The role of stroma in the development of acute myeloid leukemia (AML)

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Dedication

To the souls of my mother and father; the greatest persons in my life, who inspired me for the right way and they gave me everything above what they can.

To my lovely wife Zulfa.

To my great children Al-Zahra, Ahmed, Abdullah and Moneeb.

To all my family, especially my brother Mohammed.

To every scientist and hard-worker in the world.

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1. Abbreviations

AAM	AML-associated macrophage
AF9	alfa-fetoprotein 9
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMSC	AML-associated MSCs
ANLL	acute non-lymphoblastic leukemia
Arg1	Arginase 1
Bax	Bcl-2-associated X protein
BM	bone marrow
BMDM	bone marrow-derived macrophage
BCG	Bacillus Calmette Guerin
CMPs	common myeloid progenitors
CD	cluster of differentiation
CEBPA	CCAAT/enhancer-binding protein alpha
CLL	chronic myeloid leukemia
CML	chronic myeloid leukemia
CN-AML	cytogenetically normal –AML
CO2	Carbon diooxide
CSF	colony stimulating factor
CXCR4	CXC-Motiv-Chemokin receptor 4
DNA	deoxyribonucleic acid
DPBS	Dulbecco's' phosphate buffer saline
EDTA	Ethylenediaminetetraacetic acid
ETO	Eight Twenty One
FAB	French-American-Britisch
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc receptors	FcgRs
FLT3	fms like tyrosine kinase 3
FLT3/ITD	FLT3 internal tandem duplication
FR	favorable-risk
*GFI1*36N	a single nucleotide polymorphism of *GFI1* with the replacement of a serine by an asparagine in amino acid

	position 36
GFI1/Gfi1	Growth factor independence 1
GFP	Green fluorescent protein
GM-CSF	granulocyte monocyte –CSF
GMPs	granulocyte monocyte progenitors
Gy	Gray
H3K4	Lysine 4 of histone 3
H3K9	Lysine 9 of histone 3
HBSS	Hank’s balanced salt solution
HCL	Hydrochloric acid
HDAC	histone deacetylase
HOXD13	Homeobox D13
HSC	hematopoietic stem cell
K-ras	Kirsten rat sarcoma viral oncogene
IL	interleukin
immMSCs	Immortalized MSCs
INF	interleukin
JAK	Janus kinase
KD	knock down
KO	knock out
LAMs	Leukemia-associated macrophages
LPS	lipopolysaccharides
LSC	leukemic stem cells
LSD1	lysine demethylase1
M1	Macrophage 1
M2	Macrophage 2
M-CSF	monocyte–CSF
MDS	Myelodysplastic syndrome
MEP	megakaryocytes erthroid progenitors
MIC	Morphology, immunology and cytogenetic classification
Min	Minute
mg	milligram
MHC II	major histocompatibility complex II
ml	melliliter

Abbreviations

MLL/AF9	Mixed Lineage Leukemia/alpha fetoprotein
MM	multiple myeloma
Mo-MuLV	Moloney murine leukemia virus
MSCs	mesenchymal stromal (stem) cells
NF-kB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
ng	nanogram
NO	nitric oxide
Nos2	Nitric oxide synthase 2
NPM1	Nucleophosmin1
NSG	NOD scid gamma
NUP98	Nucleoprotien 98
OBCs	osteoblastic lineage cells
PB	peripheral blood
Pdgfrα	platelet-derived growth factor receptor-alpha
PIAS3	Protein Inhibitor Of Activated STAT 3
ROS	reactive oxygen species
RUNX1	Runt-related transcription factor 1
RT	Room temperature
Sca-1	Stem cell antigen-1
SCN	severe congenital neutropenia
SFRS2	Splicing factor, arginine/serine-rich 2
SIRPα	a signal regulatory protein α expressed mainly by myeloid cells
SNPs	single nucleotide polymorphisms
STAT	signal transducer and activator of transcription
TAMs	Tumor-associated macrophages
TGFß	transforming growth factor-beta
Tlr4	Toll-like receptor 4
TNF	tumor necrosis factor
TP53	Tumor protein 53
WHO	World Health Organization
Wnt	wingless and Int-1
WT	Wild-type

γ	Gamma
µl	microliter
µm	Micrometer
µM	Micromolar

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4. Introduction

4.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML), also called acute myelogenous leukemia or acute non-lymphoblastic leukemia (ANLL), is a malignant disorder of hematopoietic stem cells (Dohner, Weisdorf, and Bloomfield 2015; Ferrara and Schiffer 2013). It is the most common type of acute leukemia in adults and is characterized by an arrest of differentiation and an accumulation of immature myeloid progenitors in bone marrow (BM), peripheral blood (PB) and sometimes other organs such as spleen and liver (Dohner, Weisdorf, and Bloomfield 2015). The percentage of myeloblasts in the PB or BM must be more than 20% according to WHO (world health organisation) classification or more than 30% according to the French-American-British (FAB) classification (Hasserjian 2013). In contrast to acute lymphoblastic leukemia (ALL), which is more frequent in children, AML is more frequent in adults (about 80% in adult acute leukemia) (Bleyer et al. 2012; Belson, Kingsley, and Holmes 2007). AML is clinically aggressive because the accumulation of leukemic cells in the BM suppresses the normal hematopoietic development. As a result of the reduced production of normal blood cells a characteristic clinical picture of pancytopenia (anemia, neutropenia, and thrombocytopenia) is formed which together with high white blood cell counts (mostly) in the PB are the typical criteria of acute leukemia (Dohner, Weisdorf, and Bloomfield 2015). The prognosis of AML is still poor and new therapeutic approaches are urgently needed. Less than 50% of AML patients younger than 60 years and only 20% of patients older than 60 years are cured (Dohner, Weisdorf, and Bloomfield 2015).

4.2 Classification of AML

In leukemia, lymphoma and in most cancers, staging or classification is important to assess the severity and prognosis of the tumor (Hallek et al. 2008; Armitage et al. 2006; Travis et al. 2015). A correct prognosis helps to specifically address the therapeutic options for each subtype (Hasserjian 2013). For AML, there are actually three common classifications in use: the FAB, WHO, and MIC classifications ('Morphologic, immunologic and cytogenetic (MIC) working classification of the acute myeloid leukaemias). Second MIC Cooperative Study Group' 1988). In the 1970s, AML was classified by French, American, and British leukemia experts, into 7 classes

(FAB classification; table 14) depending on the cell morphology and cytochemistral staining (Bennett et al. 1985, 1976). Some AML classes have a rather good prognosis such as AML with maturation (M2) and acute promyelocytic leukemia (APML, M3), while other types have a poor prognosis such as undifferentiated leukemia (M0) and erythroleukemia (M6) (Bennett et al. 1985). In addition to the criteria used in the FAB classification, the WHO classification of AML includes other criteria such as genetic mutations, cytogenetics, clinical features, and immunophenotyping (Vardiman et al. 2009; Arber et al. 2016) (Table. 15). This classification gives some insights into the pathogenesis of the disease and thus enhances the treatment of AML patients resulting in a better outcome (Vardiman et al. 2009; Arber et al. 2016). Morphologic, immunologic and cytogenetic (MIC) classification was published in 1988 by the MIC group ('Morphologic, immunologic and cytogenetic (MIC) working classification of the acute myeloid leukaemias. Second MIC Cooperative Study Group' 1988). They added immunophenotyping to FAB-classified M1-M7 disease and put more emphasis on the importance of cytogenetics in characterization of AML (Table 16). They also added a new subgroup called M2baso that is characterized by the presence of basophilic granules in blasts and maturing granulocyte precursors.

4.3 Pathophysiology of AML

Hematopoiesis, the process of blood formation, is completely dependent on a pluripotent hematopoietic stem cell (HSC) which is considered to be the origin of all mature blood cells: erythrocytes, leukocytes, lymphocytes, megakaryocytes, and so on (Eaves 2015). The HSC can self-renew and also differentiate into different blood cells (multi-lineage differentiation). It can restore all blood components if transplanted into an irradiated recipient (Eaves 2015; Osawa et al. 1996). Under normal conditions, the process of HSC differentiation is under strict control by growth and differentiation factors and signals secreted mostly from the BM microenvironment in order to keep the level of all blood elements in the normal range, i.e. homeostasis, according to the bodies' requirements (Goodell 2015; Eaves 2015; Gottgens 2015). In AML, there is clonal proliferation of myeloblasts or myeloid immature progenitors such as promyelocytes due to defects in normal mechanisms of control such as a continuous arrest of differentiation, increased proliferation and inefficient apoptosis (Weissman 2000). This results in an accumulation of leukemic immature and non-

functional cells which replace the normal hematopoietic compartment in the BM and reduce the production of normal blood cells causing a disease phenotype (Giles et al. 2002).

4.4 Molecular pathology of AML

The abnormal proliferation of myeloid progenitors in AML has been considered to be triggered by mutations that occur in HSCs or in other more committed progenitors such as GMPs (granulocytes monocytes progenitors) or sometimes in both (Horton and Huntly 2012). These cellular leukemia-initiating lesions may include one or more cytogenetic abnormalities, genetic mutations, or epigenetic alterations (Grimwade, Ivey, and Huntly 2016; Licht and Sternberg 2005). A recent study showed that *MLL-AF9*-driven AML in a mouse model has two cellular origins, LT-HSCs (long term-HSCs) and GMPs which have different grades of aggressiveness (Stavropoulou et al. 2016). Up to now, many abnormalities have been detected in AML, which give us a better prediction of the outcome of AML treatment (Grimwade, Ivey, and Huntly 2016).

4.5 Cytogenetic abnormalities in AML

At present, cytogenetic aberrations are considered one of the most common predicting tools in AML diagnosis, treatment regimen selection, and follow-up of the patients (Grimwade et al. 1998; Byrd et al. 2002). Remarkably, the leukemic cells of the BM from 50-60% of AML patients harbour either structural or numerical cytogenetic abnormalities (Mrozek and Bloomfield 2006; Othman 2016). For example, the reciprocal translocations t(8;21)(q22;q22), which was the first cytogenetic abnormality discovered in AML in 1972 (Caspersson et al. 1972), and t(15;17)(q22;q12-21) have a good prognosis (Gowri et al. 2011). On the other hand, AML patients with a –5 karyotype or a del(7q) have a bad outcome (Mrozek and Bloomfield 2006; Othman 2016). See table 17 for the risk classification of cytogenetic abnormalities in AML patients.

4.6 Gene mutations in AML

More than 40 genes have been found to be mutated in adult *de novo* AML, which are related to the development of leukemia ('Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia' 2013; Kihara et al. 2014). Some of the

common gene mutations are present in *FLT3*, *NPM1*, *DNMT3A*, *RUNX1*, *TP53*, *SRSF2*, *CEBPA*, *KIT*, *KRAS,* and *NRAS* ('Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia' 2013; Metzeler et al. 2016; Othman 2016). According to the driving gene mutations, cytogenetically normal (CN)-AML has been classified into two risk groups, a favorable-risk (FR) group that includes either CN-AML with *NPM1* (Nucleophosmin1) but not *FLT3-ITD* (*FLT3* internal tandem duplication) mutations or with *CEBPA* mutations. In contrast, the intermediate-I-risk group includes patients with *FLT3-ITD* mutations and those with neither *NPM1* or *FLT3-ITD* mutations (Kihara et al. 2014; Shen et al. 2011; Ofran and Rowe 2013).

4.7 BM microenvironment, “BM niche”

The so-called BM niche is the closest cellular neighbourhood of normal and leukemic stem cells and contains a heterogeneous group of non-hematopoietic cells including mesenchymal stromal cells (MSC), endothelial cells, osteoblasts, osteocytes, adipocytes, and reticular cells as well as extracellular matrix elements (Jin-Xiang et al. 2004; Clark, Gallagher, and Dexter 1992). Two types of BM niches are distinguished today, the endosteal niche that is attached to trabecular or cortical bones and the perivascular niche that presents itself close to the sinusoidal vascular endothelium (Sanchez-Aguilera and Mendez-Ferrer 2016). The endosteal niche, or osteoblastic niche, contains high numbers of osteoblasts and osteocytes, and has been found to contain very primitive HSCs. This niche promotes the self-renewal and quiescence of HSCs (Mayack and Wagers 2008; Yin and Li 2006). The perivascular niche contains supportive structures such as MSCs and endothelial cells (Lane, Scadden, and Gilliland 2009). The perivascular niche plays an important role in differentiation and movement of HSCs and hematopoietic progenitor cells (Yin and Li 2006). In general, HSC growth is enhanced in the presence of stromal cells either *in vitro* or *in vivo* after co-transplantation (Sato et al. 1998; Kawada et al. 1999; Almeida-Porada et al. 1999). The self-renewal and differentiation of normal HSCs are tightly regulated and maintained and depend on secreted factors as well as signals provided by direct contact with the surrounding components of the BM niche (Martinez-Agosto et al. 2007; Sato et al. 1998; Clark, Gallagher, and Dexter 1992). Any alteration in the BM niche composition, e.g. mutations in the membrane-bound stem cell factor (*SCF*) gene, may result in an abnormal BM microenvironment and hence a disturbance of hematopoiesis *in vivo* (Barker 1994; McCulloch et al. 1965).

4.7.1 Mesenchymal stromal cells (MSCs)

MSCs (mesenchymal stromal cells or marrow stroma cells or mesenchymal stem cells) are present in almost all tissues of the body. They were first identified in the BM (Pontikoglou et al. 2011). They were firstly named as MSC in 1991 by Arnold Caplan (Caplan 1991). MSCs are spindle-shaped cells that have the capacity to adhere to plastic surfaces of culture plates and to differentiate into osteoblasts, chondrocytes, and adipocytes (Bianco, Robey, and Simmons 2008). Some authors suggested that differentiation capacity of MSCs depends on some other cell types such as endothelial cells, cardiac, neural and hepatocytes (Pontikoglou et al. 2011). There are also emerging evidences that MSCs have a self-renewal property comparable to HSCs (Sacchetti et al. 2007; Morikawa et al. 2009). Immunophenotypically, human BM-MSCs express CD73, CD91, CD44, and 105. They lack markers of HSCs including CD45, CD11b, CD14, and CD34 (Pontikoglou et al. 2011; Dominici et al. 2006). Murine MSCs express platelet-derived growth factor receptor-alpha (*Pdgfrα*), stem cell antigen-1 (Sca-1), CD44 and CD51, and they lack expression of HSC markers such as CD45 and TER119 as well as CD31 (a typical marker of endothelial cells) (Morikawa et al. 2009).

4.7.2 The role of MSCs in leukemia and lymphoma

In many solid tumors, lymphoma, and some leukemias, it has been found that the growth of malignant cells is not only the result of cell-intrinsic changes at the genetic and epigenetic level, but is also affected by the surrounding microenvironment, the stromal matrix, and the cells therein (Carlesso and Cardoso 2010; Tabe and Konopleva 2015; Hernanda et al. 2014). In the BM, the leukemic cells polarize and modify the different stromal elements of the BM niche including MSCs, endothelial cells, osteoblastic lineage cells, and immune cells to support the growth of LSCs instead of HSCs (Schepers et al. 2013; Lim et al. 2016). MSCs derived from MDS (myelodysplastic syndrome) and AML patients may bear genetic abnormalities that may have impact on the growth of leukemic cells (Blau et al. 2011). Another recent study has shown that a BM microenvironment that carries genetic abnormalities causes disturbance in the growth of HSCs and may result in AML or myeloproliferative-like disorders *in vivo* (Walkley et al. 2007; Kode et al. 2014). In *in vitro* experiments, MSCs also protect leukemic cells against chemotherapy (Ito et al.

2015; Pillozzi et al. 2011). In contrast to the supporting role of stroma in leukemia, there is an emerging evidence that MSCs can have inhibitory effects on growth of lymphoma cells but may still protect them against apoptosis by chemotherapy (Secchiero et al. 2010; Yang, Tang, and Chen 2014; Mraz et al. 2011). Thus a Janus head-like role of MSCs in leukemogenesis has emerged that demands further investigations.

4.8 Macrophages

Macrophages are a heterogeneous population of cells that form an important part of the innate immune system (Lavin et al. 2014). Macrophages are present throughout all tissues of the body with different names, e.g. Kupffer cells in the liver, microglia in the nervous system, alveolar macrophages in the lung, osteoclasts in the bone, histiocytic in the interstitial connective tissue. The cell of origin for macrophages is the monocyte, a large cell with a kidney-shaped nucleus that is present in the blood and BM (Gordon 2003). Monocytes are produced from GMPs in the BM under the control of M-CSF and GM-CSF and migrate to the PB (Gordon 2003; Gottgens 2015). Macrophages are immunophenotypically distinguished from other mononuclear cells by CD11b and F4/80 expression in the mouse and by CD14 and CD16 expression in humans (Yang et al. 2014; Zhang, Goncalves, and Mosser 2008; Bain et al. 2014). Growth and differentiation of monocytes are controlled by many cytokines such as GM-CSF, M-CSF, IL-3 (Gordon 2003). Monocytes subsequently move to the respective tissues of the body and differentiate into macrophages or dendritic cells, important antigen presenting cells of the immune system. This movement is dependent on the signals and secretions from immune cells and tissues damaged by infection (Murray and Wynn 2011). Beside macrophages, dendritic cells play also important role in innate immunity. Macrophages also stimulate the adaptive immune response through interaction with T-lymphocytes (Howard et al. 2004). The local and systemic signals determine the plasticity and heterogeneity of macrophages throughout the body (Davies et al. 2013; Ishimoto et al. 2014; Glass and Natoli 2016). The different signals, which are secreted from the surrounding tissues such as tumor cells, damaged tissues, bacteria or activated lymphocytes, stimulate macrophages to differentiate into various differentiation states with completely different characteristic phenotypes and functions (Mantovani et al. 2004). Here, the M1/M2 denomination is still one of the most common classifications for functionally

different macrophages (Mantovani et al. 2004; Murray et al. 2014), although more elaborate and distinctive classifications are proposed (Murray et al. 2014). In both activation states, there are specific profiles of gene expression, surface receptors expression and cytokine and chemokine secretions (Mantovani and Locati 2013). In response to different stimulators and signals, macrophages differentiate into either the “classical” activation state (M1 macrophages) with pro-inflammatory functions through toll-like receptor-4 (Tlr-4) or IFN receptors or into an alternative activation state (M2 macrophages) with anti-inflammatory function through IL-4/IL-13 regulation (Sica and Mantovani 2012). Due to the complexity of macrophage polarization with different cytokines and variation of expression of specific markers, some authors suggested that the activator should be added to the domination, for example M(LPS), M(IL-4) and so on (Mantovani et al. 2004). I think that a better way for nomenclature of macrophages polarization is the combination of the M1/M2 system with the above mentioned suggestion. For example, M1 macrophages polarized by LPS will be named M1(LPS) and M2 that is polarized with IL-4 will be named M2(IL-4).

4.8.1 Classically activated macrophages (M1 macrophages)

The classically activated macrophage has been first described in 1960s by enhancing of antimicrobial activity of macrophages with infection of mice with *Mycobacterium bovis bacillus Calmette Guerin* (BCG) or *Listeria monocytogenes* (Gordon 2003; Mantovani et al. 2002). This classical immune activation state (M1) of macrophages is driven either by a stimulator secreted from T helper lymphocytes 1 such as interferon-y (IFN-y), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), or by bacterial components and secretions such as lipopolysaccharide (LPS) (Sica and Mantovani 2012). Recent studies showed that GM-CSF can also polarize macrophages towards a M1 activation state (Joshi et al. 2014; Glass and Natoli 2016). IFN-y binds to its receptors on macrophages and activates STAT1 (signal transducer and activator of transcription1), while LPS binds to Tlr-4 and activates both STAT5 and NF-kB (kappa light polypeptide gene enhancer) which leads to pro-inflammatory responses (Mantovani et al. 2004; Martinez and Gordon 2014; Glass and Natoli 2016). In addition to the function of M1 macrophage as an antigen presenting cell and a potent phagocytic cell that kills invading microorganisms and tumor cells, it secretes numerous inflammatory mediators that stimulate further

immune responses such as interleukin-6 (IL-6), reactive oxygen species (ROS), and nitric oxide (NO) (Longbrake et al. 2007; Martinez and Gordon 2015). The immune response of M1 macrophages is antigen non-specific. M1 macrophages can also be differentiated from M2 macrophages by intracytoplasmic IL-10^{low} and IL-12^{high} and they do not express CD206 which is typical for M2 macrophage *in vitro* (Mantovani et al. 2004).

4.8.2 Alternatively activated macrophages (M2 macrophages)

Alternatively activated macrophages or M2 macrophages are stimulated by different cytokines such as interleukin-4 (IL-4), IL-13 or both and have anti-inflammatory activity (Mantovani et al. 2004; Murray et al. 2014). M2 macrophages produce arginase, transforming growth factor-beta (TGFß), IL-10 and metalloproteinases that subsequently result in immune suppression, angiogenesis, and tissue repair (Mantovani et al. 2002). The expression of major histocompatibility complex II (MHC II) proteins is also increased in M2 macrophages (Martinez and Gordon 2014). The alternative activation of macrophages is common in allergy, cellular and humoral responses to parasites and extracellular pathogens (Gordon 2003). In contrast to the function of M1 macrophages that inhibit tumor growth, M2 macrophages have been proven in many cases to play an important role in the development and progression of different tumors (Chittezhath et al. 2014; Franklin and Li 2014; Bingle, Brown, and Lewis 2002). According to the polarizing cytokines, M2 macrophages are further divided into M2a, M2b, M2c, and M2d macrophages (Mantovani et al. 2004; Duluc et al. 2007) (Figure 1). The best surface marker for human and murine M2 macrophages is the scavenger receptor CD206 (Joyce and Pollard 2009). M2 macrophages can also be phenotypically characterized by intracytoplasmic expression of IL-10 at high and IL-12 at low levels (Mantovani et al. 2004).

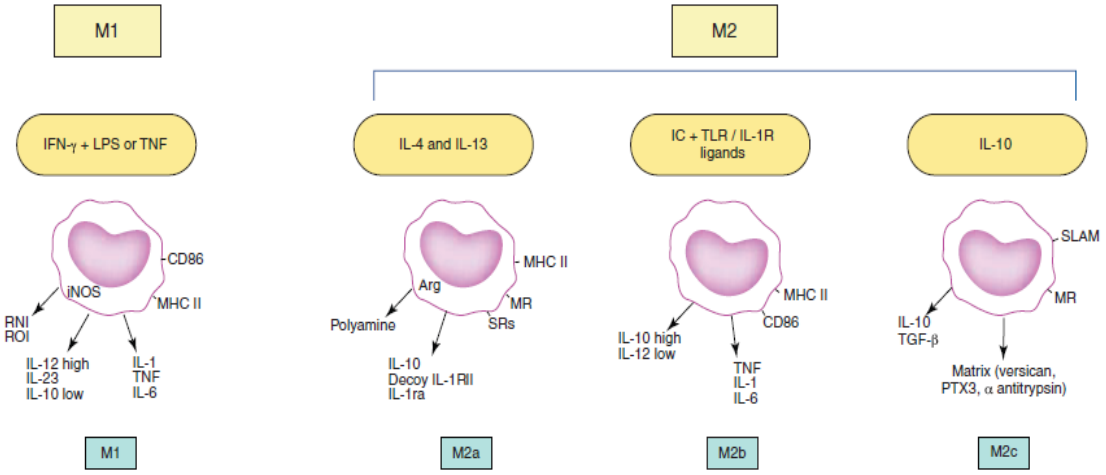

Figure 1: Classification of macrophages.

Mechanism of macrophage polarization into M1 or M2 activation states and their subclasses with different cytokines used in the polarization. Under stimulation by different cytokines, each class of macrophages will express specific cell markers for example iNOS for M1, Arg for M2a and mixed markers for M2b macrophages. Adapted from (Martinez and Gordon 2014; Mantovani et al. 2004).

4.8.2.1 M2a (alternative) macrophages

M2a macrophages are induced by treatment with IL-4 alone or with IL-13 (Mantovani et al. 2004; Murray et al. 2014). In M2a macrophages, the arginase pathway is enhanced and more ornithine and polyamines are generated (Mantovani et al. 2004). IL-4 binds to IL-4Rα1 and IL-13 binds to IL-13Rα2 receptors on macrophages. This leads to recruitment of JAK1 and JAK3 and subsequent activation of STAT6 to produce anti-inflammatory cytokines, enzymes and markers (Mantovani et al. 2004; Martinez and Gordon 2014).

4.8.2.2 M2b (Type II) macrophages

In the M2b activation state, macrophages are stimulated by immune complexes and LPS (Martinez and Gordon 2014). The activation of FcgRs (Fc receptors for IgG) on these macrophages results in reduced IL-12 expression and enhanced production of IL-10 (common markers of M2 macrophages). , But in contrast to M2 macrophages these activated macrophages still produce some inflammatory cytokines such as TNF and IL-6 (Martinez and Gordon 2014; Mosser 2003).

4.8.2.3 M2c (Deactivated) macrophages

The IL-10 cytokine is the stimulator of M2c macrophages. IL-10 binds to macrophages through IL-10R and activates STAT3, p50and NF-kB (Sica and Mantovani 2012; Martinez and Gordon 2014). Accordingly, arginase is activated and more ornithine and polyamines are generated, and pro-inflammatory cytokines are produced (Mantovani et al. 2004). The M2c macrophage plays an important role in tissue repair (Mantovani et al. 2013).

4.8.3 Tumor associated macrophages (TAM)

Recently, it has been shown that tumor cells induce the surrounding stroma, in particular immune cells therein, mainly macrophages, to produce various factors and to secrete certain cytokines which, instead of activating the immune system to attack the tumor, promote further growth and spread of tumor cells (Bingle, Brown, and Lewis 2002). The polarization of stromal cells by tumor cells is the result of a complex bi-directional interaction and communication between tumor and stroma (Bingle, Brown, and Lewis 2002). Hence, the polarized macrophages in tumors are denominated tumor-associated macrophages (TAMs). These TAMs are one of the most common stroma immune cells in the microenvironment of most solid tumors such as lung cancer, breast cancer, colorectal cancer, etc. (Chittezhath et al. 2014; Candido and Hagemann 2013; Sousa et al. 2015; Quatromoni and Eruslanov 2012; Zhang et al. 2013). It has been shown that a high macrophage density can be correlated with a poor prognosis of patients (Franklin et al. 2014). Interestingly, the depletion of macrophages in many mouse models reduced the size of tumors and slowed down the tumor progression (Laoui et al. 2014; Movahedi et al. 2010; Franklin et al. 2014). Although TAM in most tumors are potentially M2-like macrophages and show tumor-promoting functions such as induction of angiogenesis and invasion support, some studies showed that TAMs can have a gene expression profile similar to both M1- and M2-like macrophages, or they show their own specific characteristics (Laoui et al. 2014; Chittezhath et al. 2014; Chen et al. 2015). Due to the effects of signals of different tissues on macrophages, TAMs may have a variety of

immunophenotypic markers in different tumors (Laoui et al. 2014; Harris et al. 2012; Geissmann, Jung, and Littman 2003; Beider et al. 2014).

4.8.4 TAM in hematological malignancies

In acute lymphoblastic leukemia (ALL), leukemia–associated macrophages (LAMs) sorted from the BM and spleen of mice had a higher migration activity and supported the growth of leukemic cells *in vitro* (Chen et al. 2015). They also expressed higher levels of most genes specific for both M1- and M2-like macrophages (Chen et al. 2015). Clinically, it has been found in patients with classic Hodgkin's lymphoma that an accumulation of TAM in lymph nodes was related with a poor prognosis (Steidl et al. 2010). It has also been shown that macrophages play an important role in tumorigenicity of multiple myeloma (MM) where MM cells recruit macrophages and enhance their polarization into an M2-like activation state with a high expression of CD206, that in turn supports the growth of MM cells and protects them against the effect of chemotherapy (Beider et al. 2014). Interestingly, in chronic lymphocytic leukemia (CLL), macrophage depletion impaired the growth of leukemic cells and improved the survival of leukemic mice (Galletti et al. 2016). It has recently been reported that the engraftment of human LSCs in immunocompromised NSG mice was impaired by inhibition of SIRPα (signal regulatory protein α expressed mainly by myeloid cells) signalling in macrophages (Theocharides et al. 2012). In AML patients, macrophages were found to accumulate around leukemic cells (Gao, Yu, and Zhang 2014) and to support the growth of CD34-positive cells *in vitro* better than normal macrophages (Li et al. 2015).

4.9 Growth factor independence 1 (Gfi1)

4.9.1 Gfi1 gene and protein

Many years ago, the mouse *Gfi1* gene region was identified as a pro-viral insertion site in the absence of IL-2 in Moloney murine leukemia virus (Mo-MuLV)-induced rat T cell lymphoma cells (Gilks et al. 1993). The *GFI1* gene is located on chromosome 1 in humans and on chromosome 5 in mouse (Moroy 2005; Vassen et al. 2005). As a protein, Gfi1 has 423 amino acids. It has six zinc finger domains at its carboxyl terminus through which it can bind to DNA via an AATC sequence in the target genes (Moroy 2005; Vassen et al. 2005) (Figure.1- 2). From the six zinc finger, only the

third, fourth and fifth are required for DNA binding (Moroy 2005). At the amino terminal sequence, Gfi1 contains a SNAG domain consistent of 20 amino acids that is commonly found in some other proteins such as Snail and Slug (Moroy 2005) (Figure 1-2). The SNAG domain as well as the intermediate domain and the first, second, and sixth zinc finger interact with other proteins (Moroy 2005). Gfi1 acts as a transcriptional repressor by recruiting histone-modifying enzymes that add or remove acetyl or methyl groups to histone proteins resulting in silencing of the target genes.

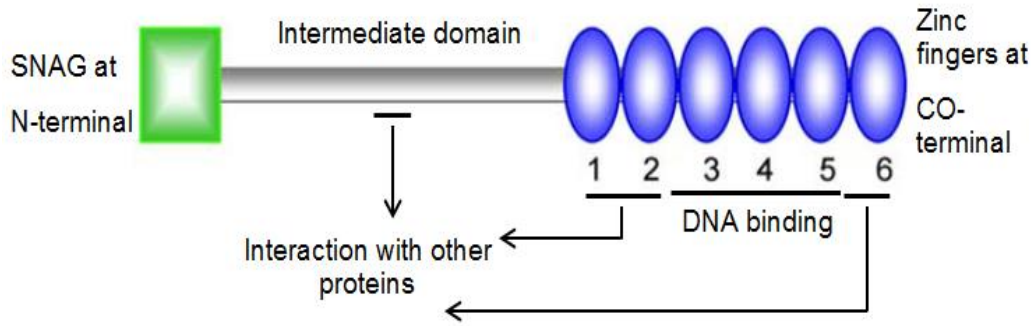

Figure 2: Gfi1 Protein.

Gfi1 is comprised of a SNAG domain (green) at the amino end which is critical for the suppressive function of Gfi1 and six zinc fingers at the carboxyl end (blue) through which it binds to the target DNA via zinc fingers 1, 2 and, 6 and interacts with other proteins via zinc fingers 3, 4 and 5. The intermediate domain (colorless) binds and interacts with other proteins. Adapted from (Moroy 2005; Moroy and Khandanpour 2011)

4.9.2 Function of Gfi1

The *Gfi1* gene is expressed in HSCs, early progenitors, CMPs (common myeloid progenitors), GMPs, MEPs (megakaryocytes erythroid progenitors) and common lymphoid progenitors as well as in mature granulocytes and monocytes (Moroy and Khandanpour 2011; Karsunky et al. 2002). In non-hematopoietic tissues, *Gfi1* is expressed in sensory epithelia of the inner ear, the central nervous system, and epithelia of the lung and intestine (Moroy et al. 2015). Gfi1 can repress its own expression through auto-regulatory feedback mechanisms (Yucel et al. 2004). Functionally, Gfi1 recruits histone-modifying enzymes such as histone deacetylases (HDAC), histone demethylases (LSD1), corepressor for REST (CoREST) and histone methyl transferases (G9a) to the target genes. These histone-modifying enzymes

alter the chromatin structure, especially at H3K4 and H3K9, resulting in silencing of the target genes (Moroy et al. 2015) (Figure 3)

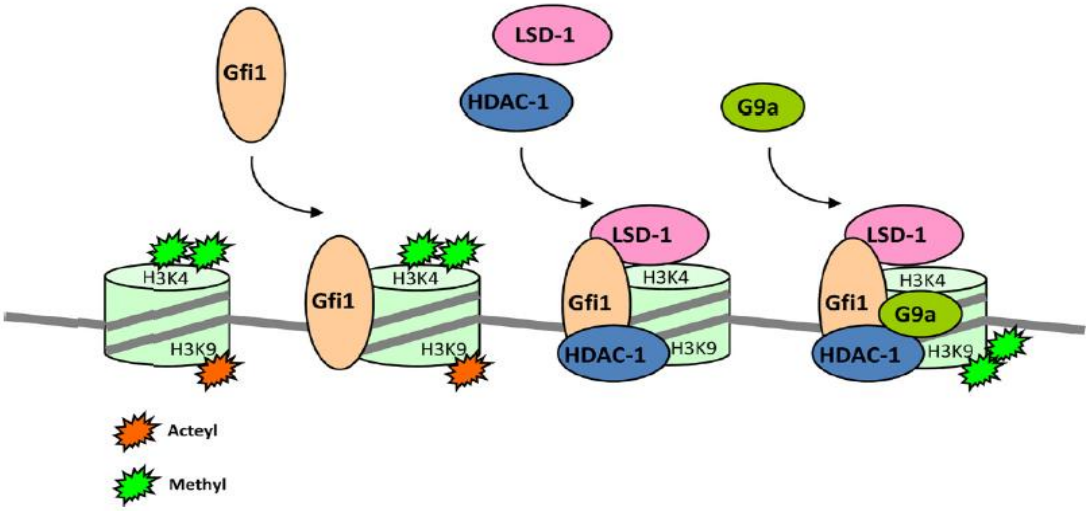

Figure 3: The mechanism of Gfi1 acting as a transcriptional repressor.

Gfi1 is a transcriptional repressor protein. The different histone modifying enzymes are recruited to DNA by the SNAG domain of Gfi1 in order to form a large functional protein complex which adds or removes acetyl or methyl groups from histones resulting in silencing of the target gene. For example, LSD-1 removes a methyl group from H3K4 (Lysine 4 of histone H3) and HDAC-1 removes acetyl groups from H3K9 ((Lysine 9 of histone H3). In the presence of G9a (histone methyl transferases), two methyl groups are added to H3K9 resulting in irreversible silencing the target gene. Adapted from (Moroy and Khandanpour 2011).

Many genes have been discovered to be targets for regulation by Gfi1 such as *E2F5*, *E2F6*, *p21*, *Myc* and *Elane* (encoding neutrophil elastase (Moroy 2005). Gfi1 is also involved in activation of STAT3 through inhibition of PIAS3 and regulation of other signalling pathways such as the *STAT5*, *IL-6, IL-10, IL-2 and G-CSF* signalling cascades (Moroy 2005). In hematopoietic tissues, *Gfi1* plays an important role in HSC maintenance and quiescence (Moroy 2005; Moroy et al. 2015; Duan and Horwitz 2005). *Gfi1* has also an anti-apoptotic function in HSCs by inhibiting pro-apoptotic gene products (e.g. Bax) (Khandanpour et al. 2011). Moreover, the differentiation of B- and T- cells, the selection of T-cells and the function of mature B cells towards antigens were all disrupted in *Gfi1*-deficient mice, indicating its role in lymphoid commitment, development, maturation and function (Moroy and Khandanpour 2011) (Moroy and Khandanpour 2011). Gfi1 plays also an important

role in the development of granulocytes and is essential for cytokine production by monocytes and macrophages (Person et al. 2003). *Gfi1*-deficient mice are characterized by reduced self-renewal of HSCs, low B- and T-lymphocyte counts, a severe neutropenia and an overproduction of TNF-α and other inflammatory mediators of macrophages exposed to bacterial endotoxin or LPS (Person et al. 2003; Moroy et al. 2008; Khandanpour et al. 2011). *Gfi1*-null myeloid cells cannot differentiate into granulocytes in the presence of G-CSF *in vitro* (de la Luz Sierra et al. 2010). Two common single nucleotide polymorphisms (SNPs) of *GFI1* have been described in patients with severe congenital neutropenia (SCN), namely N382S (as a result asparagine is replaced by serine at position 382 of the protein) (Zarebski et al. 2008) and K403R (arginine is replaced by lysine at position 403 of the protein) (Person et al. 2003). In BM stroma, *Gfi1* also plays an important role in differentiation of osteoblasts (D'Souza et al. 2011).

Gfi1b, another transcriptional repressor, is a homolog of *Gfi1* that plays an important role in erythropoiesis and megakaryopoiesis (Moroy 2005; Vassen et al. 2005; Moroy et al. 2015).

4.9.3 The role of Gfi1 in leukemia and other disorders

As mentioned above, Gfi1 is a transcriptional repressor that plays an important role in the development of normal and leukemic HSCs. Recently, it has been found that *GFI1* expression is upregulated in stromal osteoblasts in the BM of MM patients (D'Souza et al. 2011). GFI1 represses *RUNX2* expression that subsequently depresses the differentiation of MSCs into osteoblasts, inducing the common lesion in the bones of MM patients. Furthermore, *GFI1* knock down (KD) MSCs from MM patients restored their capability of differentiation into osteoblasts (D'Souza et al. 2011). Regarding lymphoid disorders, *Gfi1* is involved in the development and maintenance of T-ALL and other lymphoid leukemias, and its deletion completely prevents the development of transgenic T-ALL (Phelan et al. 2013; Khandanpour and Moroy 2013). The role of Gfi1 in the pathology of AML is still not clear, but it has been found that low expression of *GFI1* is associated with a poor prognosis in AML patients (Hones et al. 2016b). In AML mouse models, *Gfi1*-KD animals died earlier than *Gfi1*-WT (*Gfi1*-wild type) mice (Hones et al. 2016b) and in *GFI1* overexpressing transgenic mice the onset of AML was delayed (unpublished data). Moreover, the

genetic variant of *GFI1, GFI1*36N (a single nucleotide polymorphism with the replacement of a serine by an asparagine in amino acid position 36), is associated with a shorter survival in patients and mice with AML (Botezatu et al. 2016). In chronic myeloid leukemia (CML), it has been observed that Gfi1 can inhibit the proliferation and survival of CD34+ CML cells *in vitro* (Soliera et al. 2012). In non-hematopoietic malignancies, the expression of *GFI1* was recently detected in human lung tumor cells and in a pulmonary neuroendocrine cell line and its deletion depressed the proliferation of these cells (Linnoila et al. 2007).

5. The aims and hypothesis of the study

The outcome of patients with AML is still poor and only about 25 % of patients have a five-year overall survival rate (Dohner, Weisdorf, and Bloomfield 2015). As already established in many solid cancers it is likely that AML is not only caused by intrinsic genetic abnormalities of HSCs and LSCs, but also by the effects and signals produced by the surrounding microenvironment that includes different stromal cells and immune cells, mainly macrophages (Chittezhath et al. 2014; Schepers et al. 2013; Lim et al. 2016). Gfi1, a transcriptional repressor, plays an important role in the development and function of immune cells such as lymphocytes, granulocytes and monocytes (Person et al. 2003; Moroy et al. 2008; Khandanpour et al. 2011). It is also involved in differentiation of osteoblasts (D'Souza et al. 2011). Little is known about the role of MSCs and macrophages with regard to support of the growth of AML cells. Understanding the interaction between the microenvironment elements such as AAMs and AMSCs and leukemic cells could open the path to new therapeutic approaches which result in a better prognosis.

Thus, the aims of this study were the following:

- To investigate the role of macrophages in the growth of AML cells in *vitro* and *in vivo*.
- To investigate the role of MSCs in the growth of AML cells *in vitro* and *in vivo*.
- To characterize the AAMs and AMSCs.
- To study the role of Gfi1 in the polarization and function of AAMs and AMSCs.

6. Materials and methods

6.1 Materials

6.1.1 Chemicals

Table 1. List of reagents and chemicals used in this study

Reagents and chemicals	Cat. Nr.	Supplier
2-Mercapthoethanol	SHBG3397V	Sigma-Aldrich, Taufkirchen, Germany
Adipogenesis differentiation medium	A10410-01	Gibco, Life Technologies, Darmstadt, Germany
Alcian Blue	BCBP8133V	Sigma-Aldrich, Taufkirchen, Germany
Alizarin Red S	A5533-25G	Sigma-Aldrich, Taufkirchen, Germany
Alpha-MEM	12571063	Gibco, Life Technologies, Darmstadt, Germany
Ammonium chloride lysing reagent	07800	BD Biosciences, Heidelberg, Germany
Annexin V Binding Buffer	5281679	Biosciences, Franklin Lakes, United States of America (U.S.A.)
Aqua water	172328001	B. Braun, Melsungen, Germany
Baytril	KP09V34	Bayer, Leverkusen, Germany
BrdU reagents for cell cycle	552598	BD Pharmingen™, Heidelberg; Germany
Collagenase 1	LS004194	Worthington, Troisdorf, Germany
COULTER CLENZ® Cleaning Agent	50156F	Beckman Coulter, Pasadena, U.S.A.
DPBS	1869854	Gibco, Life Technologies, Darmstadt, Germany
DMEM	1858670	Gibco, Life Technologies, Darmstadt, Germany
DMEM-Glutamax	185860	Gibco Life Technologies,

		Carlsbad, U.S.A.
Absolute ethanol	SZBG2920H	Honey well, Seelze, Germany
Formaldehyde	F8775-25ML	Sigma-Aldrich, Taufkirchen, Germany
FACS Clean	340345	BD Biosciences, Heidelberg, Germany
FACS Flow	1716502821	BD Biosciences, Heidelberg, Germany
FACS Rinse	340346	BD Biosciences, Heidelberg, Germany
fetal bovine serum	P30-3702	PAN^{TM} BIOTECH, Aidenbach, Germany
HBSS	1532361	Gibco, Life Technologies, Darmstadt, Germany
H_3PO_4	12352106	Honeywell Fluka™, Bucharest, Romania
HCL	008200206816	E-Merk, Darmstadt, Germany
IL-1B ELISA	88-7013-22	eBioscience, Frankfurt am Main, Germany
IL4	130-097-757	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-6 ELISA	88-7064-22	eBioscience, Frankfurt am Main, Germany
IL-10 ELISA	88-7105-22	eBioscience, Frankfurt am Main, Germany
IMDM	1852716	Gibco, Life Technologies, Darmstadt, Germany
IFN-y	315-05	Peprotech INC, Hamburg, Germany
lineage cell depletion cocktail biotin	130-090-858	Miltenyi Biotec, Bergisch Gladbach, Germany
LPS	297-473-0	Sigma-Aldrich, Taufkirchen, Germany
Marrow Max Bone Marrow Medium	12260-014	Life Technologies, Darmstadt, Germany
Murine IL-3	130-096-687	Miltenyi Biotec, Bergisch Gladbach, Germany
Murine IL-6	130-095-365	Miltenyi Biotec, Bergisch Gladbach,

		Germany
Murine SCF	130-094-079	Miltenyi Biotec, Bergisch Gladbach, Germany
Murine M-CSF	130-101-706	Miltenyi Biotec, Bergisch Gladbach, Germany
Oil Red O	SLBH1073V	Sigma-Aldrich, Taufkirchen, Germany
Osteocyte/ chondrocyte differentiation medium	A10069-01	Gibco Life Technologies, Carlsbad, U.S.A.
Pencillin/streptomycin 10000 units pencillin and 10 mg streptomycin per ml.	025M4818V	Gibco, Life Technologies, Darmstadt, Germany
Polybrene	TR-1003-G	Merck Millipore, Billerica, U.S.A.
RLT lysis buffer	151042586	Qiagen, Hilden, Germany
RPMI-1640	RNBF9159	Sigma Aldrich, Darmstadt, Germany
Trypan blue	RNBF6596	Sigma-Aldrich, Taufkirchen, Germany
Trypsin-EDTA	1848040	Gibco, Life Technologies, Darmstadt, Germany
Tween-20	SLBN3325V	Sigma-Aldrich, Taufkirchen, Germany

6.1.2 Primers

Table 2. Tagman primers used for RT-PCR

Gene Symbol	Gene product	Primer-ID	Manufacturer
Gfi1	Gfi1 murine	Mm00515853_m1	Life Technologies, Darmstadt, Germany
GFI1	GFI1 human	Hs01090305_m1	Life Technologies, Darmstadt, Germany
IL-6	IL-6 murine	Mm00446190_m1	Life Technologies, Darmstadt, Germany
Arg1	Arginase 1	Mm00475988_m1	Life Technologies,

	murine		Darmstadt, Germany
Nos2	Nos2 murine	Mm00440502_m1	Life Technologies, Darmstadt, Germany
Gapdh	Gapdh murine	Mm03302249_g1	Life Technologies, Darmstadt, Germany
GAPDH	GAPDH human	Hs04420697_g1	Life Technologies, Darmstadt, Germany

6.1.3 Antibodies

Table 3. List of antibodies used for immunophenotyping

Antibody target	Conjugate	Cat. Nr.	Supplier
Annexin V	APC	640920	Biolegend
Annexin V	FITC	640906	Biolegend
B220 murine	PerCP	RA3-6B2	Biolegend
Brdu	APC	552598	BD Pharmingen™,
CD4 murine	PerCP	100433	Biolegend
CD8 murine	PE	100708	Biolegend
CD11b murine	PerCP	101228	Biolegend
CD14 human	PE	561707	Bioscience
CD31 murine	APC	102509	Biolegend
CD34 murine	PE	343605	Bioscience
CD34 human	PerCP	368503	Biolegend
CD45 murine	PE-Cy7	103113	Biolegend
CD45 murine	PE	103106	Biolegend
CD45 murine	PerCP	103113	Biolegend
CD45 human	PerCP	368503	Biolegend
CD73 human	APC	344005	Biolegend
CD90 human	PE	E13045-107	Bioscience
CD105 huamn	FITC	323204	Biolegend
CD163 human	APC	333609	Bioscience
CD206 murine	PE	141705	Biolegend
CD206 human	PE/Cy5	321108	Bioscience
cKit murine	PE	135106	Biolegend
F4/80 murine	APC	1231115	Biolegend
Gr1 murine	PE	108408	Biolegend
Ly6C murine	PE/Cy7	128017	Biolegend
Ly6G murine	PE	127607	Biolegend
MHC II murine	APC	107613	Biolegend
Ter119 murine	PE	116208	Biolegend
Streptavidin	PerCP-Cyanine5.5	45-437-82	Bioscience
Sca1	APC-Cy7	108125	Biolegend

CD16/32	PE/Cy7	93	Biolegend

6.1.4 Consumables

Table 4. List of consumables used in this work

Article	Cat. Nr.	Supplier
Blunt-end needles 20 G	305888	Stemcell Technologies, Köln, Germany
Blunt-end needles 25 G	305891	Stemcell Technologies, Köln, Germany
Cell Countainer with cover	5515040	Ratiolab, Dreieich, Germany
Cell culture flask, 25 cm^2 with filter cap	90025	Greiner Bio-One, Solingen, Germany
Cell culture flask, 70 cm^2 with filter cap	658175	Greiner Bio-One, Solingen, Germany
Cell culture insert 12 well-plate	353090	Greiner Bio-One, Solingen, Germany
Cell filters 40 µm (Nylon)	542040	BD Biosciences, Heidelberg, Germany
Cell filters 100 µm (Nylon)	542000	BD Biosciences, Heidelberg, Germany
Cell scraper	541070	Greiner Bio-One, Solingen, Germany
Centrifuge tubes Falcon, 15 ml, sterile	188271	Greiner Bio-One, Solingen, Germany
Centrifuge tube Falcon, 50 ml, sterile	227261	Greiner Bio-One, Solingen, Germany
Cytospin slide	27157	Thermo Scientific, Braunschweig, Germany
Graduated pipettes, 5 ml sterile, single use	606180	Greiner Bio-One, Solingen, Germany
Graduated pipettes, 10 ml sterile, single use	607180	Greiner Bio-One, Solingen, Germany
Graduated pipettes, 25 ml, sterile, single use	760780	Greiner Bio-One, Solingen, Germany
Microcentrifuge tube, 1,5ml, single use	S1615-5510	STARLAB GmbH, Hamburg, Germany
Micro-Hematocrit-capillaries, CE, heparinized	180950	BRAND GmbH; Wertheim, Germany
MicroAmp Fast 96-well reaction plate	4346907	Applied Biosystems, Darmstadt, Germany

Microtest™ U-Bottom, 96 well plates	353077	BD Biosciences, Heidelberg, Germany
Mini-Collect-blood collection tubes, K3EDTA, 0,25ml	450476	Greiner Bio-One, Solingen, Germany
Multiwell™ plate 6 well, Non-tissue culture treated	353046	BD Biosciences, Heidelberg, Germany
Multiwell™ plate 12 well, Non-tissue culture treated	353043	BD Biosciences, Heidelberg, Germany
Multiwell™ plate 24 well, Non-tissue culture treated	351147	BD Biosciences, Heidelberg, Germany
Optical adhesive cover	4360954	Applied Biosystems, Darmstadt, Germany
Pipette tips 10 µl	S1121-3810	STARLAB GmbH, Hamburg, Germany
Pipette tips 100µl	S1123-1840	STARLAB GmbH, Hamburg, Germany
Pipette tips 200µ	S1120-8810	STARLAB GmbH, Hamburg, Germany
Pipette tips 1ml	S1126-7810	STARLAB GmbH, Hamburg, Germany
Polystyrene round-bottom tube with cell-strainer cap- 5 ml, PB Falcon	352235	BD Biosciences, Heidelberg, Germany
Scalpels , sterile	I10911	Medi-ware, Wesel, Germany
Syringe 1ml	2020-02	B. Braun, Melsungen, Germany
Syringe 1ml	303173	BD Biosciences, Heidelberg, Germany
Syringe 5ml	309050	BD Biosciences, Heidelberg, Germany
Syringe 5ml	309110	BD Biosciences, Heidelberg, Germany

6.1.5 Buffers

Table 5. Lists of buffers

Buffer	Components
Annexin binding buffere	Stock solution; cat. Nr. 5281679, Bioscince, Franklin Lakes, U.S.A. Diluted 1:10 in PBS
Ammonium chloride lysing buffer	Ready to use, cat.Nr. 555899, BD Biosciences, Heidelberg, Germany Diluted 1:10 in distilled water

PD Cytofix/Cytoperm buffer	Ready to use, cat. Nr.51-2090KE, BD Pharmingen™ ,Heidelberg, Germany
PD Cytoperm permeabilization buffer plus	Ready to use, cat. Nr 51-2356KC, BD Pharmingen™ ,Heidelberg, Germany
FACS buffer	DPBS +2.5% FCS +1% Penicillin/Streptomycin
HBSS buffer	HBSS (1X) +2.5% FCS +1% Penicillin/Streptomycin
Perm/wash buffer	Raedy to use cat. Nr.51-2091KE, BD Pharmingen™ ,Heidelberg, Germany
RLT lysis buffer	Ready to use, cat. Nr. 151042586 from Qiagen, Hilden, Germany
PD Wash Buffer	1 x PBS, 0.05% Tween-20

6.1.6 Instruments

Table 6. Lists of instruments

Instrument	Supplier
Bayer HEMA-TEK2000™ Slide stainer	Bayer healthcare, Leverkusen, Germany
Beckman Coulter counter Z2	Beckman Coulter, Krefeld, Germany
Biological safety cabinet classII	NuAire, Plymouth, U.S.A
Cellsorter FACSAria II	BD Biosciences, Heidelberg, Germany
Cellsorter FACSDiva	BD Biosciences, Heidelberg, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
Centrifuge 5415C	Eppendorf, Hamburg, Germany
Centrifuge Allegra 21R	Beckman Coulter, Krefeld, Germany
Centrifuge Rotana 50RS	Hettich, Kirchlengern, Germany
Centrifuge Rotana 460R	Hettich, Kirchlengern, Germany
CO2 incubator, Water- jacketted	Thermo Scientific, Braunschweig, Germany
Cytospin 2, Shandon	Thermo Scientific, Braunschweig, Germany
Flowcytometer BD LSR II	BD Biosciences, Heidelberg, Germany
Flowcytometer BD FACScan	BD Biosciences, Heidelberg, Germany
Hirschmann automatic pipettes	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Incubator 1000	Heidolph, Schwabach , Germany
Micro-centrifuge	Hettich, Kirchlengern, Germany

Microplate reader, iMark	Bio-Rad, München, Germany
Microscope DFC 450C	Leica Biosystems, Wetzlar, Germany
Microscope DM1000LED	Leica Biosystems, Wetzlar, Germany
Microscope Olympus CK2	Olympus, Hamburg, Germany
Mini-centrifuge	Bio-Rad, München, Germany
Multi-tips Micro-pipet, 300 µl	Eppendorf, Hamburg, Germany
Neubauer Chambers 0,1 mm	Assistent, Sondheim, Germany
Pipets 10 µl, 100 µl , 200 µl and 1 ml	Eppendorf, Hamburg, Germany
StepOne Plus Real-Time PCR System	Applied Biosystems, Darmstadt, Germany
Sysmex XN-1000 blood analyzer	Sysmex Europe GmbH, Norderstedt, Germany
Thermocycler Flex Cycler	Analytik Jena, Jena, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortexer Genius 3	IKA, Staufen, Germany

6.1.7 Kits

Table 7. Lists of used kits

Kits	Cat. Nr.	Manufacturer
APC BrdU Flow-kit	552598	BD Pharmingen™ ,Heidelberg, Germany
Lineage Cell Depletion kit	130-090-858	Miltenyi Biotec, Bergisch Gladbach, Germany
Mouse IL-IB ELISA ready-SET-Go kit	88-7013-22	eBioscience, Frankfurt am Main, Germany
Mouse IL-6 ELISA ready-SET-Go kit	88-7064-22	eBioscience, Frankfurt am Main, Germany
Mouse IL-10 ELISA ready-SET-Go kit	88-7105-22	eBioscience, Frankfurt am Main, Germany
RNeasy Micro kit	74004	Qiagen, Hilden, Germany
RNeasy Mini kit	74104	Qiagen, Hilden, Germany
SMARTer® PCR cDNA synthesis kit	939506	Clonetech, Mountain View, U.S.A.
Taqman master Mix	4369016	Applied Biosystems, Darmstadt, Germany

6.1.8 Cell lines

Table 8. List of cell lines used in this study

Cells name	Cell type
C1498GFP	Murine AML cells
C-AML	Human AMSCs
KAML	Human AMSCs
NKM	Human normal MSCs
Kasumi-1	Human AML cells
Mo7e	Human AML cells

6.1.9 Analysis Programs

Table 9. List of the software programs used for analysis results in this study

Program	Manufacturer	Function
FlowJo-Software	Miltenyi Biotec,	Analysis of flow cytometry results
GraphPad Prism 5	GraphPad Software	Anaylsis of all results
StepOnePlus	Applied Biosystems	Calculation and analysis of RT-PCR results
MPM6	Bio-Rad	Calculation and analysis of ELISA results

6.2 Methods

6.2.1 Human BM samples

We collected and examined the bone marrow (BM) samples from AML patients (Table 10 and table 11) after obtaining informed consents. We carried out all experiments with human samples according to the approved protocol of the University of Duisburg-Essen ethics committee. Cytological and flow cytometric examination were performed to confirm the diagnosis of AML (Estey and Dohner 2006; Valk et al. 2004) (table 10 and 11). We defined complete remission according to the established procedures (Yates et al. 1982).

Table 10. Characteristics of patient samples used for macrophage analysis

Patient No.	Age in years	Sex	Diagnosis	Cytogenetics	Mutations
1	32	M	AML	t(2;3)(p15-22;q26)	-
2	54	M	AML	t(15;17)(q24;q21)	PML/RARA
3	60	F	AML	45,XX,-7q	-
4	65	M	AML	Normal	-
5	59	M	AML	t(15;17)(q24;q21)	PML/RARA
6	38	F	AML	Not available	

7	36	F	AML	Not available	
8	74	M	AML	Normal	-
10	31	M	Normal	-	-
11	30	F	Normal	-	-
12	51	F	Normal	-	-
13	75	M	Normal	-	-
14	70	F	Normal	-	-
15	66	M	Normal	-	-
16	66	M	Normal	-	-
17	70	M	Normal	-	-
18	74	M	normal	-	-

Table 11. Characteristics of AML patient samples used for MSC co-culture

Patient No.	Age in years	Sex	Diagnosis	Cytogenetic
1	75	M	AML, M1	46,XY[24]/47,XY,+8
2	45	M	AML,M4	47, XY, +13
3	60	F	AML, M1	44~45, XX, -4, del(5)(q13q31), -16, -

				17, +1 ~2mar, inc [cp38]
4	74	F	AML, M1	46, XX
5	85	M	AML, M3	46, XY
6	78	F	AML	46, XX
7	22	F	AML, M5	46, XX

6.2.2 Mice

6.2.2.1 Keeping and feeding mice

All mice were bred and housed in specific pathogen free conditions. They were kept under 22 °C (+/- 2 °C) with good air ventilation in specific cages in the animal facility of Essen University hospital. All animal experiments were conducted after approval of the ethics committee and local authorities of the University Hospital Essen, Germany, under permission document numbers G1196/11 and G1438/14.

6.2.2.2 C57BL/6 mice

These mice were *Gfi1*-WT and have a normal genotype. They were used in our study as controls and were provided by the animal facility of the Essen university hospital.

6.2.2.3 NUP98-HOXD13 transgenic mice

We purchased *NUP98-HOXD13*-transgenic mice from the Jackson Laboratory (Bar Harbor, ME, USA) (Lin et al. 2005). The *NUP98-HOXD13* transgenic mouse model contains the t(2;11)(q31;p15) translocation found in human AML (Lin et al. 2005). This

mouse model develops all features of human MDS including progression to AML which can be seen in a significant number of these mice (Lin et al. 2005).

6.2.2.4 Gfi1-KO mice

In *Gfi1*-KO mice, the *Gfi1* gene was deleted from murine embryonic stem cells by homologous recombination (Karsunky et al. 2002). We have obtained these mice from the laboratory of Tarik Möröy (IRCM, Montreal, Canada).

6.2.3 Transplantation experiments

The murine leukemic cells expressing *MLL-AF9* or *AML-ETO9a* oncofusion genes fused to *GFP* sequences were generated and transplanted in WT mice as previously described (Krivtsov et al. 2006; Yan et al. 2006; Hones et al. 2016a; Li et al. 2015). These murine models mimic the *MLL-AF9* and *AML1-ETO9a* subclasses of human AML (Yan et al. 2006; Krivtsov et al. 2006). *AML1-ETO9a*, the product of the t(8;21)(q22;q22) translocation, and *MLL-AF9*, the product of the t(9;11)(p22;q23) translocation, were used to model AML in mice with either good or poor prognosis, respectively (Yan et al. 2006; Krivtsov et al. 2006; Somervaille and Cleary 2006; Tan et al. 2011). Dr. Jay Hess provided us with the MSCV-*MLL-AF9-IRES-GFP* vector (Indiana University, School of Medicine, USA) while Dr. Leighton Grimes provided us with the MigR1-AML1-ETO9a-IRES-GFP vector (Cancer and blood diseases institute, Cincinnati children's hospital medical centre, USA). For primary transplantations about 5-7x10^5 *AML1-ETO9a*-transduced Lin^- BM cells or 1x10^5 *MLL-AF9*-transduced Lin^- BM cells (GFP^+) from WT mice were injected together with 1.5x10^5 competitive BM cells from WT mice into the tail vein of lethally irradiated (10 Gy) congenic recipient mice (Figure 5). After transplantation, the mice were monitored every second day and when they showed features of leukemia such as splenomegaly, tiredness, pale hands and feet and difficulty of breathing, the emerging BM leukemic cells were isolated and preserved in liquid nitrogen for subsequent experiments or directly used for secondary transplantations.

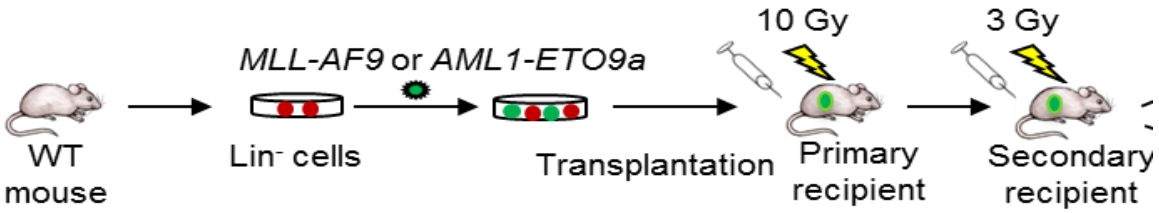

Figure 4: Generation of AML by transplantation of leukemia cells.

Lineage negative (Lin$^-$) BM cells form WT mice were transduced with retroviruses encoding MLL-AF9 or AML1-ETO9a fused to GFP and transplanted into lethally (10 Gy) irradiated mice. Upon disease development, BM leukemic cells were extracted and re-transplanted into sublethally irradiated (3Gy) recipient mice. (Published (Al-Matary et al. 2016))

For secondary transplantations, 1x10^5 *AML1-ETO9a* fresh GFP$^+$ leukemic BM cells (or 1x10^5 frozen *MLL-AF9* leukemic BM cells were transplanted into sublethally irradiated (3 Gy) *Gfi1*-WT or *Gfi1*-KO secondary recipient mice (Figure 4). In another independent experiment, we transplanted 1-4x10^4 C1498GFP cells (Zhang, Gajewski, and Kline 2009) into sublethally irradiated (3 Gy) recipient mice which subsequently developed leukemia.

6.2.4 Preparation of single cell suspensions from BM and spleen of the mice

After transplantation of WT BM cells transduced with retroviruses encoding *MLL-AF9* or *AML1-ETO9a*, the mice were monitored every second day and when they had features of leukemia like big abdomen, tiredness, paleness of hands and feet and difficulty of breathing, mice were sacrificed and BM cells were collected by flushing femurs, tibiae, and humeri as previously described (Hones et al. 2016b; Botezatu et al. 2016). After removing tissues surrounding the bones, the femur, tibia and humeri were collected in 5 ml of FACS buffer into one well of a 6 well-plate on ice. The ends of the bones were cut off and the BM was flushed by 5 ml syringe and 20G pink needle into 5 ml of FACS buffer in another well. The BM particles were re-suspended again by repeatedly pulling

the suspension up and down several times through a 5 ml syringe and a 25G yellow needle. For removing large particles such as bones or tissues, the cell suspension was filtered through 100 µm yellow filter into 50 ml Falcon tubes. In the same way, half of the spleen of the leukemic mice or the entire spleen of normal mice was crushed between the rough ends of two sterile slides into small particles. These small parts were then re-suspended again several times as described above by pulling up and down several times with a 5 ml syringe and a 25G yellow needle. The suspension was then filtered through 100 µm yellow filter into another 50 ml Falcon tube. After centrifugation of both cell suspensions of BM and spleen at 1200 rpm for 5 min at 4°C, the supernatants were discarded and red blood cells were removed by incubating the cell suspensions with 1-2 ml of lysing buffer for 7 min at room temperature (RT). After that 5 ml of FACS buffer was added and the total volume was centrifuged at 1200 rpm for 5 min at 4°C. Then supernatant was then discarded and the pellet was re-suspended in 5 ml FACS buffer. 100 µl of each suspension was added to 10 ml of coulter counting reagent to be counted automatically by Beckman Coulter counter Z2.

The endosteal stromal cells were collected as previously described with a simple modification (Schepers et al. 2013). Flushed bones were crushed by pestle and mortar into small pieces. The small pieces of the bone were then washed with 5 ml of HBSS buffer 2-3 times until the small bone chips became white (Figure 5). The endosteal stromal cells were then extracted by digestion with 1-2 ml of 3 mg/ml collagenase 1 at 37 °C for 1 hour. Afterwards, the endosteal cells were collected by washing the small bone chips 2-3 times with 5 ml of HBSS buffer. The collected volume was then filtered through a 40 µm green filter into a 50 ml Falcon tube. After centrifugation of the cell suspension at 1200 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was re-suspended again in 5 ml FACS buffer. 100 µl of the suspension was added to 10 ml of coulter counting reagent to be counted automatically by Beckman Coulter counter Z2.

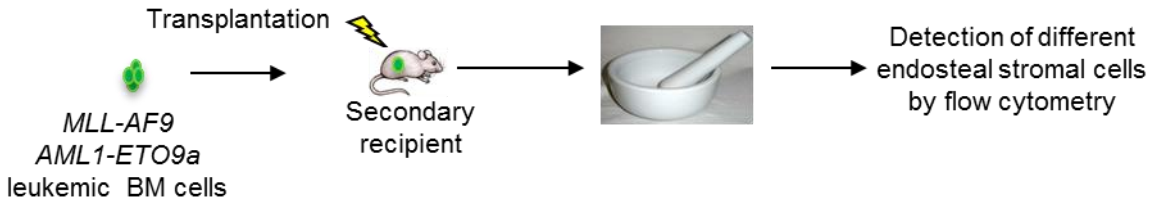

Figure 5: Isolation of endosteal cells.

MLL-AF9 or AML1-ETO9a leukemic cells were transplanted into sublethally irradiated (3Gy) secondary recipient mice. When the transplanted mice become sick, they were killed and femur, tibiae, and humeri were taken. The BM cells were collected from the bones and the bones were then crushed with mortar and pestle for extraction of endosteal stromal elements. The various cell types were then analyzed for their cell surface marker expression by flow cytometry.

6.2.5 Immortalization of MSCs from AML patients and normal donors

After informed consent, human BM stroma cells were obtained from two AML patients. Another BM sample from a healthy volunteer was purchased from Lonza Walkersville, Inc., Walkersville, MD, USA. The MSCs were prepared and cultured as previously described (Li et al. 2015) (Table 10). The cells were transduced with a lentiviral library expressing 33 potentially immortalizing genes at early passage (<3) (by Tobias May and colleagues at InscreeneX GmbH, Braunschweig, Germany). After 3 weeks colonies of outgrowing cells were pooled and expanded polyclonally. Doxycycline-resistant clones were selected, pooled, and the cells were expanded by serially dilution once per week (Kuehn et al. 2016).

Table 12 Characteristics of patient samples used for MSC immortalization

MSCs	Sex	Diagnosis	Cytogenetic
K-AML	F	AML, FAB M3	t(15;17)(q22;q21), XX
C-AML	F	AML,	46, XX

		FAB M6	
N-KM	Normal BM purchased from LONZA (Cologne, Köln, Germany)		

6.2.6 Co-culture experiments

6.2.6.1 MSCs co-culture experiments

To study the role of MSCs with regard to the growth of AML cells *in vitro*, MSCs were isolated from the BM of leukemic patients and leukemic mice. The isolated MSCs were then co-cultured with AML cell lines. We extracted BM mononuclear cells (MNCs) from the BM of AML patients and non-leukemic patients by Ficoll density gradient centrifugation. In general, BM sample was diluted 1:3 in DPBS and put in Ficoll density tube to be centrifuged at 1800 rpm for 10 min at 4 ^{o}C. Thereafter, most of the supernatant was discarded and a circle of the MNCs formed immediately above the white barrier of the Ficoll density tube. These MNCs were collected into another Falcon tubes and centrifuged at 1800 rpm for 5 min at 4 ^{o}C. Afterward, the supernatant was discarded and the red blood cells were removed by adding 5-10 ml of lysing buffer. After 7 minutes incubation at RT, 5 ml of FACS buffer was added to the falcon tube and centrifuged at 1200 rpm for 5 min at 4 ^{o}C. The supernatant was discarded and the pellet was re-suspended in 5 ml of FACS buffer and the cells were manually counted. As MSCs have a characteristic of adherence to plastic surfaces, they were isolated from human and mouse samples as previously described (Baustian, Hanley, and Ceredig 2015; Geyh et al. 2016). In brief, we cultured 5-10x10^{6} of extracted human MNCs or 0.5-2x10^{7} MNCs from mouse BM in each well of a 6 or 12-well-plate in 4 or 2 ml (respectively) of Iscove's Modified Dullbecco's medium (IMDM) containing 20% FBS and 1% penicillin/streptomycin. The MSCs cultures were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. Half of the medium was replaced twice a week. When the confluency of the MSCs in culture was approximately 80-90%, the cultures were washed with PBS and split by adding 500 µl of trypsin-EDTA for 5 minutes at R.T. After

that, 1-2 ml of medium (see above) or PBS was added to each well and the adherent cells were scraped with a sterile cell scraper. The splitted cells were collected in falcon tube and centrifuged at 1200 rpm for 5 min at 4 ^{o}C. The supernatant was discarded and the pellet was re-suspended and re-plated into another plate. After splitting for 2 or 3 times and when the MSC culture became more than 80% confluent, we discarded supernatants and washed MSCs with DPBS. Afterward, we added 5x10^{4} Kasumi-1 or Mo7e cells (human AML cell lines) to each well of human MSCs. For murine MSC, we added 5x10^{4} C1498GFP cells to each well of. As a control, we cultured 5x10^{4} of leukemic cells (5x10^{4} Kasumi-1 or Mo7e cells or C1498GFP) in the same plate without the presence of MSCs. Three days later, we added fresh medium to each well. After 6 or 7 days, we counted the total number of live CD34^{+} kasumi-1 or GFP^{+}C1498 leukemic cells. In transwell experiments, after washing 80-90% confluent MSCs, the transwell (BD Falcon) was applied for each well and then 5*10^{4} leukemic cells were cultured on the culture insert for 6 days. To avoid the effect of genetic variations of different leukemic cell lines used in the above experiment, MSCs derived from leukemic secondary transplanted recipient mice were co-cultured with 4x10^{4} GFP^{+} cells obtained from the BM of mice transplanted with *MLL-AF9* BM cells in 2 ml of IMDM containing 20% FBS, IL-3 (10 ng/ ml), IL-6 (10 ng/ml), SCF (20 ng/ml) and 1% penicillin/streptomycin in each well of 12 well-plate.

6.2.6.2 Co-culture of Macrophages with leukemia cells

In this study, I used macrophage colony stimulating factor (M-CSF) to produce bone marrow-derived macrophages (BMDMs) from BM cells *in vitro* as previously described (Longbrake et al. 2007; Choi et al. 2014) (Figure 6). I cultured 1 -2x10^{6} BM cells per well in 2-4 ml of Dulbecco's modified Eagle medium-Glutamax (DMEM-Glutamax) containing 10% FBS serum (FBS), 1% penicillin/streptomycin and 10 ng/ml M-CSF in 12 WP or 6 WP respectively. 7 days later, I harvested macrophages by using trypsin-EDTA and the purity of macrophage was determined by flow cytometry. The expression of both CD11b and F4/80 surface markers in BMDMs was higher than 95%. 2-3x10^{5} macrophages were cultured in 500 µl of DMEM-Glutamax containing 10% FBS serum (FBS) and 1%

penicillin/streptomycin in 4 wells of 24 well plate. On day 1, after discarding the supernatant, BMDMs were co-cultured with $5x10^4$ C1498GFP cells per well into three wells in the same medium and one well was used as a negative control without C1498 cells. After 6 days of incubation, I collected the supernatant cells and the non-adherent leukemic cells were collected by washing wells with DPBS and collected into a falcon tube. The adherent leukemic cells were detached by adding of 500 µl trypsin-EDTA to each well and incubating them for 5 min at RT. The cells were collected by washing with 1 ml of DPBS or medium into the same falcon tube.

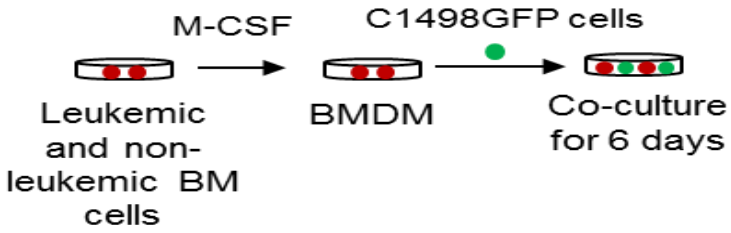

Figure 6: Co-culture of BMDM with leukima cells in vitro

BMDMs were prepared by culturing 1.5-2x10^6 BM cells in one well of 12 or 6 wellsplate in 2-4 ml (respectively) of DMEM-Glutamax containing 10% FBS serum (FBS), 1% penicillin/streptomycin and 10 ng/ml M-CSF for 7 days. 2-3x10^5 BMDMs from mice transplanted with non-transduced or AML1-ETO9a or MLL-AF9-transduced cells were co-cultured with 5x10^4 C1498GFP cells for 6 days. The number of live C1498GFP cells was calculated. (Published (Al-Matary et al. 2016))

The total cells (adherent and non-adherent) in each well were counted manually and the percentage of live C1498GFP cells was detected by a FLOWScan flow cytometer. The number of GFP^+ cells was then determined.

In a separated experiment, GFP^- macrophages ($GFP^-CD11b^+Ly6G^-$) from transplanted leukemic mice were co-cultured with AML cells (Figure 7). Briefly; I cultured $1.5x10^4$ of sorted cells per well in 48 well plates in 100 µl of DMEM-Glutamax containing 10% FBS and 1% penicillin/streptomycin.

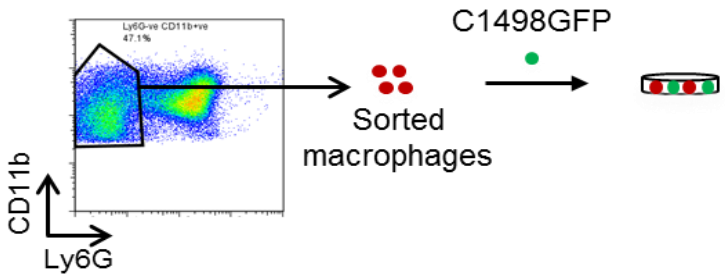

Figure 7: Sorting and co-culture of GFP$^-$CD11b$^+$Ly6G$^-$ macrophages with leukemia cells in vitro.

*GFP$^-$CD11b$^+$Ly6G$^-$ macrophages were sorted from BM of MLL-AF9 transplanted leukemic mice and from transplanted non-leukemic mice as control. 5*10^4 C1498GFP$^+$ cells were co-cultured with 1.5x10^4 sorted GFP$^-$CD11b$^+$Ly6G$^-$ cells for 24 hours. The number of live C1498GFP cells was calculated and the supernatants were collected, filtered and frozen for subsequent ELISA analysis. (Published (Al-Matary et al. 2016))*

At once, I added 5x10^4 C1498GFP cells to each well. After co-culture for 48 hours, I collected the non-adherent leukemic cells in each well by collecting supernatants and the remaining non-adherent cells using trypsin-EDTA in one tube. The cells were then manually counted and the frequency of live GFP$^+$ cells was determined by a FLOWScan flow cytometer.

6.2.7 Macrophage polarization experiments

BMDMs were produced from BM using M-CSF as described above in 6.2.7.2. The macrophage purity was set to extend 95% and this was detected as a double positive for CD11b and F4/80. 7 days later, I discarded supernatants and washed wells with DPBS. The polarization of BMDMs into M1 macrophages was done by adding 2 ml of DMEM-Glutamax supplemented with 10% FBS, 1% penicillin/streptomycin and 100 ng/ml LPS or 100 ng/ml INF-y into each well. The polarization of BMDMs into M2 macrophages was done by adding 2 ml of DMEM-Glutamax supplemented with 10% FBS, 1% penicillin/streptomycin and 20 ng/ml IL4 into each well (Ying et al. 2013; Davis et al. 2013) (Figure 8).

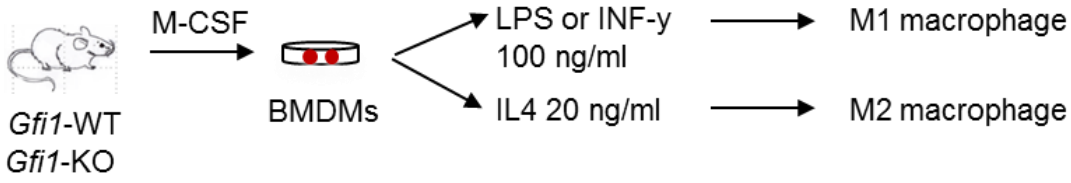

Figure 8: In vitro polarization of BMDMs into M1 and M2 macrophages

Gfi1-WT or Gfi1-KO BMDMs were stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml) respectively for 48 hours. Medium was collected and filtered for ELISA and M1 and M2 macrophages were characterized by flow cytometric and gene expression analysis. (Published (Al-Matary et al. 2016))

The polarization of M1 and M2 was examined after 48 hours by flow cytometry and RT-PCR. I collected and filtered the supernatants from each type of macrophage and frozen them for later measurement of secreted cytokines using ELISA kit.

6.2.8 Measurement of cytokine production of macrophages

To detect the secretions of M1 and M2 macrophages *in vitro*, I collected filtered and stored supernatants from both M1 and M2 macrophages in small aliquots at -80°C until subsequent analysis. In another approach, I cultured $5x10^4$ sorted macrophages (GFP^- $CD11^+Ly6G^-$) derived from transplanted leukemic and non-leukemic mice (Figure 7). After 24 hours of incubation, I collected the supernatants and stored them in small aliquots at -80°C. According to the manufacturer's instructions, the level of different cytokines of macrophages such as IL-1B, IL-6 and IL-10 was measured using the mouse ELISA kit. In brief, a specific 96 well ELISA plate was coated with 100 µl capture antibody overnight at 4 °C. Afterward, the coating antibody reagent was removed and the wells were washed 5 times with 250 µl of a washing buffer. 200 µl of 1x assay diluent (ready-to-use) was added to each well and the plate was incubated at RT for 1 hour. The supernatants were discarded again and the wells were washed with 250 µl of washing buffer for 5 times. Then 100 µl of serial dilutions of the standard was added into each well in duplicate. At the same time, our samples were also added to each well in

duplicate and the plate was then incubated at RT. After exactly 2 hours, the plate was washed 5 times as mentioned above with wash buffer to remove unbound target cytokine. Detection antibody was then added to each well and incubated for 1 hour. Thereafter, 100 µl of diluted Avidin-HRP was added to each well and incubated for further 30 minutes. The Avidin-HRP would bind to the antibody. The supernatant was then aspirated and the wells were washed as above to discard excess free Avidin-HRP. 100 µl of substrate solution was added to each solution and incubated for 15 min. 50 µl of 1M H3SO4 was then added to each well to stop the enzyme activity. Finally, the intensity of the fluorescence of the wells that depend on the concentration of the cytokine was read at 450 nm by an Imark Microplate reader.

6.2.9 Cell cycle analysis

To investigate whether the MSCs influence cell cycle progression of AML cells *in vitro*, I co-cultured Kasumi-1 and C1498GFP cells with a human normal MSC cell line or mouse MSCs, respectively, for 6 days. The cell cycle distribution was studied using APC BrdU Flow Kit according to the manufacturers' instructions. After co-culture of MSCs with leukemia cells for 6 days, 10 µl of BrdU was added to each ml of culture medium and the mixture was incubated for 30- 45 minutes (min) at 37 °C. The cells from the culture were collected in a 5 ml FACS tube and centrifuged at 1200 rpm for 5 min at 4 ºC. The supernatant was discarded and the pellet was re-suspended with 100 Cytofix/Cytoperm buffer for 30 minutes to fix the cells. The cells were then washed with 1 ml of Perm/wash buffer and centrifuged at 1200 rpm for 5 min in 4 ˚C. After which, supernatant was discarded. 100 µl of Cytoperm permeabilization buffer plus was added to the cells for 10 minutes to permeabilize the cells. The cells were then washed with 1 ml of Perm/wash buffer and centrifuged at 1200 rpm for 5 min at 4 ºC and supernatant was discarded. The cells were again re-fixed using Cytofix/Cytoperm buffer 100 µl per tube and then washed and centrifuged. Afterwards, 50 µl of diluted DNase in DPBS was added to each tube to expose incorporated BrdU. After 1 hour incubation of the mixture at 37 °C, the cells were washed and centrifuged. 50 µl of Perm/wash buffer containing 1:50 diluted anti-BrdU was added and the mixture was incubated for 20 min at RT. After washing

and centrifuging the cells with Perm/wash buffer, total DNA was stained with DAPI. Finally, the stained cells were analysed using the LSRII flow cytometer.

6.2.10 Differentiation assay of MSCs

I next examined the ability of MSC cell lines to differentiate into adipocytes, chondrocytes and osteocytes which is a characteristic important feature of MSCs (Dominici et al. 2006). The differentiation assay was applied in which MSCs were differentiated into osteocytes, adipocytes and chondrocytes depending on the differentiation media used. The assay was performed according to the manufacturer's instructions. When MSCs reached 80% confluency, the cells were harvested using trypsin-EDTA, counted and cultured in specific differentiation media.

6.2.10.1 Osteogenesis assay

For osteogenesis, $5x10^3$ cells/cm^2 were cultured in pre-warmed osteogenesis differentiation media in 12-well plates. The whole medium was replaced by fresh differentiation medium every 3 to 4 days. After 21 days of cultivation, osteocytes were stained with Alizarin Red S according to the manufacturers' instructions. In brief, the cells were washed with DPBS and then fixed with 1-2 ml of 4% formaldehyde for 30 minutes. Afterwards, the cells were washed 2 times with distilled water (DW). The osteocytes were then stained by adding 2 ml of Alzarin Red S solution for 2-3 minutes at RT. Thereafter, the cells were washed 3 times by rinsing with distilled water and the images of the stained cells were captured with a camera or a light microscope.

6.2.10.2 Adipogenesis differentiation assay

For adipogenesis, $1x10^4$ cells/cm^2 were cultured in 12-well plates containing pre-warmed adipogenesis differentiation media. Every 3 to 4 days, the whole medium was replaced by fresh differentiation medium. After 21 days of cultivation, adipocytes were stained

with Oil Red O. The cells were washed with DPBS and then fixed with 1-2 ml of 4% formaldehyde for 30 min. Afterwards, the cells were washed 2 times with distilled water (DW). For staining of adipocytes, 300 mg of Oil Red O in 100 ml of isopropanol was freshly prepared. The working reagents used for staining was 60% of Oil Red O in DW. Before staining of adipocytes with Oil Red O, 2 ml of 60% Isoprpanol was added to each well for 2-5 min. Afterwards, Isopropanol was removed and 2 ml of Oil Red O was added to each well for 5 min. The wells were then washed with tap water until the water run clear. Finally, 2 ml of hematoxylin counterstain was added into each well for 1 minute. The wells were then washed with tap water and viewed under a light microscope.

6.2.10.3 Chondrogenesis differentiation assay

For chondrogenesis, I cultured 5 µl droplets of a cell solution containing $1.6x10^7$ cells in wells of 12-well plates and incubated at 37°C and 5% CO_2. After 2 hours, a micromass was formed and chondrogenesis differentiation medium (Gibco) was added. After 14 days of incubation, with change of all medium twice a week, the supernatants were removed and the wells were washed with DPBS. The chondrogenic pellets were stained with 1% Alcian Blue prepared in 0.1N hydrochloric acid (HCL) for 30 min at RT. Thereafter, the wells were rinsed with 0.1 N HCL and then washed with water to neutralize the acidity of HCL. The images of chondrocytes stained with blue were taken by light microscope.

6.2.11 Immunophenotyping staining of BM, spleen cells and the culture cells

Each cell can be identified by specific antigens present on its surface. This antigen is called a cluster of differentiation (CD) (Chan, Ng, and Hui 1988). In our work we detected the frequency of different cells either in BM or spleen or in the cell culture by using specific antibodies (table 3) or specific combinations (table 13) for each cell type. Irrespective of the source and type of the cells, 10 µl of Fc block was added to a cell suspension in a FACS tube and incubated for at least 10 minutes at 4-8 °C in the dark to

block any non-specific binding. The cells were then washed with 1-2 ml of cold FACS buffer and centrifuged for 5 minutes at 1200 rpm and in 4 °C. Afterwards, the supernatant was discarded and the cells were stained by 80 µl of antibody mixture, for example c-kit for immature blasts or any combination of antibodies (table 13) at 4 °C in the dark. After 10-15 minutes of incubation, the cells were washed with 1-2 ml of cold FACS buffer and centrifuged for 5 min at 1200 rpm and in 4 °C. The supernatant was discarded and 250 µl of FACS buffer was added to the stained cells which were then acquired either by LSR II or FLOWScan flow cytometer. FlowJo software was used for analysis of the raw FACS data.

Table 13 antibodies combination used in immunophenotyping

Antibodies combinations	Specific cells
Gr-1PE+CD11bPerCP	Murine Granulocytes and monocytes *in vivo*
Ter-119PE+B220PerCP	Murine Erythrocytes and B- lymphocytes *in vivo*
CD8aPE+CD4 PerCP	Murine T4 and T8 lymphocytes *in vivo*
cKit PE	Blast cells *in vivo*
CD45PE-Cy7, lineage negative cocktail biotin+, Streptovidin PerCP, CD33 APC, sca-1 APC-Cy7, CD51 PE	Murine stromal cells: MSCs, ECs and OB *in vivo*
CD11b PerCPcy5.5, F4/80 APC, CD206 PE, Ly6C PEcy7	Murine M1and M2 macrophages *in vitro.*
CD11b PerCP cy5.5, F4/80 APC	BMDM
CD11b PerCPcy5.5, MHC II APC, Ly6C	Murine Macrophages and their classes

PE, Ly6C PEcy7	*in vivo*
CD14 PE, CD206 PE/Cy5, CD163 APC	Human macrophages and their classes *in vivo*
CD73 APC, CD105 FITC, CD45 PerCP cy5.5, CD90 PE	Human MSCs
FITC or APC or PerCP cy5.5 Annexin V	Detection of apoptosis

6.2.12 Differential count of peripheral blood from mice

To confirm that the sick mice, characterized by pale hands and feet, enlarged abdomen and slow movement, had leukemia or not, a differential cell count of peripheral blood was done. 0.5-1 ml Blood was taken directly from the heart of the mice by puncture of the chest using a 23G needle, transferred immediately into an EDTA blood container and mixed well. The cell count of the blood was then determined using the University.

6.2.13 Wright Giemsa and Pappenheim staining of blood films and cytospin

To further confirm the diagnosis of leukemic mice, we determined morphology of the cells in peripheral blood film and cytospin from BM and spleen of the mice by staining them by Wright Giemsa stain. The macrophages cytospin was also stained by Wright Giemsa. The cytospin of macrophages was prepared from sorted $CD11b^{+}Ly6G^{-}$ macrophages derived from MLL-AF9 transplanted leukemic mice as well as from non-leukemic mice. Before staining, the blood film or cytospin were air-dried and fixed by ethanol. The fixed blood films and cytospin were then automatically stained with Wright Giemsa stain for 10 minutes in the hematological oncology laboratory of the University Hospital Essen using slide staining machine. The stained and washed films and cytospins were then examined by the light microscope.

6.2.14 Quantitative RT-PCR

To characterize the genetic changes in macrophages and MSCs caused by leukemia cells, we used RT-PCR to detect the expression of many genes in AMSCs and AAMs compared to normal MSCs and macrophages. To this end, we extracted total RNA from human and mouse cells (10^5-$5x10^6$) and from *in vitro* cultured MSCs and macrophages using an RNeasy Mini-kit. The concentration of extracted RNA was measured by Nanophotometer. MMLV reverse transcriptase has been used for reverse transcription of 500-1000 ng of isolated RNA into cDNA using the FlexCycler apparatus for 1 hour. As previously described (Khandanpour et al. 2013), Real-Time quantitative PCR analysis was performed using the Real-Time PCR system OneStepPlus. To perform this, 1 µl of FAM TaqMan primer and 1µl of VIC GAPDH were added to 11 µl of TaqMan Gene Expression MasterMix, 3 µl of sterile DW and 4 µl of cDNA in each well of MicroAmp 96 well plate. RT-PCR was performed using the StepOnePlus™ Real-Time-PCR-Systems as the following: heating at 50 °C for 2 minutes, 10 minutes heating at 95 °C to denature the DNA double strands producing 2 single DNA strands. This was followed by one minute incubation at 60 °C to enable binding of the TagMan primer to the target sequence and then synthesizing a complementary strand. Human GAPDH (Hs04420697_g1) and mouse GAPDH (Mm03302249_g1) were used as controls.

6.2.15 Radiation of the mice

For primary transplantation, we radiated mice by 10 Gy (lethal radiation) in two stages; one is 7 Gy and another one is 3 Gy with 5 hours interval. For secondary transplantation, we sublethally radiated mice by 3 Gy. The radiation was done by the X-ray machine in the radiation unit of the University Hospital Essen

6.2.16 Statistics

Most of the results were first calculated by Microsoft excel 2010. We applied student's t-test to find out the differences between various groups. Kaplan-Meier test was used to

perform the survival analysis. A p-value of < 0.05 was considered a statistically significant difference. We applied all significance tests using the Graph Pad (version 6) software.

7. Results

7.1 The role of stroma in the development of leukemia cells

In an effort to investigate the interaction between AML cells and stroma cells in the BM microenvironment, we used different mouse AML models and we co-cultured human and murine stroma cells with human and murine AML cell lines *in vitro*. We found in general that AML cells polarized surrounding stroma elements *in vitro* and *in vivo* towards a leukemia-supporting state that supports the development and prognosis of the disease.

7.1.1 AMSCs from leukemic patients and mice support the growth of leukemia cells *in vitro* better than non-leukemic MSCs

7.1.1.1 Human primary AML-associated MSCs (AMSCs) support the growth of human AML cell lines better than normal MSCs *in vitro*

We first investigated the functional properties of MSCs from AML patients. To this end, 0.5-2x10^6 extracted human mononuclear cells (MNCs) from BM of AML patients and healthy donors (Table 11) were cultured in 4 or 2 ml of Iscove's Modified Dullbecco's medium (IMDM) containing 20% FBS and 1% penicillin/streptomycin in each well of a 6- or 12-well-plate, respectively, and incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. After splitting 2 or 3 times, we co-cultured them with Mo7e cells, a human AML cell line (Table 8) (Figure 9 A). We found that AMSCs supported the proliferation of the AML cells better than MSCs from healthy donors (Figure 9 B). Another interesting result was that MSCs from AML patients who were successfully treated and cured, did not support the growth of AML cells to the same extent as MSCs derived from BM of the same patient at diagnosis (Figure 9 B). This experiment was done by Ulrike Buttkereit and Jenny Wang in the research laboratory of the hematology department of the University Hospital Essen.

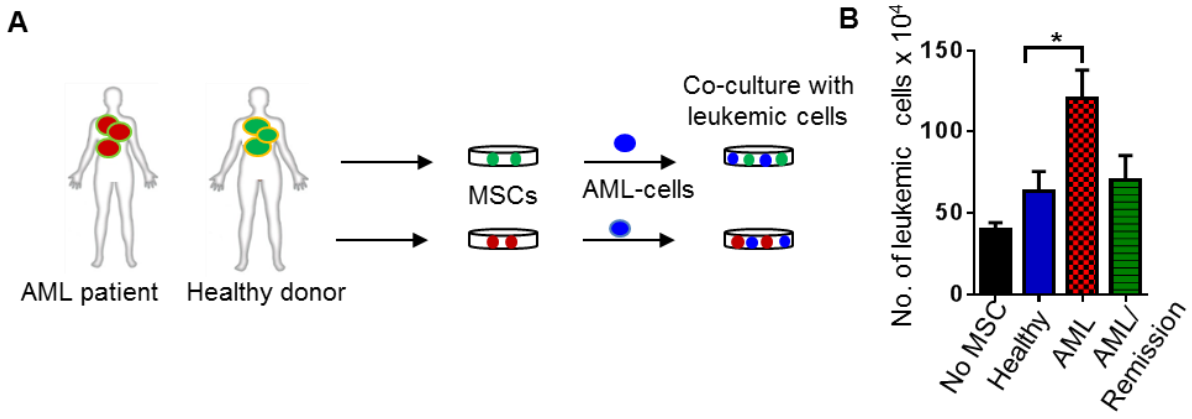

Figure 9: The human and murine AMSCs support the proliferation of AML cells in vitro

A) *Schematic representation of the outline of the co-culture experiments of MSCs from AML patients, healthy volunteers and MSC cell lines (K-AML, C-AML, N-KM and ctrl-MSCs) with the human AML cell line, Mo7e.* ***B)*** *Leukemic cell numbers after 6 days of co-culture are depicted. Leukemic cells were either cultured alone (no MSCs) or in co-culture with different MCSs originating either from healthy donors, AML patients, or from patients who achieved remission after chemotherapy. Each co-culture with MSCs from a specific patient was done in triplicate. The total number of patients for each type of MSCs was n=8 for healthy/normal MSCs, n= 4 for patients diagnosed with AML, n=4 for patients who achieved remission after chemotherapy (AML/Remission), (*p=0.02).*

7.1.1.2 Human AMSC cell lines support the growth of human AML cells *in vitro*

Since the isolation of primary MSCs from AML patients is difficult, we used immortalized MSC (immMSCs) (Lipps et al., submitted), which were derived from an AML patient or from a healthy volunteer (Table 11). To confirm that these immMSCs are really MSCs, differentiation assays into osteocytes, adipocytes and chondrocytes were performed using osteogenesis, adipogenesis and chondrogenesis differentiation media. Functionally, we found that these immMSCs still have the ability to differentiate into adipocytes, chondrocytes and osteocytes indicating their MSC potential (Figure 10).

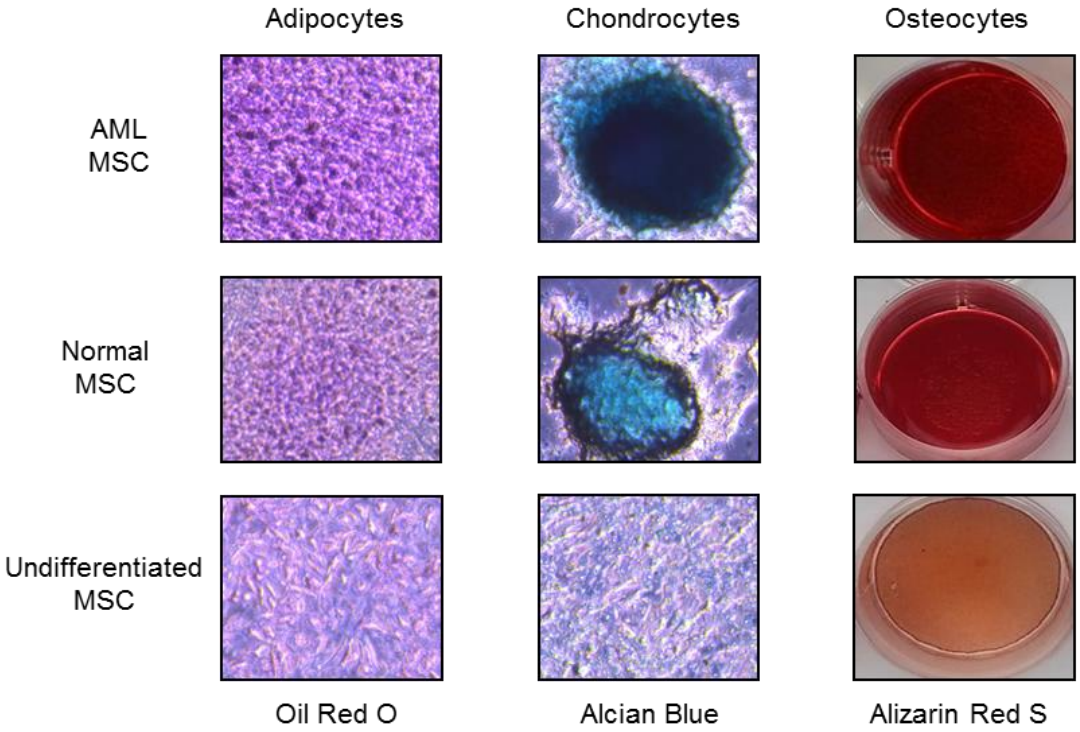

Figure 10: Differentiation assay in immortalized MSCs.

Differentiation of an AMSC cell line and a non-AML MSC cell line into adipocytes (left panel), chondrocytes (middle panel) and osteocytes (right panel). As a control for comparison, undifferentiated MSCs are shown.

We further confirmed the identity of immMSCs by flow cytometry. They expressed a combination of cell surface markers which have been shown to be characteristic for MSCs such as CD73, CD90 and CD105 (Figure 11 A) (Geyh et al. 2016; Dominici et al. 2006). At the same time, immMSCs were negative for the HSC markers CD45 and CD34 (<3%) and the endothelial cell marker CD31 (Geyh et al. 2016; Dominici et al. 2006) (Figure 11 B).

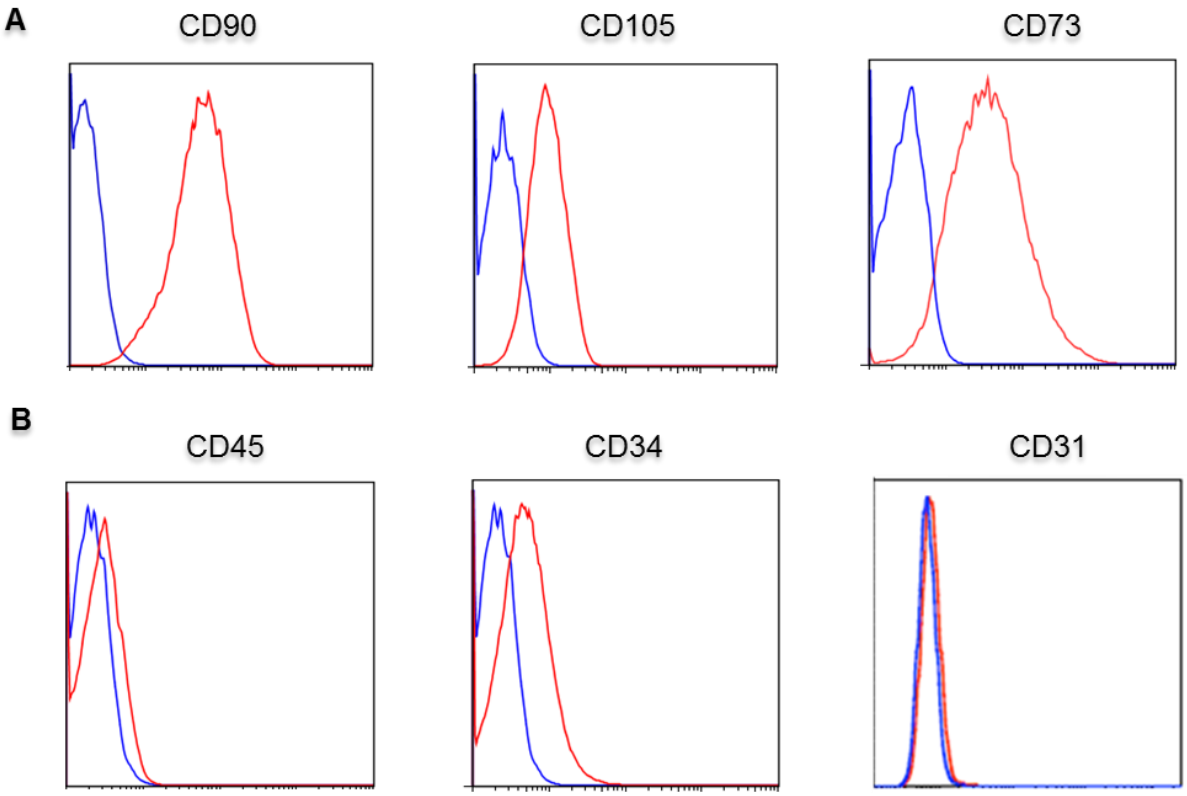

Figure 11: Immunophenotypoing of human MSCs in vitro

A) *The immortalized MSCs were stained with specific antibodies recognizing surface markers characteristic for human MSCs such as CD73, CD90 and CD105 (red line). Blue line histogram represents unstained control.* ***B)*** *The immortalized MSCs were stained with specific antibodies recognizing surface markers characteristic for hematopoietic and endothelial cells such as CD45, CD34 and CD31 (red line). Blue line histogram represents unstained control.*

In an effort to confirm our results which we obtained from co-culture of primary MSCs with leukemia cell lines, normal and AML-associated immMSCs were co-cultured with Kasumi-1 cells, another AML cell line. We tried here to prove that our observations can be also recapitulated with another AML cell line. Again, we demonstrated that the proliferation of human AML cells was supported by AMSC cell lines better than MSCs derived from healthy volunteers (Figure 12, left panel). In addition to the endogenous supporting mechanism of MSCs with respect to normal hematopoietic stem cells (Schepers et al. 2013), our results confirmed that AML cells further modulate MSCs to alter their phenotypes and function, and that subsequently these polarized cells support the growth of leukemic cells better than MSCs from non-leukemic donors. This bidirectional interaction between MSCs and AML cells was dependent on direct

cell-cell contact, since there was no significant difference in proliferation-stimulating potential after co-culture of AML cells with MSC cells separated with trans-well inserts (Figure 12, right panel).

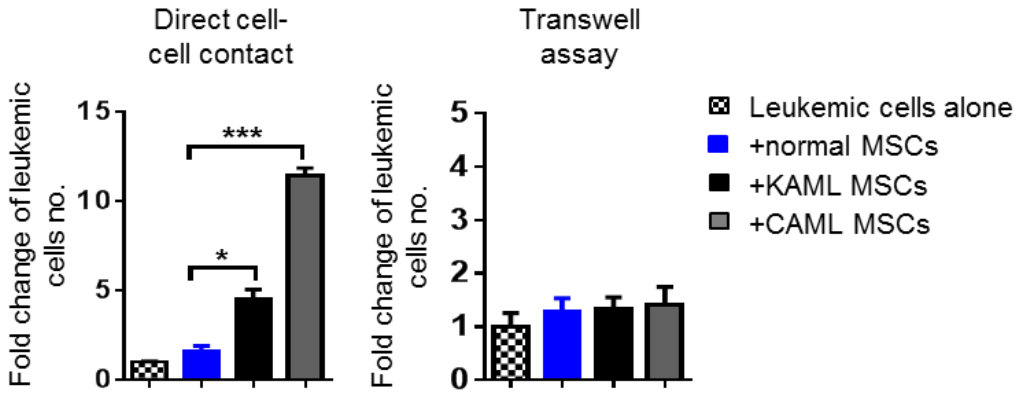

Figure 12: Human AMSC cell lines support the growth of human AML cell lines in vitro.

*Fold change of Kasumi-1 cell numbers after 6 days of culture alone or after co-culturing of 1x10^5 Kasumi-1 cells with different immortalized MSC lines in direct cell-cell contact (left panel) or in absence of direct contact by using trans-well inserts (right panel). Results of triplicates from each experiment are given. KAML and CAML are two immMSC cell lines derived from patients with AML, (*p=0.01 and ***p<0.001).*

7.1.1.3 AMSCs from transplanted leukemic mice support the growth of AML cells *in vitro* in a cell contact-dependent manner

All observations using primary cells derived from patients may be influenced by inter-individual genetic differences that may change the reaction of MSCs towards AML cells. To better understand the molecular mechanisms without being impeded by different genetic backgrounds and to confirm our results obtained from human samples of leukemic patients, we made use of mouse models, which mimic the *MLL-AF9* and *AML1-ETO9a* subtypes of human AML. *AML1-ETO9a*, the oncofusion product resulting of the t(8;21)(q22;q22) translocation, and *MLL-AF9*, the oncofusion product resulting of the t(9;11)(p22;q23) translocation, are used to model AML in mice with a good or bad prognosis, respectively (Yan et al. 2006; Krivtsov et al. 2006; Somervaille and Cleary 2006; Tan et al. 2011) (Figure 4). We performed secondary transplantation instead of primary transplantation as in this setting once full blown

leukemia is initiated; the mice die similarly at around the same time. Thus, the time span through which the MSC are exposed to the leukemic cells is approximately the same in all mice transplanted with *MLL-AF9* and *AML1-ETO9a* expressing cells. Sublethally irradiated mice transplanted with healthy, non-transduced BM cells were used as controls. When signs of leukemia were apparent such as enlarged abdomen, difficulty of breathing, paleness of hand and feet and general weakness, the mice were euthanized and the BM cells were collected and analyzed and used for the culture of MSCs (Figure 13 A). 0.5-2x10^7 MNCs from mouse BM were cultured in 4 or 2 ml of IMDM containing 20% FBS and 1% penicillin/streptomycin in each well of a 6- or 12-well-plate, respectively, and incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. After splitting the cells 1-3 times and when the MSC culture became more than 80% confluent, they were washed with DBPS and 5-10x10^4 of C1498GFP cells were added to each well in 2-4 ml of DMEM containing 10% FBS (FBS) and 1% penicillin/streptomycin. After 6 days of co-culture, I counted the live GFP^+ cells and I found that the proliferation of the murine AML cell line C1498GFP was supported by murine MSCs derived from the BM of *MLL-AF9*- or *AML-ETO9a*-transplanted leukemic mice better than MSCs derived from mice transplanted with WT non-leukemic BM cells (Figures 13 B). These results further proved the finding that MSCs were polarized by the leukemic cells *in vivo* in such a way that the MSCs subsequently support the proliferation of leukemic cells. We thus confirmed that the bi-directional interaction between MSCs and AML cells *in vitro* was dependent on direct cell-cell contact (Figure 13 C).

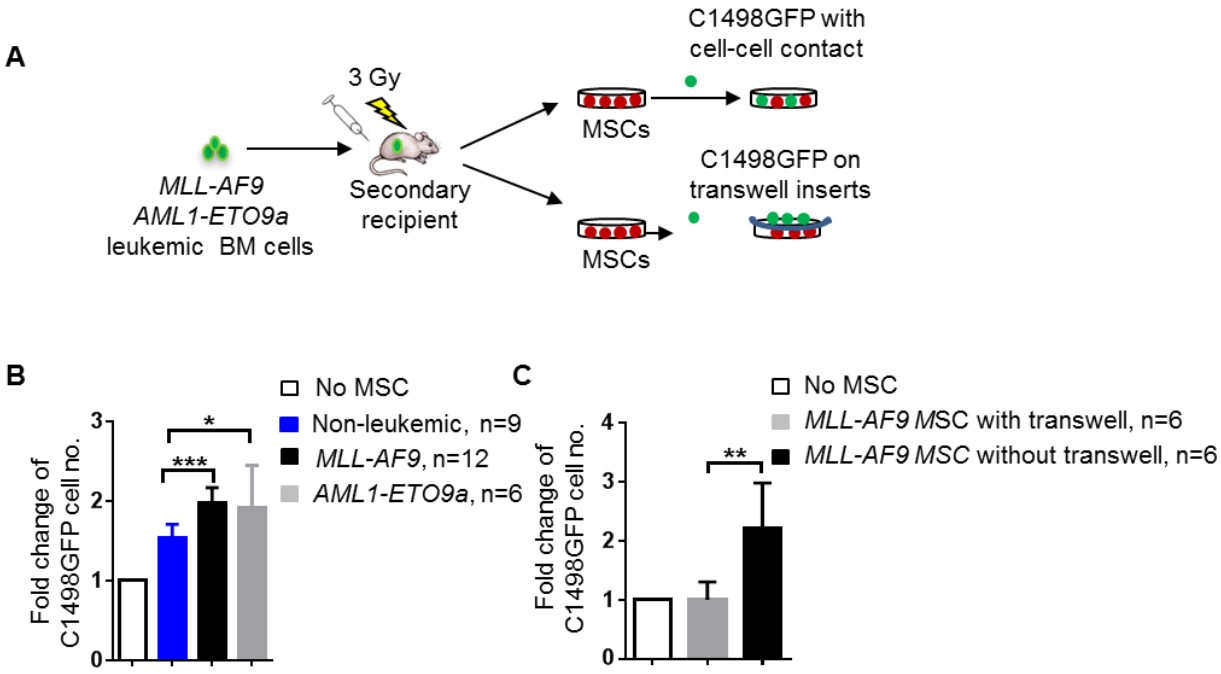

Figure 13: AMSCs from transplanted leukemic mice support the growth of a murine AML cell line in vitro

A) 1x10^5 *AML-AF9 or MLL-AF9a leukemic cells were transplanted into sublethally irradiated (3Gy) recipient mice. MSCs derived from BM of leukemic secondary transplanted recipient mice and non-leukemic mice (transplanted only with WT BM cells as control for irradiation-induced changes) were co-cultured with 5-10x10^4 of the AML cell line C1498GFP per well in direct cell-cell contact or with a trans-well insert.* ***B)*** *Fold change of C1498GFP cell numbers after co-culture with MSCs from MLL-AF9 (n=12) or AML1-ETO9a (n=6) leukemic mice compared to MSCs from sublethally irradiated mice and transplanted with WT BM cells (n=9). Results from triplicates of four independent experiments from MLL-AF9, two independent experiments from AML1-ETO9a leukemic mice and three independent experiments from non-leukemic mice were pooled and are shown here. Cells were cultured in a direct cell-cell contact (***p<0.001 and *p=0.49).* ***C)*** *Fold change of C1498GFP cell numbers after co-culturing them with MSCs derived from MLL-AF9 leukemic mice, either in direct cell-cell contact or with trans-well inserts. Results of triplicates from two independent experiments were pooled and are shown here (**p=0.005).*

The genetic variability of different cell lines might have an influence on the cell growth. To exclude the influence of this variations and to further validate the findings with the cell lines described above, I co-cultured 5x10^4 GFP$^+$ *MLL-AF9* cells, which were sorted from BM of secondary transplanted leukemic mice, with MSCs (70-80% confluent) that were also derived from *MLL-AF9* leukemic mice from the same

transplanted group for 3 days in 2 ml of IMDM containing 20% FBS, IL-3 (10 ng/ ml), IL-6 (10 ng/ml), SCF (20 ng/ml) and 1% penicillin/streptomycin in each well of a 12-well-plate (Figure 14 A). As a control, 5x10^4 GFP$^+$ *MLL-AF9* cells were co-cultured with MSCs derived from BM of non-leukemic mice. After 3 days of co-culture, I found that the growth of GFP$^+$ *MLL-AF9* leukemic BM cells was supported by MSCs from leukemic mice up to two-fold more than by MSCs from non-leukemic mice (Figure 14 B and C).

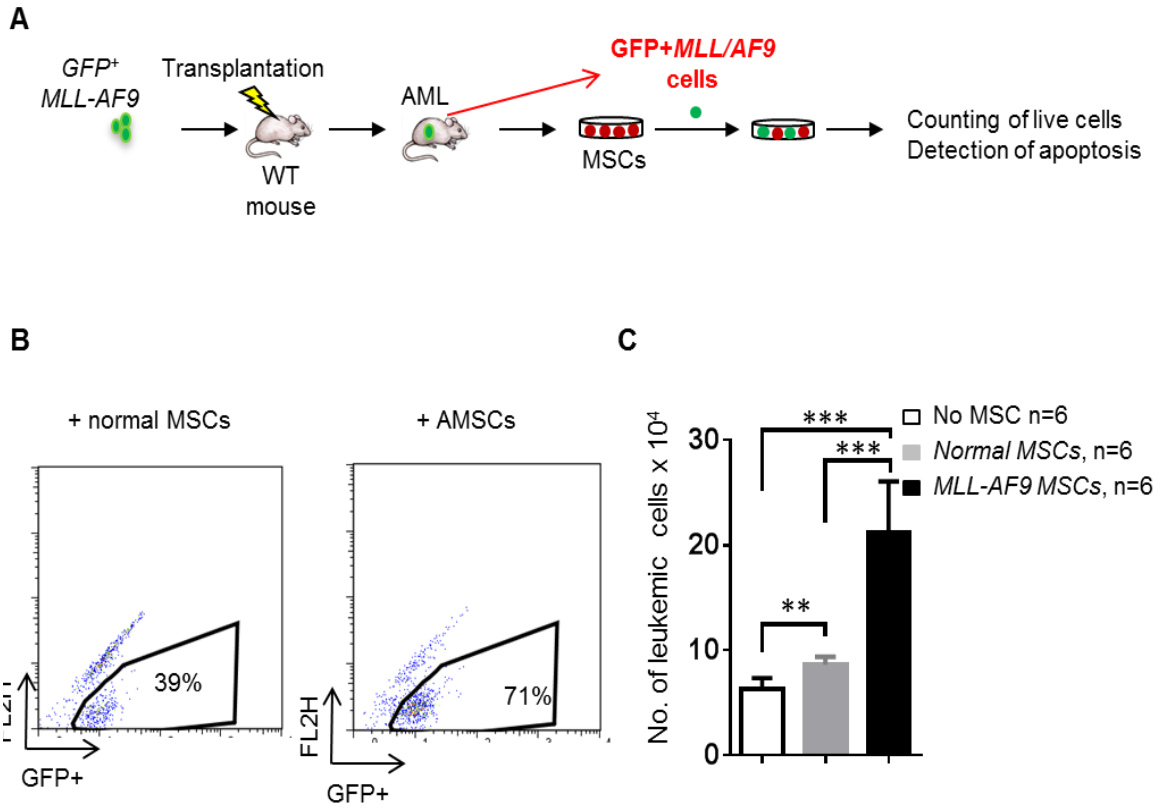

Figure 14: Murine AMSCs support the growth of murine MLL-AF9 transformed AML cells in vitro

A) *Schematic representation of the experimental design. MLL-AF9a leukemic cells derived from the BM of primary transplanted leukemic mice were re-transplanted into sublethally irradiated (3Gy) secondary recipient mice. AMSCs derived from the BM of leukemic secondary transplanted recipient mice and normal MSCs derived from the BM of irradiated mice transplanted with non-leukemic BM cells were co-cultured separately with 5x10^4 GFP$^+$ cells that were sorted from the BM of mice transplanted with MLL-AF9 BM cells. The plates of co-culture were then incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2 for 3 days* ***B)*** *Representative FACS plots showing the gating strategy of GFP$^+$ MLL-AF9 cells that were co-cultured with normal*

MSCs (left panel) or with AMSCs (right panel). ***C)*** *Fold change of GFP⁺ cell numbers after they were co-cultured with AMSCs compared to GFP⁺ cells that were co-cultured with normal MSCs. Results from triplicates of two independent experiments are shown here (**p<0.01 and ***p<0.001).*

7.1.1.4 AMSCs protect AML cells against apoptosis *in vitro*

In an effort to understand how AMSCs support the growth of leukemic cells, I investigated the role of MSCs with regard to the protection of AML cells against apoptosis. To this end, *MLL-AF9*-MSCs (cultured as described above in 7.1.1.3) and non-leukemic MSCs were co-cultured with $5x10^4$ GFP⁺ cells sorted from the BM of *MLL-AF9* secondary transplanted leukemic mice in 2 ml IMDM containing 20% FBS, IL-3 (10 ng/ ml), IL-6 (10 ng/ml), SCF (20 ng/ml) and 1% penicillin/streptomycin in each well of a 12 well-plate. They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days. Apoptosis of the GFP⁺ *MLL-AF9*-leukemic cells were determined by flow cytometry using anti-annexin V. I demonstrated that MSCs derived from transplanted leukemic mice protected the leukemia cells against apoptosis better than MSCs derived from the non-leukemic mice (Figure 15). This could be one reason that explains how AMSCs support the proliferation of leukemia cells through protecting them against apoptosis caused by exogenous triggers.

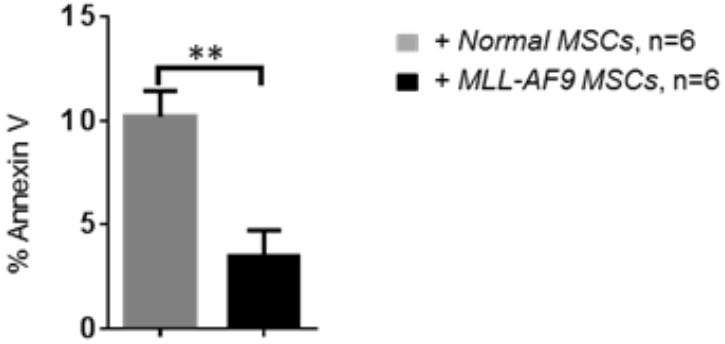

Figure 15: The level of apoptosis in leukemia cells co-cultured with AMSCs and normal MSCs

*The frequency of annexin V-positive cells in GFP+ MLL-AF9-transformed leukemic cells after they were co-cultured with MSCs derived from leukemic mice transplanted secondarily with MLL-AF9 cells and with normal MSCs derived from the BM of irradiated mice transplanted with non-leukemic BM cells for 3 days. Results from triplicates of two independent experiments are shown here (**p=0.0027).*

7.1.1.5 Co-culture of MSCs with AML cells *in vitro* leads to a shift in the cell cycle state of leukemic cells

For a better understanding of how MSCs support the proliferation of leukemic cells, I investigated the effect of MSCs on the cell cycle of an AML cell line *in vitro*. To this end, $5x10^4$ C1498GFP cells were co-cultured with MSCs derived from *MLL-AF9*-transplanted leukemic mice in 2 ml DMEM containing 10% FBS and 1% penicillin/streptomycin in each well of 12- well-plates. They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 (Figure 16 A). After 3 days of co-culture, the cell cycle distribution of leukemic cells was determined by flow cytometry using BrdU staining. I found that the cell cycle state of the leukemic cells was shifted in the presence of AMSCs, with fewer cells being in the quiescence phases G0/G1 and more cells in the DNA replication phase S (Figure 16 B and C). This result was confirmed when normal non-polarized MSC cells were co-cultured with the human AML cell line Kasumi-1. Similarly, I found that in the presence of MSCs, the DNA replication S and cell growth phases G2/M of Kasumi-1 cells were increased while the cell number in the quiescence phase (G0) was decreased compared to Kasumi-1 cells cultured without MSCs (data not shown).

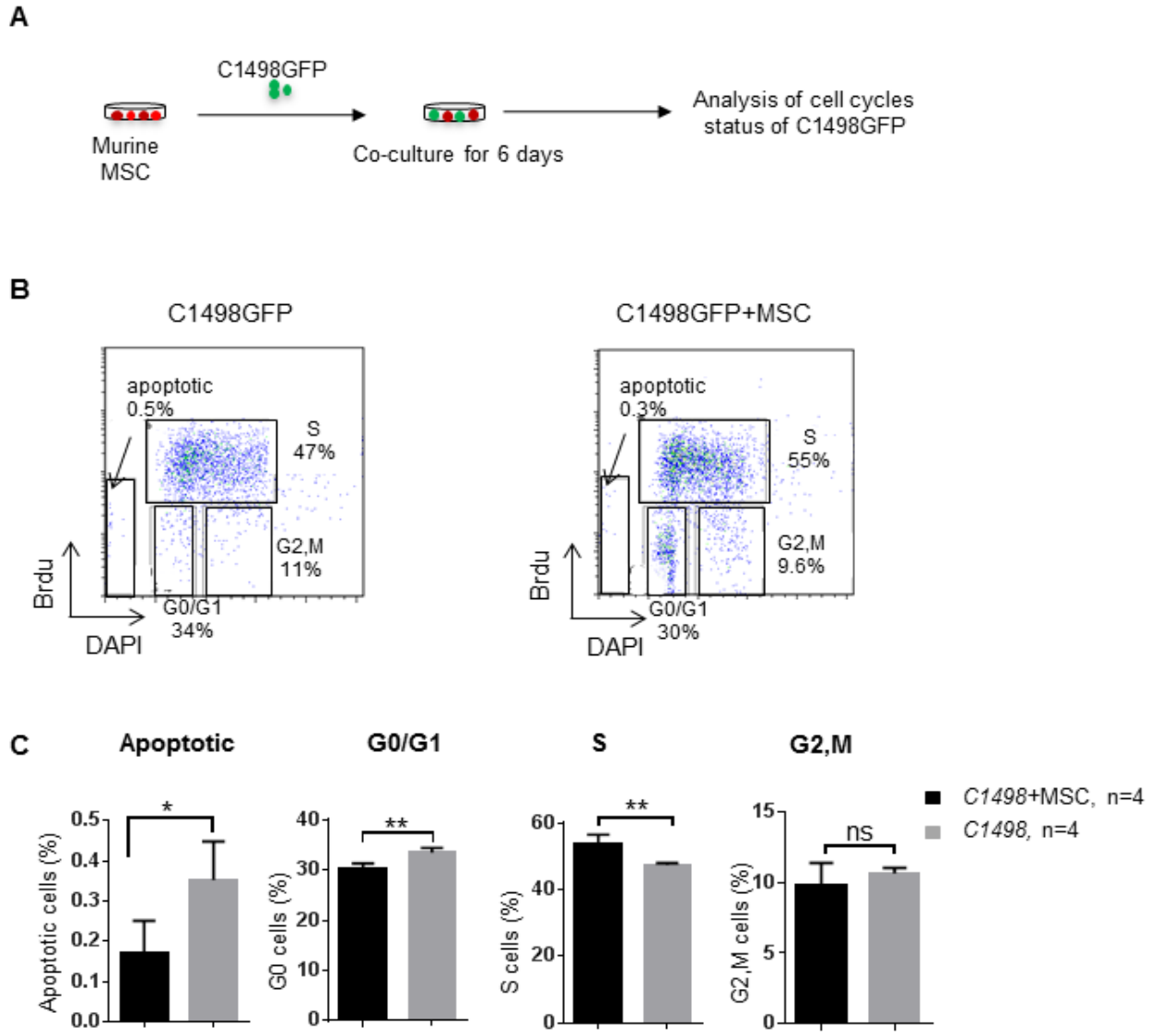

Figure 16: The effect of MSCs on the cell cycle distribution of murine C1498GFP AML cells in vitro.

A) *Schematic illustration of the experimental setup for co-culturing mouse MSCs with 5x10⁴ C1498GFP cells for 3 days. Cell cycle distribution of C1498GFP cells was studied by using BrdU APC and DAPI staining.* ***B)*** *Representative FACS figures show the gating strategy for the identification of different cell cycle stages of C1498GFP⁺ using BrdU APC and DAPI staining.* ***C)*** *The percentage of C1498GFP cells after co-cultured with (n=4) and without MSCs (n=4) (*p=0.026 for apoptotic cells, **p=0.0048 for G0/G1, **p=0.007 for S cells, ns= not significant). Results from duplicates of two independent experiments from C1498GFP⁺ with and without MSC were pooled and are shown here.*

7.1.2 AML-associated macrophages (AAMs) support the growth of leukemia cells *in vitro*

Many recent studies showed that macrophages support the growth and spread of tumor cells in many solid cancers. (Chittezhath et al. 2014; Candido and Hagemann 2013; Sousa et al. 2015; Quatromoni and Eruslanov 2012; Zhang et al. 2013). To study the molecular mechanisms and the function of monocytes/macrophages in the development of AML, we used different established murine models of human AML such as the fusion onco-proteins *AML1-ETO9a* and *MLL-AF9*, which are commonly involved in AML pathogenicity in humans (Yan et al. 2006; Krivtsov et al. 2006). A fraction of BM from leukemic and non-leukemic mice was used for the production of BMDM (Figure 6) *(Al-Matary et al. 2016)*. I then tested whether AAMs would support the growth of murine AML cells *in vitro* by co-culturing BMDMs with the murine AML cell line C1498GFP for 6-7 days. The C1498GFP cells were manually counted and the number of GFP-expressing leukemic cells was then determined by flow cytometry. I found that BMDMs derived from BM of transplanted leukemic mice supported the growth of C1498GFP cells better than BMDMs derived from BM of non-leukemic mice (Figure 17) (Al-Matary et al. 2016).

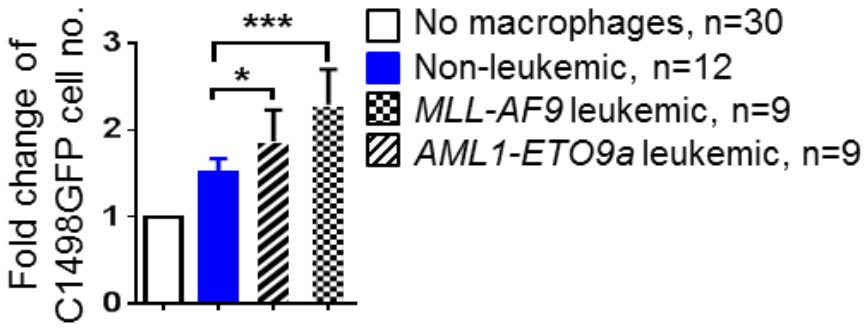

Figure 17: Murine AAMs support the growth of leukemia cells in vitro

2-3x10^5 BMDMs derived from BM of leukemic mice transplanted with non-transduced or AML1-ETO9a or MLL-AF9-transduced cells were co-cultured with 5x10^4 C1498GFP cells in 1 ml DMEM containing 10% FBS and 1% penicillin/streptomycin in each well of a 24 well-plate. They were then incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2 for 6-7 day. The fold change of C1498GFP live cell numbers is shown here. Results from triplicates of 3 independent experiments for mice transplanted with MLL-AF9 (n=9) and AML1-ETO9a (n=9) transduced cells and 4 independent experiments for mice transplanted with non-transduced cells (n=12)

*are shown. *p=0.03 for AML-ETO9a and **p<0.001 for MLL-AF9 transgenic cells). "Published data (Al-Matary et al. 2016)".*

To further investigate the role of AAMs with regard to the growth of leukemic cells *in vitro* and to avoid the artificial effect of cytokines on BMDMs polarization, we co-cultured sorted $GFP^-CD11b^+Ly6G^-$ AAMs from BM of *MLL-AF9* transplanted leukemic mice with the murine C1498GFP AML cell line for 48 hours. The proliferation of C1498GFP cells was increased up to two-fold in the presence of AAMs (Figure 18) (Al-Matary et al. 2016).

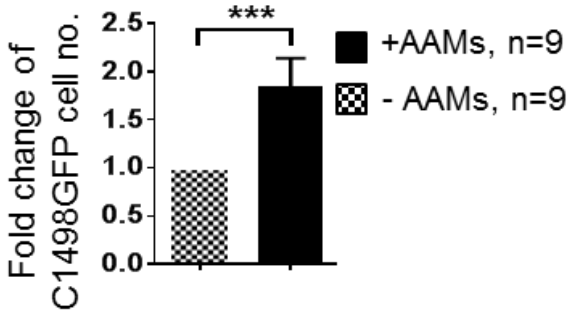

Figure 18: Murine sorted macrophages support the growth of leukemic cells in vitro

*$5x10^4$ $C1498GFP^+$ cells were co-cultured with $1.5x10^4$ GFP^-CD11b^+Ly6G AAMs sorted from the BM of MLL-AF9 transplanted leukemic mice in 0.5 ml DMEM containing 10% FBS and 1% penicillin/streptomycin in each well of a 48 well-plate. They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours (left panel). This chart shows the numbers of $C1498GFP^+$ cells in the presence (n=9) or absence (n=9) of sorted AAMs (right panel). Results of triplicates from three independent experiments are shown (***p=0.0009). "Published data (Al-Matary et al. 2016)".*

7.2 The frequency of stromal elements in leukemic patients and mice

7.2.1 The stroma elements; MSCs, endothelial cells and osteoblast lineage cells accumulate in BM of leukemic mice

It has been recently demonstrated that the BM niche was remodeled by myeloproliferative neoplasia in a way that it does not support the development of

normal HSCs well but favors the expansion of leukemic stem cells (Schepers et al. 2013). To investigate the effect of leukemic cells on the endosteal BM niche, I determined the frequency and the number of different stromal cells in hematopoietic cell-depleted, collagenase-treated crushed bones from leukemic and control mice (Figure 5). Among all stroma cells, the frequency and the number of endothelial cells ($CD45^-$ $Lin^-CD31^+Sca\text{-}1^+$), osteoblastic lineage-derivative (OBCs) cells ($CD45^-Lin^-CD31^-Sca\text{-}1^-CD51^+$) and MSCs ($CD45^-Lin^-CD31^-Sca\text{-}1^+CD51^+$) in both *MLL-AF9*- and *AML1-ETO9a*-transplanted leukemic mice were higher than in mice transplanted with WT BM cells (Figure 19 A and B).

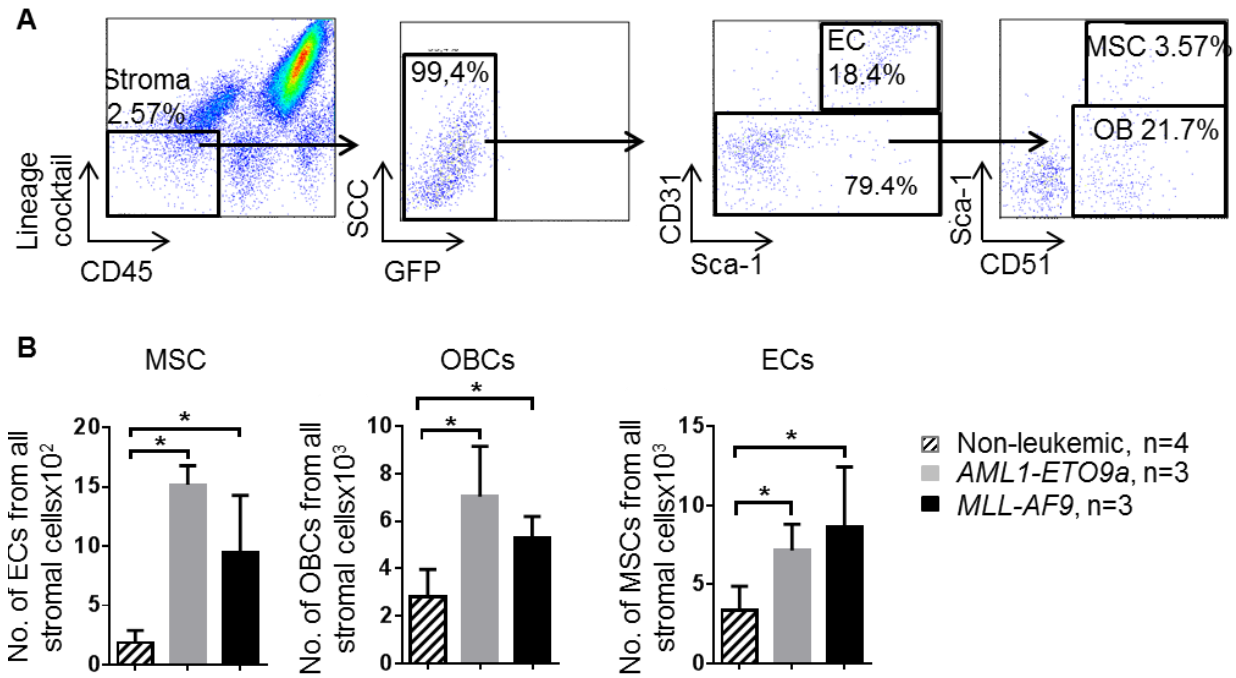

Figure 19: The frequency of stromal cells in transplanted leukemic mice.

A) *Representative FACS plots showing the gating strategy for the detection of different stromal cells in the endosteal niche. Endothelial cells (ECs) are $CD45^-Lin^-CD31^+Sca\text{-}1^+$, osteoblastic lineage cells (OBCs) are $CD45^-Lin^-CD31^-Sca\text{-}1^-CD51^+$, while mesenchymal stem cells (MSCs) are $CD45^-Lin^-CD31^-Sca\text{-}1^+CD51^+$.* ***B)*** *The absolute numbers of different stromal cells (MSCs, OBCs and ECs) in total endosteal stromal cells extracted from collagenase I treated crushed bones from MLL-AF9 (n=3) or AML1-ETO9a (n=3) transplanted leukemic mice compared to non-leukemic mice transplanted with non-leukemic cells (n=4) MSCs (left panel), OBCs (middle panel) and ECs (right panel). The numbers of all three cell types are higher in AML1-ETO9a and MLL-AF9 transplanted leukemic mice than in non-leukemic control mice.*

7.2.2 AAMs accumulate in leukemic organs of AML patients and mice

7.2.2.1 AAMs proliferate and accumulate in the BM of AML patients

CD163 has been demonstrated to be characteristic of human monocytes/macrophage cells (Nguyen et al. 2005). Recently, it has also been reported that CD163+ M2 TAMs play an important role in the development and progression of several hematological malignancies such as multiple myeloma (Beider et al. 2014) or classical Hodgkin lymphoma (CHL) (Harris et al. 2012). CD206 is another common cell surface marker of TAMs (Quatromoni and Eruslanov 2012). To investigate the ability of leukemia cells to educate and polarize macrophages, the frequency of CD163+CD206+ macrophages was determined in BM of AML patients and normal volunteers (Table 10) (Al-Matary et al. 2016). We found that the percentage and the number of CD163+CD206+ M2 like macrophages in BM of AML patients was significantly higher than in BM of healthy volunteers (Figure 20 A-C) (Al-Matary et al. 2016).

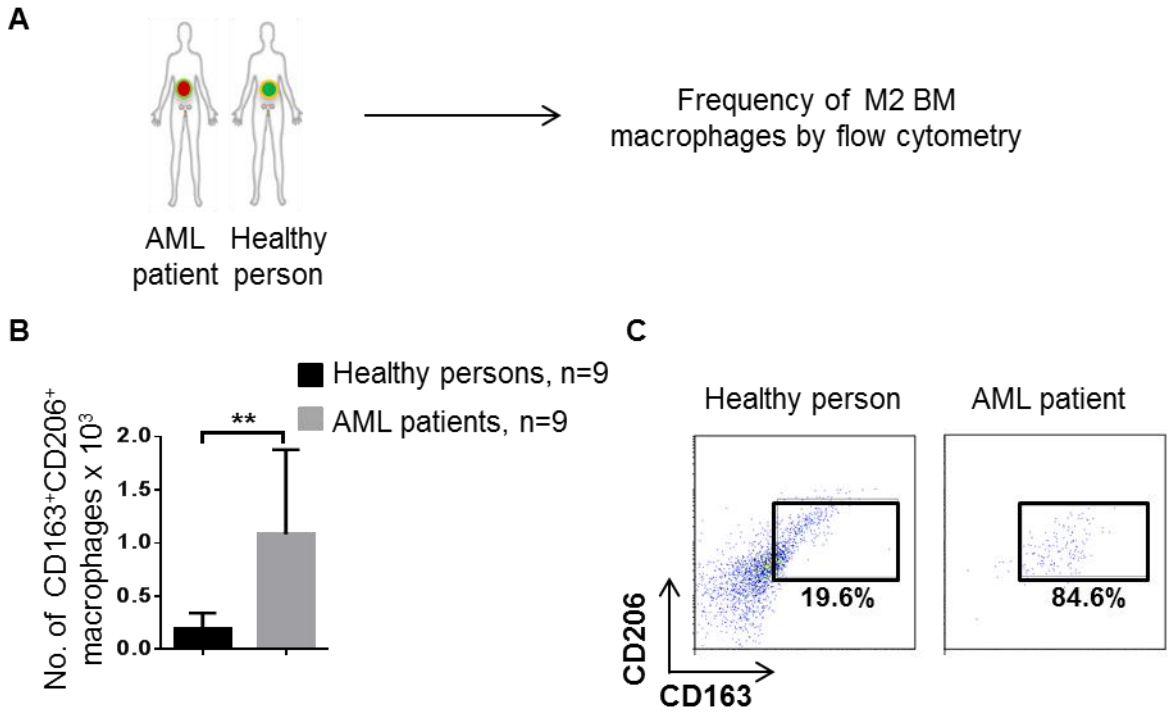

Figure 20: Accumulation of CD163⁺CD206⁺ M2 like macrophages in BM of AML patients.

A) *Schematic illustration of the experimental design. Ficoll density gradient centrifugation was used to isolate BM mononuclear cells from pre-diagnosed AML*

patients and healthy volunteers. I then evaluated the frequency of M2 macrophages in BM of AML patients and healthy volunteers by flow cytometry. ***B)*** *The absolute numbers of BM CD163^{+}CD206^{+} M2 macrophages within CD14^{+} monocytes (out of 100000 cells) determined in BM of AML patients (n=9) and healthy volunteers (n=9), (**p=0.008).* ***C)*** *Representative FACS figures show that the percentage of CD163^{+}CD206^{+} M2 macrophages (gated within total CD14^{+} monocytes) in one AML patient compared to one healthy volunteer. "Published data (Al-Matary et al. 2016)".*

7.2.2.2 Transplanted leukemic cells induce monocytes/macrophages to proliferate and accumulate in BM and spleen of recipient mice

To study the interaction between leukemia cells and monocytes/macrophages *in vivo*, we used the following two transplantation models of AML; *MLL-AF9* or *AML1-ETO9a* as described above (Figure 4). When the mice showed features of leukemia such as splenomegaly, tiredness, pale hands and feet and difficulty of breathing, monocytes/macrophages were assessed in BM and spleen of leukemic and normal mice. The GFP^{-} cells were first determined as non-leukemic cells in BM and spleen of leukemic secondary recipient mice. We then detected AAMs in this population using two different antibody combinations. In the first method, we defined AAMs as GFP^{-} CD11b^{hi}Gr1^{int} cells (Karsunky et al. 2002) (Figure 21 A), and found that the percentage of GFP^{-} AAMs in BM and spleen of leukemic transplanted mice was significantly higher than in mice transplanted with normal BM cells (Figure 21 B) (Al-Matary et al. 2016).

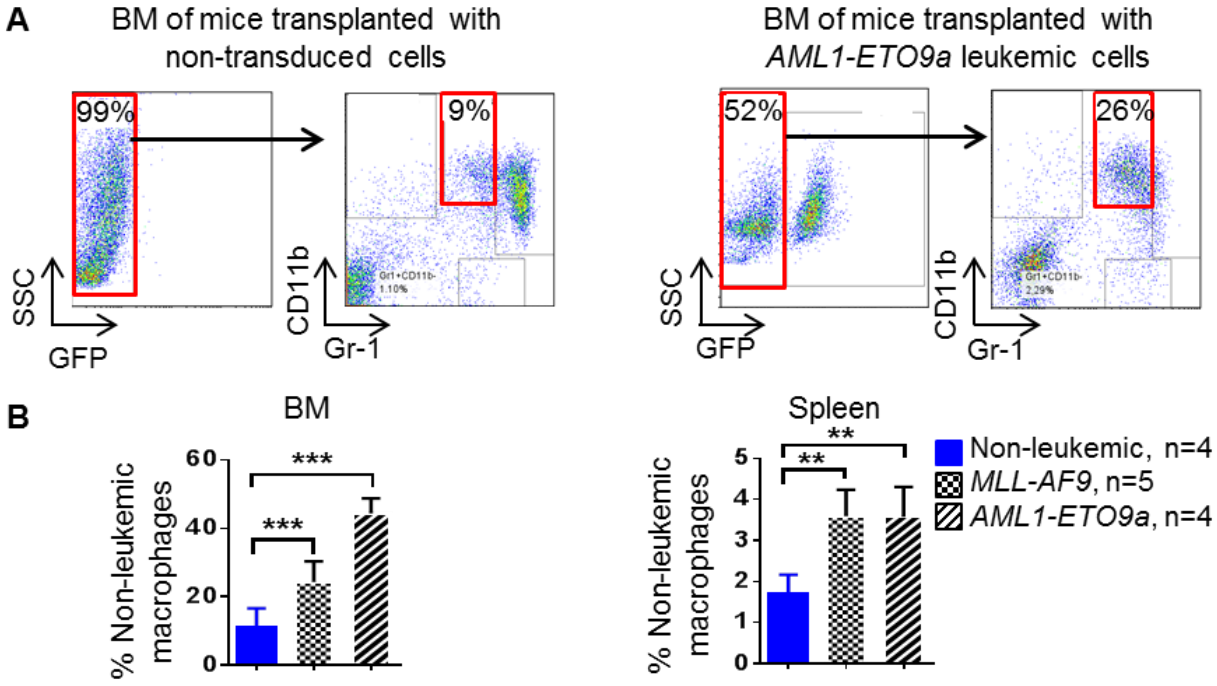

Figure 21: GFP$^-$CD11b^{hi}Gr1^{int} AAMs proliferate and accumulate in BM and spleen of AML mice

A) *Representative FACS figures show GFP$^-$CD11b^{hi}Gr1^{int} monocytes/macrophages in BM cells derived from mice transplanted with non-leukemic (left panel) or leukemic cells (AML1-ETO9a-transduced) (right panel).* ***B)*** *The percentage of non-leukemic monocytes/macrophages (GFP$^-$CD11b^{hi}Gr1$^{int)}$ in BM (left panel) and spleen (right panel) of leukemic mice transplanted with AML1-ETO9a (n=5) or MLL-AF9 (n=5) transduced cells compared to mice transplanted with non-leukemic cells (n=4), (***p<0.0008 for BM, **p<0.001 for spleen). "Published data (Al-Matary et al. 2016)".*

In the second setting, AAMs were defined as GFP$^-$CD11b$^+$Ly6G$^-$ cells (Laoui et al. 2014) in which the gating of monocytes/macrophages was better with respect to the gating described above (Figure 22 A) (Al-Matary et al. 2016). Nevertheless, we found a similar result that GFP$^-$CD11b$^+$Ly6G$^-$ cells accumulated in BM and spleen of transplanted leukemic mice (Figure 22 B) (Al-Matary et al. 2016). Thus, AML cells direct the macrophages/monocytes to proliferate and accumulate in BM and spleen of leukemic mice.

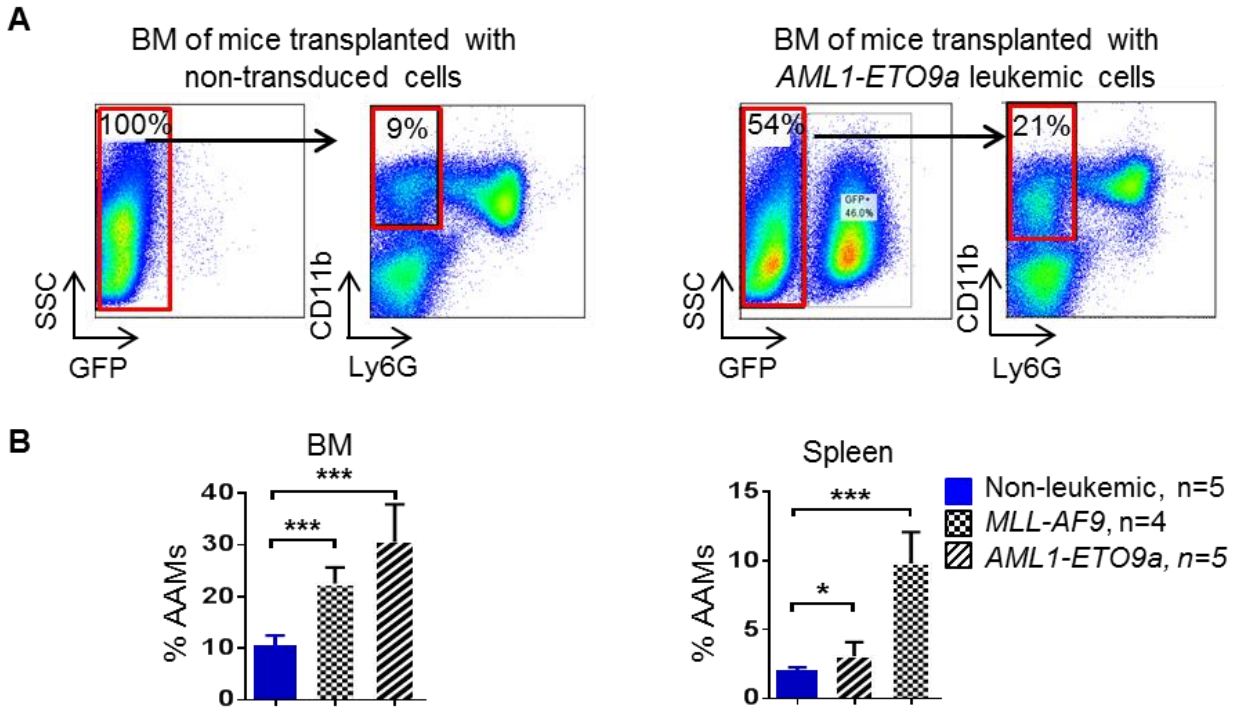

Figure 22: GFP$^-$CD11b$^+$ Ly6G$^-$ AAMs proliferate and accumulate in BM and spleen of AML mice

A) *Representative FACS plot showing the frequency of GFP$^-$CD11b$^+$Ly6G$^-$ monocytes/macrophages in BM cells derived from mice transplanted with non-leukemic (left panel) or leukemic cells (AML1-ETO9a-transduced) (right panel).* ***B)*** *The percentage of non-leukemic GFP$^-$CD11b$^+$Ly6G$^-$ macrophages in BM (left) or spleen (right) of leukemic mice transplanted with AML1-ETO9a (n=5) or MLL-AF9 (n=5) transduced cells compared to mice transplanted with non-leukemic cells (n=4) (***p<0.0001 for BM, *p=0.04 and ***p=0.0002 for spleen). "Published data (Al-Matary et al. 2016)".*

7.2.2.3 AAMs accumulate in BM and spleen of *NUP98-HOXD13* transgenic leukemic mice

To verify whether these findings are not affected by radiation, we used a third mouse model of human MDS/AML, the *NUP98-HOXD13* transgenic mouse model which mirrors human myeloid malignancies (Lin et al. 2005). These mice were controlled weekly for signs of AML such as weakness, paleness of hands and feet and hepatosplenomegaly. After developing signs of leukemia, they were killed and BM and spleen cells were prepared as described above in 6.2.4. I then detected the total

percentage of monocytes/macrophages by flow cytometry and I found a similar result as in transplanted leukemic mice, namely that that the percentage of AAMs in BM and spleen of leukemic mice was higher than in WT non-leukemic mice (Figure 23 A, B) (Al-Matary et al. 2016). We also confirmed that, phenotypically, monocytes/macrophages in both settings, GFP-CD11b^{hi}Gr1^{int} and GFP-CD11b^{+}Ly6G^{-}, express F4/80 which is a characteristic and typical marker of mouse macrophages/monocytes (Bain et al. 2014) (Figure 23 C) (Al-Matary et al. 2016).

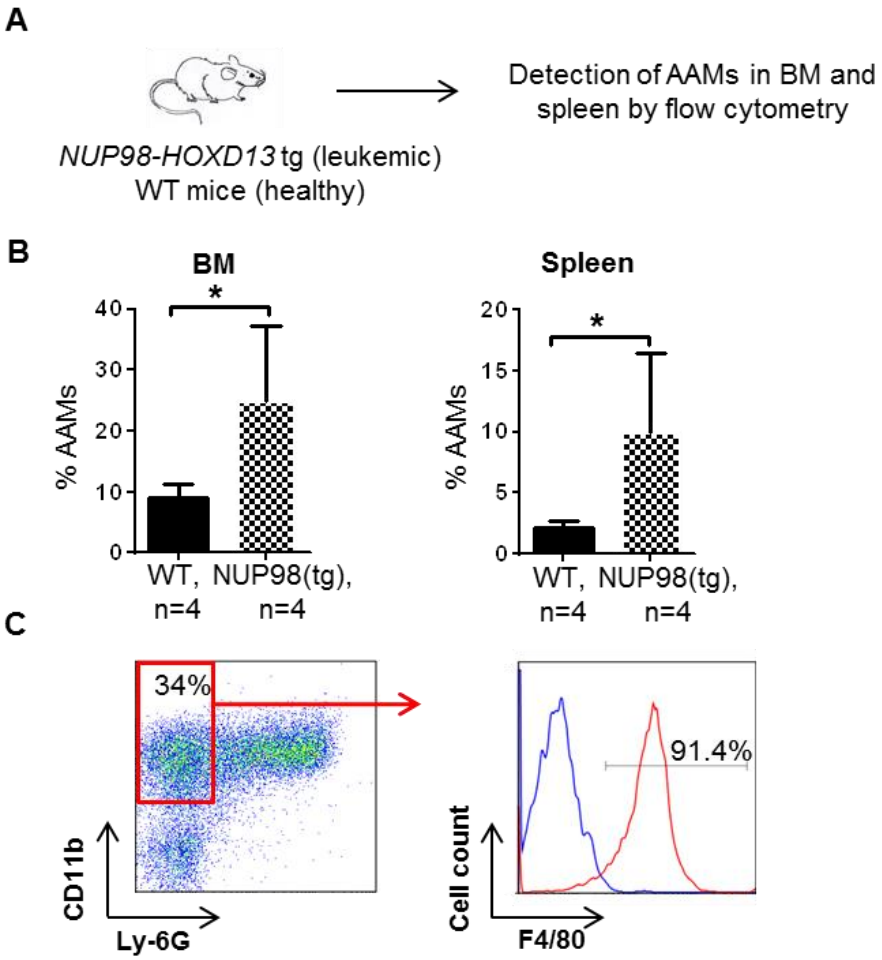

Figure 23: Increased frequency of AAMs in BM and spleen of leukemic NUP98-HOXD13 mice

A) *A representative figure of the experimental design. Leukemic NUP98HOXD13 and healthy WT mice were killed and the percentage of AAMs was detected by flow cytometry.* ***B)*** *The percentage of CD11b^{+}Ly6G^{-} monocytes/macrophages in BM (left panel) and spleen (right panel) of non-leukemic WT (n=4) and leukemic NUP98-HOXD13 (n=4) mice (*p=0.05 for BM and *p=0.04 for the spleen).* ***C)*** *Representative FACS figure from BM cells of NUP98-HOXD13. The expression of F4/80 surface*

marker in CD11b^{+}Ly6G^{-} monocytes/macrophages was more than 90%. "Published data (Al-Matary et al. 2016)".

7.3 Characterization of AML-associated stromal cells

7.3.1 Characterization of human and murine MSCs

Since isolation and expansion of primary MSCs requires a lengthy preparation time and a high number of cells and because they only have a limited capacity to be expanded in culture *in vitro*, we used immortalized MSCs from AML patients or healthy donor (Lipps et al., submitted). As I mentioned above in 7.1.1.2, I found that these immMSCs can differentiate into adipocytes, osteocytes, and chondrocytes, one important feature of MSCs (Dominici et al. 2006) (Figure 10). Furthermore, I found that the primary and immortalized human MSCs expressed CD73, CD90 and CD105, the cell surface proteins suggested to be characteristic for MSCs (Figure 11 A) (Geyh et al. 2016; Dominici et al. 2006), while the expression of CD45 and CD34 (<3%), markers for HSCs and CD31, a marker for endothelial cells (Geyh et al. 2016; Dominici et al. 2006) were absent (Figure 11 B).

I also confirmed that murine MSCs express CD51, Sca-1, CD44 and CD29, the surface markers that are characteristic for MSCs (Schepers et al. 2013; Baustian, Hanley, and Ceredig 2015) (Figure 25 A), while they were negative for the endothelial marker CD31 (Schepers et al. 2013) and markers for haematopoietic cells such as CD45, CD11b and Ter119 (Figure 25 B).

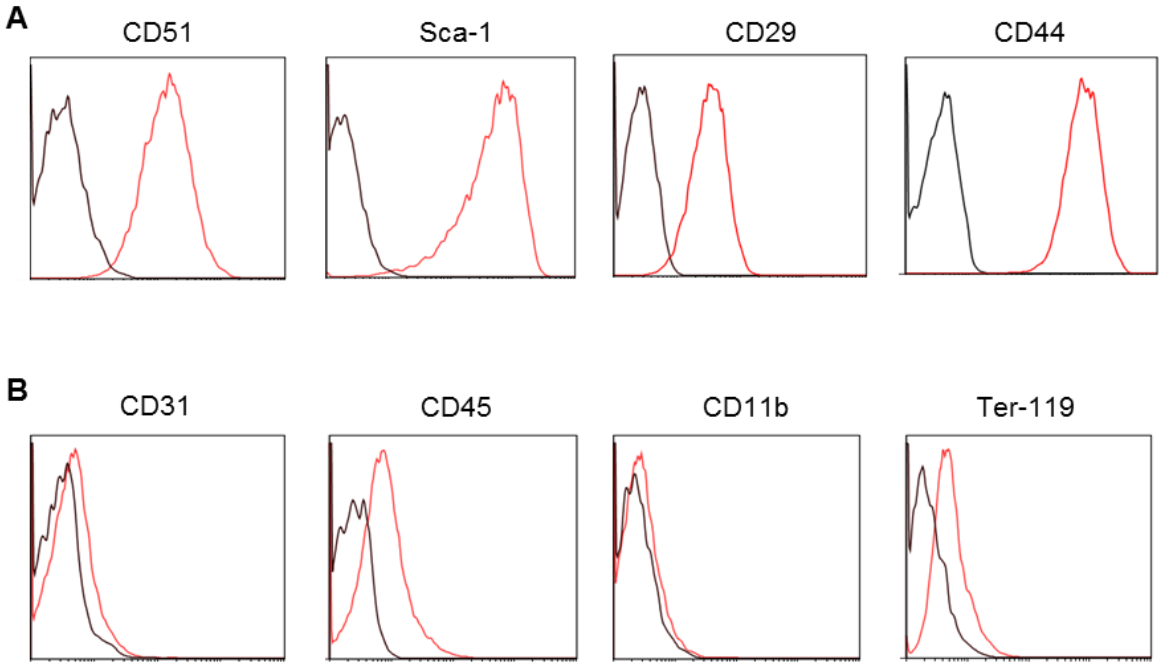

Figure 24: Immunophenotyping of murine MSCs.

A) *In vitro cultured murine MSCs were stained with specific antibodies recognizing surface markers characteristic for MSCs such as CD51, Sca-1 and CD29 (red line). Black line represents unstained control.* ***B)*** *In vitro cultured MSCs were stained with specific antibodies recognizing surface markers characteristic for endothelial cells such as CD31 or hematopoietic cells such as CD45, c-kit, CD11b and MHC II (red line). Black line represents unstained control.*

7.3.2 Characterization of AAMs

7.3.2.1 Morphology of AAMs

To evaluate the morphology of AAMs, cytospin preparations of sorted GFP$^-$ CD11b$^+$Ly6G$^-$ cells derived from BM of C1498GFP transplanted leukemic mice were stained with Wright-Giemsa and examined by light microscope. We validated here that these sorted cells were certainly macrophages (Figure 25) (Al-Matary et al. 2016).

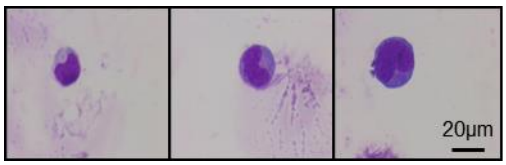

Figure 25: Morphology of AAMs

Cytospin preparation of sorted AAMs (GFP$^-$CD11b$^+$Ly6G$^-$) was stained with the wright Giemsa. Bar represents 20µm. "Published data (Al-Matary et al. 2016)".

7.3.2.2 Immunophenotyping of AAMs

The gene expression pattern, cytokine secretion and cell surface molecules are together used to characterize monocytes/macrophages (Davies et al. 2013). By using a specific gating strategy that was used in another study for classification of TAMs in lung cancer (Laoui et al. 2014), the different monocytes/macrophages subsets were quantified in BM and spleen of sublethally irradiated mice transplanted either with C1498GFP cell line or with *MLL-AF9* or *AML1-ETO9a* leukemic BM cells. GFP^- $CD11b^+Ly6G^-$ monocytes/macrophages from leukemic and non-leukemic mice were classified into six populations depending on the expression levels of Ly6C and MHCII surface markers (Figure 26) (Laoui et al. 2014; Movahedi et al. 2010; Al-Matary et al. 2016). The percentage of $Ly6C^-MHCII^-$ macrophages/monocytes in BM and spleen of transplanted leukemic mice was higher than in transplanted non-leukemic mice (Figure 27) (Al-Matary et al. 2016). According to the classification of Laoui et al, 2014, I considered these macrophages as leukemia-associated macrophage 1 (AAM1) cells which have the same phenotype as $Ly6C^-MHC\ II^-$ TAM1 in lung cancer (Laoui et al. 2014; Movahedi et al. 2010; Al-Matary et al. 2016).

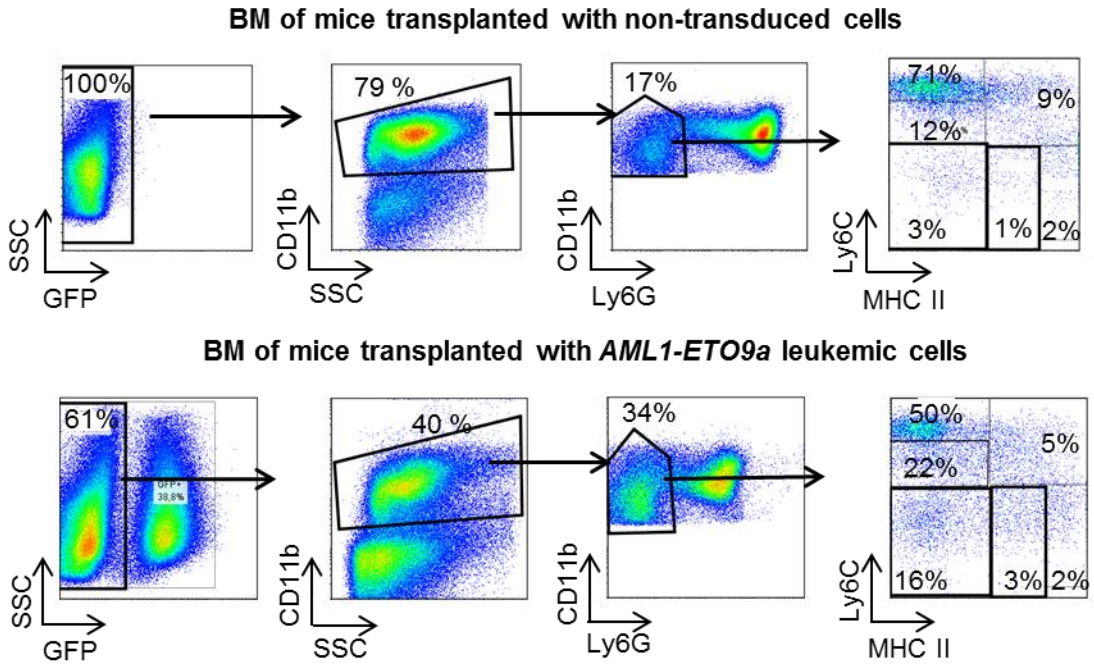

Figure 26: Gating strategy used for classification of AAMs

Representative FACS plots showing the gating strategy used for classifying different types of GFP$^-$CD11b$^+$Ly6G$^-$ monocytes/macrophages from BM of mice transplanted either with non-transduced or with AML1-ETO9a transduced BM cells according to the expression of Ly6C and MHCII markers. AAM1 cells have a GFP$^-$CD11b$^+$Ly6G$^-$ MHCII$^-$Ly6C$^-$ phenotype. "Published data (Al-Matary et al. 2016)".

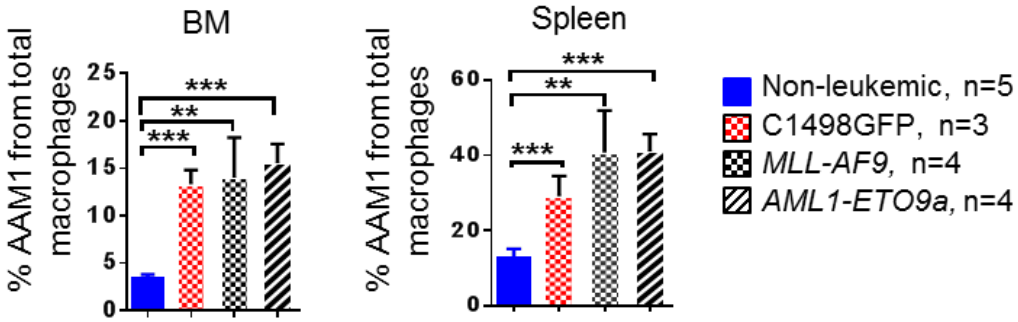

Figure 27: The frequency of AAM1 in BM and spleen of transplanted leukemic mice

*The percentage of MHCII$^-$Ly6C$^-$ AAM1 in BM (left panel) and spleen (right panel) of leukemic mice transplanted with AML1-ETO9a, (n=4), MLL-AF9 (n=4) or C1498GFP (n=3) compared to mice transplanted with non-leukemic cells, (***p<0.0001, **p=0.001). "Published data (Al-Matary et al. 2016)".*

Ly6C^{int}MHCII^{low} immature macrophages are intermediate cells between Ly6C$^+$MHCII$^-$ monocytes and other mature cells such as *MHCII$^-$Ly6C$^-$* AAM1, *MHCII^{int}Ly6C$^-$* AAM2 and *MHCII^{hi}Ly6C$^-$* dendritic cells (Movahedi et al. 2010) (Figure 28 A) (Al-Matary et al. 2016). Here I found that the Ly6C^{int}MHCII^{low} immature cells accumulated in BM and spleen of transplanted leukemic mice, whereas the percentage of other macrophage classes such as Ly6C$^+$MHCII$^-$ monocytes were decreased or not significantly changed (Figure 28 B) (Al-Matary et al. 2016).

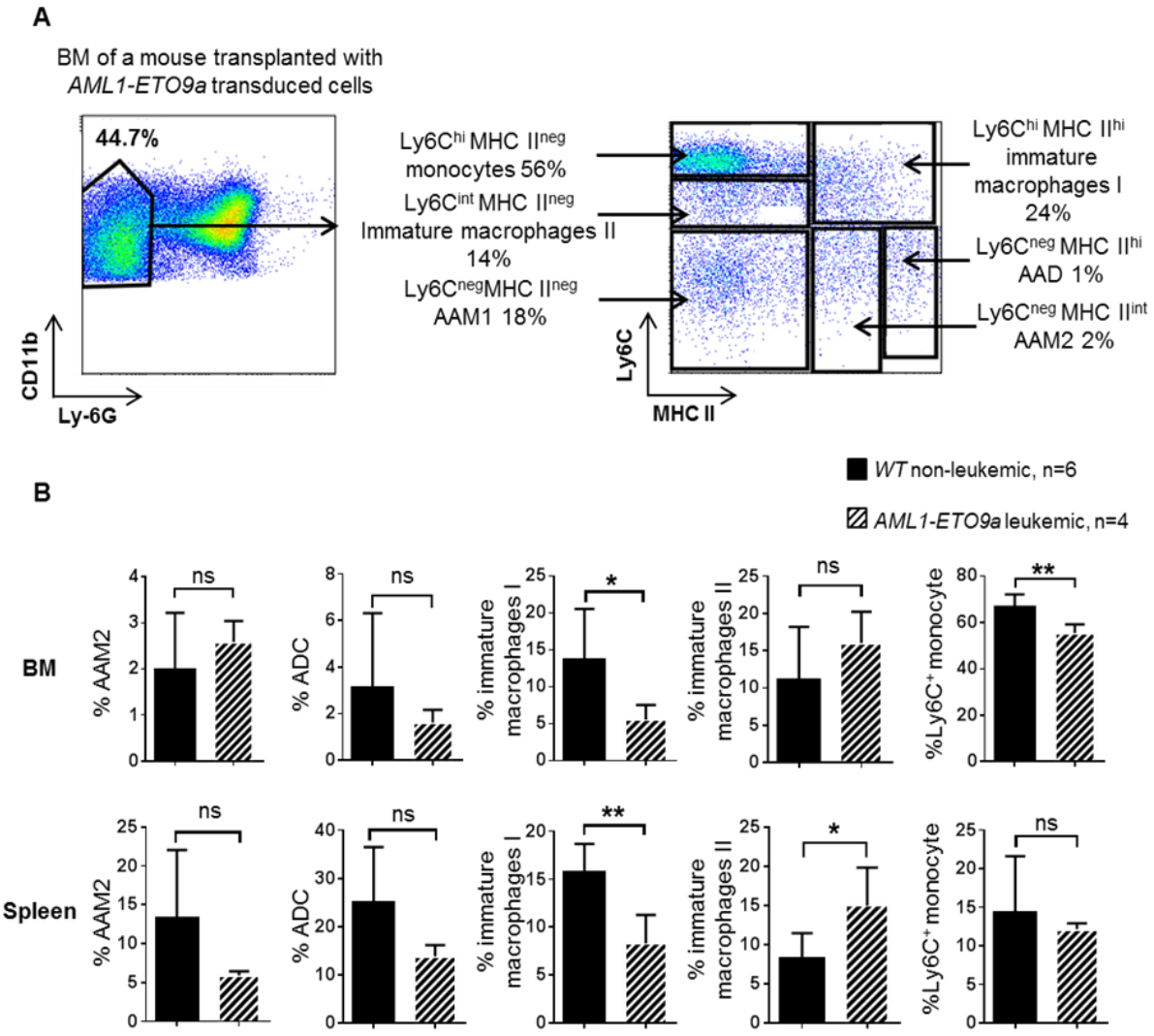

Figure 28: Distribution of different macrophage subsets in BM and spleen of AML1-ETO9a leukemic mice.

(A) *Representative FACS plot shows a gating strategy for classification of* GFP$^-$ CD11b$^+$Ly6G$^-$ monocytes/macrophages into 6 subclasses according to the expression of Ly6C and MHCII surface markers. ***B)*** *The percentage of different macrophages subsets in BM (upper panel) and spleen (lower panel) of mice transplanted with AML1-ETO9a BM cells (n=4) compared to mice transplanted with non-transduced cells (n=6) (*p=0.05 for immature macrophages I in BM, **p=0.008 for Ly6C$^+$ monocytes in BM **p=0.004 for immature macrophages I in spleen, *p=0.02 for immature macrophage II in spleen). "Published data (Al-Matary et al. 2016)".*

To validate the above results and to exclude the effect of radiation on macrophage polarization *in vivo*, I determined the frequency of AAM1 in leukemic transgenic mice. I again found that AAM1 accumulated in BM and spleen of leukemic transgenic mice compared to WT non-leukemic mice (Figure 29 A and B) (Al-Matary et al. 2016).

Interestingly, the survival of the leukemic *NUP98-HOXD13* mice was found to be inversely correlated with the frequency of AAM1 in their BM (Figure 29 C) (Al-Matary et al. 2016).

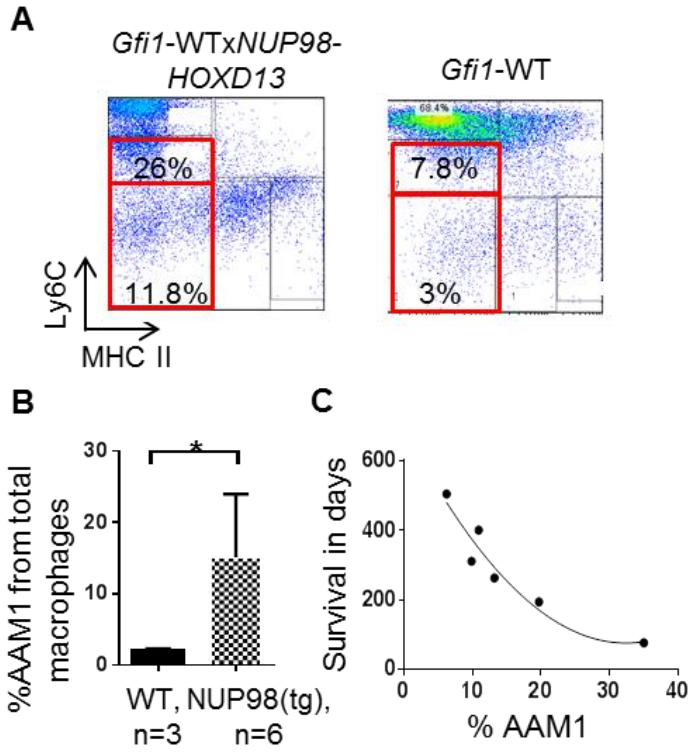

Figure 29: The frequency of AAM1 in BM of leukemic NUP98-HOXD13 mice

A) *Representative FACS figures of macrophage subclasses in BM of NUP98-HOXD13 mouse model compared to WT non-leukemic mouse.* ***B)*** *The percentage of MHCII^{-}Ly6C^{-} AAM1 cells in BM of leukemic NUP98-HOXD13 mice (n=6) in comparison to non-leukemic mice (n=3) (*p=0.04).* ***C)*** *The correlation between the frequency of AAM1 and the survival of the leukemic NUP98-HOXD13 mice (R square = 0.92). "Published data (Al-Matary et al. 2016)".*

7.3.2.3 Characterization of AAMs by RT-PCR and ELISA

To further characterize AAMs in more detail, the gene expression pattern and cytokine secretions of AAMs were assessed and evaluated (Davies et al. 2013). GFP^{-}CD11b^{+}Ly6G^{-} macrophages were sorted from the BM of leukemic mice transplanted with C1498GFP. GFP^{-}CD11b^{+}Ly6G^{-} macrophages sorted from BM of mice transplanted with non-transduced cells were used as a control (Al-Matary et al. 2016). I found that the expression of *Arg1* mRNA, a characteristic marker of M2

macrophages, was upregulated in AAMs, whereas the expression of *IL-6* and *Nos2* mRNA, which are characteristic for M1 macrophages, were downregulated (Figure 30) (Al-Matary et al. 2016).

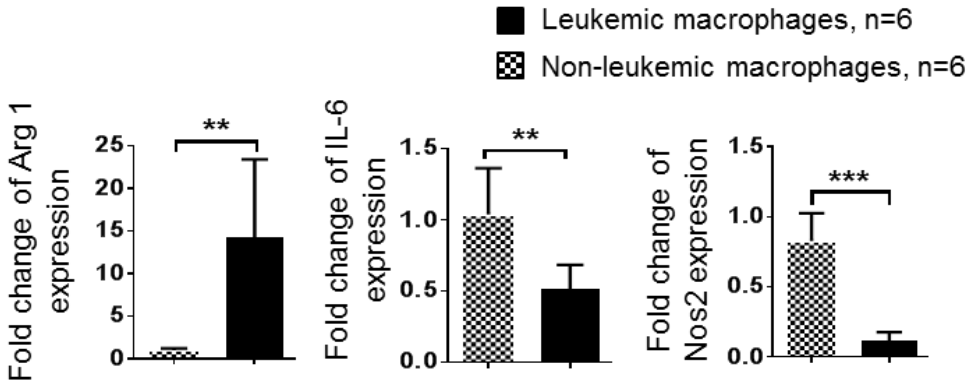

Figure 30 RNA profile of AAM

*Fold change of the mRNA expression level of Arg1, IL-6 and Nos2 in macrophages sorted from BM of non-leukemic mice transplanted with WT BM cells (n=6) and AAMs from leukemic mice transplanted with C1498GFP cells (n=6). The results were normalized to the expression of GAPDH mRNA. Results of duplicates from three independent experiments are shown (**p=0.001). "Published data (Al-Matary et al. 2016)".*

To further characterize AAMs at the secretion level, $CD11b^{+}Ly6G^{-}$ AAMs were sorted from BM of leukemic mice transplanted with C1498GFP cell and from BM of mice transplanted with non-transduced cells and then cultured in DMDM/glutamax supplemented with 10% FBS and 1% Pen/Strep. After 24 hours of incubation, the supernatants were then collected, filtered and the levels of IL-10 were determined by ELISA kit. The concentration of IL-10, another marker of M2, in AAM was higher than in macrophages derived from non-leukemic mice (Figure 31) (Al-Matary et al. 2016).

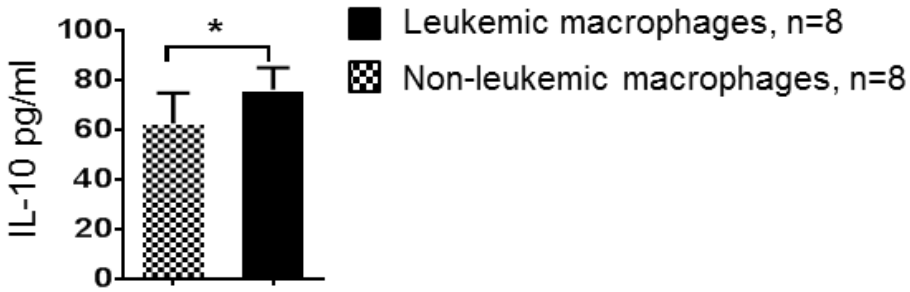

Figure 31. The concentration of IL-10 in AAMs

*$5x10^4$ AAMs sorted from leukemic mice transplanted with C1498GFP cells (n=8) or $5x10^4$ $CD11b^+Ly6G^-$ non-leukemic macrophages sorted from mice transplanted with non-leukemic BM cells (n=8) were cultured in DMDM/glutamax supplemented with 10% FBS and 1% Pen/Strep. After 24 hours of incubation, the concentration of IL-10 was measured in the collected and filtered supernatants using an ELISA kit. Results of duplicates from four independent experiments are shown (*p=0.01). "Published data (Al-Matary et al. 2016)".*

7.4 The role of Gfi1 in the polarization and function of AML-associated stroma

7.4.1 The role of Gfi1 in the polarization of AMSCs

A recent study has demonstrated that Gfi1 is a transcriptional repressor of Runt-related transcription factor 2 (Runx2) (D'Souza et al. 2011), which in turn regulates the differentiation of osteoblasts (Bruderer et al. 2014). In the same study, the GFI1 mRNA was highly expressed in MSCs derived from BM of multiple myeloma patients compared to healthy controls (D'Souza et al. 2011). Therefore we also examined the function of *GFI1* in AMSCs. *GFI1* mRNA expression was 4 to 5 fold higher in primary AMSCs as well as in AMCS cell lines than in healthy controls (Figure 32).

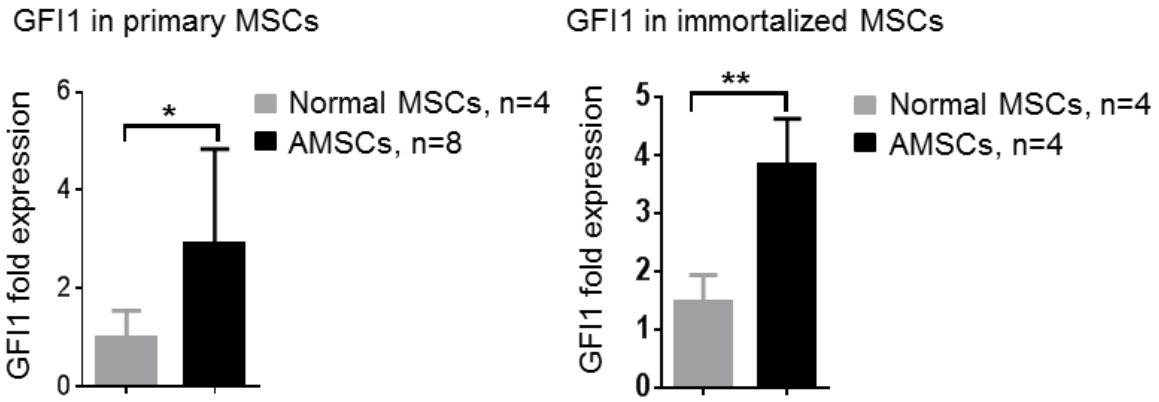

Figure 32 Gfi1 expression was upregulated in AMSCs

*Fold change of Gfi1 mRNA expression level in primary AMSCs (n=4) in comparison to MSCs from respective healthy volunteers (n=2) (**p=0.002) (left panel). Fold change of GFI1 mRNA level in MSCs from 2 AML patients and 2 normal patients (**p=0.002) (right panel). The results were normalized to human GAPDH: RT-PCR results of duplicates from each MSC line (normal vs. AML) are shown.*

Since the expression of *GFI1* was upregulated in AMSCs, we further examined the effect of loss of *Gfi1* on the function of MSCs *in vivo* with regard to the growth of leukemia cells. To this end, *Gfi1*-defcient mice *(Gfi1*-KO) mice were crossed with *NUP98-HOXD13* transgenic mice. Upon development of AML in mice, MSCs from *Gfi1*-WT and *Gfi1*-KO *NUP98-HOXD13* leukemic mice as well as from *Gfi1*-WT and *Gfi1*-KO healthy mice were isolated and co-cultured with C1498GFP cells (Figure 33 A). *Gfi1*-KO AMSCs did not support the growth of murine C1498GFP AML cells *in vitro* to the same extent as *Gfi1*-WT AMSCs (Figure 33 B).

Consistent with previous findings reported that GFI1 suppresses the differentiation of osteoblast in BM of patients with multiple myeloma (D'Souza et al. 2011) we found that the number of OBC cells in bones of *Gfi1*-KO mice was higher than in bones of *Gfi1*-WT mice (Figure 34). Additionally, GFI1 seems to have an important role in the polarization of MSCs toward AML-supporting state, since *Gfi1*-deficient AMSCs lose their capacity to support the growth of leukemic cells.

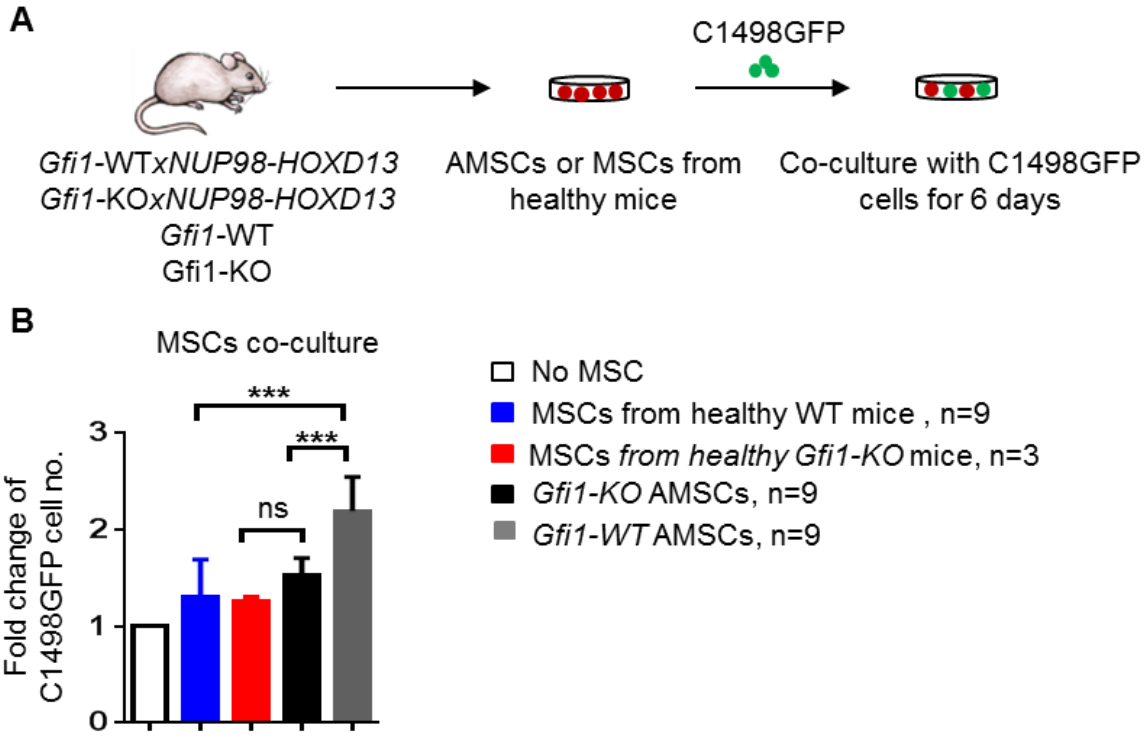

Figure 33 The role of Gfi1 in polarization and function of AMSCs.

A) *Schematic representation of the experimental setup for co-culturing MSCs from Gfi1-WTxNUP98-HOXD13 and Gfi1-KOxNUP98-HOXD13 leukemic mice with the C1498GFP cell line for 6 days.* ***B)*** *Fold change of C1498GFP cell numbers after co-culture with MSCs from Gfi1-WTxNUP98-HOXD13 (n=9) and Gfi1-KOxNUP98-HOXD13 (n=9) leukemic mice compared to non-leukemic Gfi1-WT (n=9) and Gfi1-KO MSCs (n=3) (***p< 0.001, ns= not significant). Results from triplicates of three independent experiments within cells from Gfi1-WTxNUP98-HOXD13, Gfi1-KOxNUP98-HOXD13 leukemic mice and non-leukemic Gfi1-WT and triplicates of one experiment from healthy Gfi1-KO MSCs were pooled and are shown here. Average and SD were calculated.*

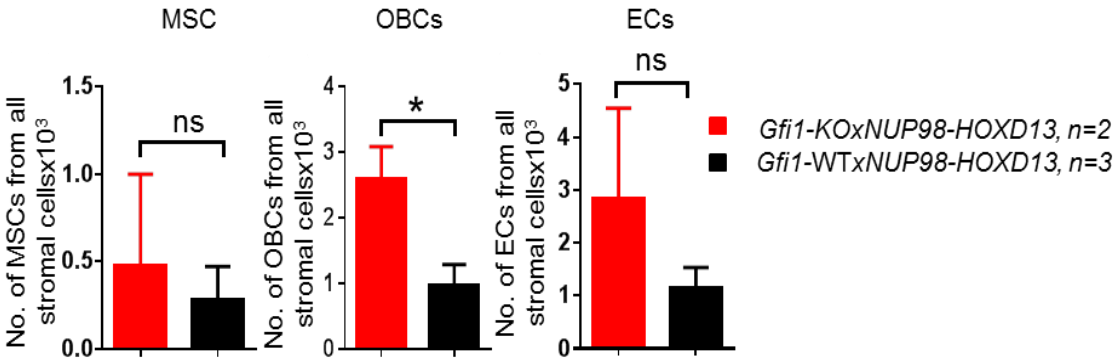

Figure 34: The frequency of stromal cells was increased in endosteal niche of Gfi1-KO mice

*The absolute number of different stromal cells in total endosteal stromal cells from bones of Gfi1-WTxNUP98-HOXD13 (n=3), Gfi1-KOxNUP98-HOXD13 (n=2) leukemic mice are given here: MSCs (left panel), OBCs (middle panel) and ECs (right panel). (*p=0.02 for OBCs, ns= not significant).*

7.4.2 The role of Gfi1 in the polarization of AAMs

7.4.2.1 Gfi1 expression was upregulated in AAMs

It has been reported that Gfi1 has an important role in the development and differentiation of granulocytes and macrophages (Phelan et al. 2010; van der Meer, Jansen, and van der Reijden 2010). In this work, *Gfi1* expression in GFP^- $CD11b^+Ly6G^-$ AAMs sorted from BM of transplanted leukemic and non-leukemic mice was determined. The expression level of *Gfi1* mRNA was about two-fold upregulated in AAMs compared to its level in macrophages derived from healthy mice (Figure 35) (Al-Matary et al. 2016).

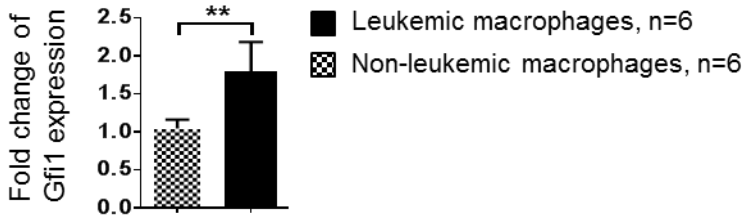

Figure 35: The level of Gfi1 mRNA expression in AAMs in vivo

Gfi1 mRNA expression level relative to the housekeeping gene *GAPDH in AAMs sorted from leukemic mice transplanted with C1498GFP cells (n=6) and non-leukemic mice transplanted with WT BM cells (n=6). Results of duplicates from three independent experiments are shown (**p=0.001). "Published data (Al-Matary et al. 2016)".*

Furthermore, the level of *Gfi1* mRNA expression was assessed in BMDM co-cultured with C1498GFP compared to BMDMs that were cultured alone (Figure 36) (Al-Matary et al. 2016). Additionally and in consistent with our previous observations, the expression of *Gfi1* was also two-fold upregulated in BMDM co-cultured with C1498GFP compared to BMDMs that were cultured alone. This suggested that higher levels of *Gfi1* could be required for the polarization of macrophages *in vivo* and *in vitro.*

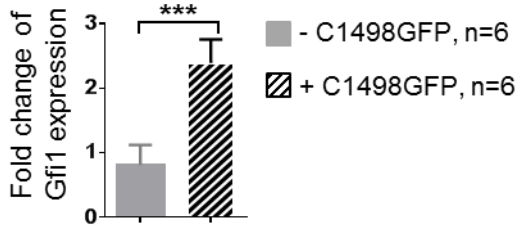

Figure 36: The level of Gfi1 mRNA expression in AAMs in vivo

The level of Gfi1 mRNA was measured in BMDMs that were co-cultured in presence or absence of C1498GFP (4*10^5) for 3 days. *The chart shows fold change of the Gfi1 mRNA expression level in BMDMs cultured in presence (n=6) or absence (n=6) of C1498GFP cells. The results were normalized to GAPDH. RT-PCR results of duplicates from three independent experiments are shown (**p<0.0001 for Gfi1). "Published data (Al-Matary et al. 2016)".*

7.4.2.2 The role of Gfi1 in polarization of macrophages *in vitro*

I next investigated the polarization of macrophage in response to M1 or M2 stimuli. To this end, I cultured *Gfi1*-KO and *Gfi1*-WT BMDMs in presence of either LPS or INF-y, which stimulate differentiation of macrophages into M1, or IL-4, which stimulate differentiation of macrophages into M2 (Mantovani et al. 2004; Mantovani et al. 2002) (Figure 8). In absence of *Gfi1,* activation of BMDM by LPS or INF-y resulted

in a four-fold increase in Ly6C^{+}CD206^{-} M1 polarization compared to BMDMs derived from *Gfi1*-WT mice (Figure 37 A and 38 A and B). Furthermore, the expression of *IL-6* and *Nos2* mRNA (Figure 37 B) as well as the secretion of IL-6 and IL-1B cytokines (Figure 37 C) in *Gfi1*-KO M1(LPS) macrophages were significantly increased compared to *Gfi1*-WT M1(LPS) (Al-Matary et al. 2016). Also, *Gfi1*-KO M1(INF-y) expressed higher levels of *Nos2* mRNA and secreted higher amounts of IL-1B than *Gfi1*-WT M1(INF-y) (Figure 38 D) (Al-Matary et al. 2016).

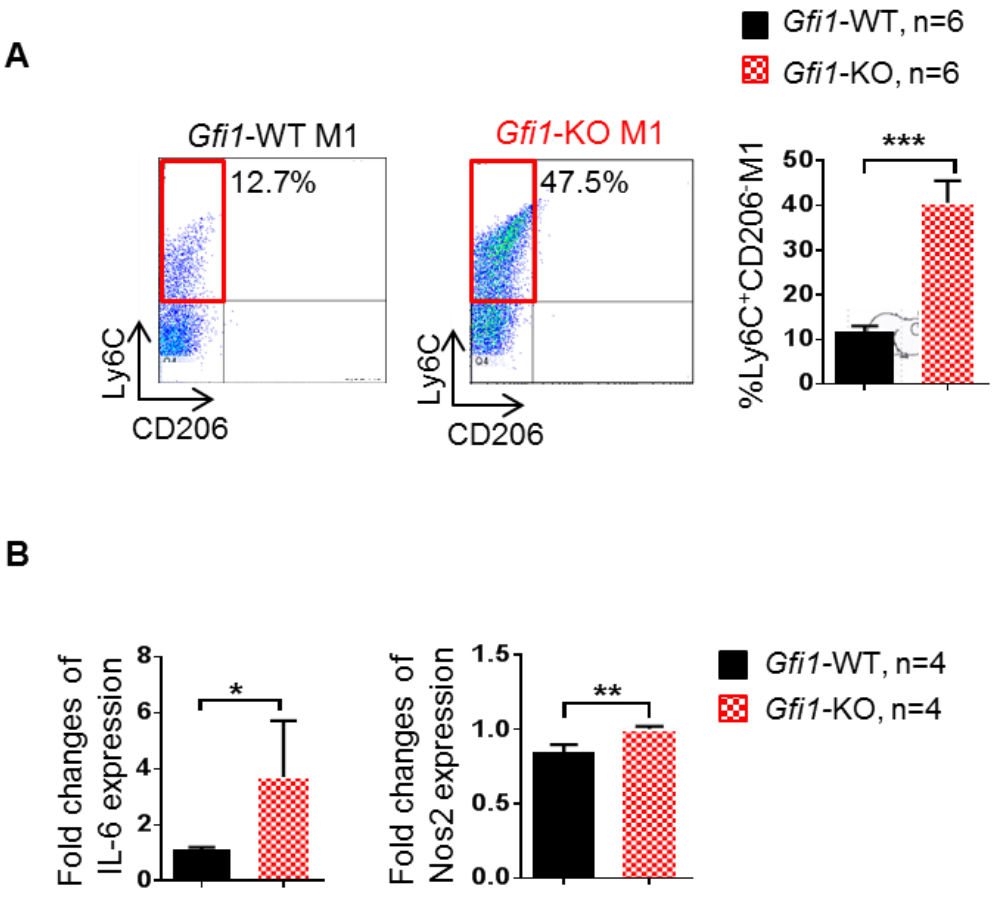

Figure 37: Gfi1 suppresses the M1 polarization of macrophage by LPS in vitro.

A) *Representative FACS figures showing Ly6C^{+}CD206^{-} M1(LPS) macrophages polarized from Gfi1-WT and Gfi1-KO BMDMs (left panel). The percentage of Ly6C^{+}CD206^{-} M1(LPS) macrophages derived from Gfi1-WT (n=6) and Gfi1-KO (n=6) BMDMs (right panel) (***p<0.0001). Results of duplicates from three independent experiments are shown.* ***B)*** *Fold change of mRNA expression level of IL-6 and Nos2 in Gfi1-WT (n=4) and Gfi1-KO (n=4) M1(LPS) macrophages. All results were normalized to mouse GAPDH. Results of duplicates from two independent experiments are given (*p=0.03 for IL-6, **p=0.002 for Nos2). "Published data (Al-Matary et al. 2016)".*

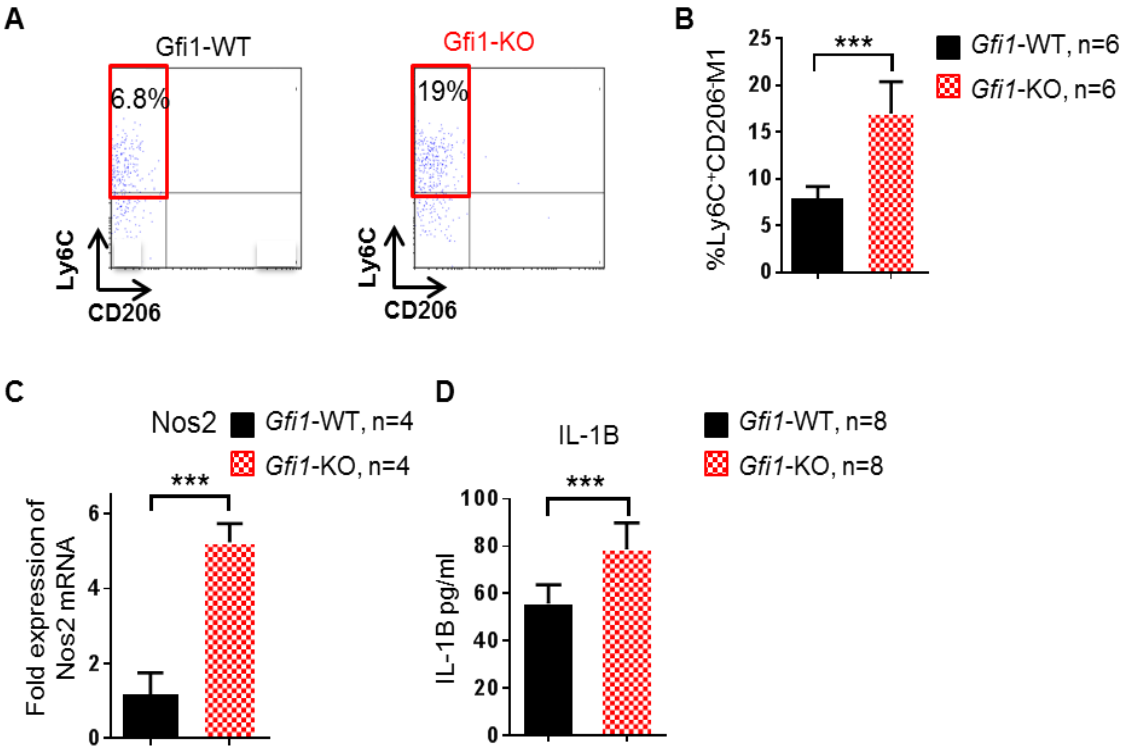

Figure 38: Polarization of M1 macrophages by INF-y

A) *Representative FACS figures of Ly6C^{+}CD206^{-} M1(INF-y) macrophages polarized by INF-y from Gfi1-WT and Gfi1-KO BMDMs.* ***B)*** *The percentage of Ly6C^{+}CD206^{-} M1(INF-y) polarized by INF-y from Gfi1-WT (n=6) and Gfi1-KO (n=6) BMDMs, (***p=0.0001).* ***C)*** *Fold change of Nos2 mRNA expression level in Gfi1-WT (n=4) and Gfi1-KO (n=4) M1 Ly6C^{+} CD206^{-} macrophages M1(INF-y). RT-PCR results of duplicates from two independent experiments are given (***p<0.0001). All results were normalized to mouse GAPDH.* ***D****) The concentration of IL-1B in mediums collected from M1(INF-y) macrophages derived from Gfi1-WT (n=8) and Gfi1-KO (n=8) mice. Results of duplicates from two independent experiments are given (p<0.0004). "Published data (Al-Matary et al. 2016)".*

Regarding M2 macrophages polarization, the results showed no difference between the frequencies of CD206^{+}M2(IL-4) macrophages derived from *Gfi1*-WT and *Gfi1*-KO mice. However, a significant increase in *Arg1* mRNA expression (Figure 39 A) and IL-10 secretion (Figure 39 B) were observed in *Gfi1*-WT macrophages stimulated by IL-4 (M2(IL-4)) compared to *Gfi1*-KO M2(IL-4) (Al-Matary et al. 2016).

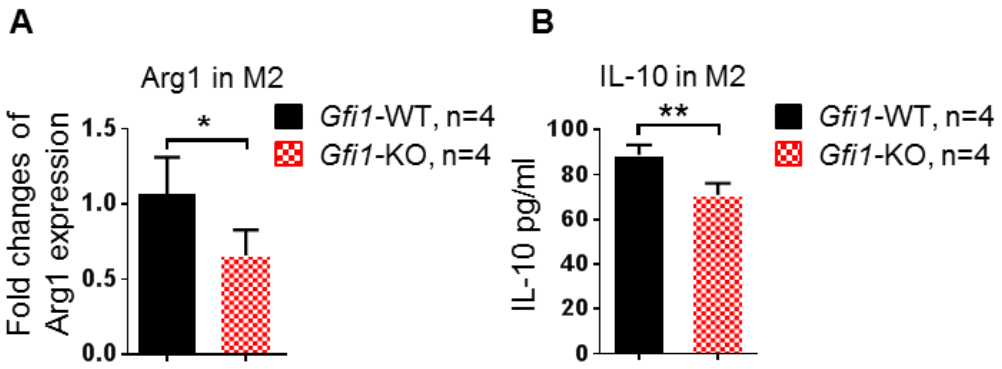

Figure 39: The role of Gfi1 in polarization of M2 macrophages in vitro

A) *Fold change of Arg1 mRNA expression level in Gfi1-WT (n=4) and Gfi1-KO (n=4) M2(IL-4) macrophages polarized by IL-10. The results were normalized to GAPDH. Results of duplicates from two independent experiments are shown (*p=0.04).* ***B)*** *The concentration of IL-10 in supernatants of Gfi1-WT (n=8) and Gfi1-KO (n=8) M2(IL-4) macrophages. Results of duplicates from four independent experiments are shown (**p=0.004). "Published data (Al-Matary et al. 2016)".*

Depending on the signals and cytokines secreted from tumor microenvironment, the polarization of M1 and M2 macrophages take place simultaneously *in vivo*. For the first time, I tried to mimic this *in vitro* by treating BMDMs derived from *Gfi1*-WT and *Gfi1*-KO with both stimuli LPS and IL-4 at the same time, followed by the examination of the polarization of macrophages through flow cytometric detection of surface markers expression levels. In the presence of both stimuli, the majority of *Gfi1*-WT BMDMs polarization into $Ly6C^{-}CD206^{+}$ M2-like macrophages with a minimum polarization into $Ly6C^{+}CD206^{-}$ M1 macrophages (Figure 40 A and B), whereas $CD206^{+}Ly6C^{-}$ M2 polarization *was* less efficient in *Gfi1*-KO BMDMs and the differentiation into $Ly6C^{+}CD206^{+}$ macrophages and $Ly6C^{+}CD206^{-}$ M1 macrophages was enhanced (Figure 40 A and B) (Al-Matary et al. 2016). Together, these results confirm that in the presence of *Gfi1,* the polarization of macrophages was directed towards M2 macrophages rather than M1 macrophages.

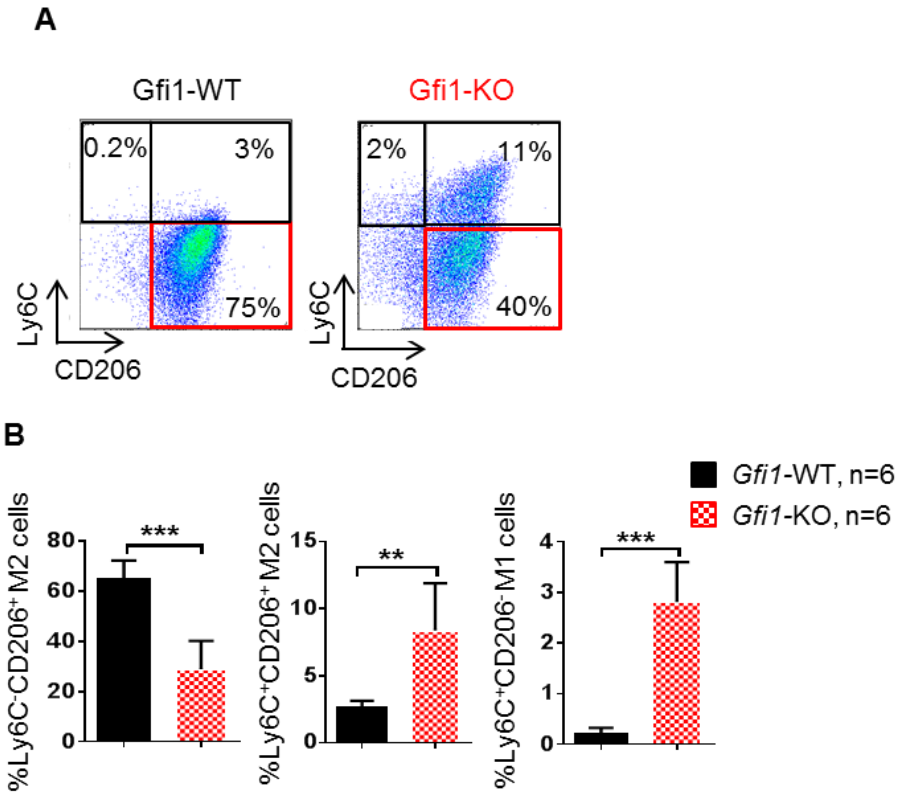

Figure 40: Polarization of macrophages by both LPS and IL-4

A) *Representative FACS figures of different macrophage subsets derived from Gfi1-WT or Gfi1-KO mice polarized by both LPS and IL-4.* ***B)*** *BMDMs derived from Gfi1-WT (n=4) and Gfi1-KO (n=4) mice were polarized for 48 hours with LPS and IL-4. The percentage of Ly6C$^-$CD206$^+$ M2 macrophages (left panel) (***p<0.0001), Ly6C$^+$CD206$^+$ macrophages (middle, **p=0.002) and Ly6C$^+$CD206$^-$ M1 macrophages (right, p<0.0001) are shown. Results of duplicates from two independent experiments are depicted. "Published data (Al-Matary et al. 2016)".*

7.4.2.3 The role of Gfi1 in the polarization of macrophages *in vivo*

In addition to the results observed *in vitro*, we examined the effect of absent *Gfi1* expression in the surrounding stroma on the growth of leukemic cells *in vivo*. To this end, we transplanted *MLL-AF9*-expressing BM cells into sublethally irradiated *Gfi1*-WT and *Gfi1*-KO mice (Figure 41 A), thus similar to the other approaches described above. We observed that *Gfi1*-KO mice transplanted with *MLL-AF9* leukemic cells survived longer (p=0.01) (Figure 41 B) and contained lower numbers of leukemic cells in BM and peripheral blood (Figure 41 C) than *Gfi1*-WT mice transplanted with *MLL-AF9*-expressing leukemic cells (Al-Matary et al. 2016).

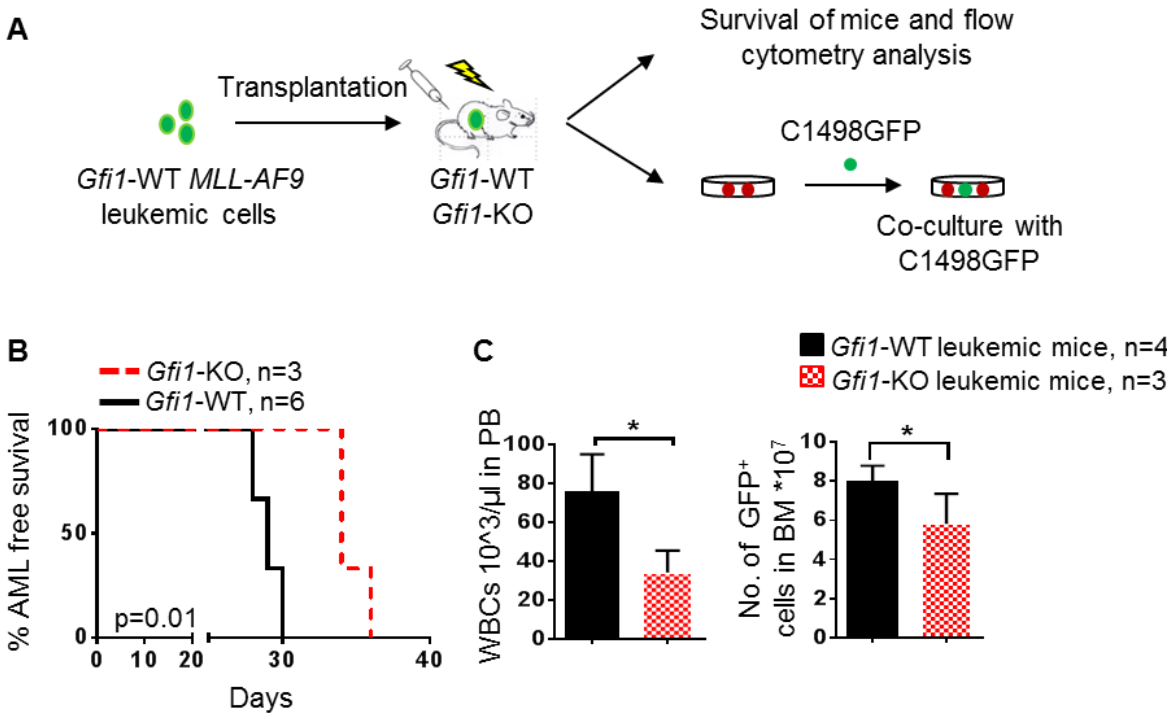

Figure 41: The role Gfi1 in polarization of AML-associated stroma in vivo

A) *Schematic illustration of the experimental design. 1x10^5 Gfi1-WT MLL-AF9 GFP$^+$ leukemic BM cells were transplanted secondarily into sublethally irradiated (3Gy) Gfi1-WT or Gfi1-KO mice. The mice were monitored and when moribund, they were sacrificed and analyzed. 5x10^5 C1498GFP AML cells were co-cultured with BMDMs from Gfi1-WT or Gfi1-KO mice. After 6 days, GFP$^+$C1498GFP cells were counted.* ***B)*** *The survival curve of Gfi1-KO (n=3) and Gfi1-WT (n=6) transplanted with Gfi1-WT MLL-AF9 leukemic cells (p=0.01).* ***C)*** *Total cell numbers of white blood cell count (WBC) in peripheral blood (left) (*p=0.02) and the GFP$^+$ leukemic cells in the BM (right) (*p=0.04) of Gfi1-WT (n=4) and Gfi1-KO (n=3) leukemic mice. "Published data (Al-Matary et al. 2016)".*

Additionally, the percentage of non-leukemic macrophages (GFP$^-$CD11b^{hi}Gr1^{int}) in BM and spleen of *Gfi1*-KO mice transplanted with *MLL-AF9*-leukemic cells was lower than in BM and spleen of *Gfi1*-WT mice (Figure 42) (Al-Matary et al. 2016).

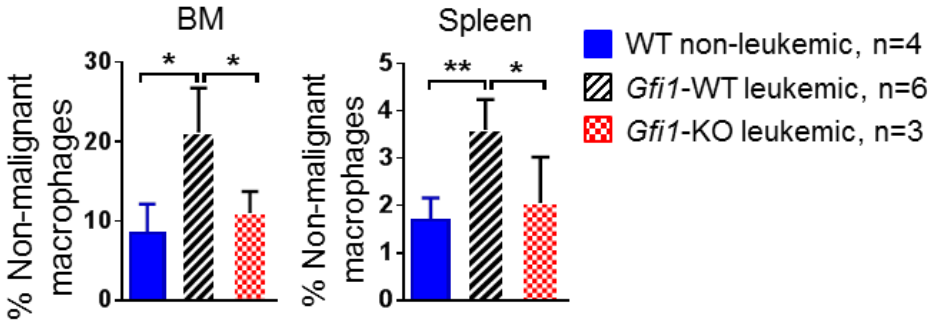

Figure 42: The percentage of non-leukemic macrophages in Gfi1-KO transplanted mice

*The percentage of GFP$^-$CD11b^{hi}Gr-1^{int} macrophages in the BM (left panel) and spleen (right panel) of Gfi1-WT (n=6) and Gfi1-KO (n=3) mice transplanted with MLL-AF9 transduced cells in comparison to mice transplanted with non-leukemic cells (n=4) (*p<0.01, **p=0.001). "Published data (Al-Matary et al. 2016)".*

To further investigate the function of Gfi1 in the polarization of macrophages, BMDMs from *Gfi1*-WT and *Gfi1*-KO leukemic mice were co-cultured with the murine AML cell line C1498GFP cells for 6 days. BMDMs derived from *Gfi1*-KO leukemic mice were inefficient in supporting the proliferation of C1498GFP compared to BMDMs derived from *Gfi1*-WT leukemic mice (Figure 43) (Al-Matary et al. 2016).

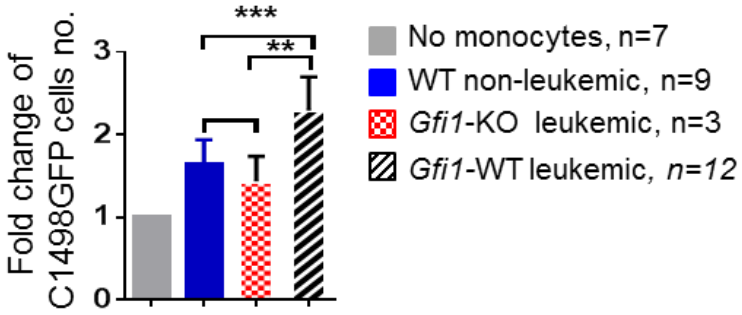

Figure 43: Gfi1-KO BMDMs lose their capability to support the growth of leukemia cells in vitro

Fold change of the cell number of GFP$^+$ C1498GFP after co-culturing with BMDMs derived from BM of Gfi1-WT or Gfi1-KO MLL-AF9 transplanted leukemic mice or from transplanted non-leukemic mice for 6 days. Results of triplicates from 3 and 4 independent experiments for Gfi1-WT leukemic (n=9) and transplanted non-leukemic

*mice (n=12) and from 1 experiment for Gfi1-KO leukemic mice (n=3) are shown (**p=0.008 and ***p=0.0004). "Published data (Al-Matary et al. 2016)".*

To further confirm the results obtained in a transplantation model of human leukemia in mice, we evaluated the effect of presence or absence of *Gfi1* in the *NUP98-HOXD13* transgenic mouse model of human MDS/AML (Figure 44 A). In consistent with the findings in *Gfi1*-KO mice transplanted with leukemic cells expressing *MLL-AF9*, the *Gfi1*-deficient(KO)*xNUP98-HOXD13* transgenic leukemic mice survived longer (Figure 44 B) and had lower numbers of WBCs in the blood and a reduced frequency of blast cells in the BM (Figure 44 C) compared to *Gfi1*-WTx*NUP98-HOXD13* leukemic mice (Al-Matary et al. 2016).

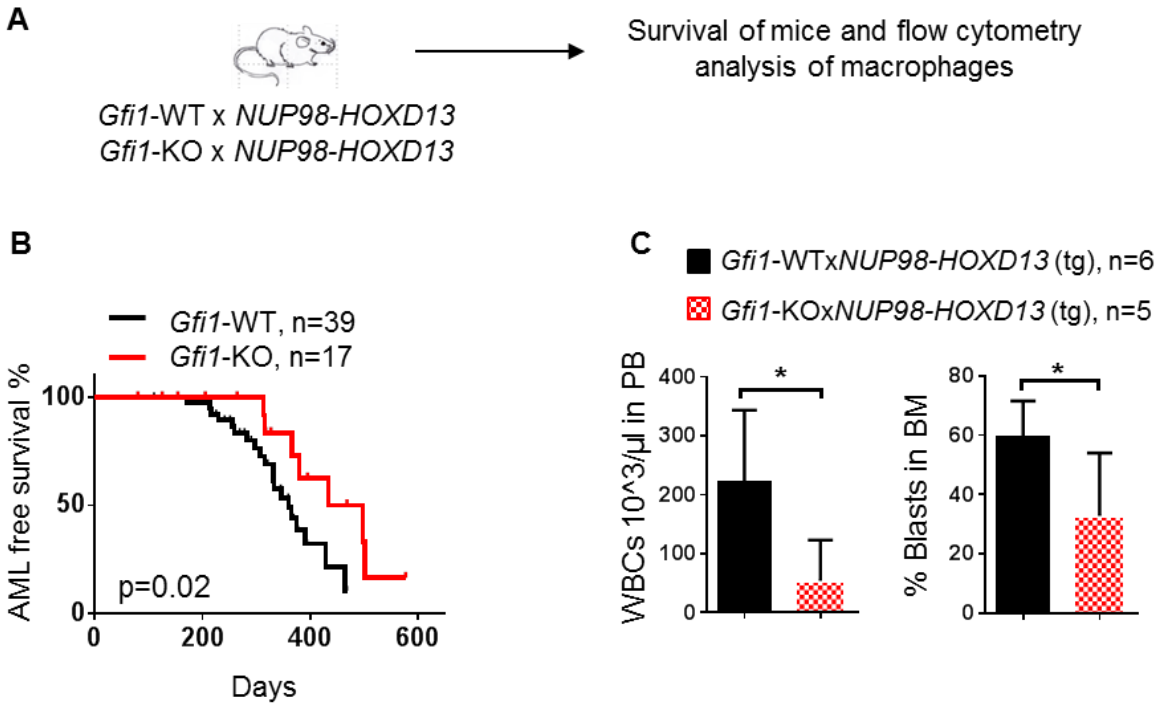

Figure 44: The role of Gfi1 in polarization of AAMs in transgenic NUP98-HOXD13 mice

A) *Schematic illustration of the experimental design. We crossed Gfi1-WT and Gfi1-KO mice with mice of the NUP98-HOXD13 MDS/AML mouse model. Gfi1-WT and Gfi1-KO- NUP98-HOXD13 mice were monitored up to the development of AML. The frequency of different macrophage types were analysed and determined in BM and spleen of leukemic mice.* ***B)*** *Survival curve of Gfi1-KO (n=17) and Gfi1-WT (n=39) xNUP98-HOXD13 AML mice (p=0.01).* ***C)*** *Total count of white blood cells (WBC) in peripheral blood (left) (*p=0.02) and the blasts frequency in the BM (right) (*p=0.04)*

of Gfi1-WT (n=6) and Gfi1-KO (n=5) xNUP98-HOXD13 leukemic mice. "Published data (Al-Matary et al. 2016)".

Additionally, the frequency of MHCII^{-}Ly6C^{-} AAM1 in BM and spleen of *Gfi1-KOxNUP98-HOXD13*-expressing leukemic mice was lower than in BM and spleen of *Gfi1*-WTx*NUP98-HOXD13*-expressing leukemic mice (Figure 45 A and B). Of note, the percentage of Ly6C^{+}MHC II^{-} monocytes, from which other macrophage populations derive (Laoui et al. 2014), was much higher in BM and spleen of *Gfi1*-KOx*NUP98-HOXD13*-expressing leukemic mice than in *Gfi1*-WT*xNUP98HOXD13* leukemic mice (Figure 45 C) (Al-Matary et al. 2016).

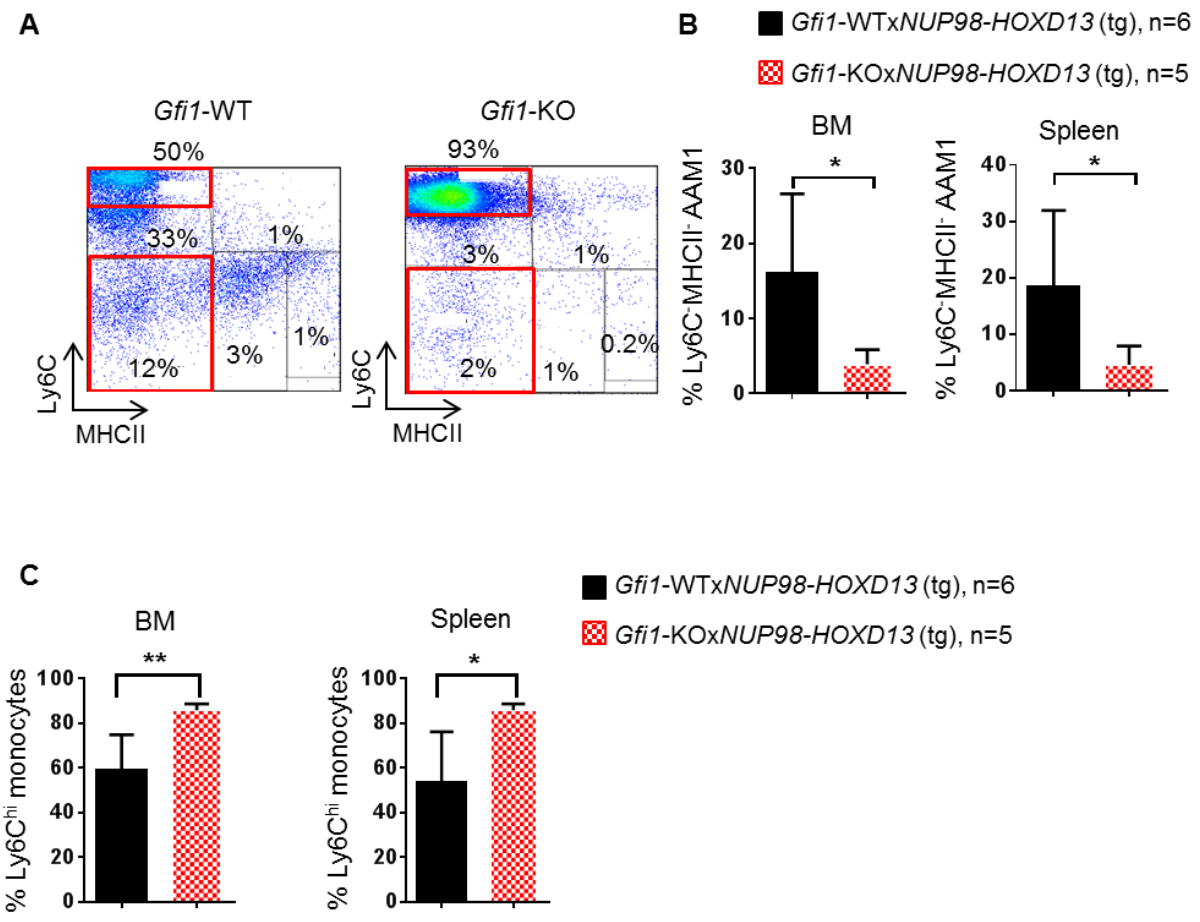

Figure 45: The frequency of AAM1 is decreased in BM and spleen of Gfi1-KOxNUP98-HOXD13 mice

A) *Representative FACS figures show gating strategy of different subsets of macrophages in Gfi1-WTxNUP98-HOXD13 and Gfi1-KOxNUP98-HOXD13-*

expressing leukemic mice. ***B)*** *The percentage of Ly6C^{-}MHCII^{-} AAM1 in the BM (right) and spleen (left) of Gfi1-WTxNUP98-HOXD13 (n=6) and Gfi1-KOxNUP98-HOXD13 (n=5) leukemic mice (*p=0.02 for BM and *p=0.05 for spleen).* ***C)*** *The frequency of Ly6C^{hi} monocytes in BM (right) and spleen (left) of Gfi1-WTxNUP98-HOXD13 (n=6) and Gfi1-KOxNUP98-HOXD13 (n=5) leukemic mice (**p=0.005 for BM and *p=0.01 for spleen) "Published data (Al-Matary et al. 2016)".*

8. Discussion

In this work, I examined the role of stromal cells, in particular, MSCs and macrophages, in the development of AML. As outlined in the introduction, stromal cells including MSCs and macrophages have a supportive effect on the growth of many solid cancers, lymphoma, and leukemias (Chittezhath et al. 2014; Schepers et al. 2013; Lim et al. 2016). The prognosis of AML patients in adults is still poor with less than 25 % of patients living longer than 5 years after diagnosis (Dohner, Weisdorf, and Bloomfield 2015). In addition to the role of the genetic and epigenetic factors in the development of AML cells, it is conceivable to hypothesize that the leukemic cells are also affected by the surrounding stromal cells in the BM niche including MSCs and immune cells (Colmone et al. 2008; Huang et al. 2015). The work presented here consists of two parts; the first is about the characterization of AMSCs and their role in the development of AML. The second part describes the role of macrophages with regard to the growth of AML cells *in vivo* and *in vitro*. Moreover and importantly, we elucidate for the first time the function of Gfi1 in the polarization and function of both AMSCs and AAMs.

8.1 The role of AMSCs in the development of AML

It has recently been shown that the functions of MSCs in myeloid malignancies is altered in a way that they do not support the function of HSCs anymore but support the leukemic HSCs (Li et al. 2015; Geyh et al. 2015; Kim et al. 2015; Falconi et al. 2016). Additionally, the prognosis of leukemia patients can also be predicted by the alterations in the stroma (Kim et al. 2015).

8.1.1 AMSCs support the growth of AML cells *in vitro*

MSCs are one component of the BM niche. Our results from co-culture experiments showed that AMSCs from AML patients and leukemic mice promote the proliferation of human leukemic cells as well as a murine AML cell line *in vitro*. Furthermore, MSCs derived from BM of AML patients who were treated successfully with complete

remission did not anymore improve the proliferation of AML cells to the same extent as MSCs from the same patient at diagnosis.

As it is difficult to isolate enough MSCs from BM of human AML patients, an external collaboration partner immortalized MSCs from healthy donors as well as MSCs from AML patients for us. I could demonstrate that these immortalized MSC cell lines (ImmMSCs) were still capable to differentiate into osteoblasts, chondrocytes and adipocytes confirming that immMSCs are indeed still MSCs. I also studied the human and murine MSCs mentioned here and showed that they express the characterizing markers of human MSCs (Pontikoglou et al. 2011; Dominici et al. 2006). Here, we confirmed again that AML immMSCs support the proliferation of leukemia cells *in vitro* better than normal immMSCs.

In vitro, I demonstrated that in the presence of MSCs the cell cycle state of AML cells shifted towards replication phase (S), with fewer cells being in the quiescence phase (G0/G1). In both human and murine MSCs, the interaction between AMSCs and AML cells was dependent on cell-to-cell contact in our experiments. Hence one possible speculation would be that the growth of leukemic cells is not only the sum of cell-intrinsic genetic and epigenetic changes but also depends on the changes found in the stroma. It has been demonstrated that MSCs protect and support the growth of normal hematopoietic stem cells (Schepers et al. 2013), and we confirmed here that leukemia cells alter MSCs quantitatively and qualitatively most probably by direct contact towards a state that these AMSCs support the growth of leukemic cells better than non-leukemic MSCs. My study was focussed solely on AML. It would be possible that in other cases of haematopoietic malignancies, the interaction between MSCs, macrophages and malignant cells might be different. This alteration was temporary and was lost when the contact with leukemic cells was abolished. This is in consistence with results from others who showed that normal BM-derived MSCs support the proliferation of leukemic lymphoid cells (Rodriguez-Pardo et al. 2013). The mechanism and molecules which are responsible for the direct cell-cell interaction between MSCs and leukemic cells have to be studied in more detail. We speculate that Notch, CXCR4 /ligand axis or Wnt signaling pathways, which play important roles in the normal HSC niche (Carlesso and Cardoso 2010) might be involved and hence explain the cell-cell dependent support of MSCs towards AML cells.

We also found out that the number of osteoblastic lineage cells (OBCs) and endothelial cells are increased in the endosteal niche of leukemic mice which could participate even partially in the development of AML. The accumulation of endothelial cells in the endosteal niche of AML mice indicates an increase of blood vessel formation (angiogenesis), which supports and maintains the growth of leukemia cells (Hussong, Rodgers, and Shami 2000).

8.1.2 Gfi1 plays an important role in polarization and function of AMSCs.

Gfi1 is a transcription factor that plays a role in the development of normal and leukemic HSCs (Moroy 2005; Moroy et al. 2015; Duan and Horwitz 2005; Khandanpour and Moroy 2013). Recently, it has been shown that Gfi1 has an inhibitory effects on the differentiation of MSCs towards osteoblasts in MM patients, which result in the common bone lesions and fractures of MM patients (D'Souza et al. 2011). In this study, we examined the role of Gfi1 in the function of AMSCs, and we found that Gfi1 expression was upregulated in AMSCs and was also required for their polarization towards a leukemia- supporting state as *Gfi1*-KO AMSCs did not support the growth of leukemic cells to the same extent as AMSCs derived from BM of *Gfi1*-WT mice. It has been found that early undifferentiated osteoblasts of the endosteal niche protect AML cells against apoptosis (Kremer et al. 2014), hence our reports would further widen the knowledge about the niche and how Gfi1 impedes differentiation of osteoblasts and hence loss of Gfi1 should lead to a higher number of osteoblast (D'Souza et al. 2011). It has also been found that the population of osteoblasts was reduced in AML patients and the loss of osteoblasts might support and maintain leukemic cells (Krevvata et al. 2014; Bowers et al. 2015). Only; we found here that the number of OBCs in *Gfi1*-deficient leukemic mice was higher than in *Gfi1*-WT leukemic mice, which might inhibit the growth of leukemia cells in the BM of *Gfi1*-KO leukemic mice. However, more studies are required to elucidate the mechanism behind the interaction of osteoblasts and AML cells as a previous finding showed that AML cells were supported and protected against apoptosis by osteoblasts at different stages of their maturation (Kremer et al. 2014). Yet it was unclear whether this is due to osteoblasts, osetocytes or osteoclasts.

8.2 The role of AAMs regarding the growth of AML cells

Besides the role of MSCs, we also investigated the interaction between AAMs and murine AML cells *in vivo* and *in vitro*. A recent study showed that macrophages derived from AML patients support the growth of CD34 positive cells *in vitro* better than macrophages from healthy controls (Li et al. 2015). We show here that monocytes/macrophages accumulated in the BM of AML patients and in the BM and spleen of several AML mice representing different mouse models. This can be explained by a mechanism whereby BM monocyte/macrophage proliferation and/or infiltration might be stimulated by the leukemic cells. A similar pattern of monocytes/macrophages infiltration was found in a *NUP98-HOXD13* transgenic MDS/AML mouse model. Moreover, we found that BMDMs derived from leukemic mice supported the growth of leukemic cells better than BMDMs derived from healthy mice. As the irradiation might have an effect on the stroma microenvironment and might change macrophages subtypes and function (Barcellos-Hoff, Park, and Wright 2005; Klug et al. 2013; Chittezhath et al. 2014) and to ensure compatibility, we always compared our findings from transplanted leukemic mice with results from sublethally irradiated mice transplanted with WT, non-leukemic BM cells. With regard to the functional characterization of AAM *in vitro*, we cannot exclude that expansion of BMDMs via M-CSF might alter their function. Yet, the same results have been observed in a murine model of AML in which AAM were sorted and co-cultured with AML cells without prior M-CSF co-culture. This is a hint that cytokine-induced polarization is not *per se* artificial.

The results from *in vivo* and *in vitro* experiments described in this thesis indicate that the presence of AML and the leukemic environment leads to an infiltration of monocytes/macrophages and promotes their differentiation into AAMs that subsequently support the leukemic cells instead of eradicating them.

8.2.1 AAMs show features of M2 macrophages

In many types of solid cancers, the supporting role of TAMs for the growth of tumor cells had been investigated and approved (Biswas and Mantovani 2010; Lewis and Pollard 2006). Although possibly being a simplification, the terms of M1 and M2

macrophages have been and are still used in determining the characterization of TAMs and other classes of macrophages and to describe the major physiological and functional differences between these populations (Biswas and Mantovani 2010; Chittezhath et al. 2014; Colegio et al. 2014; Galdiero et al. 2013). M1 macrophages secrete pro-inflammatory mediators and participate in the host defence and anti-tumor immunity while M2 macrophages are involved in immune suppression, tissue repair and pro-tumor immunity (Mantovani et al. 2002). Recently, this classification has been re-defined. It was suggested that the activator or cytokines used for polarization of macrophages *in vitro* should be added, for example M(LPS) means M1 that is polarized by LPS and M(IL-4) means M2 that is polarized by IL-4 (Mantovani et al. 2004). To better characterize different macrophage classes, distinct RNA expression profile and cytokines secretion patterns should be used (Qian and Pollard 2010). TAM have been characterized in solid tumors (Biswas and Mantovani 2010; Chittezhath et al. 2014; Colegio et al. 2014; Galdiero et al. 2013; Laoui et al. 2014). Although TAMs are characterized in many studies to be M2-like macrophages, some studies showed that TAMs have a partially characteristic profile of RNA expression of M1 macrophages (Laoui et al. 2014; Franklin et al. 2014; Chen et al. 2015). In my study, phenotyping of AAMs derived from BM of human AML patients revealed that AAMs were M2-like macrophages as they were $CD163^{+}CD206^{+}$ (Beider et al. 2014). AAMs derived from BM and spleen of leukemic mice were $Ly6C^{-}MHCII^{-}$ and they were similar to the accumulated TAM1 cells described in lung cancers (Laoui et al. 2014; Quatromoni and Eruslanov 2012). On the RNA level, AAMs from BM of leukemic mice expressed higher levels of *Arg1*, a characteristic of M2 macrophages, and lower levels of *IL-6* and *Nos2* mRNA, which are characteristics of M1 macrophages, and secreted more IL-10 than macrophages derived from non-leukemic animals. All these results together suggest that AAMs in this study were M2-like macrophages. However, more work is needed to characterize these AAMs in more detail. Moreover, lower numbers of $Ly6C^{+}MHCII^{-}$ monocytes in BM and spleen of leukemic mice in parallel with increased number of $Ly6C^{-}MHCII^{-}$ AAMs, compared to non-leukemic mice, indicate that AAMs might be derived from $Ly6C^{+}MHCII^{-}$ monocytes. This is consistent with previous studies that characterized TAM in solid cancers (Laoui et al. 2014; Movahedi et al. 2010). However, the $Ly6C^{int}MHCII^{-}$ immature macrophages which are the intermediate stage between $Ly6C^{+}MHCII^{-}$ monocytes and $Ly6C^{-}MHCII^{-}$ AAMs (Laoui et al. 2014; Movahedi et al. 2010),

accumulated in the BM and spleen of leukemic mice. This emphasizes that during leukemia development, the differentiation process of AAM phenotype and other monocytes-derived cells as dendritic cells from Ly6C^{+}MHCII^{-} monocytes is active (Figure 46).

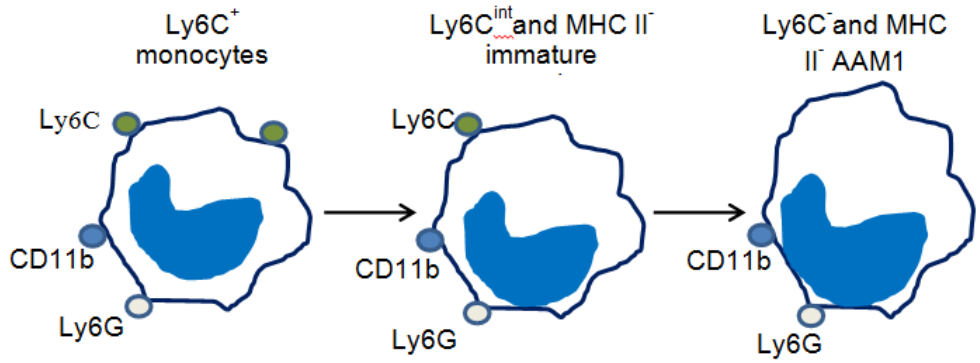

Figure 46 Theory of the differentiation process of monocytes into AAMs.

Ly6C^{+} monocytes differentiate through an intermediate immature Ly6C^{int}and MHCII^{-} cells into Ly6C^{-}and MHC II^{-} AAM1.

Here, we extend the present concepts about the role of AAMs with regard to support of the growth of leukemic cells (Gao, Yu, and Zhang 2014) (Li et al. 2015) by characterization of AAMs specifically in different AML mouse models.

8.2.2 AAMs polarization depends on Gfi1 expression

Gfi1 plays an important role in the development of granulocytes and is essential for production of cytokines by monocytes and macrophages (Person et al. 2003). Although the mRNA level of *Gfi1* expression is reduced in monocytes, Gfi1 protein level is elevated indicating that Gfi1 might play a role in the differentiation of monocytes into macrophages and maybe other monocyte-derived cells (Marteijn et al. 2007). Within the myeloid differentiation pathway, Gfi1 favours the differentiation of myeloid progenitors towards granulocytes and impedes development of monocytes (Person et al. 2003; Phelan et al. 2010; Karsunky et al. 2002; Moroy et al. 2015). In parallel with reduced production of neutrophils in *Gfi1*-KO mice, abnormal monocytes/macrophages accumulated in their BM and spleen (Sharif-Askari et al.

2010; Hock et al. 2003). An earlier work had shown that *Gfi1* is one of the upregulated genes in M2-like macrophages from BM of leukemic mice (Li et al. 2015). In this thesis, I investigated the role of *Gfi1* expression in macrophages polarization by leukemic cells *in vivo* and *in vitro*. Similarly, I found that the expression levels of *Gfi1* mRNA was upregulated in AAMs derived from BM of transplanted leukemic mice and also in BMDMs which were co-cultured with AML cells *in vitro*. In leukemic *Gfi1*-KO mice, Ly6C$^+$ monocytes accumulate in BM and spleen while the percentage of AAM1 and immature macrophages are decreased. This indicates that Gfi1 has an important role in polarization of AAMs and *Gfi1* loss results in failure of differentiation of monocytes into AAM and other monocytes derived cells as dendritic cells (Figure 47).

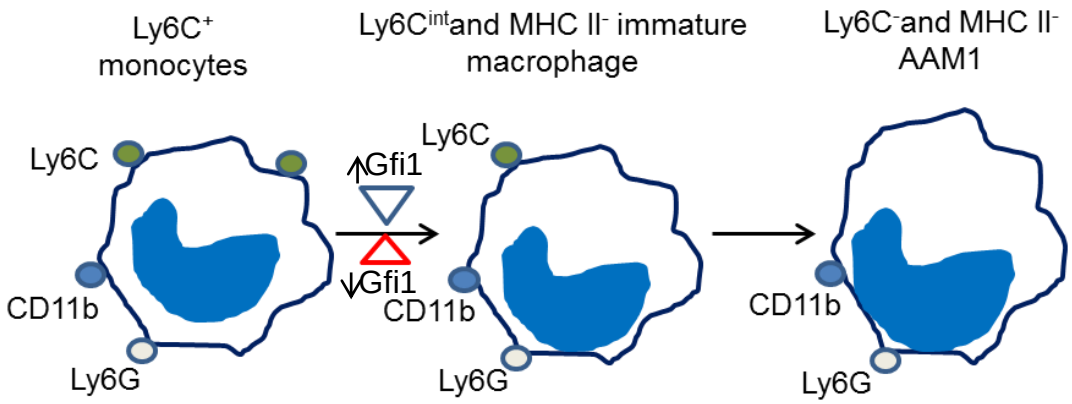

Figure 47 The role of Gfi1 in the differentiation of AAMs

Gfi1-WT Ly6C$^+$monocytes can differentiate into Ly6C$^-$and MHC II$^-$ AAMs while Gfi1-KO Ly6C$^+$monocytes fail to differentiate into Ly6C$^-$and MHC II$^-$ AAMs and other types of macrophages. This indicates that Gfi1 plays an important roles in the differentiation of monocytes into AAMs and other cells derived from monocytes. △ inhibit differentiation, ▽ enhance differentiation.

Additionally, *Gfi1*-deficient leukemic mice survived longer and contained a lower number of leukemic cells in BM and PB compared to leukemic *Gfi1*-WT mice. Although a number of publications have shown that *Gfi1*-KO macrophages might differ in part functionally from *Gfi1*-WT macrophages, they can be considered as macrophages with regards to pro-inflammatory functions of macrophages (Karsunky et al. 2002; Marteijn et al. 2007; Sharif-Askari et al. 2010; Spooner et al. 2009). In

consistence with previous reports, we found that *Gfi1*-KO M1 macrophages expressed more inflammatory mediators at both the mRNA level and protein level as well as cytokines such as *IL-6, iNos* and IL-1B (Karsunky et al. 2002; Sharif-Askari et al. 2010). One function of *Gfi1* in macrophages is to inhibit the Toll-like receptor 4 (Tlr4) pathway through antagonizing the nuclear transcription factor kappa light chain enhancer of activated B cells (NF-κB), which inhibits differentiation of macrophages towards the pro-inflammatory state (Sharif-Askari et al. 2010). This was demonstrated by hyper-reactive responses in *Gfi1*-deficient macrophages after exposure to LPS (Sharif-Askari et al. 2010). Our findings show that Gfi1 plays an important role in the polarization of AAMs and M2-like macrophages which can be explained by upregulation of *Gfi1* expression in AAMs *in vivo* and *in vitro*. Based on our findings, we suggest that Gfi1 regulates M1 and M2 polarization through its suppressive function on M1-specific pathways, mainly Tlr4 receptors and the NF-κB pathway. In another experiment, I confirmed that *Gfi1*-WT and *Gfi1*-KO macrophages treated with both stimulators of M1 and M2 polarization (LPS, IL-4) allowed only *Gfi1*-WT macrophages to differentiate into M2 macrophages while *Gfi1*-KO macrophages differentiated into M1, M2 and mixed macrophages. Furthermore, we found that M2 macrophages needed *Gfi1* expression to secrete cytokines and enzymes such as *Arg1* and IL-10 which play important roles in the suppression of the immune system. Generally, our characterization for the role of *Gfi1* displays that *Gfi1* is implicated in polarization of AAMs and other different macrophage subclasses, at least in our experimental system. Yet, in the *Gfi1*-deficient mice, Gfi1 is not only lacking in macrophages but also in the stroma and its components and hence it would be conceivable that the longer latency of AML development in *Gfi1*-deficient mice would be the sum of reduced function of MSCs, macrophages and other components of the stroma.

9. Outlook

The next steps would be the analysis of how Gfi1 induces the polarization and function of AAMs and AMSCs. Additionally, more studies are necessary to find out the mechanisms and signalling pathways involved in the interaction of stromal elements and the leukemia cells in order to find new therapeutic targets for AML. To this end, experiments should be done in which MSCs and macrophages are transplanted in parallel with leukemia cells in mice; followed by monitoring of the engraftment of leukemia cells. Furthermore, RNA microarrays of AAM and AMSCs would lead to a better characterization and identification of the mechanisms through which leukemia cells alter MSCs and macrophages in BM niche so that they can support the growth of cancer cells instead of supporting normal HSCs. In another experiment, we will study the role of stroma in resistance of AML patients to radio- and chemotherapy. Adding to this, more experiments should be done to investigate the use of M1 macrophages as a therapeutic agent for AML *in vivo* and *in vitro*. As a longterm goal, it would be desirable to have a drug targeting Gfi1.

10. Summary

Acute myeloid leukemia (AML) is a clonal malignant disorder of hematopoietic stem cells (HSCs). My goal was to dissect the interaction between AML and macrophages/mesenchymal stem cells (MSCs) and the possible molecular reasons behind this. I also investigated the role of the transcription factor Gfi1 (growth factor independence 1) in the polarization of AML-associated stroma cells, AML-associated MSCs (AMSCs) and AML-associated monocytes/macrophages (AAMs). AMSCs and AAMs supported the growth of leukemic cells *in vitro* better than normal MSCs and monocytes/macrophages. Co-culture of MSCs with AML cells led to a shift in the cell cycle state of the leukemic cells towards the DNA replication phase (S) and reduced the frequency of cells in the quiescence phases (G0/G1) *in vitro*. At the same time, AMSCs protect AML cells against apoptosis triggered by exogenous agents better than normal MSCs. Surprisingly, primary AMSCs derived from BM of AML patients who were successfully treated and cured (complete remission, CR) did not support the growth of a human AML cell line to the same extent as AMSCs derived from BM of the same patients at the time of diagnosis. The growth of leukemic cells in murine models of human leukemia is accompanied by a massive infiltration of macrophages and other stromal cells in the BM. AAMs derived from AML patients and different leukemic mice expressed the markers of M2 macrophages. We also found that the expression of the *Gfi1* gene was upregulated in AMSCs and AAMs. Moreover, BMDMs, BM-derived macrophages, and AMSCs derived from *Gfi1*-KO leukemic mice did not support the growth of AML cells *in vitro* to the same extent as the BMDMs and AMSCs derived from *Gfi1*-WT leukemic mice. The loss of Gfi1 inhibited the polarization of macrophages to a leukemia-supporting state both *in vitro* and *in vivo* and favored their differentiation into an antitumor state (M1 macrophages). In summary, I showed that macrophages and MSCs are polarized by AML cells and subsequently promote the growth of leukemic cells. The transcription factor Gfi1 plays an important role in that polarization.

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11. Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine klonale maligne Erkrankung hämatopoetischer Zellen. Die Hypothese war, dass das Voranschreiten der Leukämie nicht nur durch Mutationen in den Leukämiezellen beeinflusst wird, sondern wahrscheinlich auch durch Wechselwirkungen mit sogenannten Stromazellen in der BM-Mikroumgebung. Im Rahmen der Dissertation untersuchte ich die Rolle von Stromazellen, insbesondere MSCs und Monozyten/Makrophagen, im Hinblick auf die Unterstützung von AML-Zellen in vivo und in vitro. Darüber hinaus habe ich die Rolle des Gfi1 (growth factor independence 1) TF bei der Polarisation von AML-assoziierten Stromazellen erforscht. Ich konnte nachweisen, dass AML-assoziierte MSCs (AMSCs) und AML-assoziierte Monozyten/Makrophagen (AAMs) das Wachstum von Leukämiezellen in vitro besser unterstützen als MSCs und Monozyten/Makrophagen von gesunden Probanden oder Mäusen. Die Ko-Kultur von MSCs mit AML-Zellen führt zu einer Verschiebung des Zellzykluszustandes der Leukämiezellen in vitro hin zur DNA-Replikationsphase (S) und reduziert die Frequenz der Zellen in den Ruhephasen (G0/G1). Gleichzeitig schützen AMSCs die Leukämiezellen gegen Apoptose besser als normale MSCs. Wir zeigen auch hier, dass die Präsenz von Leukämiezellen in verschiedenen Mausmodellen der humanen Leukämie zu einer massiven Infiltration oder Expansion von Makrophagen und anderen Stromazellen einschließlich MSCs, Endothelzellen und Osteoblastenlinienzellen (OBCs) führte. Immunphänotypisch zeigten AAMs, die in AML-Patienten, aber auch im Knochenmark der leukämischen Mäuse gefunden wurden, eine Marker Kombination wie sie bei sogenannten M2-Makrophagen zu finden ist. Zusammengenommen zeige ich hier, dass AAMs M2- Makrophagen funktionell ähnlich sind. Auf molekularer Ebene fanden wir, dass die Expression des Gfi1-Gens in AMSCs hochreguliert wurde. Darüber hinaus unterstützten BMDMs, BM-abgeleitete Makrophagen und AMSCs, die von Gfi1-KO-Leukämie-Mäusen abgeleitet wurden, das Wachstum von AML-Zellen *in vitro* nicht in gleichem Maße wie die BMDMs und AMSCs von Gfi1-WT-Leukämie-Mäusen abgeleitet wurden. Der Verlust von Gfi1 behinderte sowohl *in vitro* als auch *in vivo* die Polarisation von Makrophagen zu einem Leukämie-unterstützenden Zustand und begünstigte ihre Differenzierung zu einem Antitumor-Zustand (M1-Makrophagen). Zusammenfassend konnte ich zeigen, dass Makrophagen und MSCs durch AML-Zellen beeinflusst

werden und im weiteren Verlauf das Wachstum der leukämischen Zellen fördern. Der Transkriptionsfaktor GFI1/Gfi1 spielt hierbei offensichtlich eine wichtige Rolle.

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12. Appendices

12.1 Appendix of tables

Table 14 FAB classification of AML.

Adapted from (Segeren and van 't Veer 1996; Bennett et al. 1985).

FAB class	characteristics
M0 Undifferentiated acute myeloblastic leukemia	<3%SBB+ blasts, My+,Ly-
M1 Acute myeloblastic leukemia with minimal maturation	>90% blasts
M2 Acute myeloblastic leukemia with maturation	30-90% blasts, >10% myeloid cells, <20% monocytoid cells
M3 Acute promyelocytic leukemia (APL)	Majority promyelocytes, hypergranular, Hypogranular M3V
M4 Acute myelomonocytic leukemia	>20% mature myeloid cells and >20% ANAE+ blasts ((ANAE=?)) >5x10$^{9/}$monocytes in the blood
M5 Acute monocytic leukemia	>80% monocytoid cells
M6 Acute erythroid leukemia	>50% nucleated erythroid cells >30% blasts of myeloid cells of the non-erythroid population M6b: >80% nucleated erythroid cells
M7 Acute megakaryoblastic leukemia	>30% megakaryoblasts defined by CD41/CD61

Table 15: WHO classification of AML and related neoplasms 2016.

Adapted from (Vardiman et al. 2009; Arber et al. 2016).

AML with recurrent genetic abnormalities • AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* • AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* • APL with *PML-RARA* • AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* • AML with t(6;9)(p23;q34.1); *DEK-NUP214* • AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM* • AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1* • Provisional entity: AML with *BCR-ABL1 rearrangement* • AML with mutated *NPM1* • AML with biallelic mutations of *CEBPA* • Provisional entity: AML with mutated *RUNX1*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified: • AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monoblastic/monocytic leukemia • Pure erythroid leukemia • Acute megakaryoblastic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome: • Transient abnormal myelopoiesis • Myeloid leukemia associated with Down syndrome.

Table 16 MIC classification of AML

Adapted from ('Morphologic, immunologic and cytogenetic (MIC) working classification of the acute myeloid leukaemias. Second MIC Cooperative Study Group' 1988)). GPA: glycophorin A.

MIC group	FAB	Karyotype
M2/t(8;21)	M2	t(8;21)(q22;q22)
M3/t(17;17)	M3, M3v	t(17;17)(q22;q12)
M5a/del(11q23)	M5a(M5b,M4)	del(11q)(23)
M4Eo/inv(16)	M4Eo	del/inv(16)(q23)
M1/t(9;22)	M1(M2)	t(9;22)/(q34;q11)
M2/t(6;9)	M2 or M4 with basophilia	t(6;9)(p21-22;q34)
M1/inv	M1 (M2,M4, M7) with thrombocytosis	Inv(3)(q21q26)
M5b/ t(8;16)	M5b with phagocytosis	t(8;16)(p11;p13)
M2 Baso/t(12p)	M2 with basophilia	t/del(12)(p1-p13)
M4/+4	M4(2)	+4

Appendix of tables

Table 17: Risk classification of AML patients according to karyotypes

Adapted from (Mrozek and Bloomfield 2006))

Favorable Risk Group	
Balanced structural rearrangements	t(15;17)(q22;q12-21) t(8;21)(q22;q22) inv(16)(p13q22)/t(16;16)(p13;q22)
Intermediate Risk Group	
Normal karyotype	
Balanced structural rearrangement	t(9;11)(p22;q23)
Unbalanced structural rearrangements:	del(7q) del(9q) del(11q) del(20q)
* Numerical aberrations:	-Y +8|| +11 +21
Unfavorable-Risk Group	
Balanced structural rearrangements:	Complex karyotype inv(3)(q21q26)/t(3;3)(q21;q26) t(6;9)(p23;q34) t(6;11)(q27;q23) t(11;19)(q23;p13.1)
Unbalanced structural rearrangement	del(5q)
Numerical aberrations	–5 –7

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13. Publications related to this work

Al-Matary, Y. S., Botezatu, L., Opalka, B., Hones, J. M., Lams, R. F., Thivakaran, A., Schutte, J., Koster, R., Lennartz, K., Schroeder, T., Haas, R., Duhrsen, U., and Khandanpour, C. (2016) Acute myeloid leukemia cells polarize macrophages towards a leukemia supporting state in a Growth factor independence 1 dependent manner. Haematologica 101, 1216-1227

14. Erklärung

Erklärung

Hiermit erkläre ich, gem. §6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema " Die Rolle des Stromas in der Entwicklung der akuten myeloischen Leukämie (AML)" zuzuordnen ist, in Forschung und Lehre vertrete und ich den Antrag von Yahya S. Al-Matary befürworte.

Essen, den_____ _____

PD. Dr. Cyrus Khandanpour

Erklärung

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als hier angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekeinnzeichnet habe.

Essen, den _____ _____

Yahya S. Al-Matary

Erklärung

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Essen, den _____ _____

Yahya S. Al-Matary