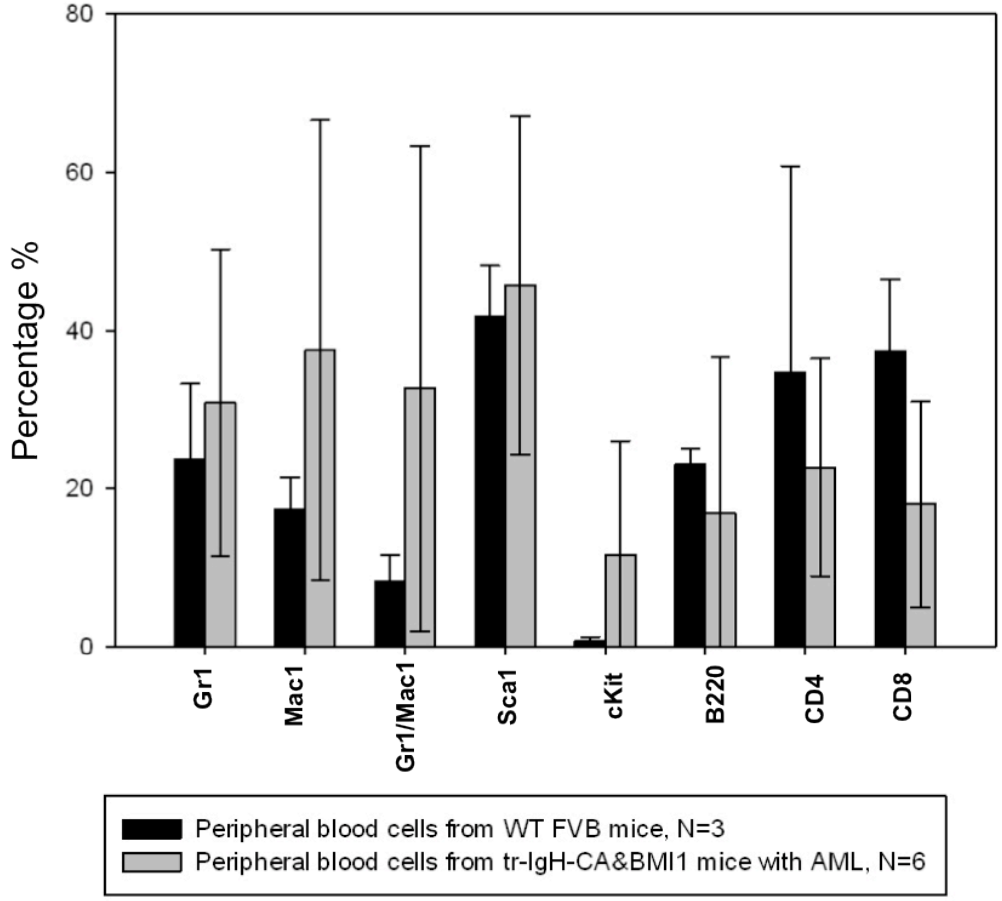

Fig 3.66: Immunophenotype of the peripheral blood cells from the six mice from the tr-IgH-CA&BMI1 group with AML.. The myeloid compartment is expanded and the percentage of cKit-positive immature cells is higher in the peripheral blood of the leukemic mice than in the wild type mice.

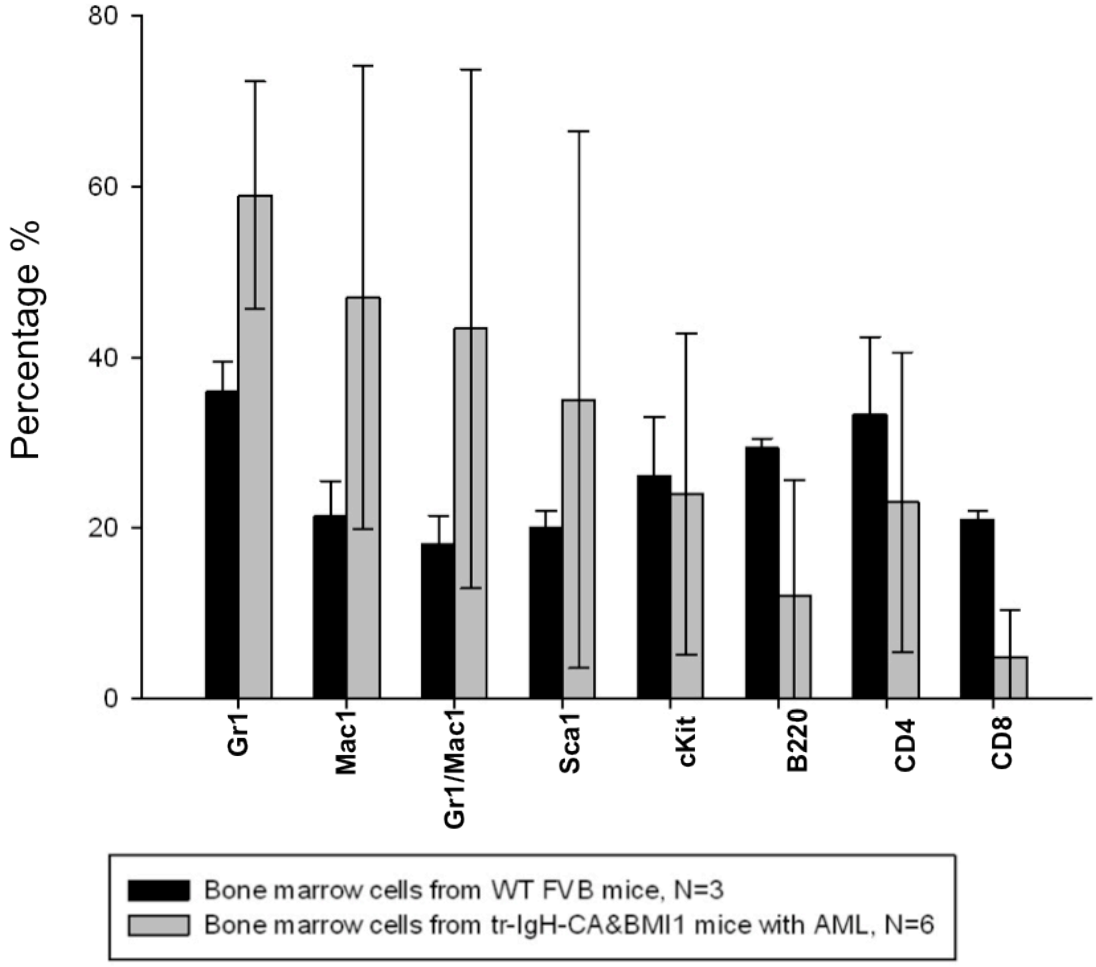

Fig 3.67: Immunophenotype of the bone marrow cells from the six mice from the tr-IgH-CA&BMI1 group with AML. The myeloid compartment in the bone marrow is greatly expanded in the leukemic mice compared to the wild type animals.

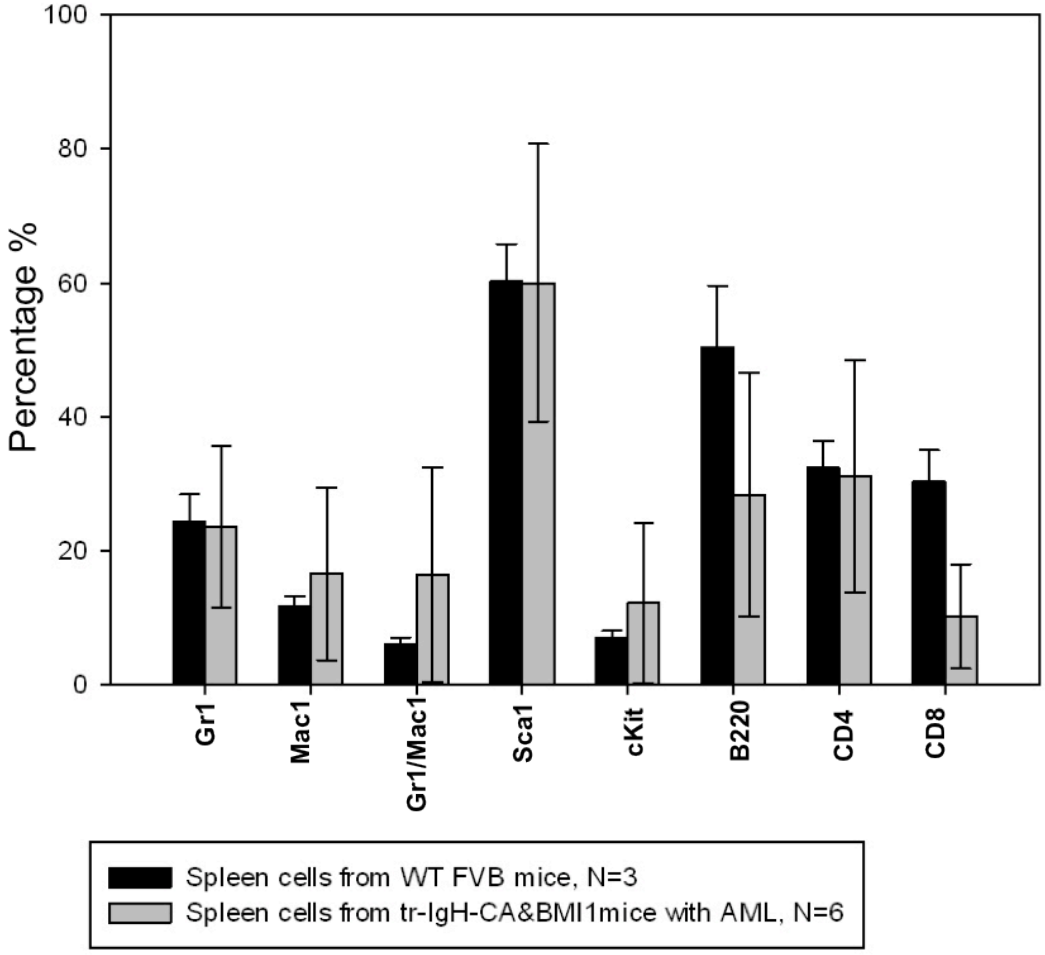

Fig 3.68: Immunophenotype of the spleen cells from the six mice from the tr-IgH-CA&BMI1 group with AML. There is some expansion of the myeloid compartement in the spleen as well.

In the peripheral blood and the bone marrow cells of the 6 mice with AML, a significant exapansion of the myeloid compartment is seen. The percentage of Gr1 and Mac1 positive cells is significantly higher in both the PB and BM.

3.2.1.10 Secondary Transplantation

The unsorted leukemic bone marrow cells from 3 sick mice (2 mice with AML, 1 mouse with T-ALL) were retransplanted into one irradiated and one non-irradiated wild type FVB recipient mouse for each leukemia sample. For the transplantation, $1x10^6$ cells from the leukemic bone marrow sample and $2x10^6$ normal bone marrow cells were injected into the tail vein.

Leukemic cells from the AML mice 5619B#1 and 5625A#1 generated acute myeloid leukemia within 20 days post transplantation proving that the leukemic cells observed in these two mice were indeed leukemic cells and capable of propagating the leukemic phenotype.

However, the leukemic cells from mouse 5625B#2, which had developed ALL, did not lead to leukemia development in two transplanted animals even after a long observation period of 1 year.

The Kaplan-Meier survival plot in figure 3.69 gives an overview of the survival of the mice in all four groups as well as the 6 animals of the secondary transplantation group. The actual days of survival of individual animals from each experimental group have been summarized in table A9 in the appendix.

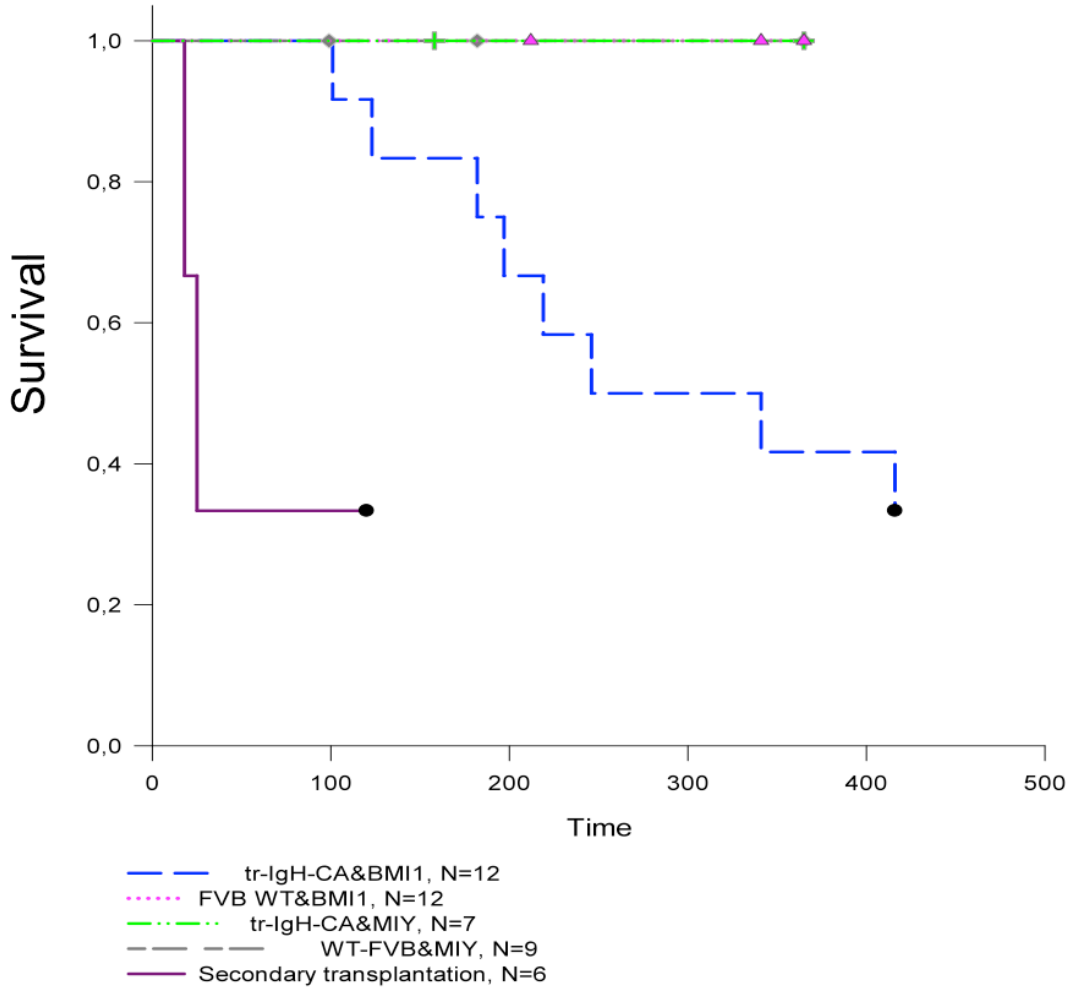

Fig 3.69: Kaplan-Meier survival plot for FVB mice transplanted with pMIY-BMI1 or pMIY transduced IgH-CALM/AF10 transgenic or wild type bone marrow cells. The survival plot of the following 5 groups is shown in the plot. The observation period was 400 days and only the mice, which died of leukemia, are counted here. The non-leukemic animals we lost due to some other reason during our observation periods are censored. 4 mice from the tr-IgH-CA&BMI1 group were alive after 400 days.

Twelve mice transplanted with bone marrow cells from transgenic IgH-CALM/AF10 mice transduced with pMIY-BMI1. This group is labeled as “tr-IgH-CA&BMI1” (n=12). Eight mice developed leukemia (6 AML and 2 T-ALL), four mice were still alive after 400 days.

Seven mice transplanted with bone marrow cells from transgenic IgH-CALM/AF10 mice transduced with pMIY. This group is labeled as “tr-IgH-CA&MIY” (n=7). None of these animals developed leukemia. After 158 days post-transplantation 1 tr-IgH-CA&MIY mouse had to be sacrificed because of infectious wound caused by fighting with another mouse. This animal was censored.

Twelve mice transplanted with bone marrow cells from wild type FVB mice transduced with pMIY-BMI1. This group is labeled as “WT-FVB&BMI1” (n=12). None of these animals developed leukemia. 341 days post-transplantation, one WT-

FVB&BMI1 mouse was sacrificed. A post mortem analysis revealed no leukemia. This animal has been censored.

Nine mice transplanted with bone marrow cells from wild type FVB mice transduced with pMIY. This group is labeled as “WT-FVB&MIY” (n=9). None of these animals developed leukemia. Two animals from this group were sacrificed 182 days after transplantation because of severe injuries due to fighting with each other. These two animals were also censored.

Six mice transplanted with the leukemic cells from the bone marrow of mice from the tr-IgH-CA&BMI1 group. Labeled as “secondary transplants”. Note that in this group only the four animals transplanted with cells from two mice with AML developed leukemia after a short latency of 20 days.

3.2.2. Approach 2: Co-transduction of wild type bone marrow cells with the CALM/AF10-minimal fusion gene and *BMI1* and transplantation into FVB wild type recipients

In our second approach to test the cooperation of CALM/AF10 with BMI1, bone marrow cells from wild type FVB mice were retrovirally co-transduced with two retroviruses, one expressing BMI1 and the other expressing the CALM/AF10 minimal fusion protein (MF). The successfully co-transduced bone marrow cells were transplanted into wild type FVB recipient mice. The workflow is shown in figure 3.70:

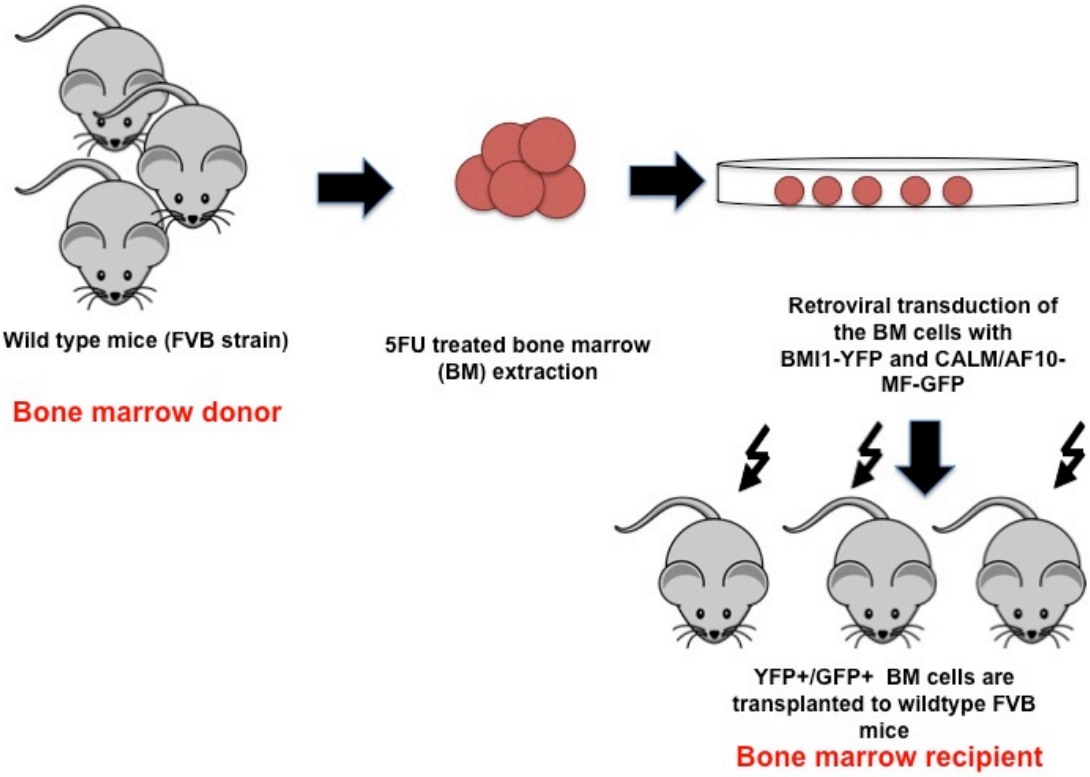

Fig 3.70: Schematic workflow of the transplantation model 2.

3.2.2.1 The CALM/AF10 minimal fusion

Deshpande *et al.* described a deletion mutant of the CALM/AF10 fusion, which contains only the 248 C terminal amino acids of the CALM protein (aa 400-648) including the clathrin binding domain, and a small portion of AF10 including the octapeptide motif/leucine zipper (OM/LZ) domain of AF10 (aa 677 to 758) *(*Deshpande *et al., 2011)* (Figure 3.71). This so-called CALM/AF10-minimal fusion (CA-MF) was very active in transforming bone marrow cells *in vitro.* In contrast to the full length CALM/AF10 fusion, CA-MF led to the development of blast colonies in CFC assays. In the bone marrow transplantation model, expression of CA-MF resulted in the

development of an aggressive AML with a similar latency and phenotype as the full-length CALM/AF10 fusion.

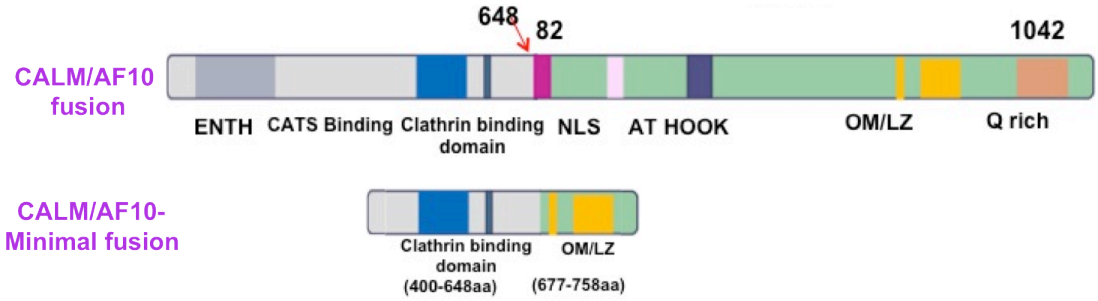

Fig 3.71: Diagrams of the CALM/AF10 full length and the CALM/AF10 minimal fusion proteins. ENTH: Epsin N-terminal homology domain; NES: nuclear export signal; OM/LZ: octapeptide motif/leucine zipper; PHD: plant homeo domain; NLS: nuclear localization signal; CATS: CALM/AF10 interacting protein expressed in thymus and spleen

The CA-MF fusion gene was used for our second approach. A stable retroviral producer cell line expressing the CA-MF, E86-CA-MF-GFP, had been established by Aniruddha Deshpande (Deshpande et al., 2011) and was used in our experiments.

3.2.2.2 Experimental procedure

To co-express CA-MF and BMI1 in bone marrow cells, we used two retrovirus producer cell lines: E86-CA-MF-GFP and E86-BMI1-YFP. These two virus producing cell lines were irradiated, mixed together according to their viral titres and cultured together. The bone marrow cells from the wild type FVB donor mice were collected and transduced using the cell mixture. Successfully co-transduced cells were positive for both YFP and GFP and were sorted using flow cytometry. $0.5x10^6$ YFP+/GFP+ cells and $0.5x10^6$ mock YFP/GFP negative cells were injected into irradiated syngenic wild type recipients. As controls, bone marrow cells transduced with only BMI1, only CALM/AF10-MF and the empty vetors were also transplanted into wild type recipients. Below is an overview of the transplantations performed.

3.5 Summary of the BMT model to transplantation experiment

Number of mice transplanted with different constructs is summarized in this table. The first column includes the different experimental groups, in the second column the constructs used for each group of experiment are listed. The third column contains the number of transplanted animals for each group of experiments. All the donors and recipients were wilt type FVB mice.

Experimental group	Construct	Number of transplants
CA-MF&BMI1	pMIY-BMI1 & pMIG-CA-MF	10
BMI1	pMIY-BMI1	12
CA-MF	pMIG-CA-MF	10
MIY	pMIY	9

3.2.2.3 Bone marrow cells transduced with pMIG-CA-MF show high engraftment potential

We transplanted the recipient mice with equal numbers of transduced and mock-transduced cells ($0.5x10^6$ cells). Mock-transduced cells are cells that had undergone the same manipulations as the successfully transduced cells but had not taken up the retrovirus. 4 weeks after transplantation, we measured the percentage of YFP/GFP positive cells in the peripheral blood of the transplanted mice. The two groups transplanted with cells expression the CALM/AF10 minimal fusion (CA-MF and CA-MF&BMI1) had slighty higher percentages of YFP/GFP positive cells (78±16.47 and 79±9.29%, respectively) indicating a growth advantage for these cells and higher engraftment potential (Figure 3.72).

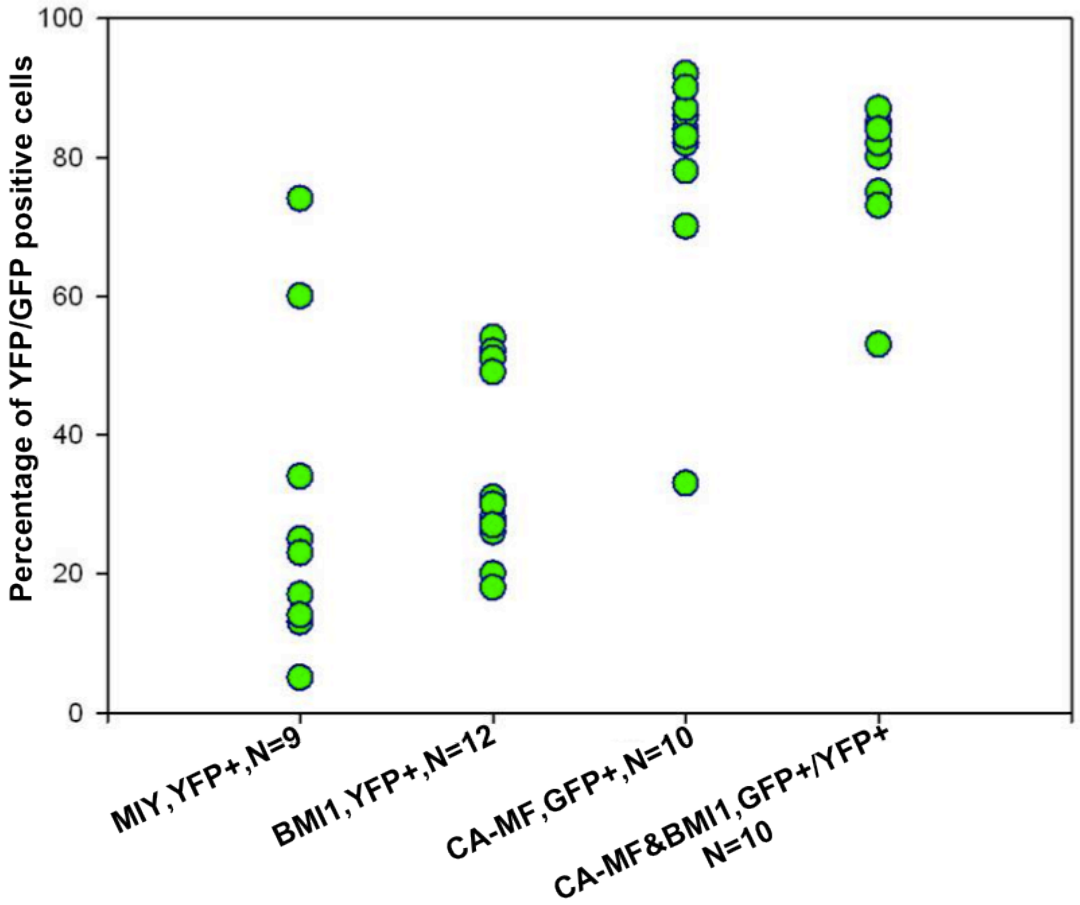

Fig 3.72: Engraftment as percentage of YFP/GFP positive cells the peripheral blood of the mice in the four experimental groups, 4 weeks after transplantation

3.2.2.4 The mice in the CA-MF and CA-MF&BMI1 groups develop an acute myeloid leukemia after a short latency

All the mice in the CA-MF and CA-MF&BMI1 groups developed an acute myeloid leukemia with a median latency of 101 and 110 days, respectively. In contrast, none of the mice from the BMI1 or MIY group developed leukemia after an observation period of more than 1 year (Figure 3.73). The leukemic cells from one leukemic mouse from the CA-MF&BMI1 group were transplanted into two secondary recipients. Only one of the recipients had received conditioning lethal irradiation before transplantation. Both secondarily transplanted animals developed AML with a short latency of 3 weeks.

There was no significant difference between the CA-MF and CA-MF&BMI1 groups in the latency time to leukemia development. The following Kaplan-Meier survival plot summarizes the results. The

percentage of survival of individual animals from each experimental group is summarized in the table A10 in the appendix.

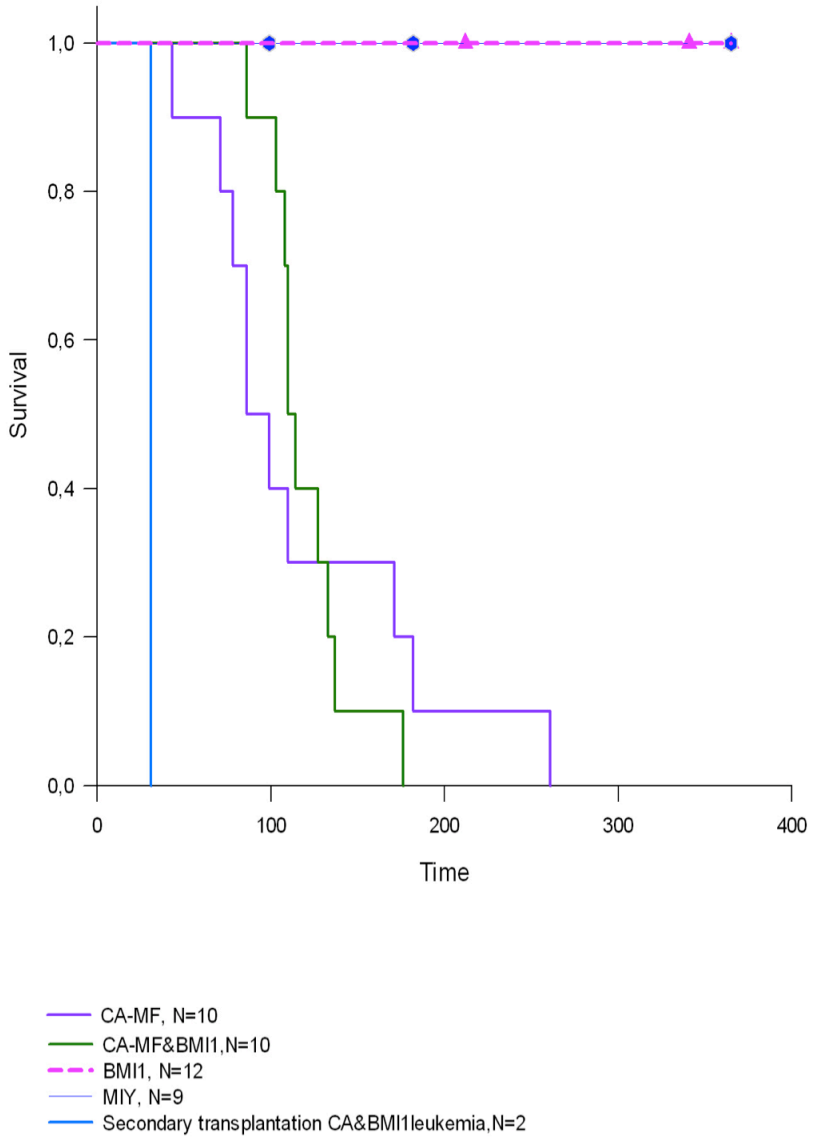

Fig 3.73: The Kaplan-meier survival curve for the CALM/AF10-MF+BMI1 mice along with the controls.

10 wild type FVB mice transplanted with CALM/AF10 minimal fusion developed leukemia with a median latency of 101 days post transplantation. This group is labeled as CA-MF.

10 wild type FVB mice transplanted with CALM/AF10-minimal fusion and BMI1 developed leukemia after a median latency of 110 days post transplantation. This group is labeled as CA-MF&BMI1.

12 wild type FVB mice were transplanted with BMI1 as control. None of them developed leukemia. The group is labeled as BMI1

9 wild type FVB mice were transplanted with empty vector MIY. None of them developed leukemia. The group is labeled as MIY. Note that two animals from this group were sacrificed 182 days after transplantation because of severe injuries due to fighting with each other. These two, non leukemic animals were censored.

3.2.2.5 All the leukemic mice show high WBC count and splenomegaly

All the leukemic mice from the CA-MF and the CA-MF&BMI1 group had splenomegaly and leukocytosis.

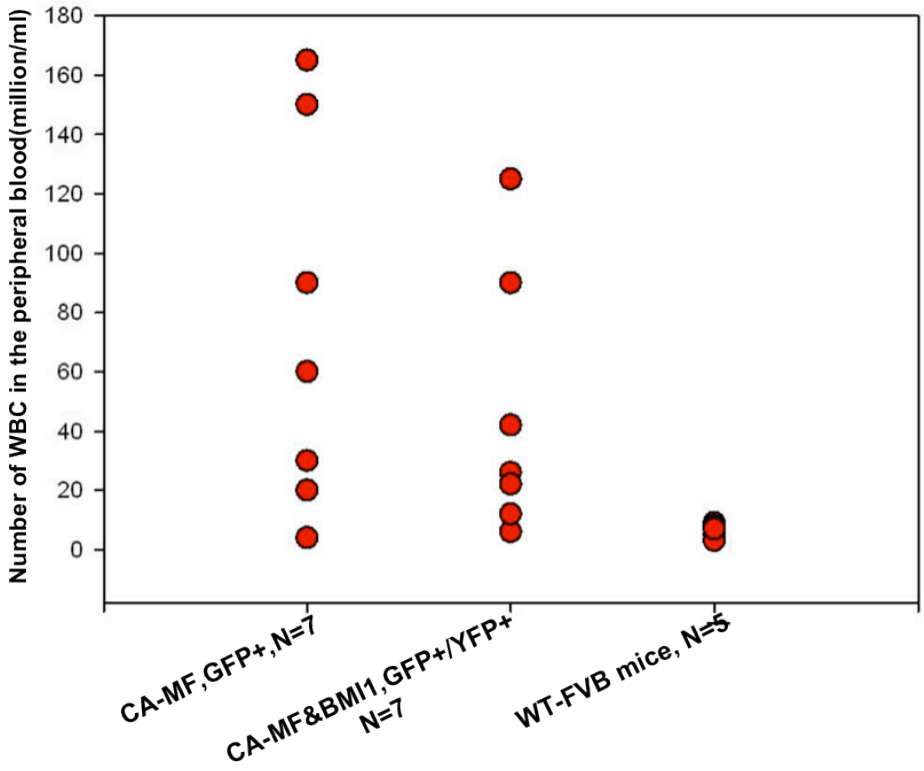

Fig 3.74: Leukocytosis in CA-MF and CA-MF&BMI1 mice. The number of WBC in the mice transplanted with BMI1 and MIY were monitored monthly till 16 weeks post transplantation and they did not show any abnormality. The WBC counts from these two groups of mice are not included in the figure above. The figure shows the WBC counts after the mice were sacrificed

3.2.2.6 All leukemic mice were highly engrafted

The leukemic mice from the CA-MF and CA-MF&BMI1 groups showed a high percentage of GFP single positive or GFP/YFP double positive cells in their peripheral blood, bone marrow and spleen. Figure 3.75 shows the average percentage of GFP positive cells from 5 leukemic mice from the CA-MF group and GFP/YFP double positive cells from 7 leukemic from the CA-MF&BMI1 group.

The average percentage of GFP positive cells in the peripheral blood of the leukemic mice from CA-MF group was 66% with a standard deviation of 22%. In the bone marrow, the percentage of GFP+ cells ranged from 36% to 73% with a standard deviation of 14%. In the spleen the average number of GFP positive cells was 30% with a standard deviation of 13.4%. In the CA-MF&BMI1 group, the average number of GFP/YFP double positive cells in the peripheral blood, bone marrow and spleen was 62% with standard deviation of 16.8%, 59% with a standard deviation of 23% and 49% with a standard deviation of 17%, respectively. The actual values of GFP positive and GFP/YFP double positive cells in different hematopoietic organs of individual leukemic animals from both groups (CA-MF and CA-MF&BMI1) are summarized in table A11 and A12 in the appendix.

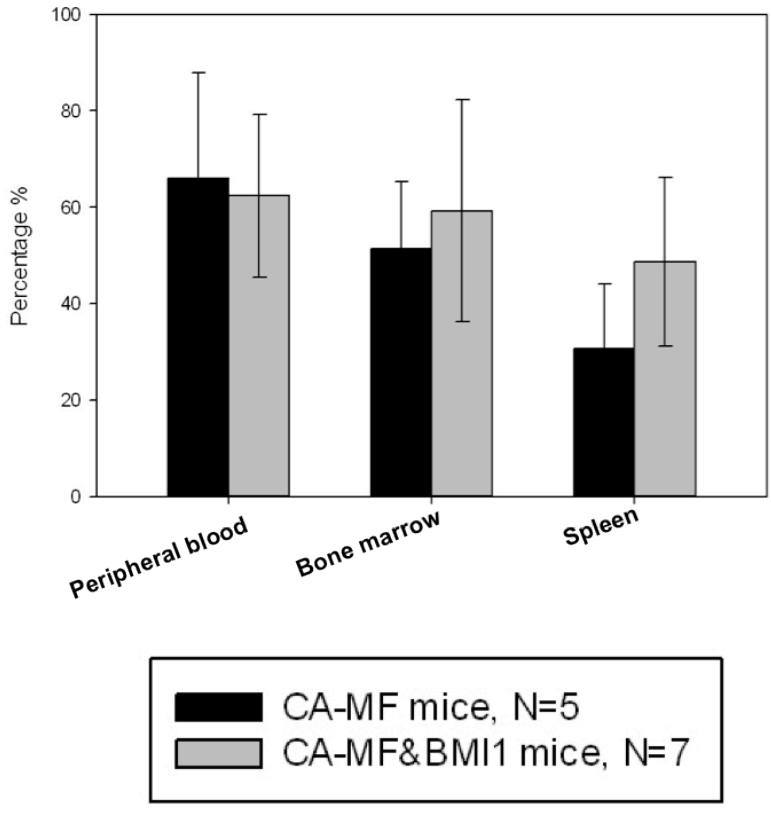

Fig 3.75: Percenatge of GFP+ cells in the peripheral blood, bone marrow and spleen of 5 leukemic mice from the experimental group CA-MF and the percentage of GFP/YFP double positive cells of 7 mice from the group CA-MF&BMI1

3.2.2.7 GFP+/YFP+ double positive leukemic cells infiltrate multiple organs in mice from the CA-MF&BMI1 group

To investigate if the transplanted cells contribute to the organ-infiltrating leukemic bulk, cells from different hematopoietic organs of the sick mice of the CA-MF&BMI1 group were prepared and characterized with flow cytometry. In all the leukemic CA-MF&BMI1 mice, the leukemic cells were GFP and YFP double positive. An example of the GFP-YFP scatter plots from the spleen cells from six CA-MF&BMI1 mice after they had developed leukemia and been sacrificed is shown in Fig. 3.76.

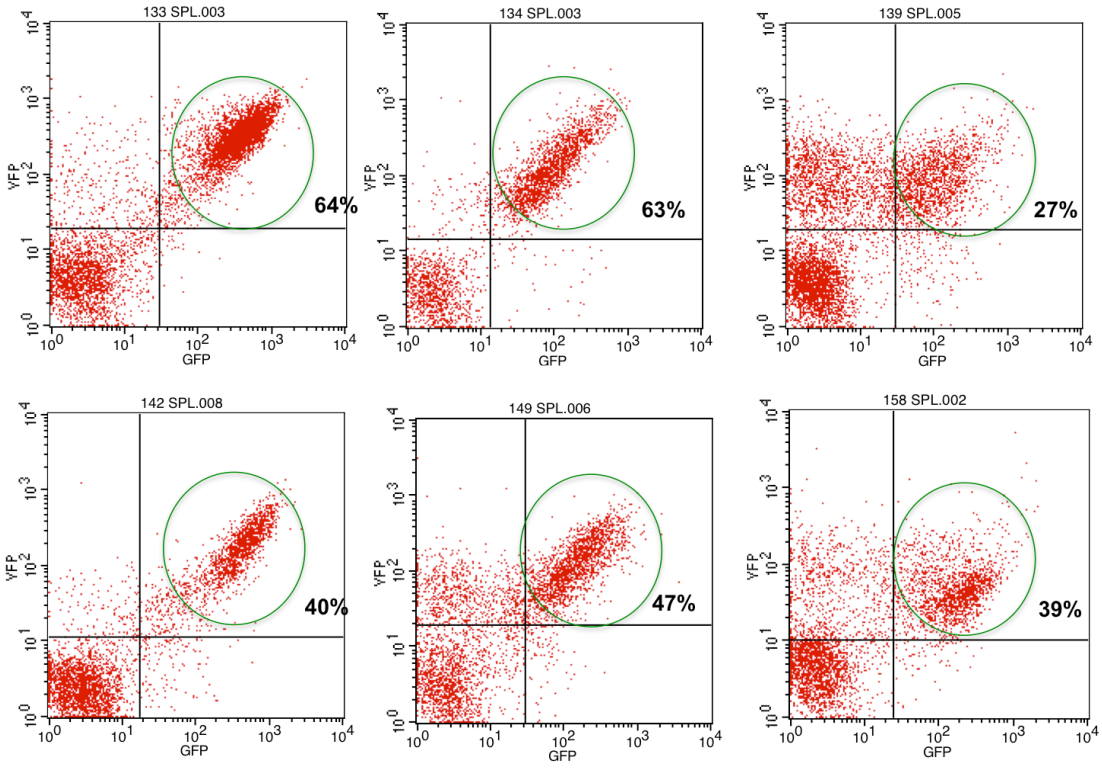

Fig 3.76: Leukemic cells from the spleen of 6 mice from the CA-MF&BMI1 group were positive for both GFP and YFP. The upper panel shows the GFP/YFP double positive population of the leukemic cells obtained from the spleens of three mice 5799A#133, 5799A#134,5807A#139. GFP/YFP double positive cells constituted 64%, 63% and 26% of all cells isolated from the spleens of these mice. In the lower panel spleen cells from mouse 5799B#142, 5807B#149 and 5788#158 are shown. Note that the GFP/YFP double positive cells constitute 40%, 45% and 39% of the total spleen cells of these three mice

3.2.2.8 Multiple organ infiltration with leukemic cells

Histological sections were prepared from formalin-fixed organs of the leukemic animals from the CA-MF&BMI1 group and analyzed by HE staining and immunohistochemitry. Leukemic blasts were found in several non-hematopoietic organs, like the liver and kidney (Figure 3.77)

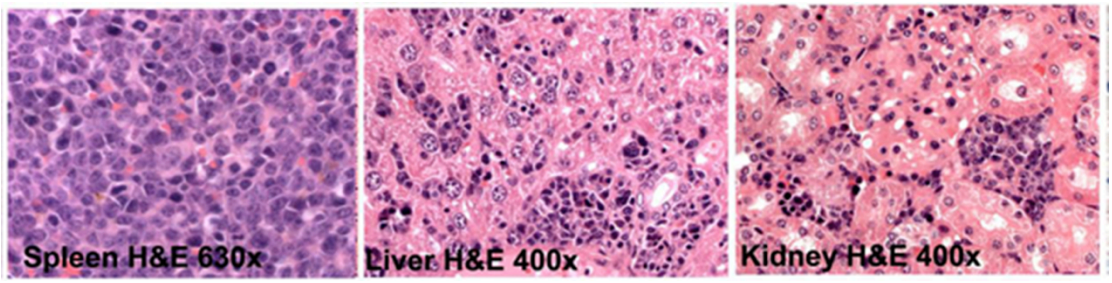

Fig 3.77: Infiltration of malignant cells in organ sections from mice of the CA-MF&BMI1 group. HE stained tissue sections of spleen, liver and kidney of a mouse with leukemia show diffuse and clustered infiltration of blast-like cells in these organs (arrows).

3.2.2.9 The infiltrating cells have myeloid characteristics

To characterize the infiltrating leukemic blasts, we performed immunohistochemistry. The infiltrating cells stained weakly positive for MPO (myeloperoxidase) and ASCDL (Chloracetate esterase) but negative for TdT. A few cells were B220 positive in the spleen, corresponding to residual B cells. These results show that the infiltrating cells are of myeloid origin and are compatible with the diagnosis of an acute myeloid leukemia in the mice of the CA-MF&BMI1 group (Figure 3.78).

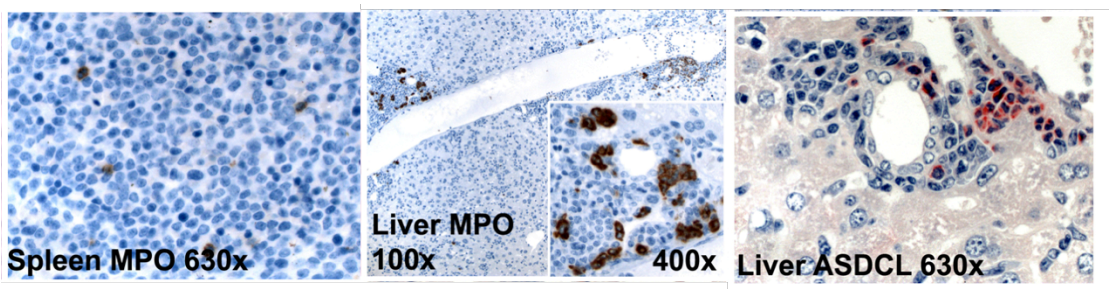

Fig 3.78: Immunohistochemistry of organ sections from leukemic mice using myeloid markers. Liver and spleen sections show weak positive staining of myeloperoxidase (MPO) and chloracetate esterase (ASDCL) staining.

3.2.2.10 The immunophenotypes of the leukemia from mice of the CA-MF&BMI1 and the CA-MF group are similar

Flow cytometric analysis of peripheral blood, bone marrow and spleen cells from leukmic animals from the CA-MF&BMI1 and the

CA-MF group with our standard panel of antibodies showed a significant increase in the percentage of cells staining positive for the myeloid maker Mac1 and a marked decrease in Sca1 positive cells and CD4 and CD8 positive T cells in comparison to cells from healthy wild type FVB mice. There was no significant difference in the immunophenotype of the leukemias from the 7 animals from the CA-MF&BMI1 group compared to the leukemias from the 5 animals from the CA-MF group (Figures 3.79-3.81). The individual values of surface marker positive cells from individual leukemic mice from both groups of mice are summarized in table A11 and A12 and A13 in the appendix.

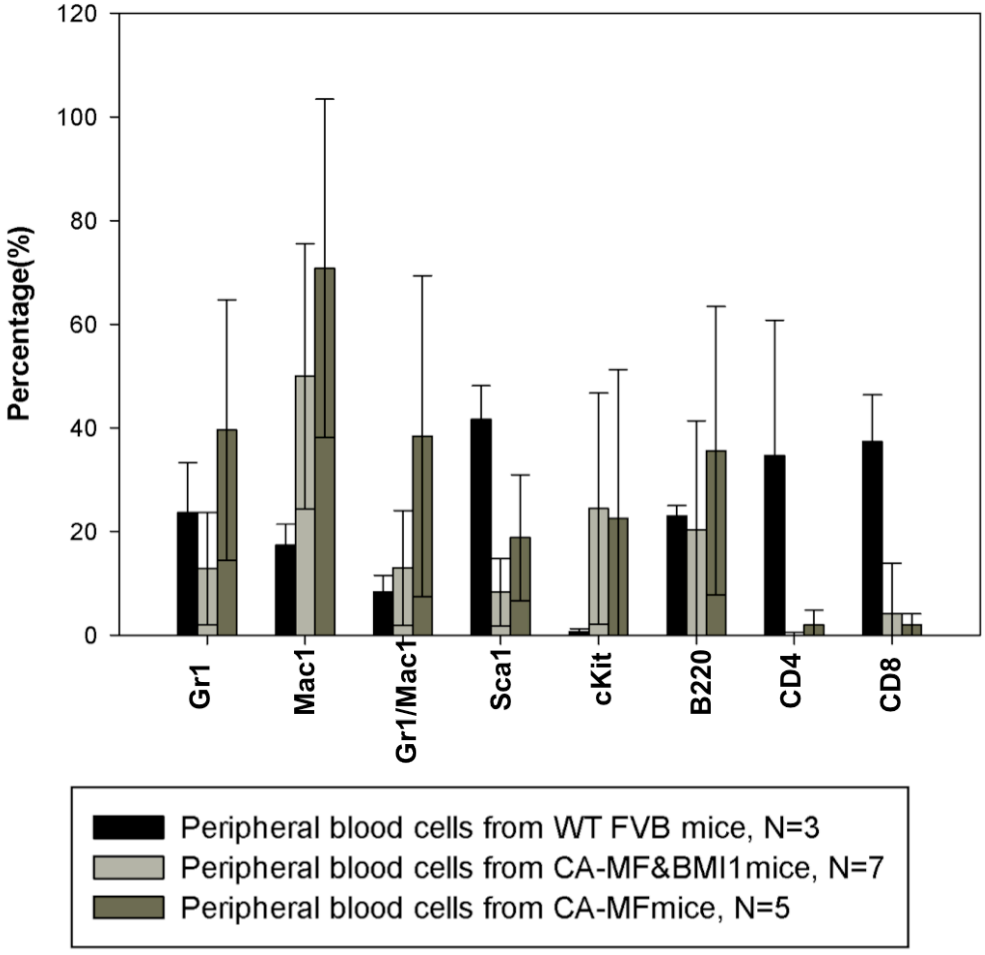

Fig 3.79: Immunophenotype of the peripheral blood mononuclear cells from 7 leukemic mice from the CA-MF&BMI1 group and 5 leukemic animals from the CA-MF group compared to cells from 3 wild type FVB, the common control used for all the analysis. The leukemic cells show a higher percentage of Mac1 positive cells as well as a significantly lower percentage of CD4 and CD8 positive T cells.

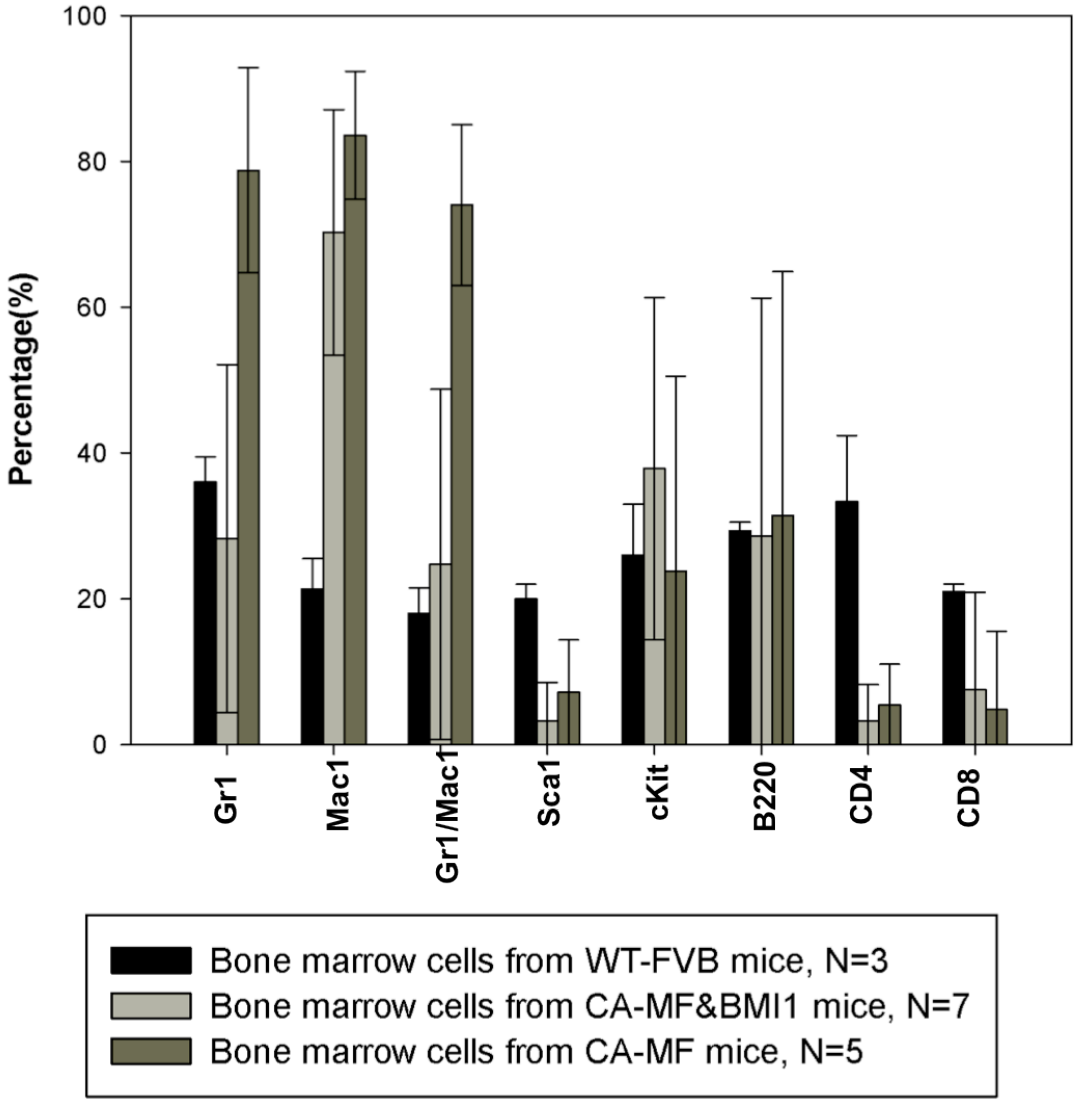

Fig 3.80: Immunophenotype of the bone marrow cells from 7 leukemic mice from the CA-MF&BMI1 group and 5 leukemic animals from the CA-MF group compared to cells from 3 wild type FVB mice. Similar to the peripheral blood, the leukemic cells show a higher percentage of Mac1 positive cells as well as fewer CD4 positive T cells.

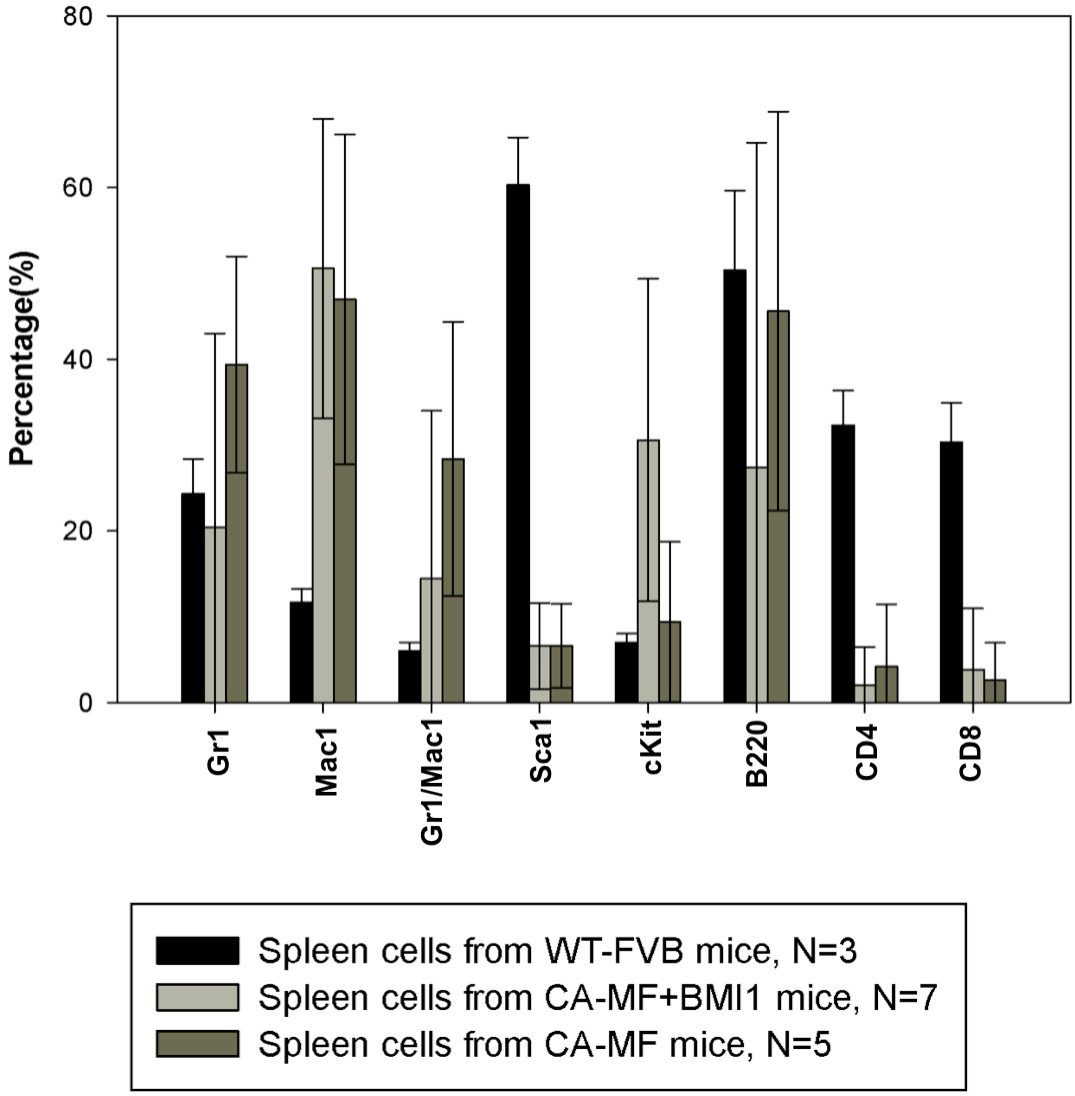

Fig 3.81: Immunophenotype of the spleen cells from 7 leukemic mice from the CA-MF&BMI1 group and 5 leukemic animals from the CA-MF group compared to cells from 3 wild type FVB mice. The leukemic cells show a higher percentage of Mac1 positive cells as well as significantly lower percentages of Sca1, CD4 and CD8 positive cells.

4. Discussion

4.1 A knock-in mouse model with tissue-specific expression of the CALM/AF10 fusion gene

The CALM/AF10 fusion gene is the result of a rare but recurring t(10;11)(p13;q14) translocation, which is found in different hematologic malignancies including T-ALL, AML, and lymphoma. The CALM/AF10 fusion gene is able to cause leukemia in a murine retroviral bone marrow transplantation model (Deshpande et al., 2006) and in a transgenic mouse model (Caudell et al., 2007). However, while the penetrance of CALM/AF10 driven leukemias is 100% in the bone marrow transplantation model with a short median latency of just 10 weeks (Deshpande et al., 2006), only 40% of the transgenic mice with a Vav promoter-driven CALM/AF10 transgene developed leukemia with a long latency of 12 months (Caudell et al., 2007). In our group, we generated CALM/AF10 transgenic mice, in which the CALM/AF10 transgene is driven by the immunoglobulin heavy chain enhancer/promoter or by the proximal Lck promoter(Krause, 2006).None of our transgenic CALM/AF10 mice developed leukemia. These models show that the expression of CALM/AF10 alone is not sufficient to cause leukemia. CALM/AF10 most likely needs to be expressed in the proper cellular context and requires additional, collaborating events to initiate leukemia development. By generating a knock-in mouse model with tissue-specific expression of the CALM/AF10 fusion, we aimed to identify the cell type most susceptible to CALM/AF10-mediated leukemia development. The Rosa26loxP-stop-loxP-CALM/AF10 (R26LSLCA) knock-in mouse line allows the tissue specific expression of the fusion protein after Cre-mediated excision of the transcriptional stop cassette from the Rosa26loxP-stop-loxP-CALM/AF10 knock-in allele. By using mouse strains, which express the Cre recombinase in different tissues, we were able to control the expression of CALM/AF10 in a tissue-specific fashion. To express the fusion gene in all hematopoietic cells, the R26LSLCA mice were crossed with Vav-Cre mice. In addition, we performed crosses with the Mb1-Cre and CD19-Cre strains to restrict the expression of CALM/AF10 to the B cell compartment starting from early B lymphoid progenitors.

4.2 A CALM/AF10 knock-in model with expression of CALM/AF10 in Vav-expressing cells leads to the development of AML with 100% penetrance.

Vav-Cre mediated excision of the stop cassette and the subsequent expression of CALM/AF10 in all hematopoietic cells resulted in the development of an acute myeloid leukemia in all mice with an

average latency of 1 year. The leukemic mice had splenomegaly, anemia and leukocytosis. Tissue sections of the leukemic mice revealed massive infiltration of MPO-positive myeloid blast in different hematopoietic and non-hematopoietic organs. The myeloid nature of the leukemia was also confirmed by flow cytometry. The leukemic cells in the peripheral blood, bone marrow and spleen of the leukemic CA+/Vav-Cre+ mice were positive for the myeloid markers Gr1 and Mac1. A total of 23 CA+/Vav-Cre+ animals were observed. A post mortem analysis was possible for only 15 leukemic animals. 8 animals were found dead in their cages and could not be analyzed in detail. 6 out of 8 dead mice had slenomegaly, which strongly suggests that they died of leukemia. In 2 dead animals we could not do any analysis due to severe autolysis.

4.3 Two phenotypically distinct types of AML developed in CA+/Vav-Cre+ mice

The leukemic cells in the CA+/Vav-Cre+ mice were positive for the myeloid markers MPO and Mac1. Interestingly, in 40% of the leukemic animals (6 mice out of total 15 leukemic mice), the leukemic cells were also positive for the lymphoid marker $B220^{+}$. The average percentage of Mac1+/B220+ cells was 40% of the total peripheral blood cells with a standard deviation of 6.43%, 36% of the total bone marrow cells with a standard deviation of 12.09% and 44% of the total cells isolated from leukemic spleens with a standard deviation of 4.58%. In contrast, in the other 9 leukemic animals, B220-positive cells were almost completely absent. Apart from the significant difference in the phenotype, no variation in the disease latency or the severity of the disease was observed between the two AML subgroups in the CA+/Vav-Cre+ mice. Interestingly, a mixed lineage or biphenotypic phenotype has been reported for some patients with the CALM/AF10 fusion (Kumon et al., 1999; Secco et al., 1996). In these cases, T lymphoid and myeloid antigens were found on the blasts. The development of a mixed myeloid/lymphoid phenotype in CALM/AF10 leukemia models is not surprising in the light of the fact that the CALM/AF10 fusion is found frequently in T-ALL patients and also in patients presenting with an AML (Bohlander et al., 2000)

4.4 How does the CALM/AF10 fusion gene cause mixed lineage phenotype leukemia?

Two alternative mechanisms for the development of the mixed lineage phenotype have been proposed, one is called ‘lineage infidelity’ theory (McCulloch, 1983) and the other is ‘lineage promiscuity’ (Greaves et al., 1986). These two alternative theories are not necessarily mutually exclusive.

According to the 'lineage infidelity' theory, unusual biphenotypic cells can arise when the transformed cells aberrantly express genes from another lineage under the influence of a particular oncogene.

In the 'lineage promiscuity' setting, it is assumed that the transformed cell itself still had multilineage differentiation potential like a hematopoietic stem cell (HSC) or a multipotent progenitor (MPP). In a murine bone marrow transplantation model reported by Chi Wai So *et al,* the MLL-GAS7 fusion transformed MPPs resulting in myeloid, biphenotypic and lymphoid leukemias (So et al., 2003).

In our CA+/Vav-Cre+ mice, the CALM/AF10 fusion gene was expressed in all hematopoietic cells that express the *Vav* gene, including fetal liver hematopoietic cells (de Boer et al., 2003; Ogilvy et al., 1999). So it is very likely that in our model the CALM/AF10 fusion transformed HSCs and MPPs with some of these transforming events leading to predominantly myeloid and other to biphenotypic leukemia. Thus the results observed in our experiments are compatible with a lineage promiscuity model giving rise to "very" myeloid leukemias or to myeloid leukemias with co-expression of lymphoid markers. The variation in leukemia phenotype might not only depend on which cell was originally transformed but also on which additional, collaborating genetic events were acquired during the long latency period.

Our immunohistochemistry analyses showed that mature granulocytes were present in both the myeloid and the biphenotypic leukemias in the CA+/Vav-Cre+ mice. 50% of the cells in the leukemic bulk stained positive for the mature monocytic/macrophage markers CD24+ and F4/80. So it appears that, although CALM/AF10 fusion impairs the proper differentiation of its target cells, these cells still retain some ability to terminally differentiate.

4.5 Expression of the CALM/AF10 fusion gene is not oncogenic in the B cell compartment

When we crossed the R26LSLCA knock-in mice with Mb1-Cre and CD19-Cre mice, no leukemia development was observed in the progeny. In the CA+/Mb1-Cre+ mice, the expression of the CALM/AF10 fusion starts at the very early pro-B cell stage with the activity of the Mb1 promoter (Hobeika et al., 2006). In the CA+/CD19-Cre+ cross, expression of the CALM/AF10 fusion starts a little later from the pre-B cell stage onward (Rickert et al., 1997).

25 CA+/Mb1-Cre+ and 20 CA+/CD19-Cre+ mice were observed for one and half years. But none of the animals developed leukemia. The presence of the CALM/AF10 transcript was determined using

real-time RT-PCR in the B cells of 6 CA+/Mb1-Cre+ mice and 3 CA+/CD19-Cre+ mice. The level of CALM/AF10 fusion transcript detected in the B cell of CA+/Mb1-Cre+ and CA+/CD19-Cre+ mice was quite comparable to the level of CALM/AF10 transcript found in the bone marrow and spleen of leukemic CA+/Vav-Cre+ mice. This result indicates that the level of CALM/AF10 fusion transcript is not critical for the development of leukemia. For leukemia development, it seems to be more important in which tissue or cell type CALM/AF10 is expressed. The CA+/Mb1-Cre+ model showed that expression of CALM/AF10 in early B cells progenitors does not lead to leukemia development.

Using a murine retroviral bone marrow transplantation (BMT) model, Deshpande *et al.* showed that the expression of the CALM/AF10 fusion gene in murine bone marrow cells led to the development of an aggressive AML with a short median latency of 15 weeks and 100% penetrance. Deshpande and colleagues were able to identify the leukemia-propagating cell in their model. While the majority of leukemic cells displayed only myeloid markers on their surface, there were some cells that had both myeloid and lymphoid surface markers and a very small percentage of cells that were myeloid marker negative but lymphoid marker B220 postive. Interestingly, the leukemia propagating cells were found with the highest frequency (about 1:34) in the only lymphoid marker positive cell population (Deshpande et al., 2006). The immunophenotype of the leukemia propagating cells was described as B220+/CD43+/AA4.1+/HAS+/CD19-. These lymphoid progenitors-like cells were able to seed leukemias that had the same, mainly myeloid, phenotype as the original leukemia. These observations led to the hypothesis that the cell, which is the target of CALM/AF10-mediated transformation, might be a lymphoid precursor cell or an early B cell progenitor (Deshpande et al., 2006).

However, our observation that the expression of CALM/AF10 in very early B cells in the CA+/Mb1-Cre+ mice does not lead to leukemia does not seem to be compatible with this hypothesis. To solve this contradiction, one has to distinguish carefully between the target cell for transformation and the leukemia propagating cell. Even though the limiting dilution assays performed by Deshpande and colleagues were able to identify the phenotype of the leukemia propagating cells, they were not designed to determine the phenotype of the target cell for transformation.

4.6 The target cell of CALM/AF10-mediated leukemogenesis is a hematopoietic stem cell

Our CA+/Vav-Cre+ model strongly suggest that the CALM/AF10 fusion needs to be expressed in a cell that is at the top of the hematopoietic hierarchy, possibly in a stem cell or in a multipotent progenitor cell, to exert its oncogenic effect. The observation that the CALM/AF10 fusion leads to bi-phenotypic leukemias in the CA+/Vav-Cre+ model is compatible with this hypothesis.

Once leukemia has been initiated by CALM/AF10 in a HSC or MPP, the phenotype of the cells changes. This explains the fact that the leukemia propagating cells in the active leukemia can have a very different phenotype from the cell that was the initial target of transformation. Thus we see a B cell progenitor-like phenotype of the leukemia propagating cells in the BMT model of Deshpande et al., but no ability of CALM/AF10 to initiate leukemia when expressed in cells of a similar phenotype and differentiation stage in our CA+/Mb1-Cre+ (or CA+/CD19-Cre) mice. The 100% leukemia penetrance in our CA+/Vav-Cre+ model clearly shows that the target for CALM/AF10-mediated transformation has to be a hematopoietic stem cell or a very early multipotent progenitor cell.

4.7 The expression of CALM/AF10 is not sufficient for leukemia development

If we take into consideration that the median latency to leukemia development in the CA+/Vav-Cre+ mice was about one year, we have to conclude that additional mutations are required for CALM/AF10 to initiate leukemia. If there were no additional mutations required, all CA+/Vav-Cre+ mice should develop leukemia at a very young age. Presently, we do not know how many additional mutations are required in this process (one, two or more). There are estimates from sequencing the genomes of human AML samples that between 5 and 8 driver mutations are required for a leukemia to develop (2013). It is highly likely that these additional mutations arise in the hematopoietic stem cells in our model, because the lifetime of the MPPs is too short to accumulate additional mutations. One would also expect, if mutations in MPPs played a prominent role, that the latency of leukemia development in the CA+/Vav-Cre+ mice would be shorter.

Some recent works show that adult hematopoietic stem cells with long-term repopulation capacity (LT-HSC) are not a homogenous population of cells. There are sub-types of HSCs showing heterogenous differentiation behaviour. Some HSCs show a low

lymphopoietic activity and were first described as 'myeloid biased' whereas some HSCs that showed a balanced output of myeloid and lymphoid cells were called 'balanced HSCs' (Muller-Sieburg et al., 2004). Single cell transplantation assays in mice have been performed with LT-HSCs and depending on the ratio of clonally derived myeloid and lymphoid cells in the peripheral blood of the transplanted animals these subtypes of HSCs were identified. Connie J Eaves and colleagues have redefined this selectively lymphoid deficient sub-group of HSCs as 'α HSCs'. β HSCs show an equal potential to differentiate into myeloid and lymphoid cells (Benz et al., 2012; Dykstra et al., 2007). The ratio of α and β HSCs also appear to be very dynamic. It has been recently shown that α HSCs become more predominant than the β HSCs during the adult hematopoiesis (Benz et al., 2012). Taking this emerging paradigm into consideration we can assume that the fate of the leukemia (lymphoid or myeloid) will be dependent on the sub-type of the transformed HSCs. In addition, the leukemia phenotype might depend on which additional mutation the HSC acquires during leukemogenesis. However, it also remains a possibility that the additional mutations are solely responsible for the phenotypic variation of the leukemia.

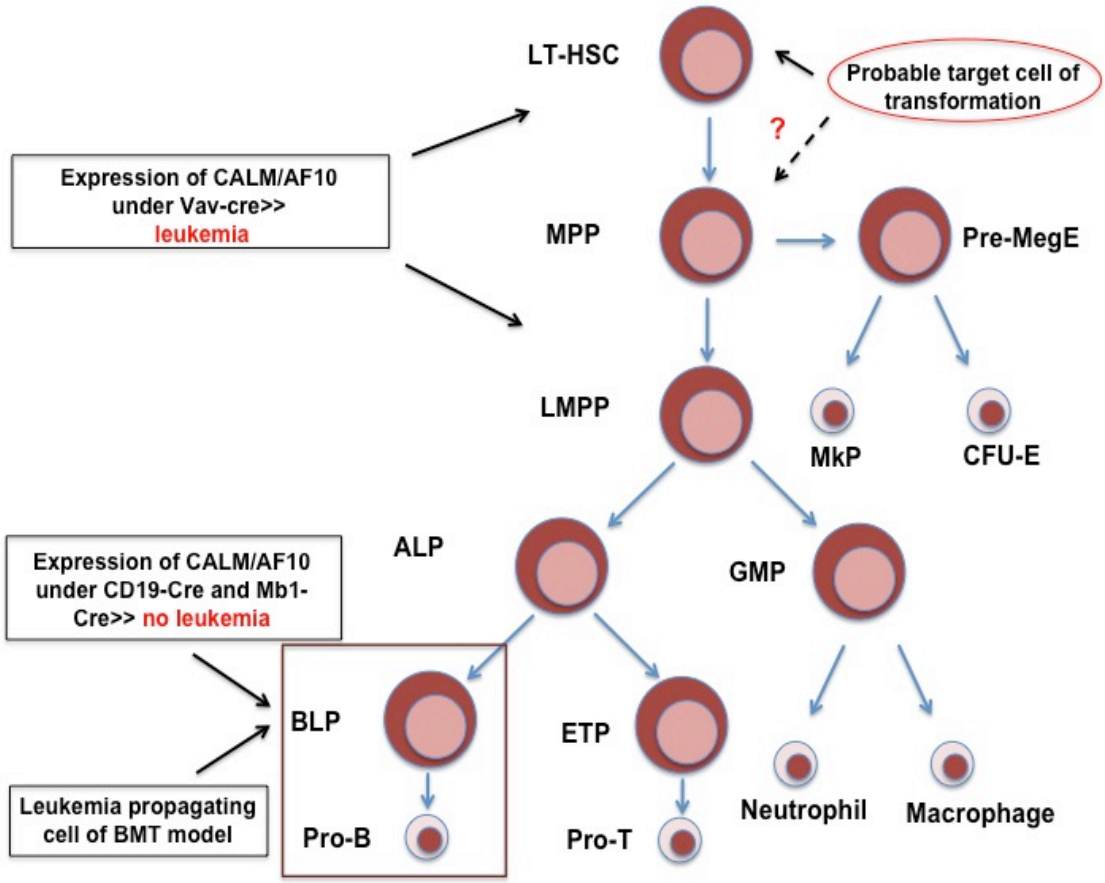

Fig 4.1: Schematic of the hematopoietic development showing the 'Leukemia initiating cell' in the R26LSLCA mouse model.

4.8 Overexpression of *Hoxa* cluster genes and *Hox* co-factor *Meis1* in CALM/AF10 fusion mediated leukemia

Dik and colleagues reported that the *Hoxa* cluster genes are highly expressed in CALM/AF10 positive T-ALL patients (Dik et al., 2005). The *Hoxa* cluster genes were also highly expressed in a transgenic CALM/AF10 mouse leukemia model (Caudell et al., 2007; Okada et al., 2006). In our CA+/Vav-Cre+ mouse model, there was also overexpression of the *Hoxa5, Hoxa7, Hoxa9 and Hoxa10* genes in the bone marrow and the spleen cells of mice with leukemia. In addition, we detected high expression levels of the *Hox* co-factor *Meis1* in this model. Interestingly, Caudell and colleagues were able to detect high expression of the *Hoxa* cluster genes and *Meis1* in the normal, non-leukemic bone marrow cells of their Vav promoter-driven CALM/AF10 transgenic mice (Caudell et al., 2007). However, we were unable to detect high *Hoxa* cluster gene or *Meis1* expression in the non-leukemic B cells from CA+/Mb1-Cre+ mice, even though the CALM/AF10 fusion transcript in the non-leukemic cells was expressed at comparable levels to expression levels in leukemic cells. These data indicate that high expression levels of the *Hoxa* genes and *Meis1* are specific to the leukemic cells in in our

CALM/AF10 knock-in mice. The *Bmi1* gene was found to be upregulated in CALM/AF10 positive human leukemias by Dik and colleagues and in our laboratory (Dik et al., 2005; Mulaw et al., 2012). *BMI1* was one of the most prominent upregulated genes in CALM/AF10 positive leukemia samples compared to several other leukemia subtypes (Mulaw et al., 2012). However, there were only 2 to 3 fold higher expression levels of *Bmi1* transcripts in the leukemic cells from CA+/Vav-Cre+ mice compared to healthy controls.

4.9 Overexpression of human *BMI1* in tr-IgH-CALM/AF10 bone marrow cells induces leukemia in a retroviral bone marrow transplantation model

BMI1 was reported to be specifically upregulated in both human and murine CALM/AF10 leukemia (Dik et al., 2005; Mulaw et al., 2012). It is known that *BMI1* is essential for the self-renewal of hematopoietic stem cells (Park et al., 2003). *BMI1* plays a role in determining the proliferating capacity of normal and leukemic stem cells (Lessard and Sauvageau, 2003) and collaborates with BCR-ABL in leukemic transformation of human CD34+ cells (Rizo et al., 2010). In a very recent study, *BMI1* overexpression has been identified as a collaborating event in the development of human and murine myelodysplastic syndromes along with AML1 mutation (Harada et al., 2013). All these finding qualifies *BMI1* as an interesting candidate to be investigated for its potential role in CALM/AF10 leukemia.

To investigate the ability of *BMI1* to collaborate with the CALM/AF10 fusion in leukemia development, bone marrow cells from transgenic mice expressing the CALM/AF10 fuison protein under the control of the immunoglobulin heavy chain enhancer/promoter (tr-IgH-CALM/AF10) were transduced with a pMIY retrovirus expressing a FLAG-tagged human *BMI1* and transplanted into lethally irradiated non-transgenic FVB mice. As mentioned earlier, the tr-IgH-CALM/AF10 mice express the CALM/AF10 fusion gene in mature B cells but do not develop leukemia. When bone marrow cells from tr-IgH-CALM/AF10 mice transduced with pMIY-BMI1 were transplanted into the lethally irradiated syngeneic recipients, 67% (8 of 12) of the transplanted mice (the "tr-IgH-CA&BMI1" group) developed leukemia within 400 days of our observation period. Transgenic bone marrow cells transduced with the empty vector or non-transgenic bone marrow cells transduced with the pMIY-BMI1 vector did not cause leukemia in the recipient mice. 5 of the 8 leukemic mice had an AML. The leukemic cells from two of the five AML mice were successfully retransplanted into the secondary recipient mice

with and without irradiation and led to the development of leukemia in the secondary recipients.

Two mice from the tr-IgH-CA&BMI1 group developed T-ALL 101 days and 219 days after transplantation. Giemsa-May-Grünwald staining of the peripheral blood smears from these mice showed immature lymphoblasts. All the hematopoietic organs in these two mice were infiltrated with CD4+/CD8+ T lymphocytes. But interestingly, the leukemic cells were YFP-negative. When unsorted bone marrow cells from one T-ALL mouse were transplanted into 2 secondary recipients, the secondary recipients failed to develop leukemia.

One mouse from the tr-IgH-CA&BMI1 group showed symptoms of leukemia like ruffled fur, difficulty in breathing and reduced movement. It was sacrificed 123 days after transplantation. The post mortem analysis revealed that the mouse had a slightly bigger spleen but high WBC counts in the peripheral blood. The cytospin preparations from the hematopoietic organs showed the presence of immature blast cells of myeloid lineage. Flow cytometric analysis showed that percentage of myeloid and lymphoid cells was normal in comparison to the wildtype FVB control mice but 41% of the total peripheral blood cells and 91% of the bone marrow cells were Sca1+. Sca1 is a marker of myeloid progenitors. The presence of a high number of Sca1+ cells in peripheral blood and bone marrow explains the immature cells identified in the blood smear and bone marrow cytospins. There were no YFP+ cells in the peripheral blood of this mouse. In the bone marrow and spleen, only 3% YFP-positive cells could be detected. The type of the hematological malignancy could not be conclusively determined by immunohistochemistry.

4.10 Both CA-MF together with BMI1 and CA-MF alone induce an aggressive AML in a murine bone marrow transplantation model

In our second approach to test the collaboration of *BMI1* with CALM/AF10 to induce leukemia, bone marrow cells from wild type FVB mice were co-transduced with pMIG-CA-MF and pMIY-BMI1. The co-transduced, GFP and YFP double positive cells were transplanted into irradiated syngeneic recipient mice. The pMIG-CA-MF retrovirus expresses a deletion mutant of CALM/AF10 (the minimal fusion), which has been shown to lead to an aggressive acute myeloid leukemia with similar latency and penetrance (100%) like the full length CALM/AF10 in bone marrow transplantation experiments (Deshpande et al., 2011). As expected, all 10 mice transplanted with CA-MF expressing bone marrow cells developed an AML with an average latency of 118 days and 100% penetrance.

Similarly, all 10 mice transplanted with bone marrow cells co-transduced with both the pMIG-CA-MF and the pMIY-BMI1 viruses also developed an acute myeloid leukemia with a similar average latency of 120 days. There was no significant difference in the phenotype, the aggressiveness or the latency of the CA-MF and the CA-MF&BMI1 induced leukemias. It is very likely that the strong leukemogenic drive of the CA-MF gene in this model prevented us from noticing any differences, like changes in the latency or aggressiveness of the leukemia when *BMI1* was coexpressed with CA-MF. Thus, this approach did not produce conclusive evidence that BMI1 is a strong collaborator of CALM/AF10-induced leukemogenesis.

4.11 Evidence for collaboration of *BMI1* with CALM/AF10 in leukemogenesis

Even though our second approach, as discussed in the previous paragraphs, did not provide evidence for collaboration between CALM/AF10 and *BMI1* in leukemogenesis, the results from our bone marrow transplantation experiments using transgenic CALM/AF10 (tr-IgH-CA) bone marrow cells (approach 1) argue very strongly in favor of a collaborative effect of the overexpression of *BMI1* in CALM/AF10-mediated leukemogenesis. Only when *BMI1* was expressed in the transgenic tr-IgH-CA bone marrow cells did we see leukemia development (tr-IgH-CA&BMI1 group). Expression of *BMI1* in non-transgenic bone marrow or transduction of tr-IgH-CA cells with an empty retrovirus did not lead to leukemia development. The penetrance of leukemia in this group was 58%, if one counts only the clear cases of leukemia. Although, most likely, the penetrance is higher because an additional mouse in this experimental group did show signs of leukemia but a clear diagnosis of leukemia could not be made.

While the CALM/AF10 fusion is frequently found in human T-ALL patients, up to now only cases of CALM/AF10-induced AML have been reported in mouse models. Interestingly, 2 mice in the tr-IgH-CA&BMI1 group developed T-ALL. However, the leukemic CD4+/CD8+ population of leukemic blast was not YFP positive as would be expected. It is thus unclear whether the ALL in these two mice originated from the transplanted cells. An explanation for the absence of the YFP protein from these T cell leukemias could be a negative selection phenomenon against GFP-like proteins in lymphoid cells and especially in T ALL cells. It is known that GFP proteins affect RING E3 ubiquitin ligase dependent processes like NF-κB signaling in cell lines and in mice (Baens et al., 2006). Baens and colleagues showed that the expression of EGFP protein inhibits NF-κB signaling. In EGFP transgenic mice, there was reduced NF-kB ubiquitination and NF-κB signaling. The structural homologue of

GFP, dsRed, had a similar effect on the NF- κB signaling *in vitro* (Baens et al., 2006). It is known that in human T-ALL, the NF-κB pathway is highly activated (Vilimas et al., 2007). T-ALL cell lines are very sensitve to the inhibition of NF-κB signalling and in Notch1-IC induced T-ALL, attenuation of NF-κB activity affects the progression and severity of the disease (Vilimas et al., 2007). Additional experiments, involving larger mouse cohorts would be required to prove that *BMI1* and CALM/AF10 expression are really responsible for the development of T-ALL in this model.

The presence of the CALM/AF10 fusion together with expression of *BMI1* in mature B-cells led to the development of myeloid leukemia (N=5) in about 1/3 of the mice of the tr-IgH-CA&BMI1 group. This is a little surprising because one would expect a B ALL phenotype. It is very likely that the expression of CALM/AF10 synergizes with *BMI1* to disturb the normal B cell differentiation pathway in these leukemias leading to a myeloid phenotype.

Rizo and colleagues showed that elevated level of *BMI1* expression acts as a collaborative event in BCR-ABL-induced transformation of human CD34+ cells (Rizo et al., 2010). Recently, *BMI1* overexpression was shown to collaborate with a RUNX1/AML1 mutation in the development of human and murine myelodysplastic syndrome (Harada et al., 2013). The mechanism by which *BMI1* contributes to oncogensis is not clear. We know, *BMI1* is essential for the self-renewal of Hsc and it is a key regulator of the proliferative potential of leukemic and normal stem and progenitor cells. (Lessard and Sauvageau, 2003; Park et al., 2003) (Iwama et al., 2004; Jacobs et al., 1999; Rizo et al., 2008). According to previous studies and the data from our mouse BMT model, it appears that expression of BMI1 alone is not leukemogenic in human CD34+ cells or in *in vivo* murine models (Jacobs et al., 1999; Rizo et al., 2010). However, overexpression of *BMI1* promotes symmetrical divisions of HSC and induces proleferation of hematopoietic progenitors (Iwama et al., 2004). It is known that BMI1 represses the p16INK4a/p19ARF locus, which is an important mechanism for bypassing the senescence of embryonic fibroblasts. Deletion of *BMI1* results in the increased expression of the cell cycle inhibitor proteins p16 and p19. However, overexpression of only BMI1 can increase number of progenitor cells, in the absence of p16 and p19 repression, indicating there must be other targets of BMI1 and that repression of the p16/p19 proteins is not solely responsible for the self-renewal stimulating property of BMI1. Thus, BMI1 could contribute to oncogenesis by bypassing senescence and increasing the life span of stem cells or by contributing to increased self-renewal of progenitors by allowing symmetrical cell division. In a recent report, a novel mechanism of BMI1-mediated inactivation of

apoptotic barrier in neuroblastoma was described. This report shows that BMI1 reduces TP53 protein levels by direct binding and directly inducing its polyubiquitination and proteosomal degradation (Calao et al., 2013), leaving the embryonic cells susceptible to oncogenesis.

The exact role of BMI1 in CALM/AF10 mediated leukemia remains elusive and requires further investigation. In a recent report Bmi1 is shown to synergise with BCR/ABL in transforming B-lymphoid progenitors by aberrant induction of HSC-specific gene expression in the mature progenitors (Sengupta et al., 2012). It is a possibility that overexpression of BMI1 along with CALM/AF10 induces aberrant expression of genes in the B lymphocytes of IgH-CA&BMI1 mice, imparting survival advantage, rendering them susceptible to acquire additional mutations which help in the transformation of these cells leading to the development of AML or T-ALL.

4.12 Is the BMI1 overexpression in CALM/AF10 fusion induced leukemia due to a position effect?

The *BMI1* gene in humans is located on chromosome 10, about 500 kbp proximal to the *AF10*. The synteny between *AF10* and *BMI1* is conserved in mice. Together with 3 other neighbouring genes (*COMMD3, SPAG6* and *DNAJC1*) *BMI1* was found to be upregulated in CALM/AF10 positive leukemia patients (Dik et al., 2005; Mulaw et al., 2012). It was speculated that this upregulation of BMI1 and its neighbours might be due to a position effect as a result of the t(10;11)(p12;q14) translocation, which brings the very active *CALM* promoter to in proximity to the 3’ portion of the AF10 and the downstream genes *DNAJC1*, *COMMD3*, *BMI1*, and *SPAG6* (Dik et al., 2005). However, the murine *Bmi1* was found to be upregulated together with *Commd3* and *Dnajc1* in samples from a murine bone marrow transplantation model (BMT) of CALM/AF10-induced leukemia (Mulaw et al., 2012). Since there is no t(10;11)(p12;q14) translocation in this BMT model, the position effect explanation for the aberrant expression of *Bmi1* could be ruled out and a role of the CALM/AF10 fusion protein itself in the upregulation of *Bmi1* can be assumed. However, the mechanism of CALM/AF10-mediated transcriptional upregulation of *Bmi1* remains unclear. In this study we were also able to detect a mild upregulation of *Bmi1* in the leukemic cells of the CA+/Vav-Cre mice. More extensive and more precise studies of the interplay of CALM/AF10 and *Bmi1* are required to clarify their role in leukemogenesis.

5. Summary

The translocation t(10;11)(p13;q14), which results in the formation of the CALM/AF10 fusion gene, is associated with variety of hematological malignancies, including T-cell acute lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), undifferentiated leukemia, and T cell lymphoma. In most cases, this translocation is associated with a very poor prognosis. Originally, the CALM/AF10 fusion was cloned from the human cell line U937, derived from a patient with histiocytic lymphoma. The CALM/AF10 fusion transcript contains the complete open reading frame of the *CALM* gene except for the last 4 c-terminal amino acids, fused in-frame to almost the complete *AF10* open-reading frame except for the first 82 amino acids. CALM is a 652 amino acid long protein, which is homologous to the neuronal specific endocytic protein AP180. CALM intereacts with clathrin and plays a role in clathrin-mediated endocytosis. AF10 is a putative transcription factor with an octapeptide motif and leucine zipper domain (OM/LZ), which was shown to be critical for CALM/AF10 mediated leukemogenesis. CALM/AF10, when expressed as a transgene in the whole hematopoietic compartment from the Vav-promoter leads to the development of AML in 40% of the transgenic mice after a median latency of 1 year. However, in a murine retroviral bone marrow transplantation model, the expression of CALM/AF10 leads to the development of aggressive AML in all the animals with a median latency of only 15 weeks. In this model, using a limiting dilution transplantation approach, Deshpande and colleagues were able to show that the frequency of the 'leukemia-propagating cell' was highest (1:34) in a lymphoid marker positive but myeloid marker negative (B220+/MM-) subpopulation. This finding led to the hypothesis that the CALM/AF10 fusion gene induces an aberrant myeloid development of B220+ early lymphoid progenitors

To investigate the tissue-specific requirement for CALM/AF10 in leukemogenesis, we have generated a Cre-inducible knock-in mouse model of CALM/AF10. In this R26LSLCA mouse line, the cDNA of human CALM/AF10 fusion preceded by a LoxP-flanked transcriptional stop cassette is knocked into the Rosa26 locus. Using mouse lines that express Cre as transgene in a tissue-specific fashion under the control of various promoters (Vav, CD19 or Mb1) or as an estrogen receptor fusion gene, which is Tamoxifen activatable, the transcriptional stop cassette can be removed, leading to the tissue-specific or temporally-controlled expression of the CALM/AF10 fusion protein. To achieve the expression of CALM/AF10 in the all-hematopoietic cells, the Vav-Cre mouse line was crossed with the R26LSLCA mice. Crosses with the Mb1-Cre and CD19-Cre transgenic lines were employed to express

CALM/AF10 in the B cell compartment from early pro-B cell onwards. When CALM/AF10 was expressed in the whole hematopoietic compartment in the cross with the Vav-Cre line, all mice developed AML after a median latency of 1 year. Leukemic mice had splenomegaly, leukocytosis, and infiltration of myeloid blast cells in hematopoietic and several non-hematopoietic organs. The leukemic cells were characterized by the expression of myeloid markers like Gr1, Mac1. In 40% of the cases, the leukemic cells also co-expressed the lymphoid marker B220 in addition to the myeloid markers. The latency or the agressiveness of the two different types of leukemia in this model were not different. However, when CALM/AF10 was expressed just in the B cell compartment in the crosses with the Mb1-Cre and CD19-Cre lines, none of the mice developed leukemia, even after a long latency of 18 months. The level of CALM/AF10 transcript was similar both in the bone marrow and spleen cells from the leukemic mice and in the B cells from non-leukemic mice. In the leukemic bone marrow and spleen cells, we detected high levels of *Hoxa* cluster gene transcripts, namely *Hoxa5, Hoxa7, Hoxa9, Hoxa10*, of the *Hox* co-factor *Meis1,* and of *Bmi1* compared to transcript levels in CALM/AF10 expressing B cells from the non-leukemic mice. From these observations we can draw the conclusion that CALM/AF10-mediated leukemogenesis is only possible if CALM/AF10 is expressed in an early hematopoietic progenitor or, most likely, hematopoitic stem cell. The relatively long latency of the onset of leukemia in Vav-Cre induced mouse cohort strongly supports the hypothesis that CALM/AF10 requires additional genetic lesions for leukemogenesis.

We used a candidate gene approach to test one putative collaborator of CALM/AF10 in leukemia development. We chose *BMI1*, a polycomb group gene, because it is specifically upregulated both in human and murine CALM/AF10 leukemia. *Bmi1* was originally identified as a co-operating factor of c-Myc in B cell lymphomagenesis. To determine the role of *BMI1* as a collaborator of CALM/AF10 in leukemogenesis, we developed a murine retroviral bone marrow transplanataion model. We used IgH-CALM/AF10 transgenic mice as bone marrow donors. As previously mentioned, these mice express the CALM/AF10 fusion in their B cells but do not develop leukemia (Krause, 2006). The bone marrow cells of the donors were transduced with a human *BMI1* expressing retrovirus and transplanted into non-transgenic, syngeneic recipients. Eight of 12 transplanted mice developed acute leukemia within 1 year after transplantation (6 mice had AML, 2 mice had ALL). Mice transplanted with empty vector transduced cells transgenic cells or with non-transgenic cells expressing *BMI1* did not develop leukemia. When we transplanted mice with wild type bone marrow

that had been retrovirally transduced to express CALM/AF10-MF (a very leukemogenic deletion mutant of CALM/AF10) alone or CALM/AF10 and *BMI1*, all transplanted animals in the two groups developed leukemia with a median latency of 10 weeks, and there was no difference in the leukemia phenotype between the two groups. Taken together, the results from these experiments strongly support the hypothesis that *BMI1* is a collaborator of CALM/AF10 in leukemogenesis.

In summary, two key assumptions are strongly supported by the results of our experiments:

1) CALM/AF10 has to be expressed in the right cell type to lead to leukemia
2) CALM/AF10 needs collaborators in leukemogenesis. It could be shown that the overexpression of BMI1 is one such collaborating event.

6. Zusammenfassung

Die t(10;11)(p13;q14) Translokation, die zur Bildung des CALM/AF10 Fusionsgens führt, findet sich bei verschiedenen, malignen hämatologischen Erkrankungen wie der akuten T-Zell lymphoblastischen Leukämie (T-ALL), der akuten myeloischen Leukämie (AML), der undifferenzierten Leukämie und bei T-Zell Lymphomen. In den meisten dieser Fälle ist diese Translokation mit einer sehr schlechten Prognose verbunden. Ursprünglich wurde die CALM/AF10 Fusion in der U937 Zelllinie kloniert, die von einem Patienten mit histiozytischem Lymphom stammt. Das CALM/AF10 Fusionstranskript besteht aus dem offenen Leserahme des CALM Gens mit Ausnahme der 4 letzten Aminosäuren, der im Leseraster mit der codierenden Region fast des gesamten AF10 Gens mit Ausnahme der ersten 82 Aminosäuren fusioniert ist. CALM ist ein 652 Aminosäure langes Protein, das Homologien zu dem Neuronen-spezifischen endozytotischen Protein AP180 aufweist. CALM interagiert mit Clathrin und spielt eine Rolle bei der Clathrin-mediierten Endozytose. AF10 is ein mutmaßlicher Transkriptionsfaktor mit einem Octapeptide/LeuzinZipper Motif (OM/LZ), von dem gezeigt wurde, dass es wichtig für die CALM/AF10 mediierte Leukämogenese ist. Wenn CALM/AF10 als Transgen in allen hämatopoetischen Zellen unter der Kontrolle des Vav-Promoters exprimiert wird, führt es zur Entstehung einer AML bei ca. 40% der transgenen Mäuse nach einer medianen Latenzzeit von einem Jahr. In einem Mausknochenmarktransplantationsmodell führt CALM/AF10 jedoch schon nach einer Latenzzeit von nur 15 Wochen zur Entwicklung einer aggressiven AML bei allen transplantierten Tieren. Desphande und Kollegen konnten in diesem Model zeigen, dass die Häufigkeit der Leukämie propagierenden Zellen mit 1:34 am höchsten in einer Zellpopulation war, die positiv für lymphatische Marker, aber negativ für myeloische Marker war (B220+/MM-). Diese Ergebnisse haben zu der Hypothese geführt, dass das CALM/AF10 Fusionsgen eine aberrante myeloische Entwicklung von B220+ frühen lymphatischen Progenitorzellen induziert.

Um zu untersuchen, welche gewebsspezifischen Voraussetzungen CALM/AF10 bei der Leukämogenese braucht, haben wir ein Cre-induzierbares Knock-in Modell für CALM/AF10 entwickelt. In dieser R26SLSCA Mauslinie ist die cDNA der humanen CALM/AF10-Fusion, der eine mit LoxP-Stellen flankierte transkriptionelle Stopp-Kassette vorangestellt ist, in den Rosa26 Locus integriert. Mithilfe von Mauslinien, die die Cre-Rekombinase als Transgen unter der Kontrolle von verschiedenen gewebsspezifischen Promotoren (Vav, CD19 oder Mb1) oder als eine Fusion mit dem Östrogenrezeptor,

der Tamoxifen aktivierbar ist, exprimieren, kann die transkriptionelle Stopp-Kassette entfernt werden, was zu einer gewebsspezifischen oder zeitlich kontrollierten Expression des CALM/AF10 Fusionsproteins führt. Um die Expression von CALM/AF10 in allen hämatopoetischen Zellen zu erreichen, wurden die R26SLSCA Mäuse mit dem Vav-Cre Mausstamm gekreuzt. Verpaarungen mit den Mb1-Cre und den CD19-Cre transgenen Linien wurden benutzt, um eine Expression von CALM/AF10 im B-Zellkompartiment ab dem frühen pro-B-Zellstadium zu erreichen. Die Expression von CALM/AF10 im allen hämatopoetischen Zellen durch die Kreuzung mit den Vav-Cre Mäusen führte zur Entwicklung von AML bei allen Mäusen mit einer medianen Latenzzeit von einem Jahr. Die leukämischen Mäuse hatten eine Splenomegalie, Leukozytose und Infiltrationen von myeloischen Blasten in allen hämatopoetischen und einigen nicht-hämatopoetischen Organen. Die leukämischen Zellen war charakterisiert durch die Expression der myeloischen Oberflächenmarker Gr1 und Mac1. Bei 40% dieser Leukämien fand sich auch eine Koexpression des lymphatischen Markers B220 zusätzlich zu den myeloischen Markern. Die Latenzzeit und die Aggressivität dieser zwei Leukämietypen unterschied sich nicht voneinander. Die Expression von CALM/AF10 nur im B-Zellkompartiment in den Mäusen der Kreuzungen mit den Mb1-Cre und den CD19-Cre führte bei keiner der Mäusen zu einer Leukämieentwicklung, selbst nach einer langen Beobachtungszeit von bis zu 18 Monaten. Die Expressionshöhe des CALM/AF10 Transkripts war sowohl in den Zellen des Knochenmarks und der Milz der Mäuse mit der Leukämie als auch in den B-Zellen der nicht leukämischen Mäuse ungefähr gleich. In den leukämischen Knochenmarks- und Milzzellen konnten wir im Vergleich zu den CALM/AF10 exprimierenden B-Zellen der nicht-leukämischen Mäusen eine hohe Expression der *Hoxa* Clustergene *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10*, des *Hox* Kofaktors *Meis1* und von *Bmi1* nachweisen. Aus diesen Beobachtungen läßt sich schlußfolgern, dass eine Leukämienentwicklung durch die Expression von CALM/AF10 nur zustande komme, wenn CALM/AF10 in einer frühen hämatopoetischen Progenitorzelle und möglicherweise einer hämatopoetischen Stammzelle exprimiert wird. Die relativ lange Latenzzeit bis zur Leukämieentwicklung in der Vav-Cre induzierten Mauskohorte deutet darauf hin, dass CALM/AF10 zusätzliche genetische Veränderungen für die Leukämogenese benötigt.

Wir wendeten einen Kandidatengenansatz an, um einen mutmaßlichen Kooperationsfaktor für CALM/AF10 bei der Leukämogenese zu testen. Wir wählten hierzu *BMI1*, ein Gen der Polycomb Gruppe, weil es spezifisch in menschlichen und murinen CALM/AF10 assoziierten Leukämien hoch exprimiert wird. *Bmi1* wurde ursprünglich als kooperierender Faktor von c-Myc bei der B-

Zelllymphomentwicklung identifiziert. Um die Rolle von BMI1 als kooperierender Faktor für CALM/AF10 bei der Leukämogenese zu untersuchen, entwickelten wir ein murines Knochenmarktransplantationsmodell. Wir verwendeten IgH-CALM/AF10 transgene Mäuse als Knochenmarkspender. Wie bereits erwähnt, exprimieren diese Mäuse die CLAM/AF10 Fusion in ihren B-Zellen, aber enwickeln keine Leukämie (Krause, 2006). Die Knochenmarkzellen der Spender wurden mit menschlichem *BMI1* exprimierenden Retroviren transduziert und in nicht transgene syngene Empfängermäuse transplantiert. Acht der 12 transplantierten Mäuse entwickelten eine akute Leukämie innerhalb eines Jahres nach der Transplantation (6 Mäuse hatten AML und 2 Mäuse eine ALL). Mäuse, die mit transgenen Knochemarkszellen, die mit dem leeren Vektor transduziert wurden, oder mit nicht transgenen Knochenmarkszellen, die mit dem BMI1 exprimierenden Vektor transduziert wurden, entwickelten keine Leukämien. Als wir Mäuse mit nicht transgenem Knochenmark, das mit CALM/AF10-MF (einer sehr leukämogenen Deletionsmutante von CALM/AF10) exprimierenden Retroviren transduziert wurde, oder mit CALM/AF10-MF und BMI1 exprimierenden Retroviren, kam es zu einer sehr schnellen Leukämieenwicklung mit einer medianen Latenzzeit von 10 Wochen in beiden Gruppen, und es zeigte sich auch kein Unterschied in dem leukämischen Phänotyp. Die Ergebnisse dieser Experimente untermauern sehr stark die Hypothese, dass *BMI1* ein Kooperationspartner von CALM/AF10 in der Leukämogenese ist.

Zusammenfassend können wir sagen, dass zwei wichtige Annahmen durch die Ergebnisse unseres Experiments untermauert werden:

1) CALM/AF10 muss im richtigen Zelltyp exprimiert werden, um eine Leukämie auszulösen.

2) CALM/AF10 braucht kollaborierende Kofaktoren für die Leukämogenese. Es konnte gezeigt werden, dass eine hohe Expression von BMI1 einer dieser Kofaktoren ist.

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8. Appendix

Table A1: Tamoxifen treated cohort of the double positive (CALM/AF10+/Cre-ERT2+) mice:

	Genotype	Mouse No	Period of Treatment
1	CALM/AF10+/ Cre ERT2 +	30	18.07.10-23.07.10
2	CALM/AF10+/ Cre ERT2 +	32	18.07.10-23.07.10
3	CALM/AF10+/ Cre ERT2 +	33	18.07.10-23.07.10
4	CALM/AF10+/ Cre ERT2 +	52	18.07.10-23.07.10
5	CALM/AF10+/ Cre ERT2 +	53	18.07.10-23.07.10
6	CALM/AF10+/ Cre ERT2 +	79	13.09.10-17.09.10
7	CALM/AF10+/ Cre ERT2 +	94	13.09.10-17.09.10
8	CALM/AF10+/ Cre ERT2 +	95	13.09.10-17.09.10
9	CALM/AF10+/ Cre ERT2 +	114	13.09.10-17.09.10
10	CALM/AF10+/ Cre ERT2 +	116	13.09.10-17.09.10
11	CALM/AF10+/ Cre ERT2 +	117	13.09.10-17.09.10
12	CALM/AF10+/ Cre ERT2 +	118	13.09.10-17.09.10
13	CALM/AF10+/ Cre ERT2 +	121	13.09.10-17.09.10
14	CALM/AF10+/ Cre ERT2 +	138	13.09.10-17.09.10
15	CALM/AF10+/ Cre ERT2 +	139	13.09.10-17.09.10
16	CALM/AF10+/ Cre ERT2 +	140	13.09.10-17.09.10
17	CALM/AF10+/ Cre ERT2 +	141	13.09.10-17.09.10
18	CALM/AF10+/ Cre ERT2 +	143	13.09.10-17.09.10
19	CALM/AF10+/ Cre ERT2 +	144	13.09.10-17.09.10

Table A.2: Tamoxifen treated cohort of only CALM/AF10 positive (CALM/AF10+/Cre-ERT2-) control mice:

	Genotype	Mouse No	Period of Treatment
1	CALM/AF10+/ Cre ERT2-	22	18.07.10-23.07.10
2	CALM/AF10+/ Cre ERT2-	23	18.07.10-23.07.10
3	CALM/AF10+/ Cre ERT2-	24	18.07.10-23.07.10
4	CALM/AF10+/ Cre ERT2-	85	18.07.10-23.07.10
5	CALM/AF10+/ Cre ERT2-	152	13.09.10-17.09.10
6	CALM/AF10+/ Cre ERT2-	153	13.09.10-17.09.10
7	CALM/AF10+/ Cre ERT2-	154	13.09.10-17.09.10
8	CALM/AF10+/ Cre ERT2-	156	13.09.10-17.09.10

Table A3: Tamoxifen treated cohort of only Cre-ERT2 positive (CALM/AF10-/Cre-ERT2+) control mice:

	Genotype	Mouse No	Period of Treatment
1	CALM/AF10-/ Cre ERT2+	30	18.07.10-23.07.10
2	CALM/AF10-/ Cre ERT2+	32	18.07.10-23.07.10
3	CALM/AF10-/ Cre ERT2+	33	18.07.10-23.07.10
4	CALM/AF10-/ Cre ERT2+	52	18.07.10-23.07.10
5	CALM/AF10-/ Cre ERT2+	53	18.07.10-23.07.10
6	CALM/AF10-/ Cre ERT2+	124	13.09.10-17.09.10
7	CALM/AF10-/ Cre ERT2+	125	13.09.10-17.09.10
8	CALM/AF10-/ Cre ERT2+	126	13.09.10-17.09.10

Table A4 Days of survival and diagnosis of the leukemic CA+/Vav-Cre+ animals

Mouse ID	Age at the time of death (days)	Comment
Vav 29	452	Analyzed bipheotypic leukemia
Vav 31	451	Analyzed, myeloid leukemia
Vav 32	352	Analyzed, bipheotypic leukemia, detailed histology and immunohistochemistry was performed.
Vav 42	414	Analyzed, myeloid leukemia, detailed histology and immunohistochemistry was performed.
Vav 51	403	Analyzed, myeloid leukemia
Vav 57	432	Analyzed, myeloid leukemia, detailed histology and immunohistochemistry was performed.
Vav 65	355	Analysed, bipheotypic leukemia
Vav 71	396	Analyzed, myeloid leukemia
Vav 72	497	Analyzed, myeloid leukemia
Vav 74	343	Analyzed, myeloid leukemia, detailed histology and immunohistochemistry was performed.
Vav 80	285	Analyzed, pre-leukemic

Vav 81	421	Analyzed, bipheotypic leukemia
Vav 84	404	Analysed, myeloid leukemia
Vav 86	293	Analyzed, bipheotypic leukemia, detailed histology and immunohistochemistry was performed.
Vav 95	261	Analyzed, bipheotypic leukemia, detailed histology and immunohistochemistry was performed.
Vav 12	559	Found dead, Splenomegaly, pale organs
Vav 14	318	Found dead, autolysis of organs,No analysis
Vav 16	354	Found dead, autolysis of organs No analysis
Vav 18	403	Found dead, Splenomegaly, pale organs
Vav 27	321	Found dead, Splenomegaly,whitish spots on spleen
Vav 45	320	Found dead, Splenomegaly, pale organs
Vav 60	261	Found dead, Splenomegaly, pale organs
Vav 66	263	Found dead, Splenomegaly, pale organs
Vav 89	349	Found dead, Splenomegaly, pale organs

Table A5 Average, standard deviation and statistical analysis of different surface markers on the cells from peripheral blood of leukemic CA+/Vav-Cre+ mice (myeloid leukemia N=7, biphenotypic leukemia N=6) and the mice from control group (N=8)

Surface Marker	Control Sample, N=8 Average %±St.deviation	Leukemic Sample Average % ±St.deviation			
		Myeloid N=7	P Value	Biphenotypic,N=6	P Value
Gr1+	15.12±8.83	45.57±12.90	0.00012	31.66±16.39	0.03
Mac1+	15.75±13.5	63±20.76	0.00015	72.66±10.48	1.93E-6
Gr1+/Mac1+	6.75±4.26	43±13.51	6.74E-6	29.66±18.93	0.005
Sca1+	26±10.86	19.14±5.63	0.158	6.16±5.19	0.001
cKit+	0.7±1.38	5.2±4.45	0.017	2.5±1.76	0.05
B220*	12±5.34	15.86±8.47	0.304	51±15.34	2.07E-5
CD43+	60±22.33	79.71±7.67	0.04	56.33±29.28	0.79
CD19+	6±3.5	8.28±7.15	0.43	3.6±2.3	0.204
CD4+	10.62±6.94	9.42±5.59	0.02	1.6±1.50	0.009
CD3+	22.25±9.79	3.42±2.57	0.009	4.66±3.01	0.001
CD8+	15.75±6.06	7±5.6	0.01	2.16±1.83	0.0002

Table A6 Average, standard deviation and statistical analysis of different surface markers on bone marrow cells from leukemic CA+/Vav-Cre+ mice (myeloid leukemia N=7, biphenotypic leukemia N=6) and the mice from control group (N=11)

Surface Marker	Control Sample, N=11 Average %±St.deviation	Leukemic Sample Average %±St.deviation			
		Myeloid N=7	P Value	Biphenotypic,N=6	P Value
Gr1+	34.54±18.56	75.85±5.87	3.77E-5	43±16.93	0.26
Mac1+	28.9±21.98	80.85±9.52	2.41E-5	75.5±12.81	0.0002
Gr1+/Mac1+	24.11±18.84	70.14±6.61	1.34E-5	36.66±15.95	0.188
Sca1+	10.63±5.27	3.14±3.18	0.003	1.16±1.16	0.0006
cKit+	18.45±2.76	15.85±9.044	0.38	11.66±5.12	0.002
B220*	9±3.12	7±3	0.206	45.33±17.44	1.26E-5
CD43+	64.9±21.54	73.57±16.0	0.381	53.83±34.67	0.44
CD19+	7.2±3.96	5.85±12.87	0.75	1.16±1.16	0.002
CD4+	8.45±4.03	2.71±1.49	0.02	1.33±1.50	0.0009
CD3+	6.02±1.21	3.71±3.45	0.08	2.5±2.07	0.0007
CD8+	2.95±1.49	0.71±0.75	0.002	0.33±0.51	0.0009

Table A7 Average, standard deviation and statistical analysis of different surface markers on the spleen cells from the leukemic CA+/Vav-Cre+mice (myeloid leukemia N=7, biphenotypic leukemia N=6) and the mice from our control group (N=11)

Surface Marker	Control Sample, N=11 Average%±St.deviation	Leukemic Sample (Average %±St.deviation)			
		Myeloid N=7	P Value	Biphenotypic ,N=6	P Value
Gr1+	14.09±7.8	37±19.40	0.002	22.5±12.46	0.105
Mac1+	10.9±9.85	32.28±12.32	0.0008	57.5±8.87	8.23E-8
Gr1+/Mac1+	5.9±6.87	24.28±13.40	0.001	20.66±14.03	0.009
Sca1+	59.63±9.35	31.57±12.43	5.14E-5	11.83±6.79	1.45E-8
cKit+	4.75±4.7	21.71±24.86	0.03	7.33±3.50	0.25
B220*	30.27±12.58	25.14±12.37	0.408	57.5±6.44	0.0001
CD43+	68±10.56	64.14±19.30	0.602	39.33±27.17	0.008
CD19+	23.3±12.41	11.28±7.91	0.04	8.5±6.09	0.01
CD4+	21.09±9.57	13.14±6.93	0.003	5±3.16	0.001
CD3+	42.4±17.29	7.71±4.15	0.0007	7.66±5.31	0.0003
CD8+	19.54±7.04	7.14±4.87	0.0009	3.33±1.75	6.49E-5

Table A8: The latency of the leukemia, YFP positivity and immunophenotype of the hematopoietic tissues of the individual leukemic animals from tr-IgH-CA&BMI1 experimental group. PB-peripheral blood, BM- bone marrow, Spl-spleen

Mouse Id	Latency (days)		GFP %	Gr1 %	Mac1 %	GM %	Sca1 %	cKit %	B220%	CD4 %	CD8 %	CD4/ CD8 %
5619A#1	219	PB	1	13	0	0	28	0	6	98	75	99
		BM	18	58	17	17	27	11	3	53	4	4
		Spl	14	45	4	4	66	5	13	92	63	63
5619A#2	246	PB	9	55	65	65	53	37	50	30	38	
		BM	4	80	78	80	25	44	35	26	16	
		Spl	17	22	26	26	88	14	63	56	25	
5619B#1	341	PB	42	46	76	75	42	0	2	16	3	
		BM	43	55	66	73	51	1	3	27	1	
		Spl	23	35	37	44	65	0	23	23	4	
5619B#2	461	PB	32	24	46	29	66	2	23	38	9	
		BM	12	62	50	37	11	4	6	10	2	
		Spl	28	15	13	9	60	1	33	23	8	
5625A#1	101	PB	21	8	11	1	8	12	1	7	10	
		BM	12	62	50	37	11	4	6	10	2	
		Spl	28	15	13	9	60	1	33	23	8	
5625A#2	197	PB	30	41	19	19	65	18	25	36	25	
		BM	86	65	13	13	91	34	22	54	3	
		Spl	52	41	7	7	73	11	20	48	6	
5625B#1	123	PB	0	11	8	7	40	1	0	9	23	
		BM	3	51	60	51	7	19	0	4	4	
		Spl	3	10	14	12	29	14	17	10	12	
5625B#2	101	PB	0	10	1	0	85	0	0	79	77	77
		BM	3	26	6	0	16	23	4	19	8	2
		Spl	23	9	0	0	75	3	24	37	13	6

Table A9: Summary of the percentage of survival of the leukemic and non leukemic animals from the following experimental group: tr-lgH-CA&BMI1,WT-FVB&BMI1,WT-FVB&MIY, tr-lgH-CA&MIY and the secondary transplanted animals.

Days (Post transplantation)	Survival IgH-CA&BMI1 (N=12) (%)	Survival WTFVB&BMI1 (N=12) (%)	Survival WTFVB&MIY(N=9) (%)	Survival IgH-CA&MIY(N=7) (%)	Survival Secondary transplant(N=6) (%)
0	100	100	100	100	100
18	100	100	100	100	66.7
25	100	100	100	100	33.4
101	83.34	100	100	100	33.4
123	75.01	100	100	100	33.4
158	75.01	100	100	85.7 (censored)	33.4
182	75.01	100	77.78 (censored)	85.7	33.4
197	66.68	100	77.78	85.7	33.4
219	58.35	100	77.78	85.7	33.4
246	50.02	100	77.78	85.7	33.4
341	41.69	75.01 (censored)	77.78	85.7	33.4
416	33.36	75.01	77.78	85.7	33.4

Table A10: Summary of the percentage of survival of the leukemic and non leukemic animals from the following experimental group: CA-MF, CA-MF&BMI1, WT-FVB&BMI1, WT-FVB&MIY

Days (post-transplantation)	Survival CA-MF N=11 (%)	Survival CA-MF&BMI1,N=10 (%)	Survival WTFVB&MIY(%), N=9	Survival WTFVB&BMI, N=7 (%)	Survival Secondary transplant,N=2 (%)
0	100	100	100	100	100
31	100	100	100	100	0
43	90.91	100	100	100	0
71	81.82	100	100	100	0
78	72.73	100	100	100	0
86	63.64	90	100	100	0
86	54.33	90	100	100	0
99	45.46	90	100	100	0
103	36.37	80	100	100	0
108	36.37	70	100	100	0
110	27.28	50	100	100	0
114	27.28	40	100	100	0
127	27.28	30	100	100	0
133	27.28	20	100	100	0
137	27.28	10	100	100	0
171	18.19	0	100	100	0
176	18.19	0	77.78 (censored)	100	0
182	9.1	0	77.78	100	0
261	0	0	77.78	100	0

Table A.11: Summary of the latency of leukemia, GFP positivity and the immunophenotype of the cells from different hematopoietic organs of mice transplanted with CALM/AF10-minimal fusion. PB-peripheral blood, BM- bone marrow, Spl-spleen

Mouse Id	Latency (days)		GFP %	Gr1 %	Mac1 %	GM %	Sca1 %	cKit %	B220%	CD4 %	CD8 %
5781A#120	110	PB	84	22	61	6	8	0	17	6	2
		BM	36	88	80	74	2	0	0	2	0
		Spl	43	42	32	20	2	0	10	1	0
5800#144	71	PB	60	60	98	72	27	34	66	4	3
		BM	49	84	95	84	18	61	76	14	24
		Spl	32	51	76	51	12	17	74	17	10
5800#143	86	PB	92	68	87	62	35	6	17	0	0
		BM	73	91	82	82	0	0	11	3	
		Spl	10	50	32	30	1	0	44	0	0
5800#145	86	PB	56	40	90	46	17	68	66	0	0
		BM	44	75	89	74	6	41	58	0	0
		Spl	42	33	57	33	10	20	45	0	0
5806#147	103	PB	38	8	18	6	7	5	12	0	5
		BM	55	56	72	56	10	17	12	8	0
		Spl	26	21	38	8	8	10	55	3	3

Table A.12: Summary of the latency of leukemia, GFP positivity and the immunophenotype of the cells from different hematopoietic organs of mice transplanted with CALM/AF10-minimal fusion and BMI1 (CA-MF&BMI1)

PB-peripheral blood, BM- bone marrow, Spl-spleen

Mouse Id	Latency (days)		GFP %	Gr 1 %	Mac 1%	GM %	Sca 1%	cKit%	B220 %	CD4 %	CD8 %
5799A#133	86	PB	53	23	85	23	22	16	32	0	0
		BM	45	25	84	25	15	38	74	13	21
		Spl	32	14	69	14	17	26	85	12	18
5799A#134	110	PB	86	8	66	0	9	61	0	0	0
		BM	83	4	62	0	2	75	0	0	0
		Spl	79	6	54	2	4	60	5	0	0
5799B#142	133	PB	60	2	62	11	2	50	20	0	0
		BM	51	4	47	4	0	53	15	0	0
		Spl	44	0	32	0	4	38	0	0	0
5788A#158	127	PB	64	6	8	6	7	5	0	0	0
		BM	83	42	54	42	2	29	0	0	0
		Spl	64	68	57	57	7	26	11	0	0
5807A#138	103	PB	65	16	37	16	7	4	0	0	0
		BM	59	11	90	9	1	17	0	0	0
		Spl	49	10	24	4	8	15	8	0	0
5807A#139	108	PB	76	4	33	4	7	21	39	0	26
		BM	74	46	69	25	2	48	44	4	32
		Spl	43	20	70	12	4	45	80	2	9
5807A#149	110	PB	33	31	59	31	4	14	51	1	3
		BM	20	66	86	68	1	5	67	0	0
		Spl	30	25	48	12	2	4	3	0	0

Table A13: Summary of the average percentage and the standard deviation of different surface markers in the different hematopoietic tissues from the wild type controls and the leukemic mice from group CA-MF&BMI1 and CA-MF

PB-peripheral blood, BM- bone marrow, Spl-spleen

Surface marker		CA-MF&BMI1, N=7	CA-MF, N=5	WT-FVB, N=3
	PB	12.81±10.87	39.60±25.16	23.66±9.60
Gr1	BM	28.29±23.85	78.80±32.61	36±3.46
	Spl	20.43±22.58	39.40±12.58	24.33±4.04
	PB	50±25.59	70.80±32.61	17.33±4.01
Mac1	BM	70.29±16.84	83.60±8.79	21.33±4.16
	Spl	50.57±17.45	47.00±19.18	11.66±1.52
	PB	13±11.08	38.40±31.00	8.33±3.21
Gr1/Mac1	BM	24.71±24.01	74.00±11.05	18±3.46
	Spl	14.43±19.56	28.40±15.98	6±1
	PB	8.29±6.47	18.80±12.13	41.66±6.50
Sca1	BM	3.29±5.22	7.20±7.16	20±2
	Spl	6.57±5.03	6.60±4.88	60.33±5.50
	PB	24.43±22.28	22.60±28.67	0.66±0.57
cKit	BM	37.86±23.47	23.80±26.73	26±6.92
	Spl	30.57±18.80	9.40±9.32	7±1
	PB	20.29±21.08	35.60±27.83	23±2
B220	BM	28.57±32.69	31.40±33.45	29.33±1.15
	Spl	27.43±37.81	45.60±23.27	50.33±9.29
	PB	0.14±0,38	2.00±2.83	34.66±26.10
CD4	BM	3.29±4.92	5.40±5.64	33.33±9.07
	Spl	2±4.47	4.20±7.26	32.33±4.04
	PB	4.14±9.70	2.00±2.12	37.33±9.01
CD8	BM	7.57±13.31	4.80±10.73	21±1
	Spl	3.86±7.08	2.60±4.34	30.33±4.61

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