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Investigation of phenotypic AML stem and non-stem cell populations in patient-derived xenotransplantation (PDX) models

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Abbreviations

AA:	aplastic anemia
ADH-AML:	antecedent hematological disorder AML
AF700:	Alexa Fluor 700
ALL:	acute lymphoblastic leukemia
AML:	acute myeloid leukemia
APC:	antigen-presenting cell
APC:	allophycocyanin
APC-Cy7:	allophycocyanin-cyanine 7
B7-H3:	B7 homolog 3 protein
BCAM:	basal cell adhesion molecule
BM:	bone marrow
BMP:	bone marrow punction
BV:	Brillant Violet
CD:	cluster of differentiation
CD45RA:	CD45 restricted molecule in which the exon A is expressed
CEBPA:	CCAAT enhancer binding protein
CLL-1:	C-type lectin-like receptor
CML:	chronic myeloid leukemia
CR:	complete remission
CTLA4:	cytotoxic T-lymphocyte-associate protein 4
DNMT3A:	DNA-methyltransferase 3A
ELN:	European leukemia net
ESMO:	European Society for Medical Oncology
FAB:	french-american-british
FACS:	fluorescence activated cell sorting
FC:	flow cytometry
FCS:	fetal calf serum
FDA:	Food and Drug Administration
FITC:	fluorescein isothiocyanate
FLT3:	fms-like tyrosine kinase 3
FMO:	fluorescence-minus-one
G-CSF:	granulocyte colony stimulating factor
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GP:	glycoprotein

GPR56:	G-protein coupled receptor 56
GvL:	graft-versus-leukemia
hGM-CSF:	human granulocyte-macrophage colony-stimulating factor
hIL3:	human interleukin 3
hSCF:	human stem cell factor
HSCs:	hematopoietic stem cells
HSPCs:	hematopoietic stem and progenitor cells
HSCT:	hematopoietic stem cell transplantation
IDH:	isocitrate dehydrogenase
IF:	intrafemoral
IL:	interleukin
IL-2Rγ:	interleukin-2 receptor subunit gamma
IL-3Rα:	interleukin-3 receptor alpha chain
ITD:	internal tandem duplication
IV:	intravenous
LMO-2:	lim domain only 2
LSC:	leukemia stem cell
M-CFS:	macrophage colony-stimulating factor
MDS:	myelodysplastic syndrome
MISTRG:	M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRPa$^{h/h}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL2Rg$^{-/-}$
MLL:	mixed lineage leukemia
MPN:	myeloproliferative neoplasm
MRC2:	mannose receptor c-type 2
MRD:	minimal residual disease
NAD+:	nicotinamide adenine dinucleotide
NCAM:	neural cell adhesion molecule
NCCN:	National Comprehensive Cancer Network
NK-κB:	nuclear factor kappa B
NK:	natural killer
NKG2D:	natural killer group D
NKG2DL:	natural killer group D ligand
NPM1:	nucleophosmin 1
NOD/SCID:	non obese diabetic/ severe combined immunodeficient mice
NSG:	NOD/SCID/IL2Rγnull
PARP1:	poly-ADP-ribose polymerase 1

PB:	peripheral blood
PBMC:	peripheral blood mononuclear cells
PBS:	phosphate-buffered saline
PD-L1:	programmed death ligand 1
PDX:	patient-derived xenotransplantation
PE:	phycoerythrin
PECy5/PECy7:	phycoerythrin-cyanine 5/7
PerCP:	peridinin-chlorophyll-protein
PTPRC:	protein tyrosine phosphatase receptor type C
RAG2:	recombination activating gene 2 protein
RT-PCR:	real time polymerase chain reaction
RUNX1:	Runt-related transcription factor 1
SCID:	severe combined immunodeficient mice
SIRPα/SIRPγ:	signal recognition particle α/γ
t-AML:	therapy-related AML
TEL/ETV6:	Ets family transcription factor
TF:	transcription factor
TPO.	thrombopoietin
TP53:	tumor protein 53
VCAM-1:	vascular cell adhesion molecule 1
VLA-4:	very late antigen 4
WHO:	world health organization
WT:	wild-type

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1. Introduction

1.1 Healthy Hematopoiesis

Hematopoiesis describes the process of blood formation during the life span of living organisms and is organized as a hierarchy with hematopoietic stem cells (HSCs) on top. HSCs, which reside mainly in the bone marrow (BM), are characterized by their ability to self-renew and maintain the hematopoietic system by generating all mature blood cells. They give rise to more differentiated progenitor cells which eventually mature into specialized cells along different pathways. (Orkin und Zon 2008; Orkin 2000). Mature blood cells are rather short-lived; therefore, organisms have to produce an estimated amount of 4 x 10^{11} differentiated hematopoietic cells per day (Pinho und Frenette 2019). These precursor and mature cells only exhibit restricted self-renewal properties and only limited potential to differentiate (Pinho und Frenette 2019).

Hematopoiesis occurs in so-called two waves at different sites during the mammal's development. In order of occurrence, these are the yolk sac, the aorta-gonad-mesonephros region, the fetal liver, thymus, spleen and lastly, the bone marrow (BM). In the yolk sac, hematopoiesis is called "primitive" because the main function is to produce red blood cells which ensure tissue oxygenation. This system is quickly replaced by the "definite" wave of hematopoiesis (Orkin und Zon 2008).

HSCs in adult mammals are mostly found in a special microenvironment called the BM niche. This niche ensures hematopoietic homeostasis by regulating highly proliferative processes, including the regulation and differentiation of HSCs and progenitor cells in quiescence and in response to a triggered immune system e.g. infections or injury (Pinho und Frenette 2019).

HSCs are further regulated by intrinsic factors such as transcription factors and DNA-binding proteins. The function is often altered by somatic mutations or chromosomal translocations that occur in human hematopoietic malignancies, indicating that the fate of the HSC is strongly intertwined with the induction of

leukemia (Orkin und Zon 2008). For example, the translocations SCL/tal1 (stem cell leukemia/T-cell acute lymphoblastic leukemia) and LMO2 (Lim Domain only 2) can deregulate expression of the locus, whereas leukemias associated with MLL (Mixed Lineage Leukemia), Runx1 (Runt-related transcription factor 1) or TEL/ETV6 (ETs family transcription factor) are characterized by respective chimeric fusion proteins.

1.2 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a malignant disorder of the blood maturation process commonly caused by abnormalities in growth and differentiation of genetically altered hematopoietic stem and progenitor cells (HSPCs). Subsequently, immature myeloid precursors (myeloblasts) accumulate in the BM and eventually the peripheral blood (PB) and extramedullary tissues (Khwaja et al. 2016). AML usually presents with a rapid onset of symptoms with patients suffering from BM failure within a couple of weeks. AML patients often display fatigue symptoms and shortness of breath due to anemia, higher susceptibility to bleed due to thrombocytopenia and infections because of neutropenia (Khwaja et al. 2016). AML affects both children and adults, but it is more commonly seen in elderly who also have worse clinical outcome when compared to people of younger age. Leukemia in elderly people may show worse prognosis because it occurs in HSPCs that already carry age-related mutations (Erba 2007), as opposed to leukemia in children and young adults which lack such mutations (Creutzig et al. 2018). Additionally, when compared to younger patients, older patients are less fit for aggressive treatments which further negatively impacts their prognosis (Webster und Pratz 2018; Erba 2007; Creutzig et al. 2018).

Similar to healthy hematopoiesis, AML is organized hierarchically with the leukemic stem cells (LSCs) residing on top of the pyramid. LSCs share common traits with their healthy counterparts such as the ability to self-renew, to occupy

the BM niche and to give rise to more differentiated leukemic progenitors and AML blasts which are responsible for the above-mentioned symptoms (Khwaja et al. 2016). In contrast to these more differentiated cells, LSCs display enhanced resistance to chemotherapy. Thus, LSCs are not only responsible for leukemia initiation, but also for disease relapse after treatment.

LSCs are defined functionally, but over the last years have been increasingly characterized biochemically, molecularly and by their surface markers. They are most commonly found in the more immature CD34+/CD38- compartment, but in some AML also within CD34+/CD38+ subpopulations. In some cases, LSCs have also even been detected in CD34-non-expressing AML cases or in CD34- cells of normally CD34+ AML (Eppert et al. 2011). The surface markers will be discussed in detail later in this work. Biochemically, LSCs are known to have impaired DNA repair due to damages caused by ROS (reactive oxygen species), which then leads to accumulation of mutations. On a molecular level, LSCs show mRNA or epigenetic signatures showing similarities with their cells of origin, the HSCs or multipotent hematopoietic progenitor cells. Somatic DNA mutations in HSCs are acquired over time and they can be divided into “early” and “late” mutations. “Early” mutations most often occur in genes that are known to encode epigenetic regulators (e.g., DNMT3A, TP53) and lead to improvement of self-renewal abilities and disablement of differentiation (e.g. IDH1, IDH2). “Late” mutations occur mainly in signaling pathways (e.g., FLT3) that further improve proliferation thereby facilitating leukemia onset (Vetrie et al. 2020). Through impairment of differentiation, the LSCs give rise to undifferentiated leukemic blasts, which is considered one of the hallmarks of AML. Overall, features as e.g., cell cycle activity, LSC frequency and immunophenotype of LSCs are very heterogeneous regarding intra- and inter-patient comparisons (Chopra und Bohlander 2019).

1.2.1 Incidence

As described above, AML affects both children and adults with acute leukemia. The median age at diagnosis ranges at about 70 years with a slight male dominance (1.1-1.8: 1) (Khwaja et al. 2016). In the last century, an increment of incidence could be observed, but the numbers stopped rising in the first decade of the twenty-first century and persist to be stable since (Khwaja et al. 2016). Additionally, AML seems to be strongly influenced by the patient´s ethnicity. AML is more common in European countries and the United States, than in Asian countries. In addition, the white residents of the United States are also more likely to develop AML than citizens of other ethnic origins regardless of their birth place (Khwaja et al. 2016).

The overall five year survival rate in adults across all persons and AML subtypes is only 25 percent (Narayanan und Weinberg 2020) with survival being even lower in patients treated after relapse with only less that 20 percent, as reviewed by Thol and Ganser (Thol und Ganser 2020). It is also worth noting that there is a significant difference in survival between older and younger patients if they are assigned to the same group, meaning that both should be considered independently (Mrózek et al. 2012).

When compared to younger patients, elderly patients show particularly low survival due to worse tolerance of therapy but also differences in disease biology (Creutzig et al. 2018; Webster und Pratz 2018). As such, older patients have a higher probability of acquiring somatic DNA mutations over the course of the years. These mutations may not only predispose to AML development but can also induce an inferior prognosis (Brower 2016; Chopra und Bohlander 2019).

1.2.2 Diagnosis and Classification of AML

The molecular characteristics of AML serve as a tool to risk-stratify patients and evaluate appropriate treatment strategies. Many expert panels and organizations work together to continuously improve molecular classification tools for the disease. It includes the WHO (World Health Organization), NCCN (National Comprehensive Cancer Network, ESMO (European Society for Medical Oncology) and the ELN (European LeukemiaNet). Although the guidelines published by the organizations vary in some aspects, they are all based on the concept of cytogenetic abnormalities and mutations as the most important factor in risk-stratification (Narayanan und Weinberg 2020).

In laboratory settings, the diagnosis of AML is achieved through morphological analysis of BM aspirates or biopsies, immunohistochemistry, flow cytometry, cytogenetic analysis and molecular mutation analysis (Narayanan und Weinberg 2020). To distinguish AML from other hematological malignancies, the clinical appearance, medical history, and symptoms are taken into consideration. In addition, one of the two following criteria has to be fulfilled to diagnose AML:

a) Detection of ≥20% of myeloid blasts in the BM or the peripheral blood. The blasts are detected by morphology and enumeration based on specific criteria nicely described by Narayanan and Weinberg in 2020. (Narayanan und Weinberg 2020)
b) Detection of disease-defining molecular abnormalities such as: t(8;21)(q22:q22.1), inv(16)(p13.1q22) or t(15;17)(q24.1;q21.2) (Narayanan und Weinberg 2020)

The most used guideline to identify AML subtypes is the ELN guideline updated in 2017 and, most recently, in 2022 (Döhner et al. 2022), which divides patients into three different molecular risk groups affecting the overall survival rate and the outcome. These risk groups are called “favorable“, “intermediate” and “adverse“ and are defined at disease onset based on cytogenetic abnormalities and mutations detected in the leukemic cells (see Table 1).

These mutations include genes that are associated with epigenetic modulations, promotion of cell survival and proliferation, tumor suppression and the ability to evade differentiation and apoptosis (Narayanan und Weinberg 2020). In the following, I will address the four most important factors. TP53 (tumor protein 53) is described as the most important risk factor for adverse risk group classification. It is also often associated with complex cytogenetics as a mutation and functions as a tumor suppressor protein in healthy cells. In contrast, patients with AML carrying mutated NPM1 (Nucleophosmin) or bi-allelic CEBPA (CCAAT enhancer binding protein) mutations are most likely considered favorable risk. Detection of an internal tandem duplication (ITD) of FLT3 (fms-like tyrosine kinase 3) is also considered unfavorable but only if the ratio of mutated to unmutated is >0.5 (Estey 2018). However importantly, the effect of a mutation almost never stands for itself but is dependent on the other mutations (Estey 2018).

Table 1: 2017 ELN risk stratification by genetics (adapted from (Estey 2018))

Risk category*	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} – allelic ratio < 0.5
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high} – allelic ratio > 0.5
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD^{high†}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

In 2020, Eisfeld *et al*. investigated the prognostic relevance of the ELN Classification in a large group of patients. They showed that combined presence of e.g. WT1 and NPM1 mutations are associated with inferior prognosis when compared with single mutation detection. Therefore, the classification needs to be refined and survival analysis tends to be a suitable tool to analyze prognostic markers in large cohorts of patients (Eisfeld et al. 2020).

In 2022, Tazi et al. presented a refined model for classification and risk-stratification which includes 16 molecular classes. The molecular classes are associated with clinical presentation, response to treatment, risk of relapsing and overall survival over time. The model is not only based on cytogenetic abnormalities but also on molecular biomarkers and identifies new genomic regions relevant for prognosis and also provides improvement in accuracy of prognosis and class membership. This biologically informed classification system enables the emergence of stratified treatment decisions and further studies to detect class-specific associations (Tazi et al. 2022).

1.2.3 Risk Factors

In most cases it is not possible to identify the causes leading to AML development (Khwaja et al. 2016). However, older age, male gender, family history and history of exposure to substances damaging the DNA (e.g. chemotherapeutic agents, cigarette smoke, radiotherapy) as well as certain inherited syndromes (e.g. Down syndrome, Fanconi anemia, Bloom syndrome, severe congenital neutropenia etc.) have been reported as risk factors for AML (Khwaja et al. 2016; Narayanan und Weinberg 2020).

De novo AML arises in patients without previous hematologic disease or exposure to previous chemo- or radiation therapy. In contrast, AML occurring after such therapies (therapy-related AML (t-AML)) or arising from antecedent hematologic disorder (ADH-AML) such as myelodysplastic syndrome (MDS),

myeloproliferative neoplasm (MPN) or aplastic anemia (AA), are called secondary AML. Secondary AML generally show worse survival and treatment outcome than *de novo* AML (Kim et al. 2020).

1.2.4 Treatment Strategies

Given the heterogeneity of AML, treatment strategies vary from patient to patient and are adapted to the molecular characteristics of the disease as well as the fitness and age of the patient. Treatments include intensive chemotherapy, hypomethylating agents, BCL2 protein inhibitors, molecular targeting agents, antibody treatments, and finally hematopoietic stem cell transplantation (HSCT) (Khwaja et al. 2016).

One major challenge is that even patients achieving apparent complete remission (CR) are at high risk of relapse. Furthermore, primary resistance and treatment-related mortality are major challenges especially in older patients (Khwaja et al. 2016).

The cytotoxic chemotherapy treatment of AML can be divided in three different stages namely induction, consolidation, and maintenance phase. The main objective of the induction phase is achieving CR, while the goal of consolidation and maintenance is to sustain remission and eliminate minimal residual disease (MRD). Standard protocols for remission induction include a chemotherapy backbone containing 7 days of cytarabine plus 3 days of anthracycline. Additional drugs like granulocyte colony stimulating factor (G-CSF), FLT3-3 inhibitors or the BCL-2 inhibitor Venetoclax can increase the efficacy.

Allogeneic HSCT is used in many patients to add an immunotherapy as consolidation therapy. In this procedure, an HLA-matched donor is identified to donate HSPCs (by collection from the peripheral blood after mobilization with GM-CSF injections or alternatively from the BM). These healthy donor HSPCs

are given to the patient after chemotherapy conditioning (Khwaja et al. 2016) and will generate a donor-based immune system that induces important immune effects against residual AML cells (graft-versus-leukemia effects, GvL).

Although antibody treatment appears to be a promising approach to treat AML, it is highly challenging because there are no lineage or differentiation markers that would selectively target AML cells. Instead, markers found on AML cells are also found on regular HSCs so targeting them could induce severe side effects. Since AML cells are more likely to express higher levels of such markers, some of them are used as therapeutic targets although not specific for AML (Khwaja et al. 2016) .

Targeted therapies, second HSCTs or cytotoxic chemotherapy can be discussed as therapeutic options in patients showing relapse after HSCT (Thol und Ganser 2020).

Whenever possible, patients should be included into clinical trials (Estey 2018). HSCT also represents an option which is more suited for patients considered as intermediate or adverse risk in comparison to receiving more courses of chemotherapy.
It should also be mentioned, that supportive care alongside the treatment helps to reduce side effects and maintain the quality of living for the patients (Khwaja et al. 2016).

1.2.5 Monitoring and Relapse

In the follow-up process after treatment patients need to be monitored closely. To assess relapse or MRD, the threshold blast count lies at 5 percent. The blast count is assessed via morphological evaluation and cell enumeration in the BM. Additionally, in laboratory settings, real time polymerase chain reaction (RT-PCR), next-generation sequencing (NGS) or flow cytometry (FC) are used to

detect MRD and chemotherapy-resistant clones (Narayanan und Weinberg 2020)
.

When a patient relapses after chemotherapy, the option of HSCT as a curative treatment remains if the fitness of the patient allows this procedure. After HSCT as the initial treatment, approximately 40 percent of patients also experience relapse. The earlier the recurrence, the worse the prognosis. Targeted therapies, HMAs, secondary HSCTs or cytotoxic chemotherapy can be discussed as therapeutic options. Targeted therapies, HMAs, low intensity cytoreduction and best supportive care are possible options for unfit elderly relapsed or refractory patients. (Thol und Ganser 2020).

1.3 Phenotypic markers of AML

AML cells (over)express a variety of markers that can be used for diagnostics and classification of therapeutic targeting. In this project, we focused on three different marker groups, namely LSC markers (CD34, CD38, CD117, CD123, CLL-1, NKG2DL, CD200, CD45RA, GPR56), immune checkpoint (IC) markers (CD80, CD86, CD276, PD-L1) and adhesion markers (MRC2, CD56, CD47, VLA-4, CD49f, CD239). Some of these markers are already described to be important in AML whereas others are not as commonly known in hematological malignancies. The following paragraphs will provide an overview over the markers used for this project and their importance regarding AML.

1.3.1 Leukemic stem cell markers

In order to avoid leukemia progression and relapse, LSCs must be targeted. Thus, identifying new LSC targets would open the door to better treatments of AML (Arnone et al. 2020). John Dick et al. demonstrated for the first time the existence of human LSCs back

in 1994. They used an *in vivo* assay to show that CD34+ leukemic cells were able to induce leukemia in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice whereas CD34- cells from the same donor failed to induce the disease (Lapidot et al. 1994). Follow-up work has confirmed the notion that CD34+ populations contain LSCs (Bonnet und Dick 1997). However, about 30 % of AML cases lack expression of CD34. These cases are termed CD34-non-expressing AML (Taussig et al. 2010; Arnone et al. 2020). The identification of selective and specific LSC markers is subject of ongoing research and will be reviewed below.

1.3.1.1 CD34 and CD38

In CD34 expressing AML, LSCs were described to often reside within the subpopulation of CD34+/CD38- cells (Zahran et al. 2018). But this distinction does not capture all the LSCs as also AML cases with CD34+/CD38+ LSCs have been described. Moreover, CD34 non-expressing AML naturally contain CD34- LSCs (Taussig et al. 2010).

In CD34 expressing AML, there is a negative association between the frequency of CD34+/CD38- cells and patient survival (Zahran et al. 2018). The functions of CD34 include cell proliferation, adhesion, trafficking, and inhibition of differentiation and it is found mostly on multipotent precursors, mast cells and eosinophils but also on vascular endothelial cells and fibrocytes (Nielsen und McNagny 2008).
CD38, on the other hand, is mainly found on more differentiated cells (among which T and B cells, monocytes, NK (natural killer) cells, granulocytes). Its main functions are to regulate calcium levels and NAD+ homeostasis. In the context of cancer, CD38 may further regulate interactions with the tumor microenvironment (Hogan et al. 2019) and low CD38 levels were associated with worse patient outcome and patients that express low levels of CD38 should be considered for more intense therapy (Keyhani et al. 2000).

1.3.1.2 CD117 (cKit)

CD117 is expressed on HSCs and other healthy tissues and its functions include the HSC expansion by binding the stem cell factor as its receptor. Moreover, it is a proto-oncogene that encodes a transmembrane tyrosine kinase receptor which is found on AML blasts in more than half of the cases and it is more frequently upregulated in t(8;21) AML (Sperling et al. 1997). CD117 mediates proliferation in AML cells, but its prognostic value remains still unclear as there are conflicting reports on the expression of CD117 and patient outcome (Ashman et al. 1988; Wells et al. 1996; Tsao et al. 2004).

1.3.1.3 CD123

CD123 (interleukin-3 receptor alpha chain (IL-3Rα)) is normally found on plasmacytoid dendritic cells, eosinophils, and neutrophils and in hematological malignancies on subsets of both AML blasts and LSCs. The expression levels at diagnosis and relapse are highly variable (Bras et al. 2019). Recent studies also have shown that CD123 is a marker found on LSCs. The CD34+/CD38-/CD123+ phenotype has been detected in all AML blasts and depicts an interesting therapeutic target (Al-Mawali et al. 2017). However, targeting CD123 will lead to hematotoxicity because of the known expression of healthy hematopoietic cells (Haubner et al. 2019). Furthermore, high levels of CD34+/CD38-/CD123+ cells at diagnosis are associated with poor prognosis, inferior survival, and higher probability of resistance in patients (Goardon et al. 2011).

1.3.1.4 CLL-1

CLL-1 (C-type lectin 1) is a type II transmembrane glycoprotein which modulates immune response and is expressed on AML blasts, as well as on healthy monocytes and granulocytes (van Rhenen et al. 2007). CLL-1 is preferentially expressed on CD34+CD38- AML cells enriched for LSCs, and has been further used to detect MRD (van Rhenen et al. 2007). The expression of CLL-1 can be divided into two groups: CLL-1 low and CLL-1 high. The outcome of patients belonging to the CLL-1 low group were significantly worse than the outcome of the patients in the CLL-1 high group, independent of other variables (Wang et al. 2021).

1.3.1.5 NKG2DL

The ligands of the natural killer group 2D (NKG2DL) are not found on healthy tissues and their upregulation on infected or malignant cells leads to their clearance by natural killer cells (Arnone et al. 2020; Paczulla et al. 2019). Paczulla *et al.* used xenograft and syngeneic models to demonstrate that the absence of NKG2DL on AML cell surface enriches LSCs, as only the NKG2DL negative population gave rise to leukemia. The AML cells that exhibit LSC properties such as disease induction, relapse and self-renewal are characterized by their lack of NKG2DL. The NKG2DL negative cells evade the immune-mediated NK cell killing. Interestingly, PARP1 (poly-ADP-ribose polymerase 1) inhibits expression of NKG2DL so pharmacological inhibition of PARP1 can induce the expression of NKG2DLs on the surface of LSCs to enable NK cell killing and to suppress leukemogenesis (Paczulla et al. 2019).

1.3.1.6 CD200

CD200 is a molecule whose receptor is found on myeloid cells as well as T and B cells. CD200 can identify LSCs on both CD34 expressing and CD34 non-expressing AML. With the addition of other markers like CD33 and CD45RA, CD200 positive HSCs can be discriminated from CD200 positive LSCs. Interestingly, more than 75% of the samples that contained CD200+ LSCs further exhibited NPM1 mutations, raising the question whether CD200 is a targetable LSC marker in this AML subset (Ho et al. 2020; Ngwa und Liu 2019).

1.3.1.7 CD45RA

CD45RA is encoded by the PTPRC (protein tyrosine phosphatase receptor type C) gene and is mostly found on T and B cells as well as progenitor cells where it functions as a cell signaling molecule.
In AML, high levels (>90%) of CD45RA in the CD34+/CD38- subpopulation could identify LSCs in comparison to low or intermediate levels of CD45RA which were not able to discriminate between LSCs and HSCs. Interestingly, LSCs that exhibit CD45RA rather associated with favorable molecular risk. In addition to other markers, e.g. CD34, absence of CD38, CD123, CD45RA could be used as a tool to detect LSCs more accurately (Kersten et al. 2016; Hardwick et al. 2010; Goardon et al. 2011).

1.3.1.8 GPR56

G-protein coupled receptor 56 (GPR56) is expressed on HSCs, T cells as well as cells of the central nervous system. It regulates frontal cortex development, immune regulation and HSC function (Huang und Lin 2018). Within the LSC compartment, GPR56 expression was highest within the CD34+/CD38-

subpopulations suggesting GPR56 as a marker to identify LSCs at the beginning of the disease. Indeed, high GPR56 expression correlated with a worse clinical outcome and inferior survival (Daga et al. 2019). Moreover, it has been shown that GPR56 is able to define an LSC compartment other than the CD34+/CD38- compartment because of its potential to engraft in the CD34 expressing and non-expressing compartments. GPR56 regulates key features of LSCs such as the ability to self-renew and repopulate the BM (Pabst et al. 2016).

1.3.2 Immune Checkpoints

Targeting immune checkpoint molecules became a promising therapeutic approach for a variety of solid cancers in the last decade. Here we thus propose to further study their expression in AML.

1.3.2.1 CD80 and CD86

CD80 (B7-1) and CD86 (B7-2) belong to the family of costimulatory molecules. They provide a T-cell activating signal via CD28 (stimulatory) and CTLA4 (inhibitory) (Costello et al. 1998).
CD86 is mostly present on antigen-presenting cells (APCs) like activated B cells, dendritic cells, and monocytes. It shares its main function with CD80. AML cells were shown to express CD86, especially the blasts and cells that co-express CD86 with CD34 or CD14, identifying them as leukemic progenitors. CD86 could be of use to identify AML of monocytic/dendritic lineage (Re et al. 2002). CD80 and CD86 provide costimulatory signals necessary for activating T-cells for lysis of leukemic blasts (Li et al. 2003).

1.3.2.2 CD276

CD276, also known as B7-H3, is an immune checkpoint marker which has inhibitory and stimulatory functions regarding T-cell immune response. It is expressed on cells of monocytic lineage, e.g., neutrophils, dendritic cells, macrophages and Th17 cells. CD276 expression has been further documented in some cases of AML and Hodgkin lymphomas and they are associated with poor clinical outcome in patients (Zhang et al. 2021). CD276 expression was found to correlate with some AML risk factors e.g., age, TP53 mutations or wild-type WT1 and CEBPA mutations (Zhang et al. 2021). Recently, it has been shown that, by blocking the activity of CD276 via monoclonal antibodies, natural killer (NK) cell-mediated cytotoxicity could be amplified *in vivo* and *in vitro* (Tyagi et al. 2022).

1.3.2.3 PD-L1

Programmed death-ligand 1 (PD-L1) is found on tumor cells, healthy stromal tissues and macrophages. It binds its receptor, programmed death-1 (PD-1) which is found on activated T cells. The interaction of PD-1 and PD-L1 results in a suppression of the T-cell immune response against tumors and high levels of PD-L1 are associated with poor outcome in patients with solid tumors (Zhang et al. 2009). Moreover, it is associated with poor prognosis and low TP53 in AML, so PD-L1 blockade appears as an interesting therapeutic option. Nivolumab, Pembrolizumab and Atezolizumab are FDA-approved options for therapeutic use in patients with cancer (Haroun et al. 2017).

1.3.3 Adhesion Markers

Adhesion molecules play an important role in normal hematopoiesis, but also in AML as they are expressed on leukemic blasts. They mediate migration, homing and quiescence within the healthy BM niche, and are involved in the homing and metastasis of AML blasts by interactions with the niche. Response to chemotherapy can also be influenced by the adhesion molecules, indicating these as potentially promising therapeutic targets but there is still much to be investigated in this field (Gruszka et al. 2019).

1.3.3.1 MRC2

The mannose-receptor C-type 2 is an endocytic receptor which is involved in collagen metabolism by internalizing intact and degraded extracellular matrix collagen. MRC2 mediates efficient ligand internalization, recycling and lysosomal degradation. It shows expression on a variety of non-epithelial cancers and subsets of AML cells (Bagger et al. 2016; Nielsen et al. 2017). Increased levels of MRC2 were shown to associate with cancer metastasis and thus bad prognosis in several solid cancers such as hepatocellular carcinoma, prostate, and breast cancer as well as tumors of the head and neck (Gai et al. 2014; Nielsen et al. 2017).

1.3.3.2 CD56

CD56, which is also known as NCAM1 (neural cell adhesion molecule 1), is widely expressed on natural killer (NK) cells, as well as on T and B cells, dendritic cells and neural or mesenchymal stem cells and belongs to the family of adhesion molecules. One of the most important functions are neurogenesis and neural outgrowth. The expression of CD56 in AML is associated with poor survival, high

relapse rate and inferior outcome. In diagnostics, the marker is widely used to identify MRD. Its highest expression is observed in t(8;21) AML and acute promyelocytic syndromes, although CD56 levels only range between 15-20%. Therapeutic targeting via Trametinib showed promising preclinical results in CD56+ AML (Sasca et al. 2019).

1.3.3.3 CD47

CD47 is a membrane receptor found on HSCs as well as other healthy cells and is known to bind to SIRPα and SIRPγ to enable cell-to-cell communication and to thrombospondins to enable cell- extracellular matrix communications. It is also involved in other cellular processes like apoptosis, proliferation etc. (Sick et al. 2012).

Through its interaction with SIRPα, CD47 prevents normal, healthy cells from phagocytosis. Neoplastic cells from myeloid malignancies were shown to overexpress CD47 and thereby evade phagocytosis-mediated killing. Most leukemias exhibit very high levels of CD47, conferring them with immune-evasive properties (Jaiswal et al. 2009). CD47 could also be seen as an immune checkpoint marker, but since we stained the marker with the adhesion protocol, we decided to include it with the adhesion markers in the following paragraphs. For the discussion and analysis, the immune-evasive properties will be taken into consideration and included accordingly.

1.3.3.4 VLA-4

Very late antigen 4 (VLA-4) is an adhesion molecule most often found on leukemic cells. In hematologic malignancies, VLA-4 expression was shown to bind to VCAM-1 molecules and mediate downstream signaling via interaction with osteopontin, fibronectin and initiates NF-κB as well (Deak et al. 2021). High VLA-4 expression was described particularly in AML and ALL cases which

affect the central nervous system and are associated with chemotherapy resistance and inferior patient outcome (Deak et al. 2021). Additionally, higher levels of VLA-4 are associated with a bad prognosis and relapse in pediatric B-cell precursor acute lymphoblastic leukemia (Shalapour et al. 2011).

1.3.3.5 CD49f

CD49f, also known as α6 integrin, is a laminin-binding receptor that works via formation of heterodimers with β1 or β4 integrins (Velázquez-Quesada et al. 2020). Therefore, the integrin-complexes that inhibit CD49f can modulate the interaction between the microenvironment and cancer cells. High levels of CD49f are associated with enhanced invasion and migration ability in addition to higher metastases formation in breast tumors. CD49f surface expression was also shown to enrich subpopulations of cells with stemness features in breast cancers (Velázquez-Quesada et al. 2020). CD49f is a stem cell marker indicating enhanced self-renewal, proliferation, and tumor initiation capacity in several cancers (Krebsbach und Villa-Diaz 2017).

1.3.3.6 CD239

Basal cell adhesion molecule (BCAM) is expressed throughout most healthy tissues and functions as a molecule involved in migration, invasion, and cell adhesion (Jin et al. 2020). CD239 expression on cancer cells was shown to associate with poor clinical outcome in solid cancers like gastrointestinal cancer, skin tumors or bladder cancer (Jin et al. 2020). CD239 is under-investigated in hematological cancers.

1.4 *In Vivo* Experimental Models to Study AML

In 1994, Lapidot and his colleagues were the first to demonstrate the existence of LSCs in AML in an *in vivo* experimental model. They sorted human CD34+ and CD34- AML cells from the same AML patient and transplanted them separately into severe combined immunodeficient (SCID) mice and were able to show that only the CD34+ subpopulation was leukemogenic and therefore able to initiate the disease while the CD34- cells did not show these characteristics (Lapidot et al. 1994).

Over time, further experimental *in vivo* models and stem cell markers were investigated, and all confirmed the notion that leukemia is selectively induced by subpopulations of leukemic stem cells (LSC). Besides patient-derived xenograft (PDX) models, there are many different approaches to generate AML in mice and to provide a base to study the disease, e.g., induction of AML in (syngeneic) murine models by viral infection, chemicals or irradiation or via transferring HSCs that express an AML-associated proto-oncogene.

Syngeneic mouse models are another powerful tool to explore AML (Almosailleakh und Schwaller 2019). They are generated by introducing leukemia-inducing genetic events in mouse cells. These tumor cells are collected, cultured and multiplied *in vivo*. The resulting cells are then injected into the immune-competent mice of the same inbred strain (Syngeneic Mouse Models | Charles River (criver.com), website, consulted on the 28.09.2022). Syngeneic models are able to mimic the pathomechanism and response to treatment in more detail because they have functioning immune cells, as opposed to studies of human AML that have to be performed in xenograft models. The immune cells have a significant effect on treatment response and overall outcome (Zhao et al. 2014). But, in contrast to PDX models, syngeneic models cannot fully depict the AML heterogeneity found in patients.

PDX-models were used as the surrogate assay for this project and will thus be explained more thoroughly.

There are different strains of immunodeficient animals that were established over time. The SCID mice do not express mature functioning T and B cells but exhibit remaining NK-cell function. By combining the SCID strain with the non-obese diabetic (NOD) strain, engraftment rates could be increased further because the NOD/SCID mice not only lacked functioning T and B cells but also showed reduced function of NK cells and macrophages. To further decrease the immune status of these mice, they were combined with IL2Rγnull (Interleukin-2-receptor gamma deleted) mice which led to the NSG mice. These mice showed superior engraftment rates to the other models due to the almost complete depletion of the murine immune system (Almosailleakh und Schwaller 2019). The NSG model thus issues an improved environment for development and growth of human cells (Sanchez et al. 2009).

Generally, animals are sacrificed after 10 to 16 weeks post-injection which leads to engraftment in only about less than a half of transplanted samples with them belonging mostly to the intermediate or poor risk group. In 2017, Paczulla *et al*. could show that long-term observation for up to 1 year after transplantation in comparison to the standard endpoint of 10 to 16 weeks enables engraftment in almost every case (95%). The favorable risk subpopulations took longer to engraft than the intermediate and poor risk subtypes which then allowed the study of AML samples from different genetic backgrounds and even samples that did not engraft after regular time points. Altogether, this shows that the severity of the disease in human is similarly mimicked in animals, which is a powerful tool to study AML and its heterogeneity (Paczulla et al. 2017). Taken together, NSG mice mirror the biology and heterogeneity of AML samples and even the favorable patient samples will engraft after a longer period of time, meaning that the NSG mice are a very valuable tool to study the disease and its heterogeneity. Hence, it is important to keep in mind that all these studies are evaluated in the absence of an immune system.

Further developments include the idea to humanize the model by crossing the NSG strain with mice harboring knock-ins of human cytokines (hIL3, hGM-CSF and hSCF). These mice are called NSG-S mice. These “humanized mice” display

increased engraftment rates because they further stimulate leukemic cell growth by expressing fully compatible human cytokines. Additionally, β2-macroglobulin-depleted NSG-β2 mice were developed to further reduce the reactivity of the human immune cells against the host tissue. Although this does not affect the transplantation of AML cells, it shall be mentioned for the sake of completeness (Almosailleakh und Schwaller 2019).
Since cytokine-levels remained very high in these mice, normal human HSPCs were shown to exhaust. To solve this problem and to provide more physiological levels of human cytokines, a new generation of knock-in mice has been developed, e.g., MISTRG mice. These mice have been genetically modified and developed to exhibit several different genes like human macrophage-stimulating factor, IL-3, SIRPα, thrombopoietin and GM-CSF. By expressing those factors, HSPCs failed to exhaust that easily and human cells can evolve more efficiently but these models are still subjects to research (Abarrategi et al. 2018). To enable the development of a humanized mouse model that displays a more complete human immune system, the NOD,B6.SCID IL-2rγ $^{-/-}$Kit$^{W41/W41}$ (NBSGW) immunodeficient mouse strain was developed by adding a knock-in of kit alleles to the NSG (Adigbli et al. 2021).

Taken together, we want to focus on the heterogeneity in primary AML and thus use PDX models. The interaction with the immune system cannot be monitored, but they better depict AML heterogeneity and show high engraftment rates, making them the preferred option.

1.5 Aim of this Study

To cure AML, we need to target phenotypically heterogeneous LSCs. Studies with xenotransplantation models of human AML cells injected into immunocompromised mice allow us to gain insights into the heterogeneity amongst patient samples.

In this project, we decided to focus on the phenotypic characterization of BM cells from NSG animals injected with primary AML samples to investigate potential LSC markers in different AML risk groups. This is a retrospective analysis of samples injected by a former PhD student from our lab (Dr. Pauline Hanns).

We aim to potentially detect new correlations between cell surface markers and the respective risk group as well as survival of the mice. We analyzed a large panel of markers (see table 2, 3 and 4) involved in the LSC biology as well as immune interactions to especially interrogate the potential interactions between leukemic and immune cells. Since we analyzed samples from all AML risk groups and a larger cohort of patients, we could analyze the cell surface expression of markers in different AML subtypes and perform comparisons between groups.

Primary patient samples collected from patients were furthermore phenotyped and compared to corresponding cells derived from engrafted NSG mice, to investigate whether there are changes induced by the mouse incubation period.

With this retrospective analysis we want to gain a deeper understanding of the PDX model and its advantages and limitations regarding its function as a surrogate assay for AML. Additionally, we want to detect variables that could identify LSCs in different AML subtypes and inform on leukemic interactions with immune cells.

2. Material and Methods

2.1 Primary AML cells and PDX-models

This project is a follow-up of the project of a former PhD student in our lab, Dr. Pauline Hanns. Peripheral blood (PB) samples from AML patients or healthy donors were collected following informed consent (in accordance with the declaration of Helsinki) approval by the Ethics Review Board of the University Hospital of Basel and Tuebingen and enriched for peripheral blood mononuclear cells (PBMCs) using gradient centrifugation. These PBMCs were frozen in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20 % fetal calf serum (FCS; Sigma-Aldrich) and 10 % dimethyl sulfoxide solution (DMSO; AppliChem, Darmstadt, Germany) and stored in the vapor phase of liquid nitrogen.

NSG mice were purchased from Jackson Laboratory and bred in-house under pathogen-free conditions according to Swiss federal state regulations (Veterinäramt Basel-Stadt, 2972_30222).

Primary AML cells were thawed and transplanted intrafemorally into sublethally irradiated (225 cGy, 24 hours prior to transplantation) 6 to 10-week-old female mice. Mice were anesthetized using an intraperitoneally injected mixture of ketamin (65 mg /kg, Streuli pharma, Uznach, Switzerland) and xylazine (13 mg/kg, Streuli pharma). The femur was disinfected using povidone-iodine solution (betadine, Mundipharma, Frankfurt, Germany) and then the transplantation was performed using 5x10*5 primary AML cells resuspended in 30 µL of PBS. The transplantation process was performed according to previously published protocols (Paczulla et al., 2017; Paczulla et al., 2019). Mice were monitored via routine BM punctures at defined time points (16, 26- and 39-weeks post-transplantation), followed by flow cytometric detection for human CD33.

Mice were sacrificed if either more than 1 % human leukemic among total cells were detected in the PB or BM of transplanted mice, or if the latter showed signs of disease e.g., weight loss, ruffled coat, or reduced mobility inducing stop of the

experiment. If none of these criteria occurred, mice were sacrificed at the end point of the study, which was one year after transplantation. After the mice were euthanized, BM was isolated, washed with PBS, erythrocytes were lysed using ACK buffer (155 mM Ammoniumchloride, 9,98 mM Potassiumhydrogencarbonate, 0,4 mM 0,5 EDTA, MilliQ Water; Tuebingen, Germany) and analyzed via flow cytometry for human CD33 and cryopreserved. For each patient, 1 to 6 mice were injected and sacrificed afterwards.

2.2 Antibodies for Flow Cytometry

For the analysis of human AML cells transplanted in NSG mice, fluorescently labeled antibodies against surface markers were used. We divided the markers into three different panels each of them focusing on another aspect of interest. The antibodies are listed in the tables 2, 3 and 4. Antibodies were titrated prior to the usage and were used in the following concentrations.

2.2.1 LSC Panel

Table 2: Detailed information on the surface markers in the LSC panel

Surface Marker	Conjugate	Clone	Supplier	Concentration
CD33	BV421	WM53	BD Bioscience, Franklin Lakes, NJ, USA	0,5/100
Viability	BV510	Catalogue number: L34957	ThermoFisher, Waltham, MA, USA	0,5/100
CLL-1	BV605	50C1	BD Bioscience	4/100
CD200	BV711	OX-104	Biolegend, San Diego, CA, USA	4/100
CD34	FITC	581	BD Bioscience	2/100
CD45RA	PerCP	HI100	Biolegend	1/100
NKG2DL	Not conjugated, secondary streptavidin-PE, see below	/	R&D // ThermoFisher	1/100
CD117	PE-Cy5	104D2	Biolegend	2/100
CD38	PE-Cy7	HIT2	BD Bioscience	0,5/100
GPR56	APC	4C3	Biolegend	1/100

CD123	APC-Cy7	REA918	Miltenyi, Bergisch-Gladbach, Germany	2/100

2.2.2 Immune Checkpoint Panel

Table 3: Detailed information on the surface markers in the IC panel

Surface Marker	Conjugate	Clone	Supplier	Concentration
CD33	BV421	WM53	BD Bioscience	0,5/100
Viability	BV510	/	ThermoFisher	0,5/100
CD80	BV711	2D10	BD Bioscience	4/100
CD34	FITC	581	BD Bioscience	2/100
NKG2DL	PE	/	R&D // ThermoFisher	1/100
CD276	PE-Cy7	MIH42	Biolegend	2/100
PD-L1	APC	MIH1	BD Bioscience	1/100
CD86	Alexa Fluor 700	2331	BD Bioscience	1/100

2.2.3 Adhesion Panel

Table 4: Detailed information on the surface markers in the AP panel

Surface Marker	Conjugate	Clone	Supplier	Concentration
CD33	BV421	WM53	BD Bioscience	0,5/100
Viability	BV510	/	ThermoFisher	0,5/100
CD49d (VLA4)	BV605	L25	BD Bioscience	1/100
CD47	BV711	CC2C6	BD Bioscience	1/100
CD34	FITC	581	BD Bioscience	2/100
CD280 (MRC2)	PE	E1/183	BD Bioscience	1/100
NKG2DL	PE-Cy5	/	BD Bioscience/ ThermoFisher	1/100
CD239	PE-Cy7	A1	Biolegend	2/100
CD56	APC	581	BD Bioscience	1/100
CD49f	APC-Cy7	GoH3	Biolegend	2/100

<u>Antibodies:</u>

PE: Phycoerythrin

PE-Cy5: Phycoerythrin-Cyanine 5

PE-Cy7: Phycoerythrin-Cyanide 7

APC: Allophycocyanin

APC-Cy7: Allophycocyanin-Cyanide 7

FITC:	fluorescein isothiocyanite
PerCP:	Peridinin-Chlorophyll-Protein-Complex
BV421:	Brillant Violet 421
BV510:	Brillant Violet 510
BV605:	Brillant Violet 605
BV711:	Brillant Violet 711

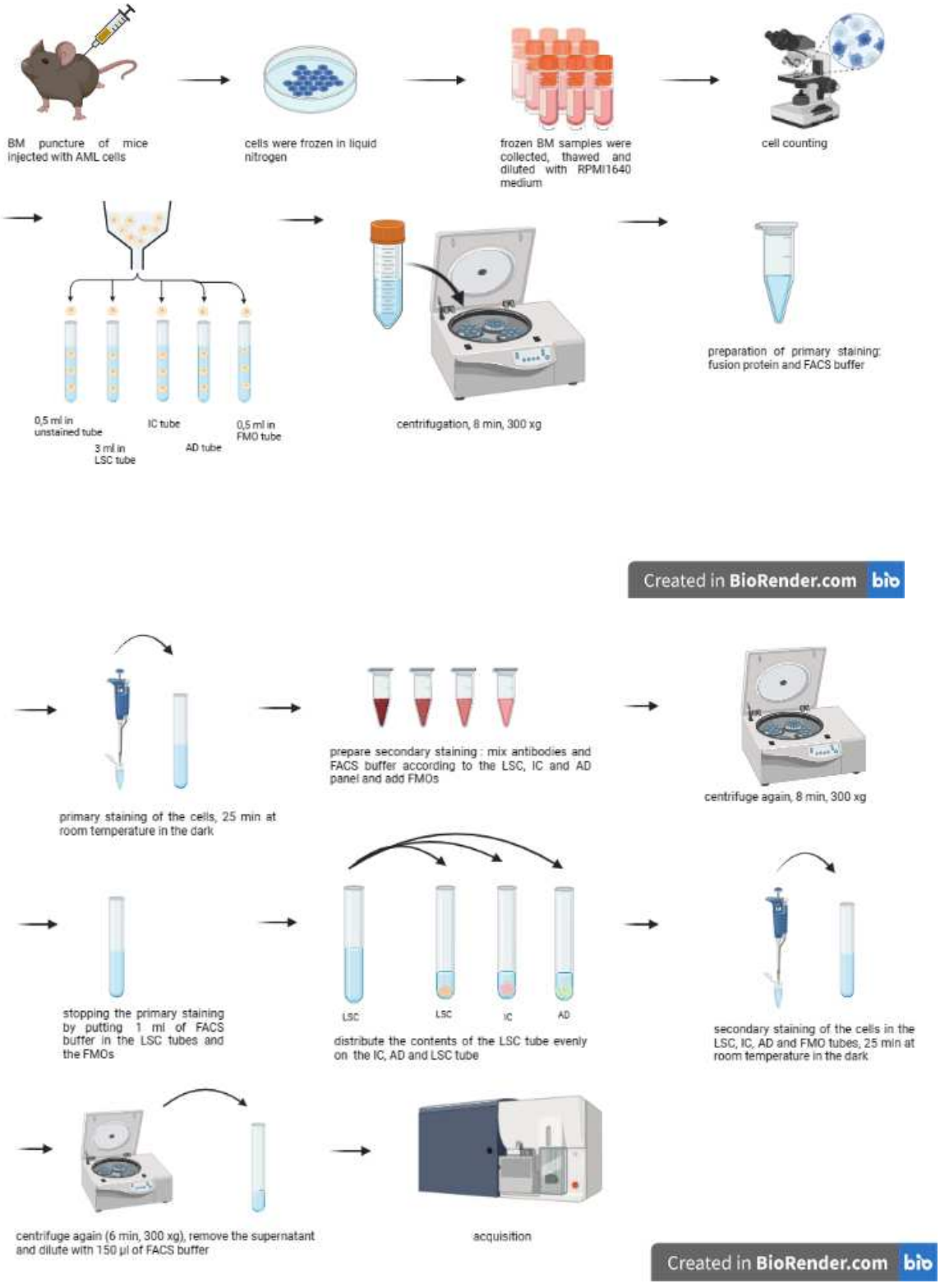
Analyses were performed on a BD LSR II Fortessa and analyzed using FlowJo (BD Biosciences, version 10). Viability dye was used to discriminate between living and dead cells.

2.3 Antibody Staining for Flow Cytometry

For the antibody staining frozen BM samples were always thawed freshly and then transferred into 4 ml of RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal calf serum and 1% penicillin/streptavidin (P/S, Sigma-Aldrich) heated to 37 °C. Figure A below shows the staining procedure. The diluted samples then were transferred into FACS tubes that were labeled correctly prior to the staining. The tubes for the staining were labeled with unstained, LSC, IC, AD and FMO. 0.5 ml of the diluted samples were transferred to the tubes for the unstained samples, 3 ml were put in the tubes for the LSC panel, and the rest was used for the FMOs. Then the samples were centrifuged for 8 minutes at 300 xg. In the meantime, the mix for the primary antibody staining had to be prepared. The primary staining is needed to enable a staining for NKG2DL later on. Therefore, the fusion protein (Streptavidin R-PE, ThermoFisher, Waltham, MA, USA) and FACS (10 % FBS, 2 mM EDTA; Tuebingen, Germany) buffer had to be mixed in a 1/10 dilution and kept in the dark until usage.
After centrifugation of the samples, the supernatant had to be removed after making sure there was a pellet in the tube. Then 50 µl of the primary mix were

added to the LSC tubes and the FMOs. The samples were vortexed quickly and incubated in the dark for 25 minutes at room temperature.
In the meantime, the remaining unstained tubes were diluted with 100 µl of FACS buffer and the secondary staining had to be prepared. Therefore, antibodies were added in an appropriate concentration to an appropriate amount of FACS buffer (depending on how many samples had to be stained) and kept in the dark until the primary staining was done. Afterwards, the primary staining was stopped by adding 1 ml of FACS buffer in every tube and then the samples were distributed evenly to the AP- and IC- tubes so we had the same number of cells in the tubes for the three panels. They were centrifuged again for 8 minutes at 300 xg. Then the supernatant had to be removed again and 50 µl of the secondary antibody mix was added to the correct tubes. The samples then need to stain for approximately 30 minutes in the dark at room temperature. Afterwards, the staining was stopped by putting 1 ml of FACS buffer in every tube. They were centrifuged again for 6 minutes at 300 xg. Then the supernatant was discarded again, and the samples were diluted with 150 µl of FACS buffer for acquisition.

Staining of the primary AML patient samples was performed by PhD student Marlon Arnone, staining of the mice-derived samples was performed by me. Figure conceptualization was also performed by Marlon Arnone, the analysis and the making of the figures was performed by me.



Flow Chart 1: Modus operandi: primary samples were injected in NSG animals and after sacrifice bone marrow cells were collected and viably frozen. After sample thawing, cells were divided in different FACS tubes, span down and stained with accordingly prepared antibody mixes prior to flow cytometry acquisition. Created with BioRender.com (Toronto, Ontario, Canada)

2.4 Compensation Controls and FMOs (Fluorescence-minus-one-controls)

The flow cytometer has to detect multiple colors through multiple lasers during the acquisition of stained samples. To ensure that the device only detects the real positive signal of one laser without overlapping in other channels, compensation controls must be established. It is possible to compensate with beads (small Styrofoam pellets) or cells. They are used to correct the fluorescence spillover, e.g., removing the signal of any fluorochrome from all detectors except the one responsible for measuring that color. Each fluorescent molecule emits light within a specific spectrum and these emission spectra can overlap, e.g., PE (phycoerythrin) and FITC (fluorescein). This overlap is called spillover and it is not practical to design panels that don't show any spillover. The beads or cells are stained with the according fluorescent antibodies (single stains) and are acquired in the device in the according compensation program. The FACS software is now able to calculate and correct the compensation matrix, which ensures correct data analysis downstream.

FMOs are a type of control used to facilitate the gating of the acquired signals. Sometimes it can be difficult to decide if the signal of a marker is positive or at least partially positive so FMOs are used to make sure that only the real positive population is gated. FMO controls are stained for every marker in the antibody mix except for one, so the signal for the marker is for sure negative. FMO controls consist of the cells of interest, and they are prepared according to the protocol. By acquiring the FMO control, a gate can be established at the threshold between positive and negative and then be used for all the other, fully stained acquired samples. FMOs were performed and gating was done accordingly.

2.5 Gating Strategy

After acquisition of the samples, gating was performed on all of the samples in the same way. Viable cells were distinguished from the dead cells and exclusion of doublets was performed via FSC-A and FSC-H. Afterwards, every sample was gated for CD33 to evaluate the engraftment rate. If CD33 was ≥ 1 %, the sample was considered engrafted and gated according to the protocol of markers shown above.

2.6 Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Results were tested for Gaussian normality distribution using normality and lognormality test (D´Agostino & Pearson test; Anderson-Darling test, Shapiro-Wilk test; Kolmogorov-Smirnov test). Comparisons were performed using unpaired Student t test or one-way ANOVA. Associations between two markers were measured using unpaired Student t test or Mann-Whitney-U test depending on the results of the normality analysis. Survival analysis were performed using Kaplan-Meier. All statistical analyses were performed with GraphPad Prism v9.4.1 (GraphPad Software, San Diego, CA, USA) and statistical significance is defined as $p < 0.05$. Only results with alpha < 0.05 are displayed in this work.

3. Results

3.1 Engraftment of various ELN molecular risk AML in PDX models

We stained bone marrow (BM) of 101 mice transplanted with cells derived from 46 AML patients. The mice were transplanted with all ELN molecular risk groups (Khwaja et al. 2016), distributed as follows: out of 101 samples, 45 (45.45 %) were “favorable”, 44 (44.44 %) were “intermediate” and 15 (15.15 %) were “adverse” ELN risk samples. Engraftment was defined as ≥1 % of human leukemic cells among total BM cells in the analysis following the staining and acquisition. Per definition, 51 out of 101 transplanted mice (51.51 %) were considered engrafted after the analysis (n = 51 engrafted mice from n = 46 transplanted patient samples engrafted in n = 1-5 mice per patient). The 51 engrafted BM were derived from 25 out of 46 (54 %) transplanted patient samples, of which 10 were of “favorable”, 10 were of “intermediate” and 5 were of “adverse”. Out of the engrafted 51 mice, 23 (11.73 %) were of “favorable”, 18 (9.81 %) were of “intermediate” and 10 (5.1 %) were of “adverse”.

The lowest and highest engraftment values as resulting from CD33 positivity evaluation were 1.06 % and 100 %, respectively. The average engraftment level for all the samples is 54.85 ± 40.08 % and the mean engraftment rate for the risk groups are 62.92 ± 39.36 % for the “favorable” group, 56.79 ± 39.97 % for the “intermediate” group and 32.82 ± 37.50 % for the “adverse” group (figure 1A). To rule out that our findings are dependent on the number of animals per sample, we calculated the mean expression of CD33, and in the following for all of the other markers in the panel, in the respective mice. The highest engraftment rate was now observed in the “intermediate” group (51.94 ± 40.73 %), followed by the “favorable” group (47.71 ± 39.93 %) and the “severe” group (27.45 ± 30.56 %; figure 1B).

CD34 represents the gold standard to enrich LSC subpopulations and distinguish them from non-LSCs. However, CD34 is not robustly expressed in all AML and

around 30 % of all AML cases are so-called “CD34-negative AML” lacking CD34 expression completely. Our lab previously showed that instead of CD34, absence of NKG2DL expression can be used to enrich LSCs across all AML subtypes (Paczulla et al. 2019). Here we evaluated that expression of CD34 was lost for the mouse-derived samples ($p = 0.0037$; figure 1C). NKG2DL were expressed on 86.27 % of our samples (figure 1D) with an average expression of 41.00 ± 31.98 % among all engrafted BMs. We observed the highest count of NKG2DL positive cells in the “favorable” risk group with a mean expression of 49.11 ± 30.70 %, followed closely by the “intermediate” and “adverse” group (40.05 ± 34.40 % and 24.06 ± 25.57 % respectively). These findings were also observed when we analyzed the mean expression of NKG2DL per patient (“favorable”: 48.85 ± 28.95 %; “intermediate”: 42.10 ± 33.12 %; “adverse”: 28.25 ± 31.28 %; figure 1E). Interestingly, a significant increase of NKG2DL was noted after transplantation ($p = 0.0152$; figure 1F).

Taken together, since we defined LSCs by the absence of NKG2DL, these data suggest that the favorable risk AML show highest NKG2DL expression and thus lower LSC numbers, while the adverse risk AML contain higher percentages of (NKG2DL negative) LSCs.

3.2 Description of The Patient Cohort

In the second part of this project, we aimed to assess if there would be phenotypic changes of AML cells before and after transplantation and assess if the phenotypic identity of LSCs remain similar. We want to observe if the phenotype is affected by passage in the animal and if so, we want to examine the plasticity of the LSC phenotype. We aimed for a translational analysis by staining a total of 16 primary patient samples according to the methods described in the paragraph above. The ELN Classification was used to discriminate between favorable, intermediate and poor prognosis subpopulations based on molecular and cytogenetic abnormalities (Khwaja et al. 2016). To rule out the possibility that our

observations are cohort-dependent, we included 28 more AML patient samples collected in Tuebingen (table 5).

Out of the 16 primary patient samples, 12 were considered leukemic based on the criteria mentioned in the paragraphs above and 4 samples couldn´t be analyzed due to a bad cell recovery. These 4 samples consisted mostly of dead cells and could therefore not be used for the analysis. Because of the small number of samples, we refrained from further distributing the samples into risk groups for the analysis. These primary AML patient samples correspond to the mouse-derived samples as the originate from the same patients.

The 28 AML patient samples collected in Tuebingen originate from bone marrow or peripheral blood from non-corresponding AML patients and were stained according to the methods described above. The only difference was that the panels for the patient cohort were slightly different than for the pre- and post-transplantation cohort, e.g., CD56, CD49f, CD239 and CD47 were not included in the staining protocol for the patient cohort. For the analysis, we pooled all the patient samples. The mean expression of CD33 in the primary samples lies at 66.23 ± 33.68 % with numbers ranging from 0.44 to 99.30 % (figure 1G).

The expression of NKG2DL ranges from 0.00 to 99.60 % with a mean expression of 36.68 ± 35.34 % (figure 1H).

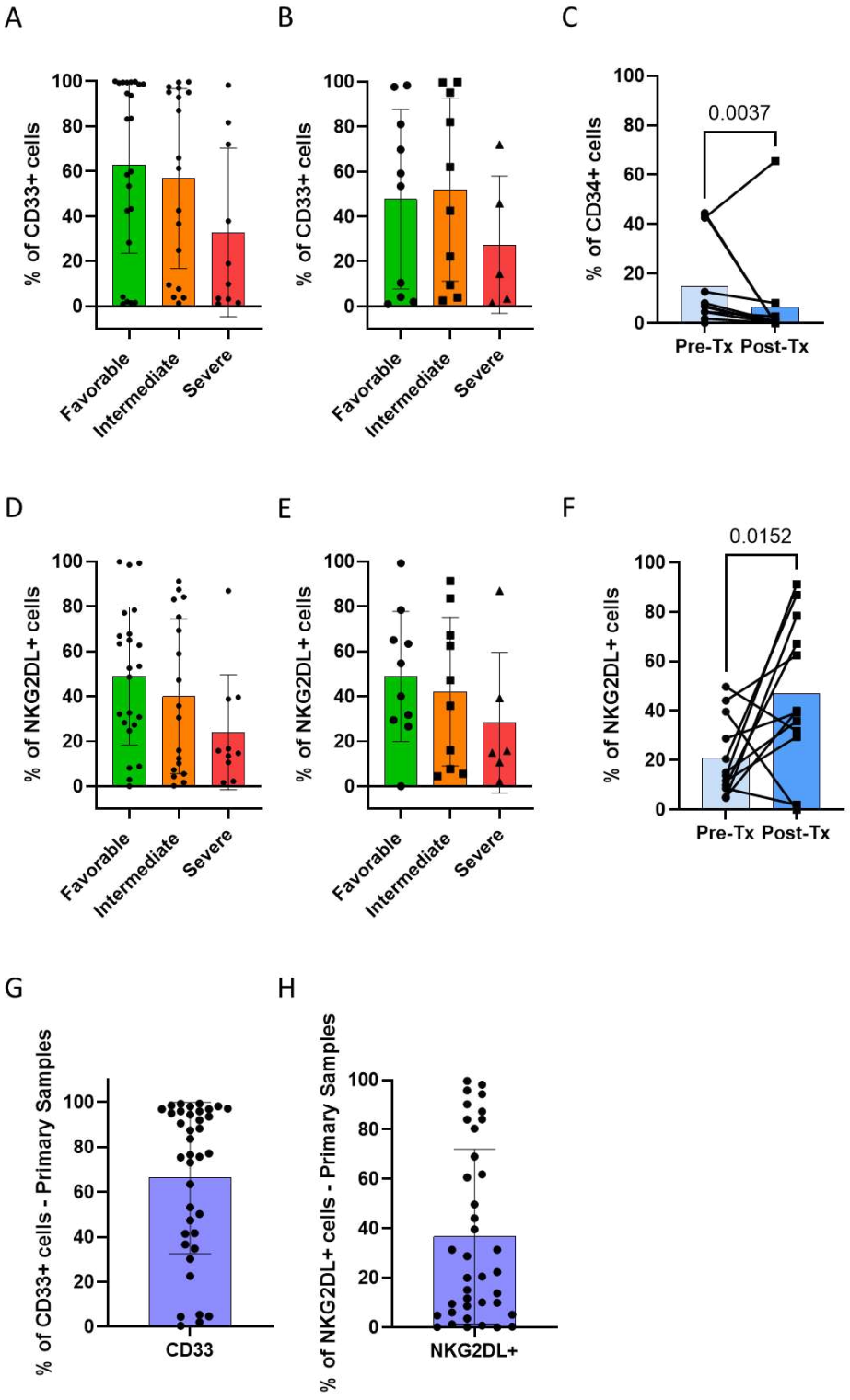


Figure 1: Characterization of the murine samples and the primary samples based on the expression of CD33, NKG2DL and CD34 (A) Expression of CD33 distinguished for the risk groups in the murine samples (E) Mean expression of CD33 per patient sample distinguished for the risk groups (C) Comparison of the expression of CD34 pre- and post-transplantation (D) Expression of NKG2DL distinguished for the risk groups in the murine samples (E) Mean expression of NKG2DL per patient sample distinguished for the risk groups (F) Comparison of the expression of NKG2DL pre- and post-transplantation (G) Expression of CD33 in primary patient samples (H) Expression of NKG2DL in primary patient samples.

3.3 Description of the Leukemic Stem Cell Markers

3.3.1 CD34

CD34+ AML cells are described to possess leukemia-initiating properties and LSCs to reside within the CD34+/CD38- compartment (Lapidot et al. 1994). CD34 was reported to associate with worse clinical outcome in patients (Zahran et al. 2018). Out of the 51 engrafted mice-derived samples derived from 46 transplanted patient samples engrafted in n = 1-5 mice per patient, only 13 showed expression of CD34 with the average expression being 11.04 ± 24.56 %. The highest expression of CD34 was found in the “adverse” risk group (mean = 25.73 ± 31.57 %) with the expression in the “intermediate” and “favorable” group being rather low (6.23 ± 19.81 % and 8.41 ± 23.35 %; figure 2A). When looking at the mean expression of the marker in all the mice derived from one patient, we noted similar observations (“favorable”: 7.27 ± 17.63 %; “intermediate”: 9.25 ± 25.99 %; “adverse”: 27.68 ± 30.79 %; figure 2B). We further investigated the co-expression with NKG2DL and no difference between the two groups was detectable, although, as displayed in figure 2C, the trend suggests that it is more likely correlated with the NKG2DL negative population. This result may be due to the small sample size in our analyzed CD34 positive cohort. Taken together, we could show that CD34 is most likely associated with an LSC phenotype in mouse-derived samples.

The expression of CD34 in the primary samples derived from patients directly was higher as in the mice-derived samples, as shown before (figure 1C; mean pre-Tx: 14.71 %; mean post-Tx: 6.43 %; p = 0,0037). We did not find any further associations regarding this marker in primary samples (data not shown).

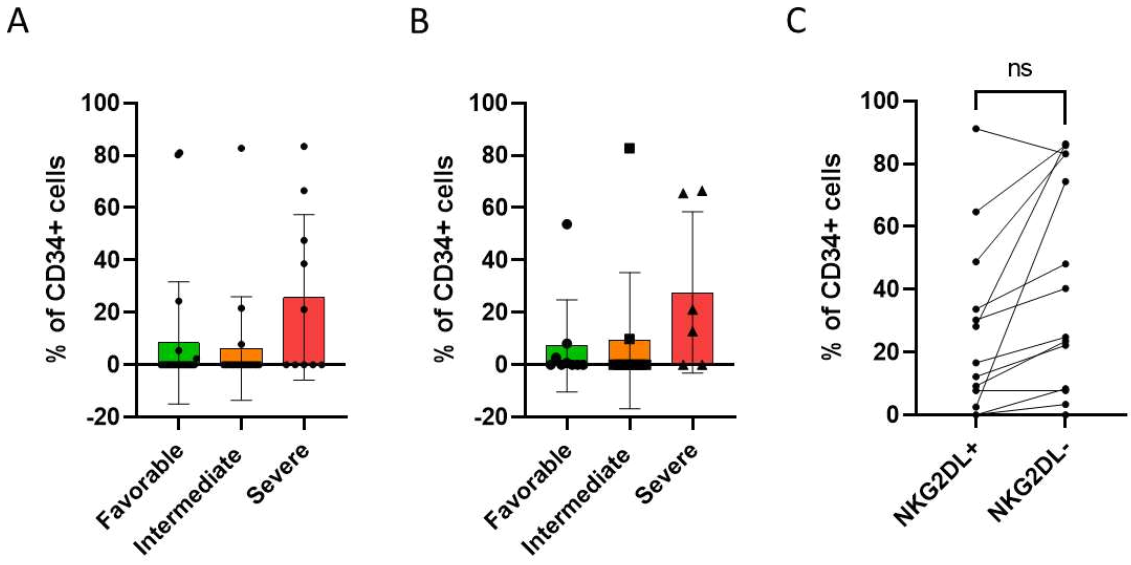


Figure 2: Characterization of CD34 in mouse-derived samples (A) Expression of CD34 distinguished for the risk groups in the murine samples (B) Mean expression of CD34 per patient sample distinguished for the risk groups (C) Co-expression of CD34 with NKG2DL in the murine samples

3.3.2 CD38

In contrast to most of the markers described in this paragraph, low levels of CD38 were associated with worse outcome in patients (Keyhani et al. 2000). LSCs were described to reside within the CD34+/CD38- compartment, explaining the prognostic relevance of this marker (Zahran et al. 2018). Interestingly, mouse-derived samples, showed high levels of CD38 for the majority of the samples (mean expression = 71.19 ± 30.04 %; expression in 49 of 51 cases) without a difference between ELN risk groups (“favorable” = 71.63 ± 29.42 %; “intermediate” = 73.20 ± 36.39 %; “adverse” = 68.57 ± 22.48 %; figure 3A). The results of the mean expression per patient were very similar (“favorable”: 69.09 ± 22.71 %; “intermediate”: 69.00 ± 34.14 %; “adverse”: 69.71 ± 23.05%; figure 3B). CD38 significantly associates with NKG2DL co-expression in mouse-derived samples, consistent with the notion that it marks non-LSCs (p = 0,0010; figure 3C),

In our cohort, CD38 expression was similar in primary patient derived (mean = 71.45 ± 27.43 %; figure 3D) and mouse-derived samples as displayed in figure 3E (p = 0.3685) and CD38 in primary samples associated with a more committed phenotype (p = 0.0051; figure 3F) suggesting CD38 as a consistent marker indicating differentiation in patients as well as the engrafted PDX settings.

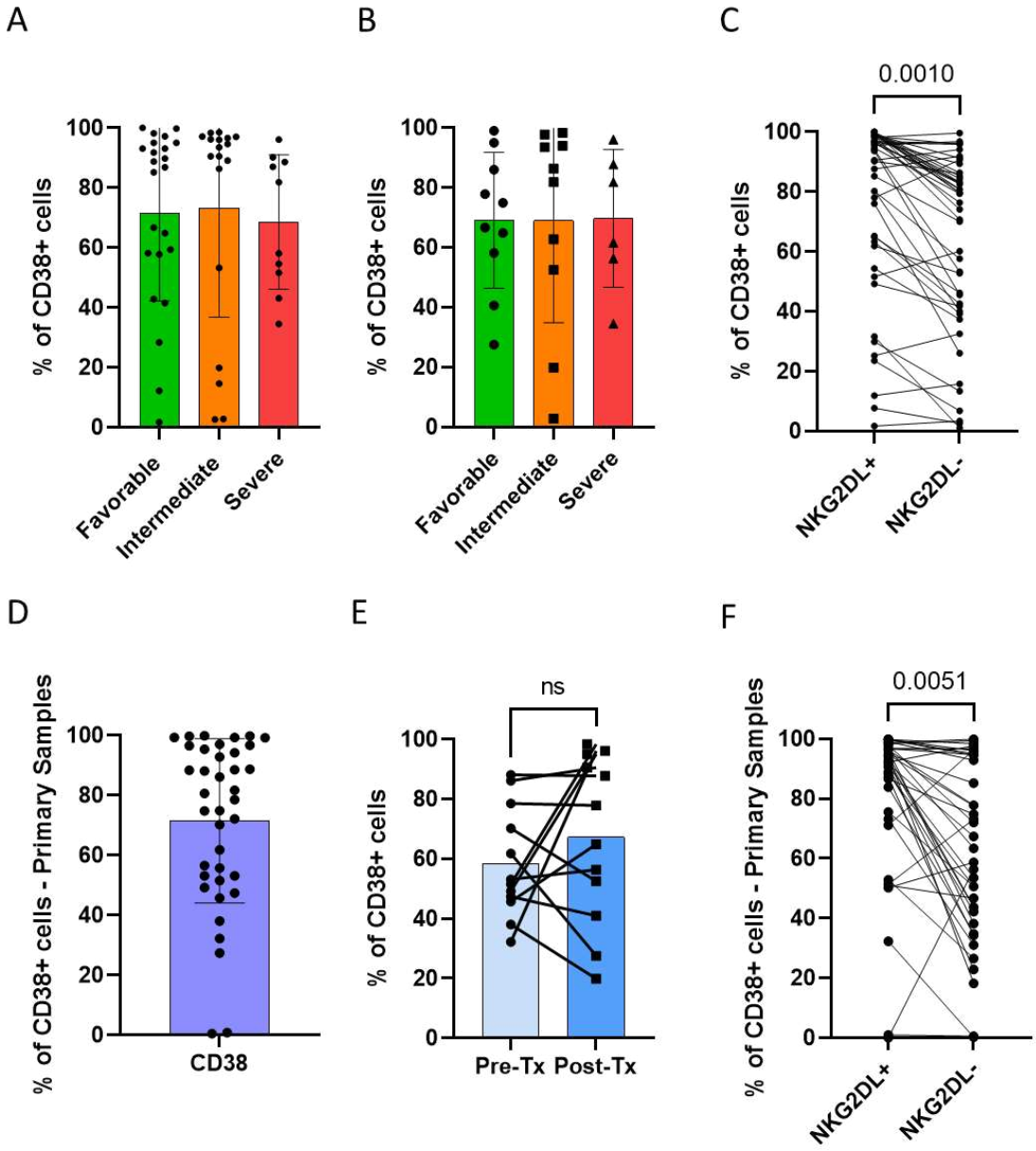


Figure 3: Characterization of CD38 in mouse-derived and primary patient samples (A) Expression of CD38 distinguished for the risk groups in the murine samples (B) Mean expression of CD38 per patient samples distinguished for the risk groups (C) Co-expression of CD38 with NKG2DL in the murine samples (D) Expression of CD38 in primary patient samples (E) Comparison of the expression of CD38 pre- and post-transplantation (F) Co-expression of CD38 with NKG2DL in primary patient samples

3.3.3 CD117

The average expression of CD117 was 50.07 ± 35.16 % with the highest number of positive cells being observed in the “adverse” risk group (66.53 ± 23.38 %), followed by the “intermediate” and the “favorable” risk group (56.56 ± 35.52 %; 33.78 ± 32.38 %; figure 4A). These results could be confirmed by analyzing the mean expression of the marker per patient sample (“favorable”: 32.01 ± 33.78 %: “intermediate”: 55.66 ± 35.02 %; “adverse”: 62.06 ± 27.54 %; figure 4B).

Interestingly, the vast majority of CD117+ cells were NKG2DL negative (figure 4C). Moreover, we appreciated a negative correlation between CD117, an LSC marker, and the expression of NKG2DL, as a non LSC marker (Spearman $r = -0.7049$; $p < 0.0001$; figure not shown). In order to further enrich for LSC, we used the NKG2DL-/CD117+ population and could appreciate a significant higher proportion of such cells in the “adverse” (mean: 84.59 %; $p = 0.0185$) and in the “intermediate” (mean: 77.39 %; $p = 0.0186$) population in comparison with the “favorable” populations (mean: 49.40 %) (figure 4D).
Thus, our data reinforces CD117 as a marker strongly associated with an LSC phenotype.

For CD117 in primary patient samples, we observed mean values of 41.21 ± 34.83 % (figure 4E), which did not differ significantly from the values in the mouse-derived samples ($p = 0.5512$; figure 4F), suggesting that CD117 is also conserved during the transplantation process.
Additionally, we could show that there is a significant association with NKG2DL negativity ($p < 0.0001$; figure 4G) in primary patient samples as well.

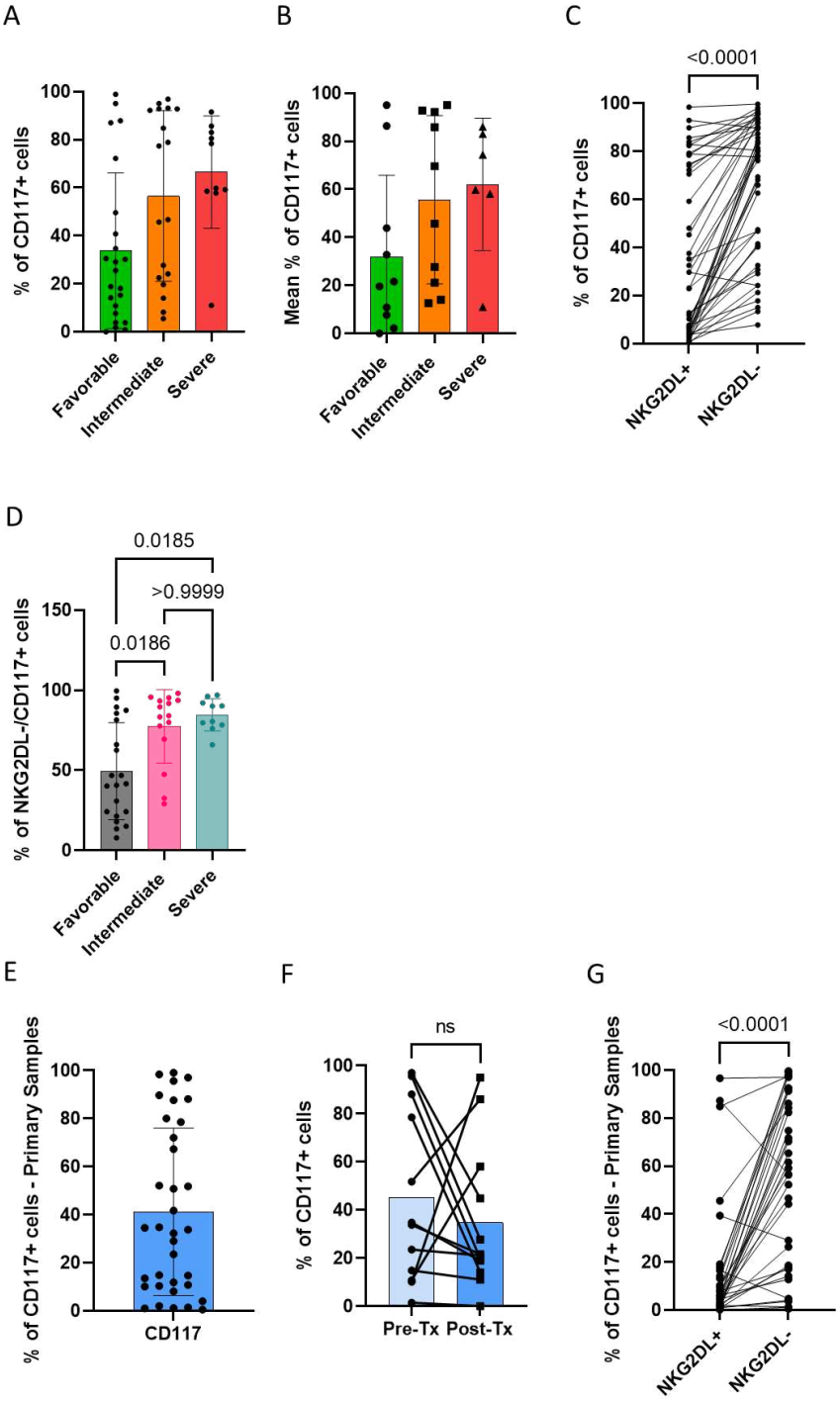


Figure 4: Characterization of CD117 in mouse-derived and primary patient samples (A) Expression of CD117 distinguished for the risk groups in the murine samples (B) Mean expression of CD117 per patient samples distinguished for the risk groups (C) Co-expression of CD117 with NKG2DL in the murine samples (D) Expression of CD117+/NKG2DL- distinguished for the risk groups in the murine samples (E) Expression of CD117 in primary patient samples (F) Comparison of the expression of CD117 pre- and post-transplantation (G) Co-expression of CD117 with NKG2DL in primary patient samples

3.3.4 CD123

In our mouse-derived samples, we could detect an average CD123 expression of 39.54 ± 29.97 %. The highest amount of CD123+ cells was found in the “intermediate” risk group (48.58 ± 31.20 %), although there were no significant differences regarding the distribution throughout the risk groups (“favorable”: 34.41 ± 30.42; “adverse”: 35.04 ± 24.69; figure 5A). In mouse-derived samples, CD123 showed higher expression in the “intermediate” risk group (mean = 44.91 ± 23.48 %; figure 5B) than in the “favorable” (mean: 23.48 ± 2.,74 %) or “adverse” group of our cohort (mean: 32.69 ± 22.06 %) (figure 5B). In order to observe an association with an LSC phenotype, the co-expression of CD123 and NKG2DL was determined, but no correlation of these two markers was observed ($p = 0.2478$; figure 5C).

In patient derived samples, the mean expression of CD123 was 32.03 ± 33.52 % (figure 5D). As displayed in figure 5E, we compared matched patient and mouse-derived samples pre- to post-transplantation. The expression of CD123 showed no significant change ($p = 0.7440$) between both groups, meaning that its expression is unaffected by an incubation period in NSG animals.

As for the post-transplantation samples, no significant co-expression with NKG2DL ($p = 0.2061$; figure 5F) was found.

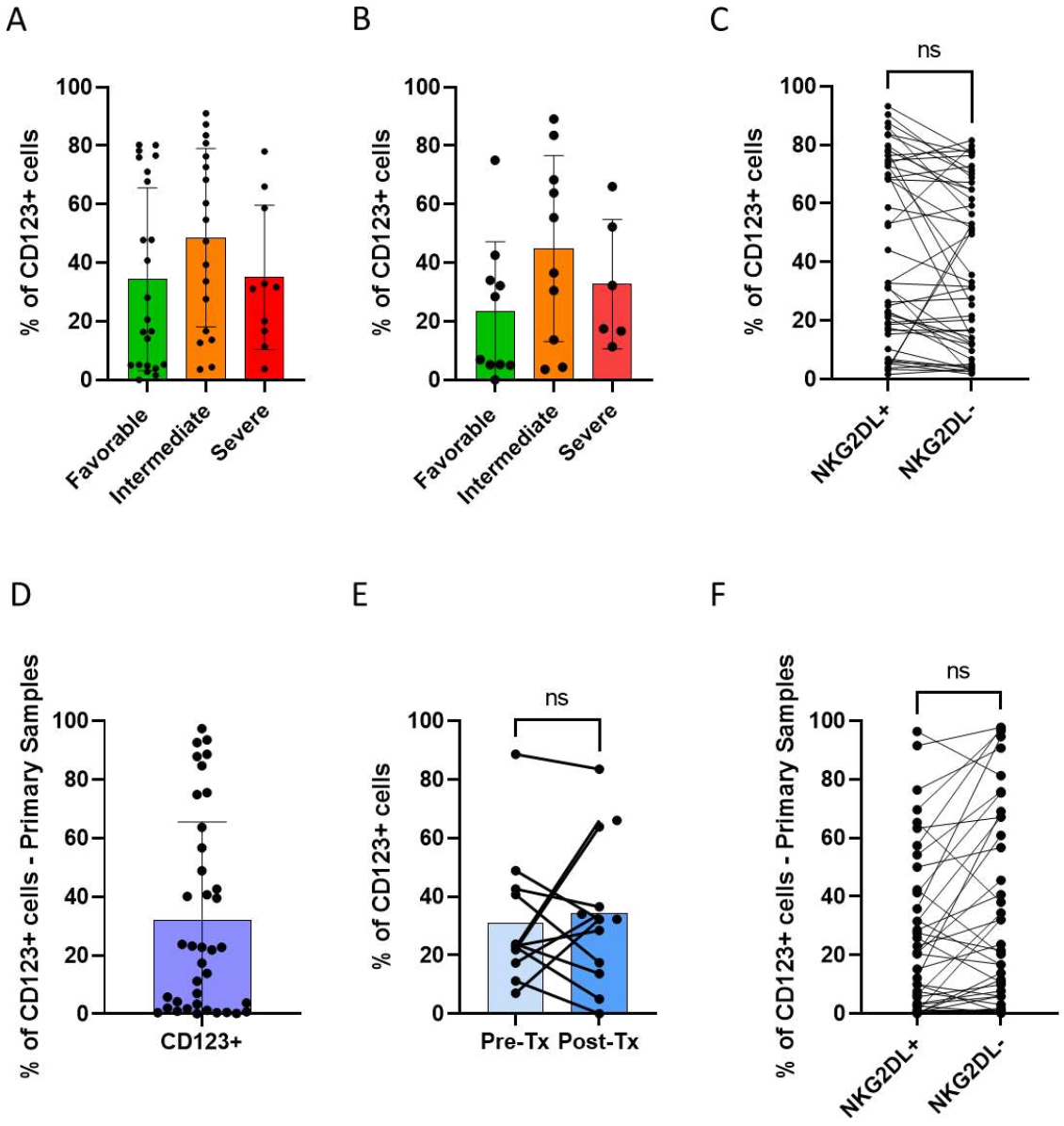


Figure 5: Characterization of CD123 in mouse-derived and primary patient samples (A) Expression of CD123 distinguished for the risk groups in the murine samples (B) Mean expression of CD123 per patient samples distinguished for the risk groups (C) Co-expression of CD123 with NKG2DL in the murine samples (D) Expression of CD123 in primary patient samples (E) Comparison of the expression of CD123 pre- and post-transplantation (F) Co-expression of CD123 with NKG2DL in primary patient samples

3.3.5 CLL-1

As described above, CLL-1 expression can be divided into two distinct groups, CLL-1 low and CLL-1 high that were reported to impact overall survival and outcome in patients (Wang et al. 2021).
We observed two distinct positive populations for CLL-1, one of which was high expressing and one of which was low-expressing which is why we termed the populations CLL-1 high and CLL-1 low. We observed average values of 51.24 ± 31.59 % CLL-1 expression and by trend a higher percentage of CLL-1 low cells in the “adverse” risk group (61.52 ± 38.95 %) compared to the “favorable” (mean: 47.63 ± 27.87 %) and “intermediate” group of our cohort (mean: 50.20 ± 32.65 %) (figure 6A). These results were consistent in all mice transplanted with cells from one patient (“favorable”: 51.30 ± 27.29 %; “intermediate”: 47.80 ± 30.86 %; “adverse”: 69.03 ± 35.43 %; figure 6B). Additionally, CLL-1 low cells were enriched in NKG2DL negative subpopulation ($p < 0.0001$; figure 6C) and the size of this subpopulation strongly negatively correlated with NKG2DL expression (Spearman $r = -0.6967$; $p < 0.0001$; figure not shown).

In comparison, the CLL-1 high population is found mostly in the “favorable” risk group (49.41 ± 26.35 %; figure 6D), followed closely by the “adverse” (40.33 ± 43.16 %) and “intermediate” (30.32 ± 32.06 %) risk groups with an average expression of 41.62 ± 31.86 %. Interestingly, we could not validate these findings by looking at the mean expression of the marker per patient as the numbers in the risk groups were very similar (“favorable”: 38.17 ± 28.60 %; “intermediate”: 37.46 ± 34.12 %; “severe”: 39.45 ± 44.78 %; figure 6F).
In the mouse-derived samples, the percentages of CLL-1 positive cells correlated with the percentage of NKG2DL positive cells, and overall CLL-1 expression associated with NKG2DL expression ($p < 0.0001$; figure 6G; Spearman $r = 0.7400$; $p < 0.0001$; figure not shown).

These observations support the notion that suppressed CLL-1 expression associates with an LSC phenotype, whereas CLL-1 high cells rather display a committed, non-LSC phenotype.

The patient derived cohort of 16 AML samples showed an average CLL-1 expression of 60.57 ± 38.32 % (figure 6G) with no clearly distinguishable subpopulations of CLL-1 high and low cells, meaning that we had only one positive population and not two as for the mouse-derived samples. However, CLL-1 and NKG2DL negative co-expression was equally observed in this cohort ($p < 0.0001$; figure 6H).

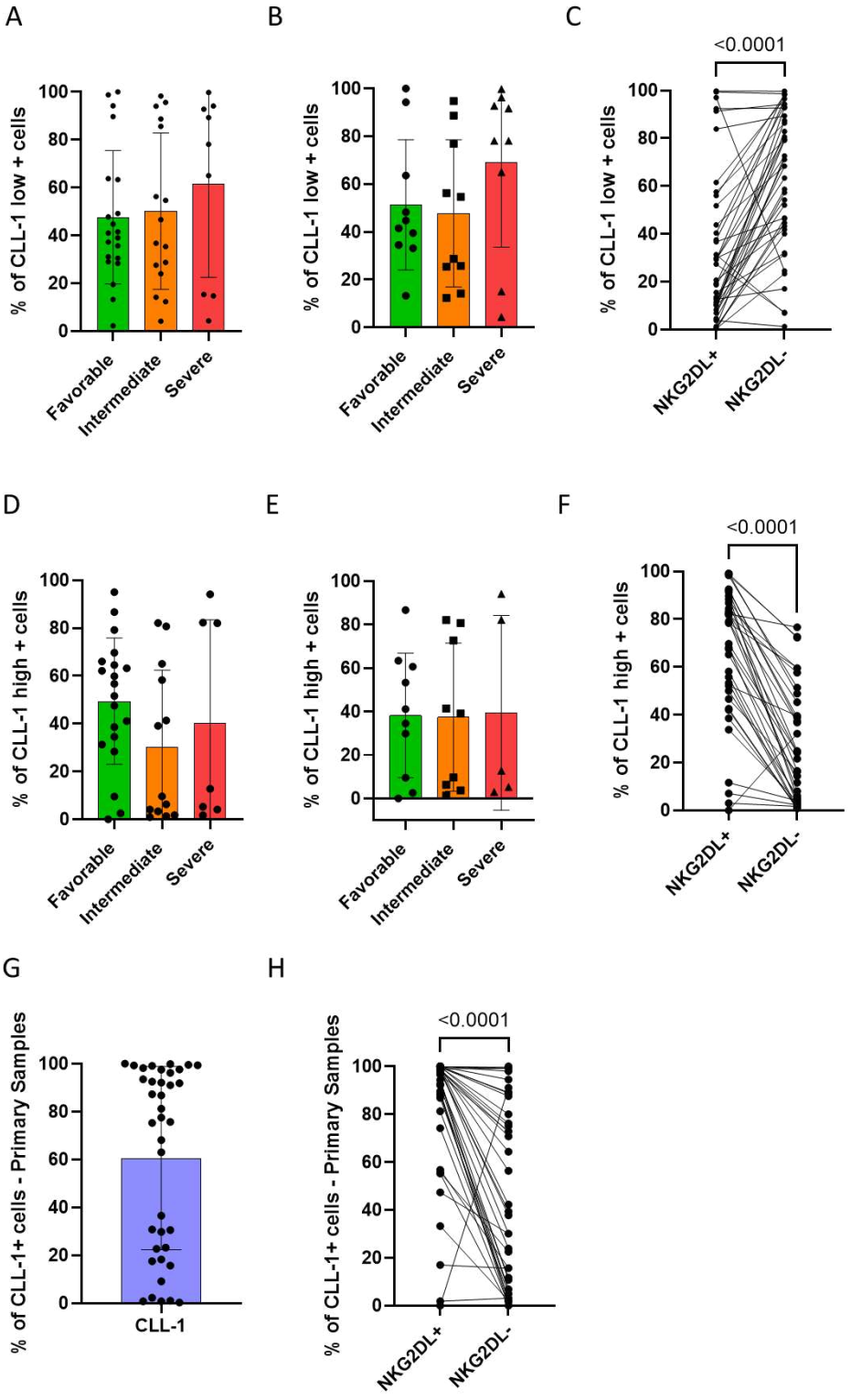


Figure 6: Characterization of CLL-1 in mouse-derived and primary patient samples (A) Expression of CLL-1 low distinguished for the risk groups in the murine samples (B) Mean expression of CLL-1 low per patient samples distinguished for the risk groups (C) Co-expression of CLL-1 low with NKG2DL in the murine samples (D) Expression of CLL-1 high distinguished for the risk groups in the murine samples (E) Mean expression of CLL-1 high per patient samples distinguished for the risk groups (F) Co-expression of CLL-1 high with NKG2DL in the murine samples (G) Expression of CLL-1 in primary patient samples (H) Co-expression of CLL-1 with NKG2DL in primary patient samples

3.3.6 CD200

CD200 was only detected at very low levels (mean = 10.65 ± 22.91 %) in the mouse-derived cohort. The highest expression was observed in the “adverse” risk group (14.11 ± 20.17 %), although there were no significant differences with both other groups detectable (“favorable”: 12.92 ± 26.19 %; “intermediate”: 5.84 ± 20.03 %; figure 7A). These results did not differ significantly from the mean expression per patient in our cohort (“favorable”: 10.27 ± 19.62 %; “intermediate”: 9.62 ± 26.83 %; “adverse”: 17.29 ± 25.13 %; figure 7B). Nevertheless, we could show that CD200 co-expresses with NKG2DL negative populations ($p = 0.0038$; figure 7C), supporting the notion that it enriches LSCs.

Overall, primary patient samples had slightly higher levels of CD200 (14.88 ± 23.23 %; figure 7D), than mouse-derived cells which overall showed also dimmer CD200 levels ($p = 0.0205$; figure 7E). This suggests that its expression was lost during the incubation process of the samples in the xenotransplant setting, consistent with the notion that the PDX environment in NSG mice might less well support the persistence of undifferentiated LSCs. Similarly, to mice-derived samples, we did not find significant associations with NKG2DL expression ($p = 0.5233$; figure 7F).

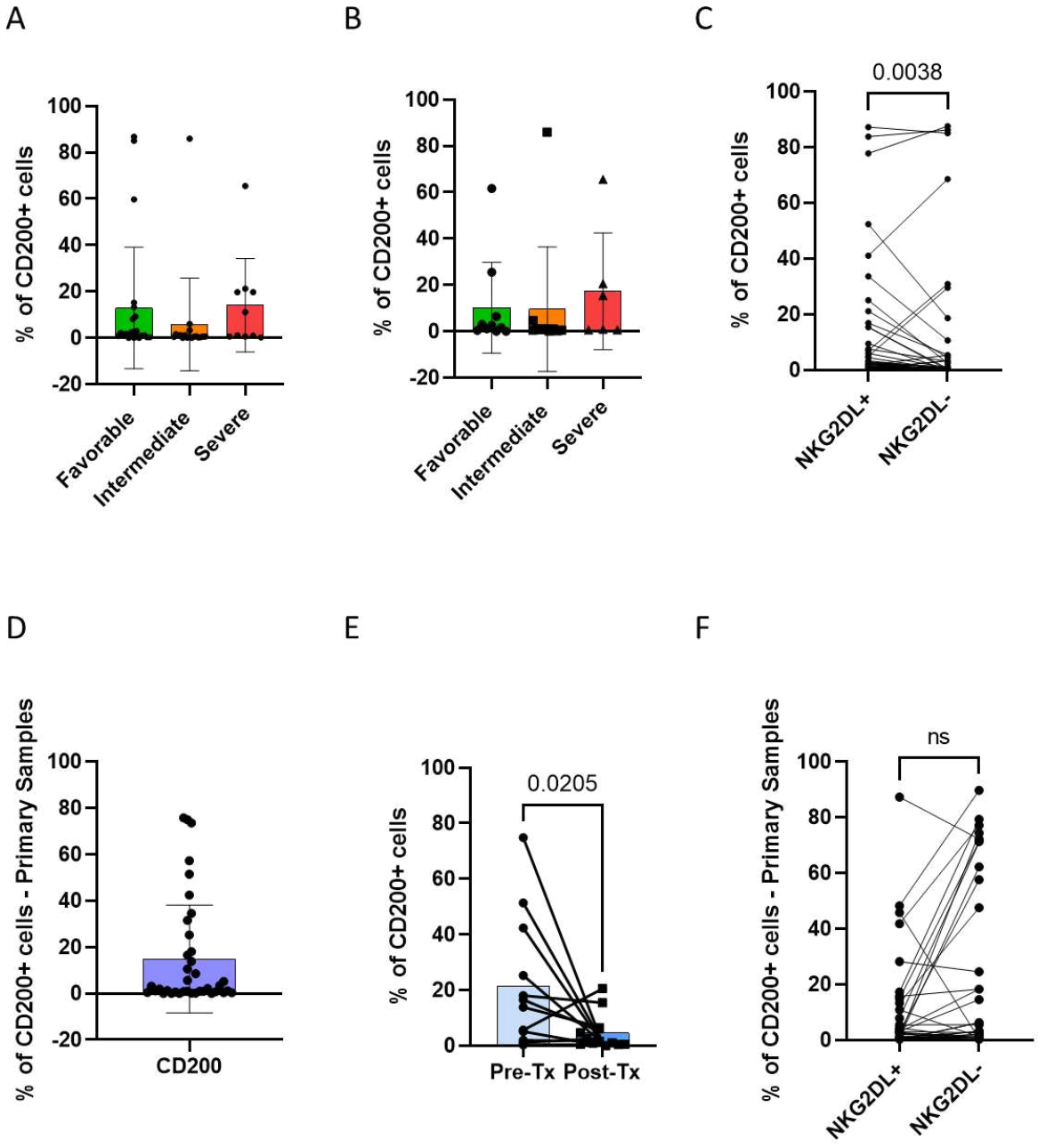


Figure 7: Characterization of CD200 in mouse-derived and primary patient samples (A) Expression of CD200 distinguished for the risk groups in the murine samples (B) Mean expression of CD200 per patient samples distinguished for the risk groups (C) Co-expression of CD200 with NKG2DL in the murine samples (D) Expression of CD200 in primary patient samples (E) Comparison of the expression of CD200 pre- and post-transplantation (F) Co-expression of CD200 with NKG2DL in primary patient samples

3.3.7 CD45RA

For CD45RA was expressed in AML cells of both patient or mouse sources (mean = 33.75 ± 26.31 %; figure not shown), but showed no further significant correlations with other markers (data not shown). The values for the risk groups lie at 28.42 ± 21.79 % (“favorable”), 38.22 ± 27.47 % (“intermediate”) and 37.96 ± 33.59 % (“adverse). In the mouse-derived cohort, no significant correlation with NKG2DL was detectable ($p = 0.3998$).

3.3.8 GPR56

GPR56 was described as an LSC and HSC marker (Daga et al. 2019). In our mice-derived samples, we had only very few samples that expressed detectable levels of GPR56 with an average expression of 17.39 ± 18.68 %. The highest expression was observed in the “intermediate” risk group (24.90 ± 25.88 %; figure 8A) although there were no significant differences regarding the distribution among the risk groups (“favorable”: 12.42 ± 11.27 %; “adverse”: 15.84 ± 12.95 %; figure 8A). These observations could be validated by looking at the mean expression of the marker per patient (“favorable”: 10.40 ± 7.64 %; “intermediate”: 20.22 ± 22.41 %; “adverse”: 17.43 ± 16.11 %; figure 8B). But regardless of the dim expression, we could appreciate that GPR56 correlates with NKG2DL negative cells ($p < 0.0001$; figure 8C), supporting the notion that GPR56 marks LSCs.

The average expression of GPR56 in primary patient samples lies at 18.83 ± 29.35 % (figure 8D) and resembles the expression levels of mouse-derived samples. There was no significant difference in the expression between the matched samples of the patient and mouse-derived cohorts ($p = 0.6707$; figure 8E). In the patient derived samples, GPR56 is also associated with NKG2DL negative populations ($p = 0.0183$; figure 8F).

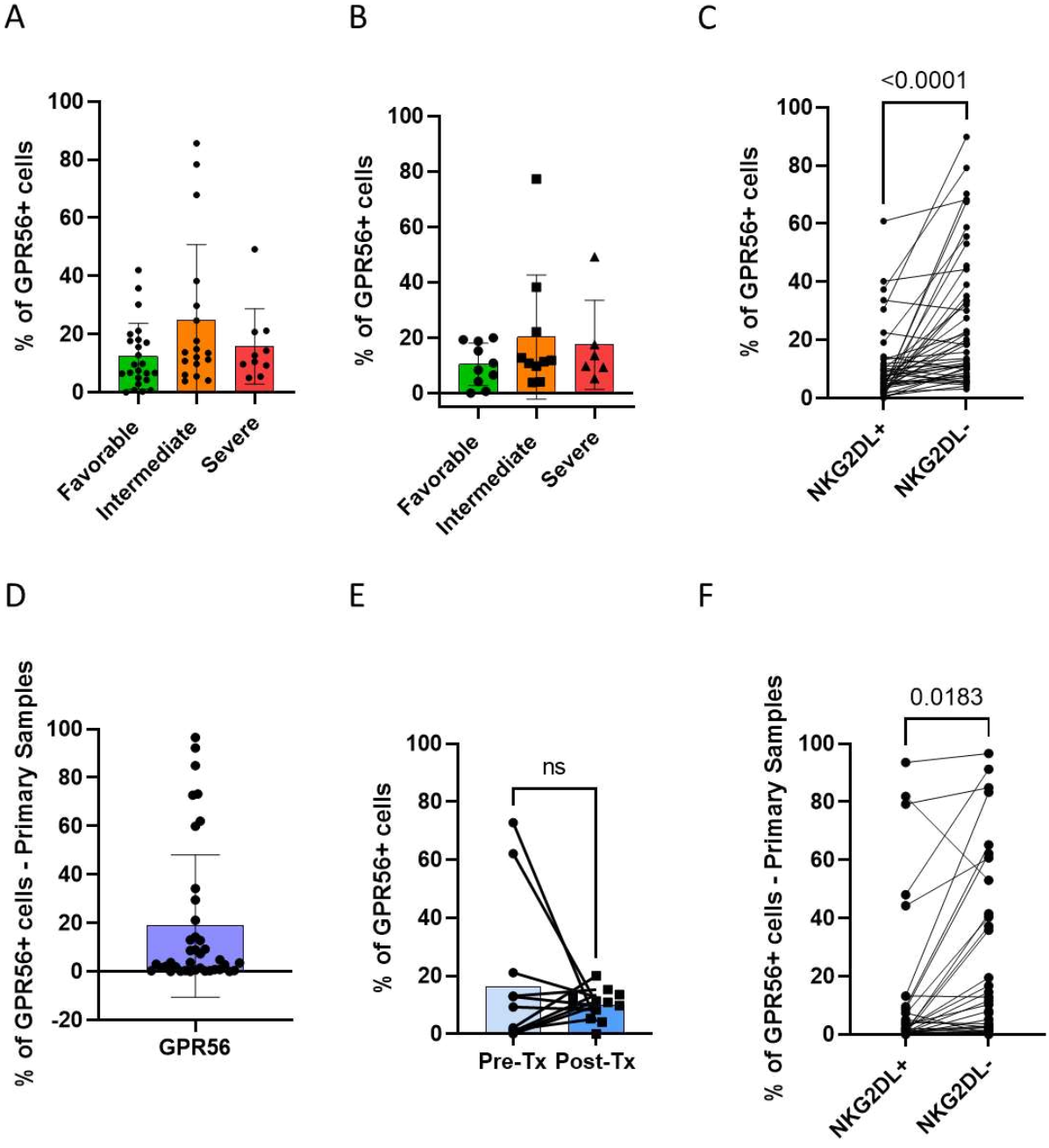


Figure 8 Characterization of GPR56 in mouse-derived and primary patient samples (A) Expression of GPR56 distinguished for the risk groups in the murine samples (B) Mean expression of GPR56 per patient samples distinguished for the risk groups (C) Co-expression of GPR56 with NKG2DL in the murine samples (D) Expression of GPR56 in primary patient samples (E) Comparison of the expression of GPR56 pre- and post-transplantation (F) Co-expression of GPR56 with NKG2DL in primary patient samples

3.4 Description of the Immune Checkpoint Markers

Immune evasion is a known hallmark of cancer and we and others could show that LSCs are able to evade immune-mediated killing in order to e.g. favor relapse after apparent remission (Paczulla et al. 2019). Therefore, we added more immune checkpoint markers to the analysis to improve our understanding of how to manipulate the immune system to kill cancer cells.

3.4.1 CD80

CD80 is an immune checkpoint marker that plays an important part in allogenic immune recognition (Costello et al. 1998). CD80 was expressed on a great proportion of AML cells in our mouse-derived cohort with an average expression of 59.46 ± 31.84 %. The highest number of CD80+ cells was found in the “favorable” risk group (71.30 ± 27.02 %; figure 9A), followed by the “adverse” (53.93 ± 30.63 %) and the “intermediate” (47.41 ± 34.34 %) population (figure 9A). The same trend was observable for the mean expression per patient, although we also had no significant difference between the risk groups (“favorable”: 69.55 ± 23.01 %; “intermediate”: 51.04 ± 34.57 %; “adverse”: 50.73 ± 19.80 %; figure 9B).

In figure 9C, we could show that CD80 is co-expressed with NKG2DL ($p = 0.0002$). The proportion of NKG2DL+/CD80+ cells was significantly enhanced in the “favorable” risk group ($p = 0.0204$; figure 9D).
To summarize, we could show that CD80 associates it with a non-LSC phenotype. This also matches the observation that CD80 is mostly found in the “favorable” risk group.

CD80 was expressed only very weakly in the primary patient samples with a mean expression of only 8.19 ± 20.93 % (figure 9E) which is a very significant difference in comparison with the matched samples of the mouse-derived group

where we had much higher levels of the respective marker ($p < 0.0001$; figure 9F). The cells may have experienced some differentiation or alternatively plasticity during the transplantation process, enabling them to gain CD80 and express it on the surface. We could also show that CD80 is associated with a more committed, non-LSC phenotype in primary samples as it co-expresses with NKG2DL ($p = 0.0033$; figure 9G).

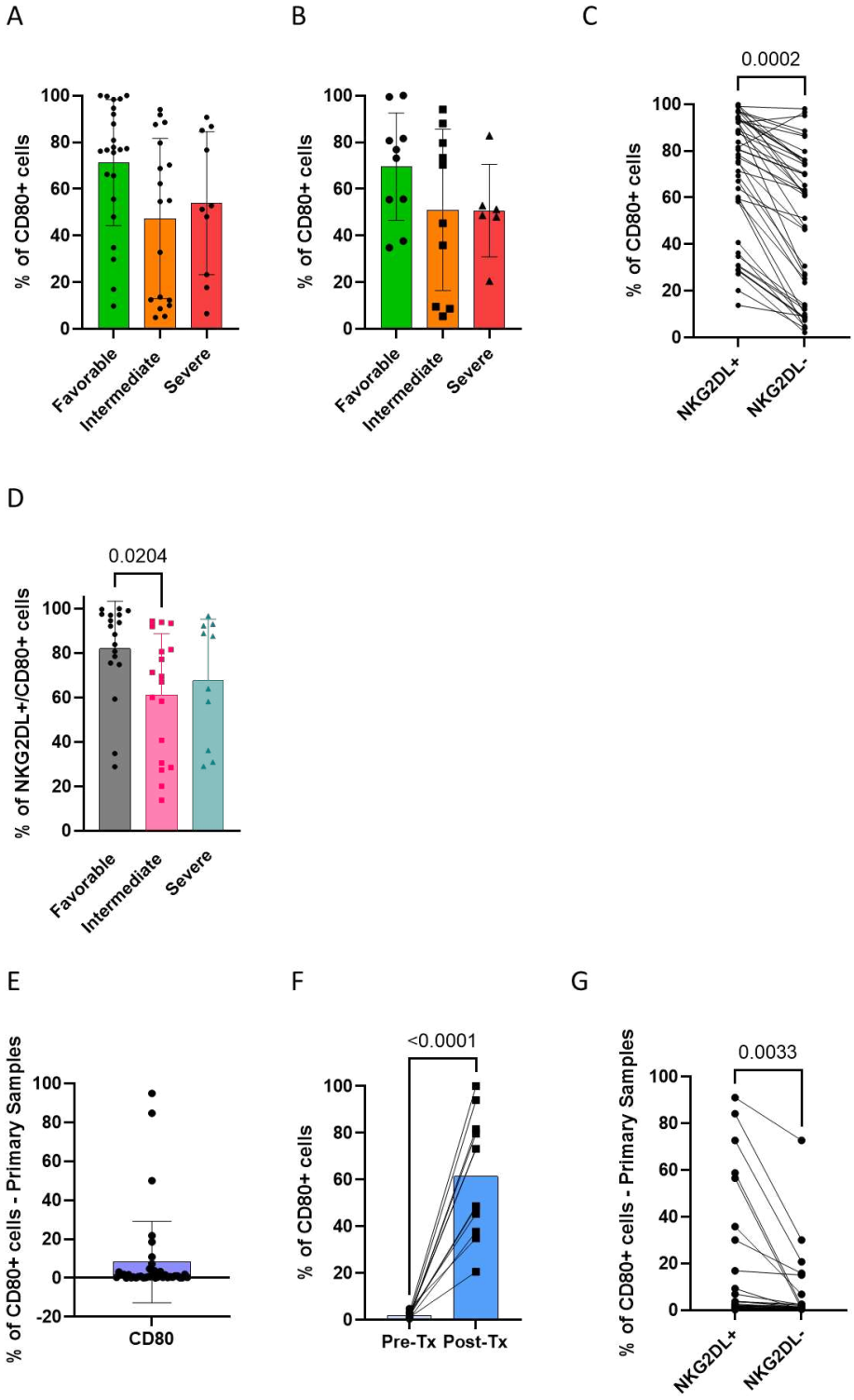


Figure 9: Characterization of CD80 in mouse-derived and primary patient samples (A) Expression of CD80 distinguished for the risk groups in the murine samples (B) Mean expression of CD80 per patient samples distinguished for the risk groups (C) Co-expression of CD80 with NKG2DL in the murine samples (D) Expression of CD80+/NKG2DL+ distinguished for the risk groups in the murine samples (E) Expression of CD80 in primary patient samples (F) Comparison of the expression of CD80 pre- and post-transplantation (G) Co-expression of CD80 with NKG2DL in primary patient samples

3.4.2 CD86

Similar results as for CD80 were observed for CD86. The expression of CD86 was generally lower among the mouse-derived samples with an average expression of 29.80 ± 25.85 %. The expression in the “intermediate” and the “favorable” risk group were almost similar (31.78 ± 32.36 %; 31.60 ± 23.29 %; figure 10A), only the expression in the “adverse” risk group differed from the others (22.10 ± 15.72 %), although not significantly (figure 10A), possibly reflecting the higher LSC content in the latter. When checking for the mean expression of the marker per patient, we could validate these findings (“favorable”; 33.61 ± 27.25 %; “intermediate”: 32.47 ± 31.01 %; “adverse”: 22.44 ± 14.90 %; figure 10B). CD86 is also significantly associated with NKG2DL ($p < 0.0001$; figure 10C) which suggests that CD86 also seems to be associated with a non-LSC phenotype in the PDX model, although we could not detect a significant correlation to a specific risk group.

CD86 showed average numbers of 35.90 ± 32.36 % (figure 10D) in the primary patient samples, which is not significantly different from what we noted for the matched samples of the mouse-derived cohort ($p = 0.4403$; figure 10E). But, as for the mouse-derived cohort, we were able to show that the marker is co-expressed with the NKG2DL ($p < 0.0001$; figure 10F), meaning the phenotypic identity of the marker is consistent.

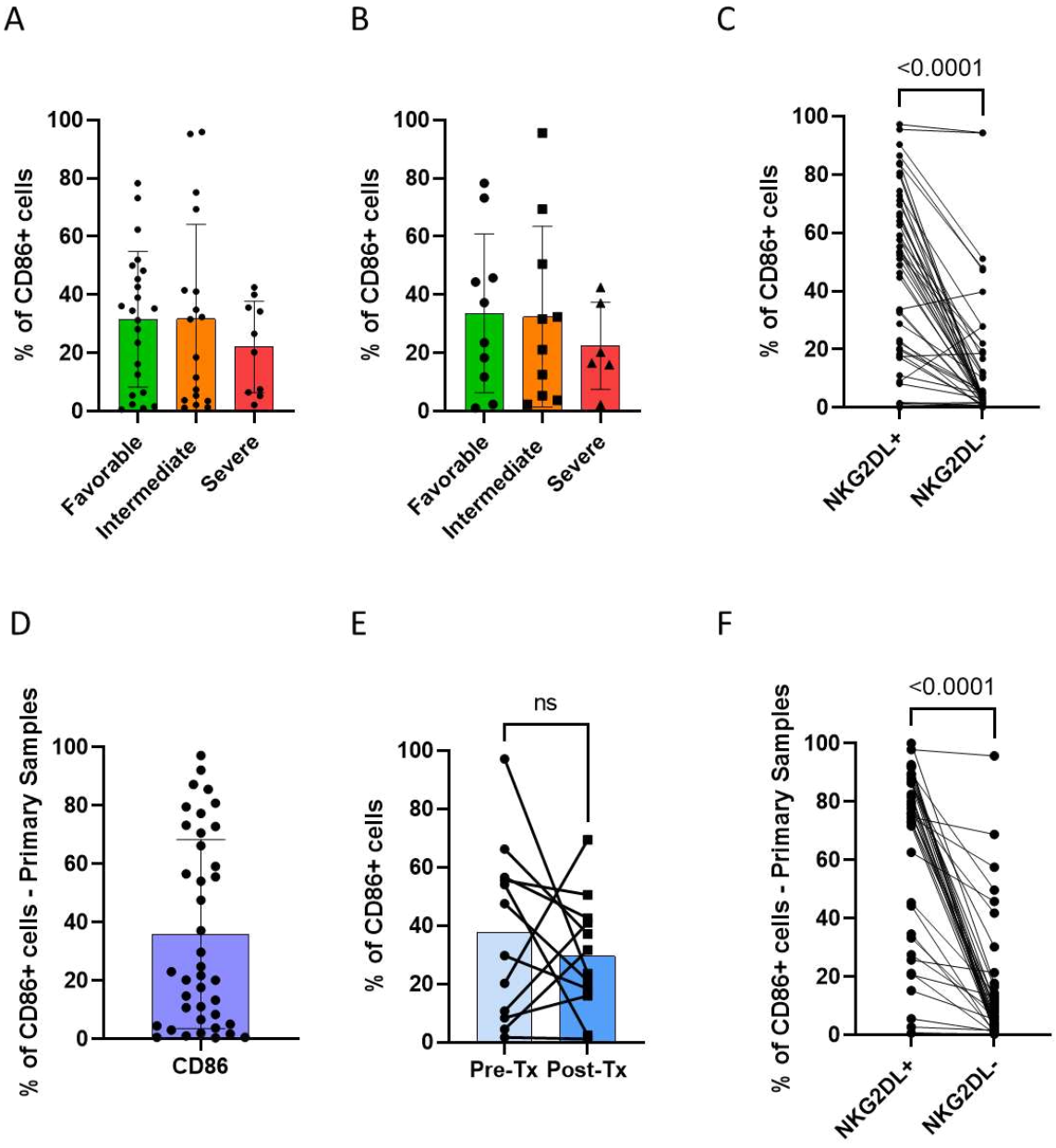


Figure 10: Characterization of CD86 in mouse-derived and primary patient samples (A) Expression of CD86 distinguished for the risk groups in the murine samples (B) Mean expression of CD86 per patient samples distinguished for the risk groups (C) Co-expression of CD86 with NKG2DL in the murine samples (D) Expression of CD86 in primary patient samples (E) Comparison of the expression of CD86 pre- and post-transplantation (F) Co-expression of CD86 with NKG2DL in primary patient samples

3.4.3 CD276

CD276 was already described as a marker of inferior prognosis in patients with AML (Zhang et al. 2021). In our mouse-derived cohort, we observed average values of 40.92 ± 27.56 % for the molecule. Interestingly, we found the highest proportion of CD276 positive cells in the “favorable” risk group (48.54 ± 31.00 %; figure 11A), although there were no significant differences when compared to both other groups (“intermediate”: 30.56 ± 22.99 %; “adverse”: 42.04 ± 22.55 %; figure 11A). Similar results were observed when analyzing the mean expression of the marker per patient sample (“favorable”: 45.97 ± 30.65 %; “intermediate”: 29.62 ± 23.39 %; “adverse”: 36.67 ± 20.38 %; figure 11B). Like CD80 and CD86, CD276 strongly correlated with NKG2DL ($p < 0.0001$; figure 11C), confirming the Non-LSC phenotypic identity of the marker.

The patient derived samples, by average showed 38.47 ± 29.56 % of CD276 expression (figure 11D) with no significant changes to the mouse cohort ($p = 0.2215$; figure 11E) but we could appreciate a strong association with NKG2DL also for the primary patient samples ($p < 0.0001$; figure 11F). This suggests that CD276 also is conserved during the transplantation process and preserves its phenotypic identity.

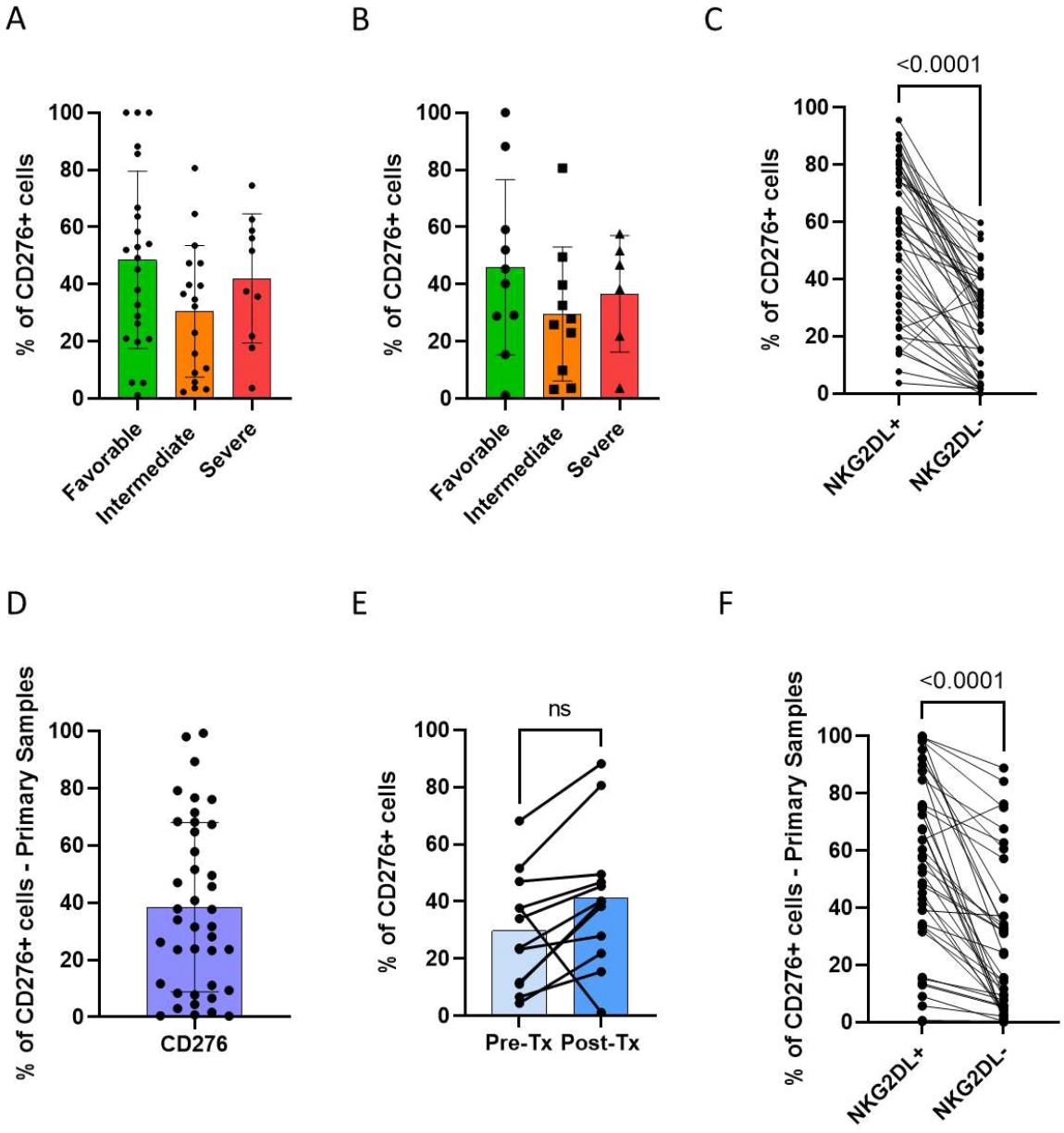


Figure 11: Characterization of CD276 in mouse-derived and primary patient samples (A) Expression of CD276 distinguished for the risk groups in the murine samples (B) Mean expression of CD276 per patient samples distinguished for the risk groups (C) Co-expression of CD276 with NKG2DL in the murine samples (D) Expression of CD276 in primary patient samples (E) Comparison of the expression of CD276 pre- and post-transplantation (F) Co-expression of CD276 with NKG2DL in primary patient samples

3.4.4 PD-L1

PD-L1 is used as an immunotherapy target in several solid cancers and its expression was reported in AML (Zhang et al. 2009). In our mouse derived cells, we were only able to detect one murine BM sample that expressed PD-L1 positive cells (18.7 %) which belonged to the “favorable” risk group. Therefore, we couldn’t make any further statements or gain any further insights into PD-L1 and its interactions with other potential tumor markers (no figure attached).

3.5 Description of the Adhesion Markers

3.5.1 MRC2

We found MRC2 in our previous screen that compared LSC from non-LSC and its functions are of particular interest. These functions include migration, invasion and collagen interaction. MRC2 is a cancer stem cell marker in different solid cancers but not yet described in leukemia, so it was the perfect hit for us (Gai et al. 2014; Nielsen et al. 2017).
We observed a mean expression of 41.59 ± 25.24 % throughout the mouse-derived samples with the highest number of MRC2 positive cells being expressed in the “intermediate” risk group (50.22 ± 18.02 %; figure 12A). The expression in the risk groups did not differ significantly (“favorable”: 36.21 ± 26.96 %; “adverse”: 39.28 ± 29.89 %; figure 12A). These results were confirmed by checking the mean expression of the marker per patient, indicating that the results are not a cohort-dependent phenomenon (“favorable”: 30.23 ± 22.25 %; “intermediate”: 49.51 ± 19.53 %; “adverse”: 31.04 ± 21.68 %; figure 12B).
No association was detected between MRC2 and NKG2DL in mouse derived samples ($p = 0.6288$; figure 12C).
Taken together, we could show a robust expression of the marker in our cohort.

Patient derived samples showed a mean expression of 32.21 ± 28.87 % (figure 12D) which did not differ significantly from the expression of the matched samples in the mouse-derived cohort ($p = 0.4276$; figure 12E). In contrast to the mouse-derived cohort, we were able to appreciate a significant association with NKG2DL negativity, which phenotypically makes MRC2 an LSC marker in patient-derived samples ($p = 0.0002$; figure 12F). This underlines our assumption that MRC2 could be subject to plasticity during the engraftment in mice.

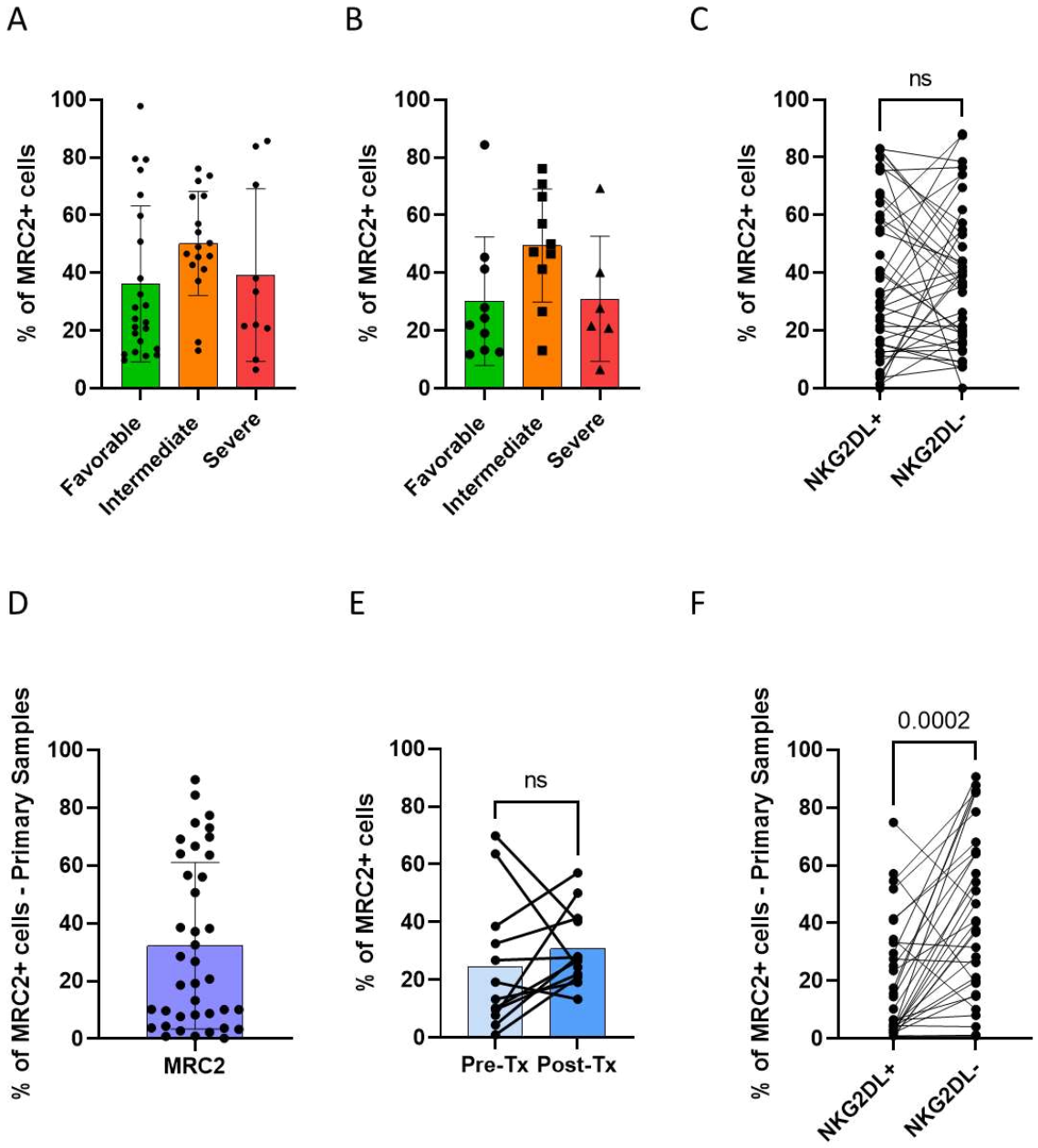


Figure 12: Characterization of MRC2 in mouse-derived and primary patient samples (A) Expression of MRC2 distinguished for the risk groups in the murine samples (B) Mean expression of MRC2 per patient samples distinguished for the risk groups (C) Co-expression of MRC2 with NKG2DL in the murine samples (D) Expression of MRC2 in primary patient samples (E) Comparison of the expression of MRC2 pre- and post-transplantation (F) Co-expression of MRC2 with NKG2DL in primary patient samples

3.5.2 CD56

CD56 was only noticed at very dim signals in our mouse-derived cohort with a mean expression of 10.39 ± 24.15 % and highest expression in the “favorable” risk group (19.85 ± 33.22 %; figure 13A) with almost no expression in the other two groups (“intermediate”: 2.14 ± 2.94 %; “adverse”: 3.26 ± 6.90 %; figure 13A). These observations were also true for the mean expression of the marker per patient (“favorable”: 16.14 ± 29.89 %; “Intermediate”: 2.12 ± 2.70 %; “adverse”: 3.85 ± 8.03 %; figure 13B). A significant co-expression of CD56 with NKG2DL was furthermore detected ($p = 0.0006$; figure 13C).

The patient derived cohort expressed significantly higher levels of CD56 (20.03 ± 30.65 %; figure 13D; $p = 0.0028$; figure 13E) when compared to the mouse-derived cohort which however also revealed an association of CD56 and NKG2DL ($p = 0.0145$; figure 13F).

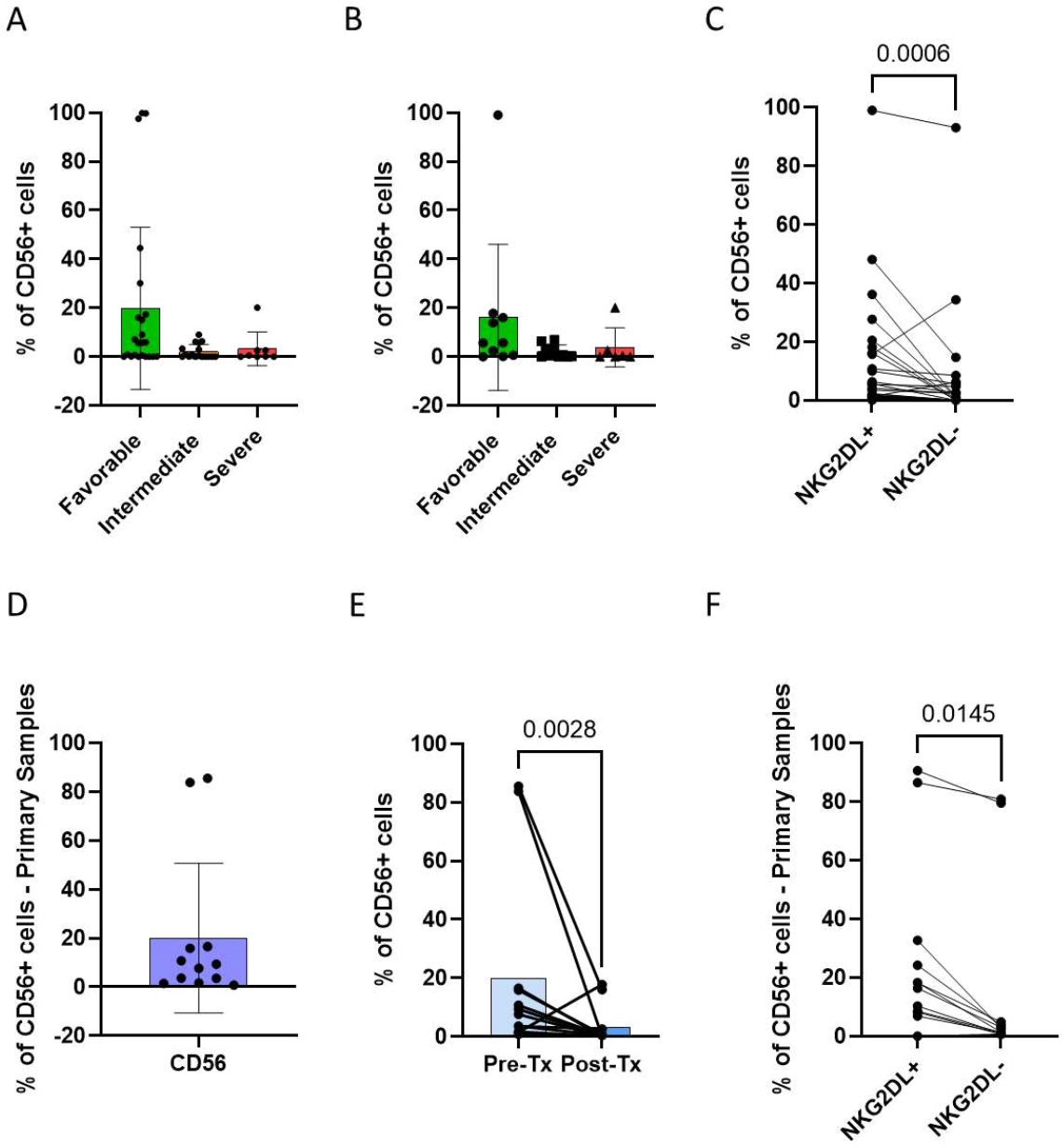


Figure 13: Characterization of CD56 in mouse-derived and primary patient samples (A) Expression of CD56 distinguished for the risk groups in the murine samples (B) Mean expression of CD56 per patient samples distinguished for the risk groups (C) Co-expression of CD56 with NKG2DL in the murine samples (D) Expression of CD56 in primary patient samples (E) Comparison of the expression of CD56 pre- and post-transplantation (F) Co-expression of CD56 with NKG2DL in primary patient samples

3.5.3 CD47

CD47 is known to be overexpressed on most leukemia cells and confer them immune-evasive properties (Jaiswal et al. 2009). In our cohort CD47 was expressed on mouse-derived samples with an average expression of 89.55 ± 20.60 %. The expression in the risk groups did not differ significantly (“favorable”: 89.03 ± 20.82 %; “intermediate”: 95.25 ± 5.61 %; “adverse”: 78.56 ± 33.32 %; figure 14A) and similar results were documented for the mean expression per patient (“favorable”: 88.85 ± 15.19 %; “intermediate”: 95.46 ± 4.01 %; “adverse”: 81.09 ± 31.14 %; figure 14B). We detected co-expression with NKG2DL ($p = 0.0224$; figure 14C), indicating a non-LSC phenotype.

Similarly, we experienced very high levels of CD47 in most of our patient derived samples (89.59 ± 18.94%; figure 14D) without significant differences between the two groups ($p = 0.7987$; figure 14E). As displayed in figure 14F, we again could show a co-expression of the marker with NKG2DL ($p = 0.0035$), questioning CD47 as a marker primarily expressed on LSCs.

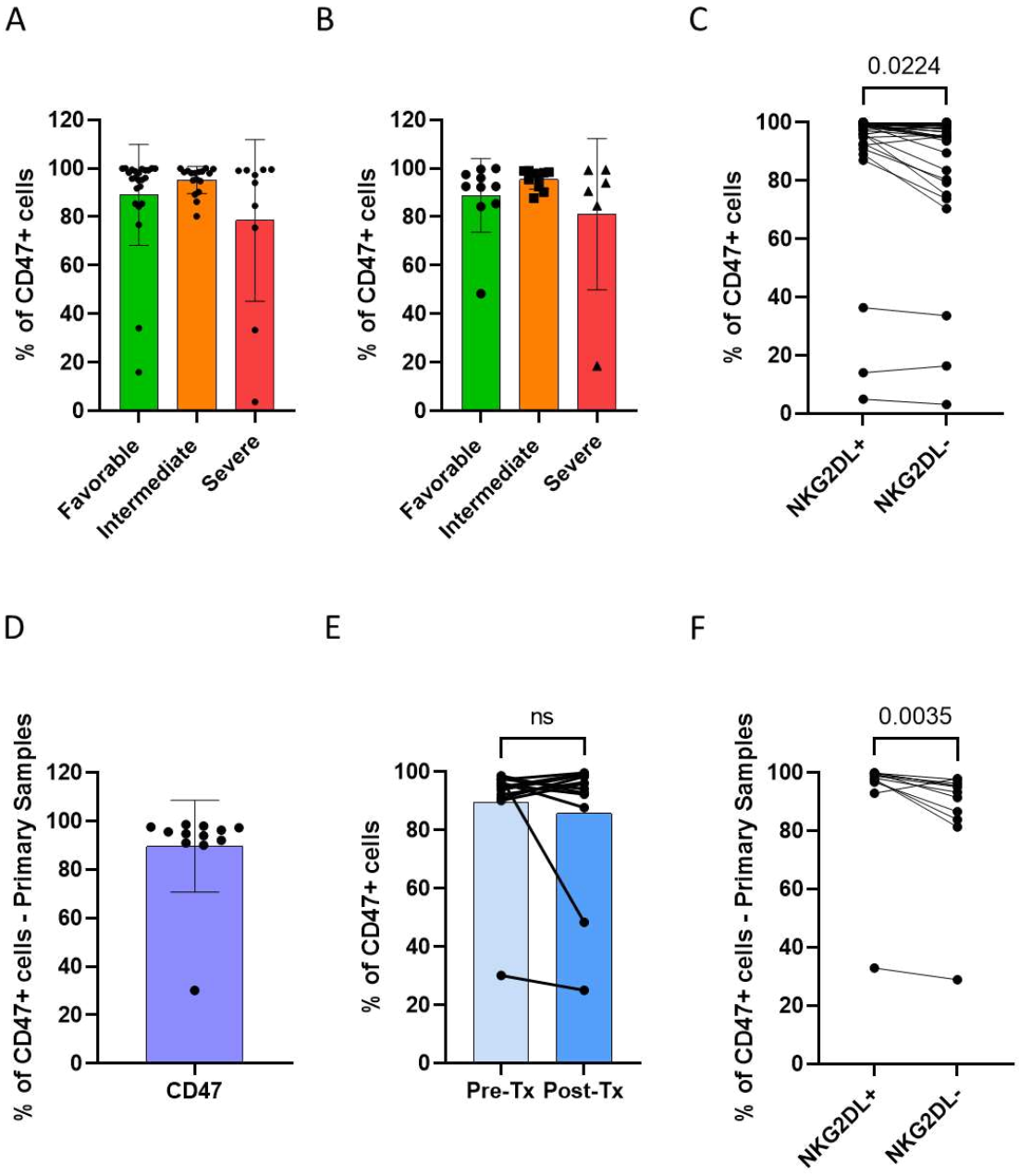


Figure 14: Characterization of CD47 in mouse-derived and primary patient samples (A) Expression of CD47 distinguished for the risk groups in the murine samples (B) Mean expression of CD47 per patient samples distinguished for the risk groups (C) Co-expression of CD47 with NKG2DL in the murine samples (D) Expression of CD47 in primary patient samples (E) Comparison of the expression of CD47 pre- and post-transplantation (F) Co-expression of CD47 with NKG2DL in primary patient samples

3.5.4 VLA-4

VLA-4 was expressed on most of the mouse-derived samples with an average expression of 60.23 ± 34.96 %. The highest levels of VLA4 were observed in the “favorable” risk group (67.40 ± 31.67 %; figure 15A) with no significant differences to the other two groups (“intermediate”: 62.63 ± 33.29 %; “adverse”: 35.21 ± 41.86 %; figure 15A). Interestingly, we observed differences for the mean expression per patient. The “adverse” (30.81 ± 27.58 %; figure 15B) group has significantly lower expression of VLA4 in comparison to the “favorable” (68.65 ± 18.22 %; $p = 0.0233$; figure 15B) and the “intermediate” (66.59 ± 30.45 %; $p = 0.0329$; figure 15B) group. VLA4 positive cells are significantly associated with NKG2DL ($p = 0.0199$; figure 15C), suggesting a non-LSC phenotypic identity.

We experienced lower levels of the marker in the patient versus mouse-derived samples (28.54 ± 24.25 %; figure 15D; $p = 0.0050$; figure 15E), meaning that the cells acquired the ability to express the marker during expansion in the mouse. In mouse-derived samples, we could show an association of the marker with a more committed phenotype ($p = 0.0332$; figure 15F), indicating VLA4 as a non-LSC marker in both sample cohorts.

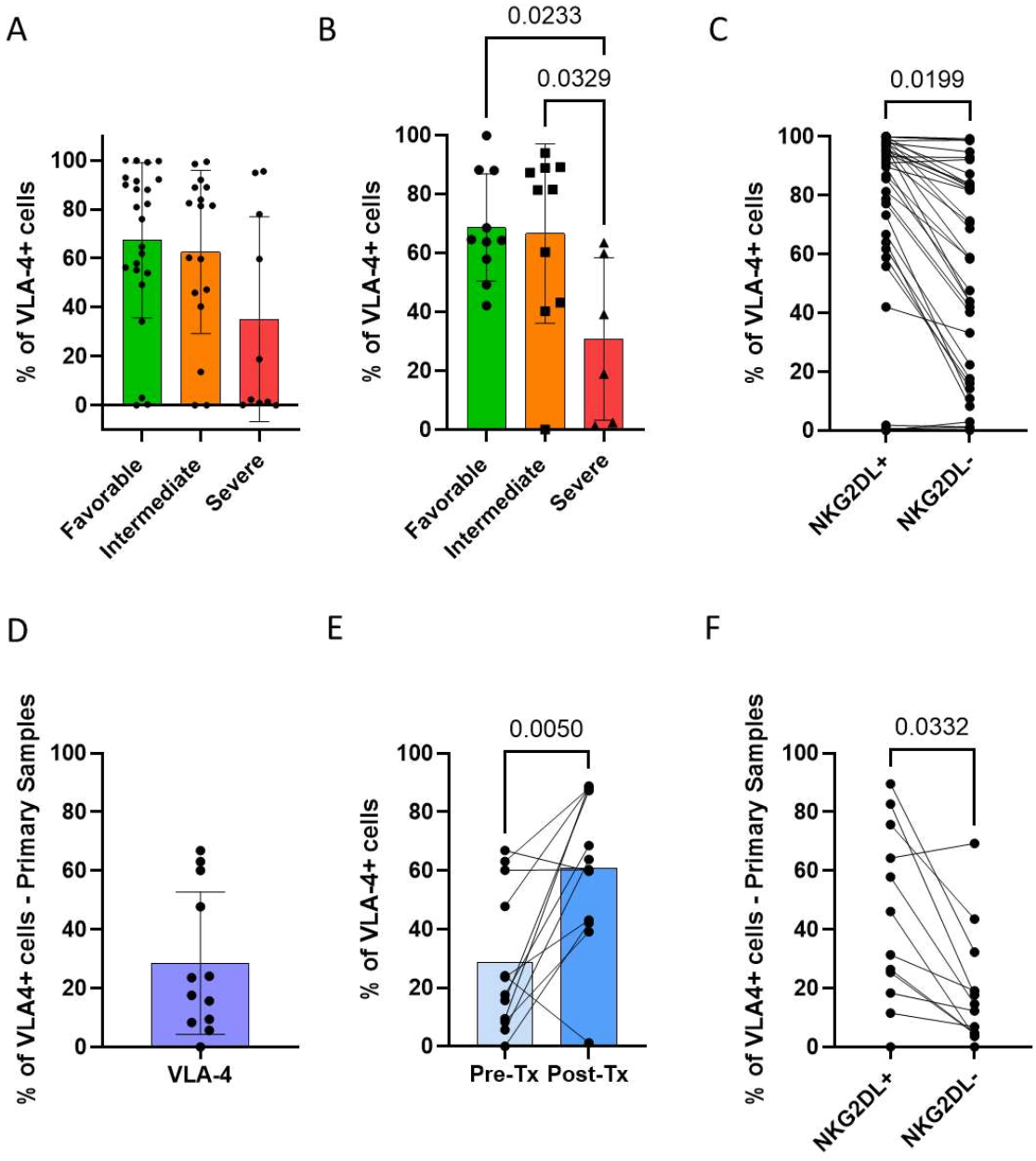


Figure 15: Characterization of VLA4 in mouse-derived and primary patient samples (A) Expression of VLA4 distinguished for the risk groups in the murine samples (B) Mean expression of VLA4 per patient samples distinguished for the risk groups (C) Co-expression of VLA4 with NKG2DL in the murine samples (D) Expression of VLA4 in primary patient samples (E) Comparison of the expression of VLA4 pre- and post-transplantation (F) Co-expression of VLA4 with NKG2DL in primary patient samples

3.5.5 CD49f

The average expression in the mouse-derived cohort was 19.92 ± 18.90 % with highest expression detected in the “favorable” (27.35 ± 22.59 %; figure 16A) versus “intermediate” (12.48 ± 11.70 %; p = 0.0447; figure 16A) and “adverse” risk groups (15.49 ± 12.77 %; figure 16A). This significant difference could not be observed when analyzing the mean expression of every patient (n = 22 patients; “favorable”: 24.55 ± 22.20 %; “intermediate”: 14.94 ± 12.06 %; “adverse”: 19.47 ± 13.55 %; figure 16B). CD49f co-expresses with NKG2DL (p = 0.0027; figure 16C), suggesting CD49f as a marker for non-LSCs in the mouse-derived samples.

CD49f was significantly lower expressed in the patient derived samples (12.79 ± 9.98 %; figure 16D; p = 0.0275; figure 16E) but we could also observe co-expression of CD49f with NKG2DL (p = 0.0364; figure 16F).

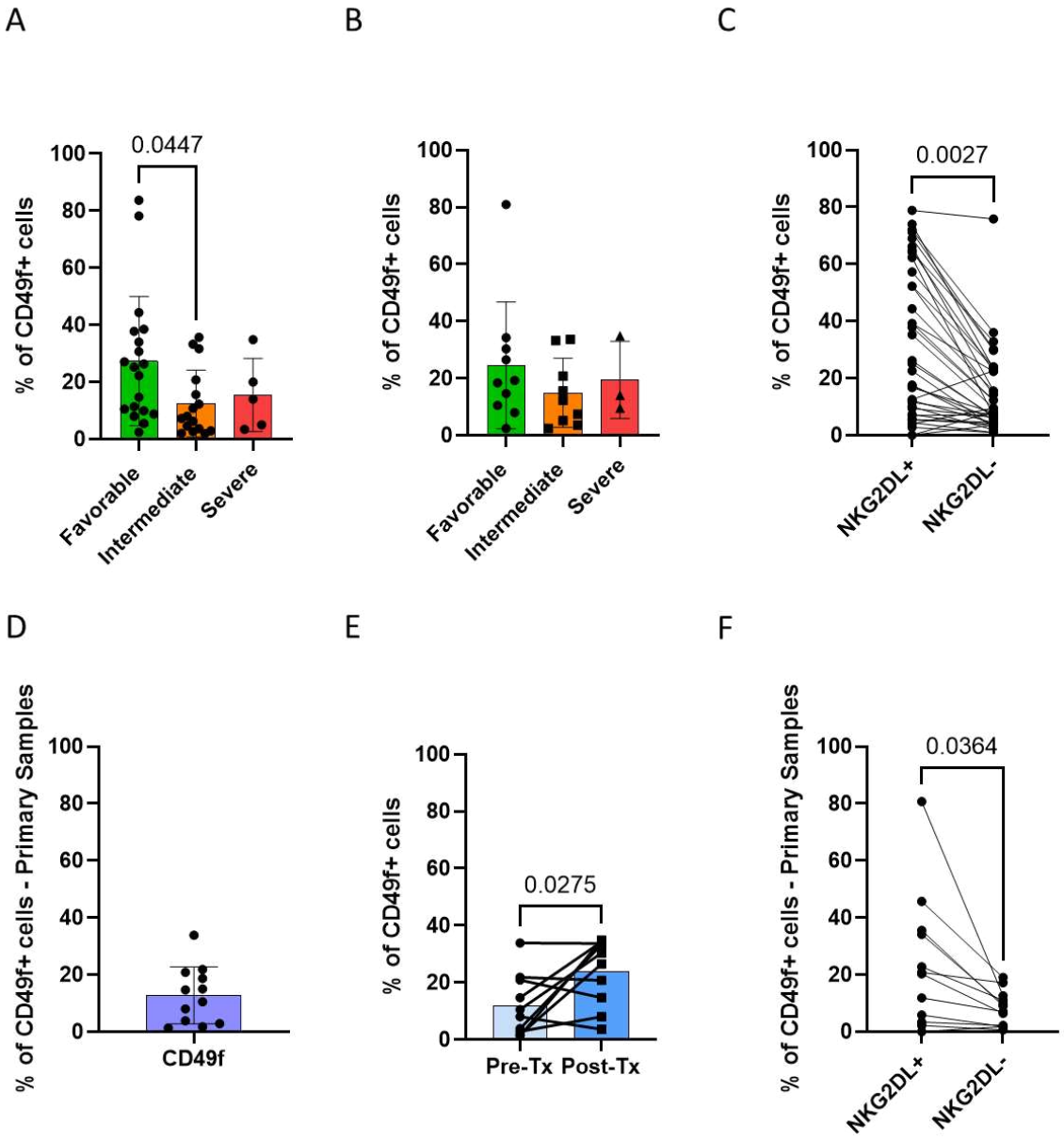


Figure 16: Characterization of CD49f in mouse-derived and primary patient samples (A) Expression of CD49f distinguished for the risk groups in the murine samples (B) Mean expression of CD49f per patient samples distinguished for the risk groups (C) Co-expression of CD49f with NKG2DL in the murine samples (D) Expression of CD49f in primary patient samples (E) Comparison of the expression of CD49f pre- and post-transplantation (F) Co-expression of CD49f with NKG2DL in primary patient samples

3.5.6 CD239

We were not able to detect CD239 in mouse derived AML cells (mean = 6.30 ± 11.16 %; figure not shown). However, technical reasons might be involved since the addition of CD49f to our panel might have involuntarily suppressed the signal of CD239. The antibodies for CD49f and CD239 likely compete, so that the antibody for CD239 cannot longer bind to its target structure after treatment with the CD49f antibody was applied. Because of this possibility, we cannot make a reliable statement about the expression of CD239 in our cohorts.

3.6 Mouse- Versus Patient-derived Samples

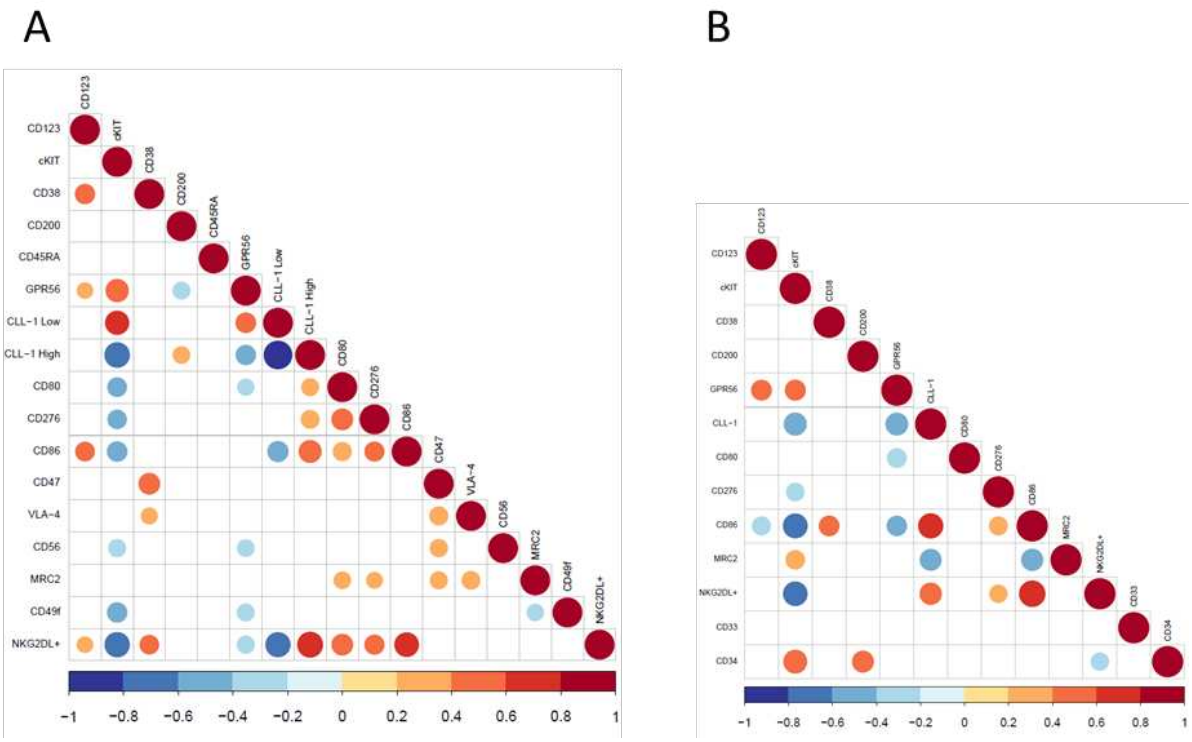


Figure 17: Correlation matrices displaying the markers in the murine cohort and the primary patient cohort (A) Correlations of the LSC, immune checkpoint and adhesion markers in the post-transplantation cohort, sorted by the strength of the correlation (B) Correlations of the LSC, immune checkpoint and adhesion markers in the primary patient samples, sorted by the strength of the correlation

To initiate leukemia, LSCs need to evade immune surveillance (Paczulla et al. 2019; Chao et al. 2019) but also to furthermore induce processes like adhesion, migration and invasion (Khwaja et al. 2016). To further explore the relationship between markers associated with these processes, we created correlation matrices (figure 17A and B). Absence of NKG2DL was used as the most robust marker to enrich LSCs. Our data reinforce that NKG2DL is a robust surrogate distinguishing LSCs from Non-LSCs in the mouse derived cohort (figure 17A). Additionally, LSC markers, including CD117, are most likely to associate with each other. Furthermore, the three immune checkpoint markers, CD86, CD80 and CD276 seem to rather express on non-LSC subpopulations from mouse-derived samples. As shown in figure 17A, they seem to strongly co-express with NKG2DL expressing non-LSCs. Notably, we also found that the three markers

correlate with each other as well as with other non-LSC markers and, as explained before, they were found most represented in the “favorable” risk group. The adhesion markers by trend correlated with a non-LSC phenotype and co-expressed with NKG2DL (CD49f and CD56 vs. GPR56 and CD117). For other markers, namely CD123 and CD47, it seems that they directly correlate with other markers, LSC or non-LSC, making them ambiguous to follow-up. Our results suggest that the markers involved in specific operations, e.g. joint pathways, are more likely to correlate with each other and we also observed strong correlations of LSC markers with non-LSC markers (e.g., NKG2DL+ vs. CD117, CLL-1 low, GPR56, CD200) (figure 17A). We concluded that, in our *in vivo* settings, LSCs and non-LSCs are well represented, distinct populations that can be studied in further detail.

Because we made these observations and correlations in samples collected from transplanted NSG mice, we wanted to know if this would also be true in primary AML samples. To rule out the possibility that our results are cohort-dependent, we relied on a new cohort of patient-derived samples and analyzed them together with our initial cohort of patient-derived samples. When creating the second correlation matrix, we noticed that some of our observations are also true for the patient-derived samples. The phenotypic LSC signature of e.g., CD117 and GPR56 was observed in the second correlation matrix together with a conserved positive association of CD86 and CD276 with a non-LSC phenotype as they are strongly correlated with NKG2DL expression (figure 17B). Interestingly, because CD80 was significantly lower expressed in the primary samples, we could not find an association between CD80 and NKG2DL. But nevertheless, as shown before, CD80 co-expresses with NKG2DL (figure 9B, 9G and 9J), meaning that all three immune checkpoint markers in our dataset behaved identically in human and mouse-derived samples. Overall, we observed that non-LSCs are defined by their expression of immune checkpoint markers. This is a novel, yet unreported, important observation.

As mentioned earlier, there were no two distinct positive populations for CLL-1 in the patient-derived cohort and its expression is in part negatively correlated with LSC markers such as CD117 and GPR56 challenging previous reports indicating

CLL-1 as an LSC marker (figure 6A-C and 6E-G), but confirming CLL-1 as being expressed on more committed cells (figure 6J and 6L) (Daga et al. 2019). In contrast to our post-transplantation samples, we could rely on CD34 in this cohort, which, to no surprise, correlated with the LSC markers (CD117, CD200 and negatively with NKG2DL+). Overall, the observations we made in the mouse-derived cohort showed similar trends where LSC and non-LSC markers would correlate with each other, respectively GPR56, CD117 and CD34 for LSC association and CD80, CD86 and CD276 for non-LSC (figure 17B).

In contrast to our correlation from the engrafted samples, MRC2 showed opposite trends. After the transplantation, the associations suggested a non-LSC phenotype (figure 17A), which is unexpected because the primary patient samples display a positive correlation with the LSC marker CD117. This led us to the conclusion that some markers are more prone to plasticity than others and that some markers may switch from LSC markers to markers that can no longer define the LSC population during propagation in mice. We compared the expression of all the markers pre- and post-transplantation, and we noted that some are prone to change (e.g., CD200, CD49f, CD56 and VLA4) and some not. This was especially interesting for CD80, which is barely expressed in primary patient samples, but instead was specifically induced in leukemic cells growing in NSG mice.

Taken together, our approach robustly demonstrated that some of our observations and correlations made in NSG mice are reproducible in human samples.

4. Discussion

We and others have used xenotransplantation models for the purpose of understanding and studying the biology of AML, more specifically the NSG mouse strain that was used for the mouse-derived samples of this work. One important disadvantage of this approach is the complexity of predicting engraftment and the generally rather low engraftment rate and long latency. By using a larger cohort of samples and extending the incubation period to up to 52 weeks, we were able to counteract these two factors and produce reliable results (Paczulla et al. 2017). The NSG mice are heavily immunocompromised, making the model unsuitable to depict interactions of leukemic cells with the immune system (Abarrategi et al. 2018; Almosailleakh und Schwaller 2019). Syngeneic models would have been the preferred option if studying the interaction with the immune system was the aim of the study. In this work, with the focus on the heterogeneity of AML, we preferred the NSG model depicts the properties of the primary tumor of a patient (Okada et al. 2019). To further study the interaction of tumor cells and the immune system as well as response to immunotherapy, humanized PDX are an option (Okada et al. 2019).

Previously, we have developed and optimized an *in vivo* xenotransplantation model to study human primary AML samples from different genetic backgrounds, which represents a powerful tool in AML research by extending the incubation period (Paczulla et al. 2017). This enabled us to further investigate associations with the AML molecular risk groups. We ruled out the common problem of the “favorable” risk group being considered unable to engraft (Sanchez et al. 2009; Rombouts et al. 2000) and thus we were also able to gain further insight in the specific expression of the markers in all risk groups.

In AML, LSCs are known to be a rare cell population with the ability to initiate the disease and enable relapse after apparent remission (Vetrie et al. 2020). We

detected LSCs within all three risk groups but noted a lower frequency in the “favorable” group which is in line with former published results (Ailles et al. 1999; Rombouts et al. 2000). In the literature, it is clearly stated that CD34 is a reliable tool to distinguish between LSCs and non-LSCs (Bonnet und Dick 1997; Zeijlemaker et al. 2019), but some of our mouse-derived samples lost the expression of the marker during the mouse-expansion period. We hypothesize that only fitter LSCs may conserve CD34 which could be true as most of the samples still expressing CD34 are derived from the “adverse” risk group, but this remains to be functionally tested.

Furthermore, reports from our group showed that the absence of NKG2DL defines LSCs and favors immune evasion (Paczulla et al. 2019), underlining the importance of NKG2DL in AML. In this study we showed that NKG2DL expression negatively associated with other LSC markers like CD117 or GPR56, emphasizing the role of NKG2DL in the distinction between LSCs and non-LSCs. Hence, we used the absence of NKG2DL to distinguish LSCs from non-LSCs in our *in vivo* settings because its expression is robust and remains conserved in the PDX setting.

After staining the BM samples and acquiring the results, we proved that - for the mouse-derived samples - the majority of the LSC markers associated with absence of NKG2DL expression. For example, CD117, GPR56, CD200 and CLL-1 low are co-expressed with NKG2DL negative populations and CLL-1 high associates with NKG2DL positive populations, underlining the assumption that the two distinct positive populations of CLL-1 are independent predictors of a patient´s outcome (Wang et al. 2021). CD38 is co-expressed with NKG2DL consistent with previous studies reporting high levels of CD38 expression to associate with better outcome (Keyhani et al. 2000). Interestingly, we also found that the highest level of CD117 was expressed in the “adverse” risk patients, confirming the importance of CD117 as an LSC and thus poor prognosis marker (Tsao et al. 2004; Wells et al. 1996). We also enriched for LSCs by combining markers co-expression and their distribution among the risk groups, and we could show that the NKG2DL-/CD117+ phenotype was shown overrepresented in the “adverse” risk patients, in line with the assumed LSC properties associated with

these markers and with their previously reported prognostic influence. Regarding the other markers, we could not find significant associations with the risk groups, although the overall trend suggests that the LSC frequency tend to be expressed mostly in the “intermediate” or “adverse” group, indicating that the LSC markers frequency are directly associated with the risk groups. Of note, our adverse risk AML group was particularly small and contained only 10 patient samples.

Further investigations in larger cohorts are required to further validate these results and especially to reliably compare intermediate with adverse risk AML.

Given the reported negative prognostic influence of CD123 expression (Testa et al. 2002), we assumed that CD123 would co-express with the NKG2DL negative population, which however did not hold true in our cohorts. Consistent with with the literature, CD123 was not only present on the LSCs but also on blasts with a more committed phenotype (Testa et al. 2019).

Interestingly, the immune checkpoint markers CD80, CD86, CD276 as well as CD47 were shown to positively correlate with NKG2DL expression, suggesting that they are primarily expressed by non-LSC blasts in both mouse- and patient-derived samples. We observed that the four markers show highest expression in “favorable” (CD86, CD276) and “intermediate” (CD80, CD47) risk group AMLs. Furthermore, NKG2DL+/CD80+ and CD86+/CD276+ double positive cells were significantly overrepresented in “favorable” risk AMLs. This is particularly interesting, as we have found no former reports of such observations and thus this could be very interesting to investigate in further *in vivo* and *in vitro* settings to gain insight into the mechanisms involved in immune evasion and immune checkpoints.

Finally, Adhesion markers seemed to correlate with a more committed, non-LSC phenotype, e.g., CD56, VLA4, and CD49f. MRC2, on the other hand, did neither correlate with the presence, nor the absence of NKG2DL in the mouse-derived setting, suggesting ambiguous, perhaps plastic expression. Interestingly, these four markers are also represented highest in the “favorable” and the “intermediate” risk group, suggesting them as potential non-LSC markers as well.

Overall, the mouse-derived samples showed us impressively that the markers known for their LSC properties exhibit an LSC phenotype (e.g., CD117, CD200, GPR56) and that the immune checkpoint markers (e.g., CD80, CD86 and CD276) all possess an association with a more committed phenotype, meaning they display a non-LSC phenotype and could even define non-LSCs by their expression. The Adhesion markers surprisingly also mostly correlated with a non-LSC phenotype after transplantation (e.g., CD49f, VLA4, CD56), although MRC2 seems to exhibit some stemness characteristics as well.

To further validate our results, we also analyzed matching patient derived samples and an additional patient cohort. Interestingly, when we compared the markers pre- and post-transplantation, we found significant differences for some of the markers that will now be discussed in detail. We noticed the number of NKG2DL positive cells rose significantly during the transplantation process which could indicate differentiation induction in the PDX model. Alternatively, NKG2DL could be upregulated on blasts (Paczulla et al. 2019) given that there are no functionaö NKG2D receptor expressing T or NK cells in these mice, blasts can multiply without any external intervention. This could be a reason why we detected more NKG2DL positive cells in mouse-derived versus patient-derived samples. In contrast, we experienced lower levels of CD200 after the transplantation. CD200 is overexpressed on AML LSCs compared to the blasts and is therefore a valuable surrogate to distinguish between LSCs and non-LSCs (Herbrich et al. 2021), but after transplantation, the amount of CD200 decreases. Due to that, we could not use CD200 to distinguish between LSCs and non-LSCs in the post-transplantation cohort. It is possible that this effect relies on plasticity and the expression is lost after the transplantation, as for MRC2 as well. It can also indicate that non-LSCs (NKG2DL positive cells) survive better in mice because they are not immunologically cleared, and thus we have lower percentages of LSCs (and thus also of CD200 expression).

For CD117, CD123, GPR56 and CD38, no significant difference before and after transplantation could be observed, meaning that the markers stay consistent and thus are robustly expressed in both groups. The robust expression before and after transplantation tells us that the markers are unaffected by the influences of the expansion in mice, meaning this enables us to study the markers *in vivo* and that they are comparable. Interestingly, CD34 was downregulated in leukemic cells expanded in mice, with the highest amount of conserved CD34 expression detectable in AML cells of the “adverse” risk group. This led us to the suggestion, that the CD34+ blasts from the “adverse” patient samples in the NSG mice were fitter and could be rather serially transplanted as opposed to the CD34+ blasts from the “intermediate” and “favorable” patient samples which could not maintain their CD34 expression (La Díaz de Guardia et al. 2021). It is known that exogenous cytokines can increase the engraftment of CD34 cells, although inadequate, (Bonnet et al. 1999) and that some mouse strains are more capable at hosting CD34+ cells than others (McDermott et al. 2010), but generally, CD34+ seem to be rather reduced than increased after transplantation in PDX models (Wunderlich et al. 2018). It is also possible that CD34+ cells from “intermediate” or “favorable” risk patients exhibit a decreased ability to self-renew after transplantation, but it is more likely that the self-renewal ability remains untouched by the process (McDermott et al. 2010). Lastly, it cannot be ruled out that the effect of increased CD34+ cells for the “adverse” risk group could be due to specific niche interactions which promote leukemic growth (La Díaz de Guardia et al. 2021). Considering the relevance of these findings for experimental settings, this could be of particular interest to pursue in further *in vivo* or *in vitro* studies. Considering CLL-1, we could not compare the expression before and after transplantation as we did for the other markers because we did not observe two distinct populations in the matched primary patient samples. To clarify, we observed two separate positive populations in the mouse-derived cohort but only one positive population in the primary patient samples. Thus, we could not compare CLL-1 high/low cells before and after transplantation. Our observations in the mouse-derived samples indicate that the CLL-1 low

population is associated with an LSC phenotype, which can explain why CLL-1 low is associated with poor prognosis in other scenarios (Wang et al. 2021), whereas the association with the NKG2DL positive population in the patient group did favor another assumption. CLL-1 in primary samples inversely correlates with the LSC phenotype, indicating that here, we captured more differentiated cells (Daga et al. 2019). It seems possible that the CLL-1 low population exhibits LSC properties after transplantation, whereas CLL-1 high is more likely a non-LSC marker which is expressed on blasts in primary patient samples.

For the immune checkpoint markers, we also had no significant differences before and after transplantation for CD86 and CD276, meaning that the expression of the markers also is very robust throughout the transplantation process. On the other hand, CD80 had significantly lower levels in the pre-transplantation cohort and in primary samples in general, meaning that somehow during expansion in mice, the cells have gained the ability to express this marker. This could be the case because the immunocompromised mice enable the blasts to upregulate the immune checkpoint markers. But still, CD86 and CD276 showed no significant difference, so maybe CD80 is particularly induced via a separate, yet unknown mechanism. *Hicks et al.* demonstrated, that CD80 can e.g. be induced on blasts cultured with different cytokines (e.g., GM-CSF, IL-3, IL-6), but this seems rather unlikely in the chosen *in vivo* setting (Hicks et al. 2001) . Indeed, if it is of interest to study CD80 on primary AML cells, we could think first of having the primary samples transplanted in NSG mice, then collecting the cells prior for further testing. Such scheme works also for markers that would be gained during their incubation period in NSG animals.

For the Adhesion marker MRC2 and the immune checkpoint marker CD47, they seemed to be expressed robustly before and after transplantation, meaning that these markers did not change much during the engraftment process, enabling us to study them in detail in primary as well as in mouse propagated samples. In contrast, CD49f, VLA4 and CD56 changed very significantly in our setting. VLA4 and CD49f seem to be upregulated after transplantation, suggesting that the markers are able to evolve more in the NSG mice. The downregulation of CD56 in the post-transplantation setting was surprising because we had a very robust

expression of the marker in the primary patient cohort. It is already known that positivity for CD56 is associated with a higher probability of infiltration of extramedullary tissues, thus correlating the marker with an inferior prognosis (Deak et al. 2021). Certain cytokines may be needed for proper expression of CD56, which could be a reason why the marker was downregulated in cells collected from immunocompromised mice (Deak et al. 2021). On the other hand, the study states that CD56 can be associated with CD34 (Deak et al. 2021), which was also downregulated in the post-transplantation cohort, indicating potential the co-dependency between these markers.

Regarding the co-expression with NKG2DL, the results in the patient derived cohort met our expectations. Interestingly, we could not associate CD123, CD200 and GPR56 with an LSC phenotype, although by trend correlations of GPR56 and CD200 with the NKG2DL negative population were observed. CD200 and GPR56 were reported as LSC markers (Herbrich et al. 2021; Daria et al. 2016), a higher cohort size will be perhaps required to observe significant effects.

CD80, CD86, CD276 and CD47 associated with a non-LSC phenotype in the two primary patient cohorts, stating the importance of the expression of the immune checkpoint markers for the definition of non-LSCs. Because of the rather specific expression on non-LSCs, the three markers represent themselves as promising therapeutic targets (Lichtman et al. 2021; Shi et al. 2018; Hardwick et al. 2010). CD86, previously shown to identify AML cells of monocytic or dendritic lineage (Costello et al. 1998; Hirano et al. 1996) is of particular interest. Cancer cells are known to evade immune-mediated killing by expressing CTLA4, a co-inhibitory receptor of CD86. This axis results in T-cell anergy in the end and by blocking this axis, promising results *in vitro* have been accomplished (Korman et al. 2005) and it also correlates with long-term response in human trials against solid malignancies while however showing less response in leukemia (Tabata et al. 2021; Specenier 2016; Davids et al. 2016).

The Adhesion markers VLA4, CD49f, CD56 interestingly associated with a more committed phenotype in the patient derived cohort (Chao et al. 2019; Zaidi et al. 2016; Rashidi und Uy 2015; Yamakawa et al. 2012), while MRC2 co-expressed

with the NKG2DL negative population, but as explained before, this correlation was lost after transplantation, suggesting that MRC2 is prone to plasticity. MRC2 still associated with an LSC phenotype in patient derived samples, suggesting that the marker could be of use to distinguish between LSCs and non-LSCs (personal communication by Marlon Arnone, not shown).

The correlation matrices mentioned above held some interesting observations as well. The immune checkpoint markers as well as the Adhesion markers are not only associated with NKG2DL, but also with each other, suggesting a synergistic effect and maybe even codependency in the murine cohort. The LSC markers, on the other hand, correlate with the NKG2DL negative population and also with each other (e.g., CD117 with CLL-1 low and GPR56), indicating co-expression on the same cellular population. These phenotypes were also observed in the second correlation matrix consisting of the primary patient samples, although not displayed as clearly as in the first matrix because some correlations were lost during the transplantation. For example, CD200, as explained before, lost its phenotypic LSC signature, whereas CD80, which was barely associated with other markers in primary samples, turned out to be the opposite from the engrafted sample perspective. The two independent matrices complement each other and further confirm the identified correlations.

Overall, this study provides a better understanding of the phenotypic heterogeneity of human AML pointing out interesting correlations between surface markers that can generate hypotheses for further studies. Interestingly, immune checkpoint markers were found to define non-LSCs. There is still much to be investigated, but this study has produced reliable comparisons which can be of use for further studies in the future.

5. Abstract

Acute myeloid leukemia (AML) is a malignant neoplasia of the blood system showing highly heterogeneous molecular properties and a broad range of different surface markers. Murine experimental models report a hierarchical organization of AML with the capacity of leukemia initiation, maintenance and relapse being confined to rare subpopulations of so-called leukemia initiating-cells (LICs) or leukemic stem cells (LSCs).

Here we used patient-derived xenotransplantation (PDX) in immunodeficient NSG mice in order to in-depth characterize the phenotypic heterogeneity of primary human AML, and to especially gain a better understanding of the correlation between surface and molecular markers in this disease.

Additionally, we stained primary AML samples from patients to compare the potential phenotypic changes after propagation in NSG animals.

We observed that the highest frequency of LSCs, as determined by the CD34 positivity or the absence of NKG2D ligands, was found in the “adverse” molecular risk group and that LSC markers such as CD117, CLL-1 low, CD200 and GPR56 co-associated with NKG2DL negativity, whereas immune checkpoint markers like CD80, CD86 and CD276 were predominantly expressed on NKG2DL positive non-stem cell populations. The Adhesion markers also shared non-LSC characteristics. Interestingly, few markers were prone to change from pre- to post-transplantation.

In conclusion, our study shows that the NSG-derived PDX model reliably recapitulates outcomes observed in patients. The expansion of AML cells in mice allows us to study their phenotype over time and enables us to gain deeper understanding of the underlying mechanisms.

6. Zusammenfassung (Deutsch)

Akute myeloische Leukämie (AML) ist eine maligne Neoplasie des blutbildenden Systems und stellt sich als eine höchst heterogene Krankheit dar und, mit besonderem Augenmerk auf die molekularen Eigenschaften und einer breiten Vielfalt an Oberflächenmarkern. Experimente im Mausmodell zeigen eine hierarchische Organisation von AML, wobei die Fähigkeit, Leukämie zu initiieren, aufrecht zu erhalten und Rückfälle zu provozieren einer seltenen Subgruppe vorbehalten ist, den sogenannten Leukämie-initiierenden Zellen (LICs) oder auch Leukämische Stammzellen (LSCs).
In unserer Analyse nutzten wir patient-derived xenotransplantation (PDX) in immunosupprimierten NSG Modellen, um eine tiefergreifende Charakterisierung primärer humaner AML-Zellen zu erreichen. Dadurch wollen wir ein besseres Verständnis der Krankheit und der molekularen Risikogruppen etablieren. Zusätzlich analysierten wir eine Kohorte an Primärproben von AML Patienten um den Phänotyp in Patientenzellen mit dem nach Propagierung dieser Zellen in den NSG Mäusen zu vergleichen.
Wir beobachteten, dass die höchste Anzahl an leukämischen Stammzellen (LSCs), welche entweder durch CD34 Positivität oder die Abwesenheit von NKG2D Liganden (es wurde gezeigt, dass sie die Immunevasion von LSCs identifizieren können) definiert wurden, in der “ungünstigen” molekularen Risikogruppe aufzufinden war und dass LSC-Marker wie CD117, CLL-1 niedrig, CD200 und GPR56 die phänotypischen Merkmale von LSCs teilen, wohingegen Immun Checkpoint-Marker wie CD80, CD86 und CD276 mit einem Nicht-LSC Phänotyp assoziiert sind. Die Adhäsions-Marker sind überraschenderweise ebenfalls auf Nicht-LSC erhöht. Interessanterweise wurden diese phänotypischen Merkmale über den Transplantationsprozess zumeist konserviert, sodass kaum phänotypische Unterschiede resultierten.
Zusammenfassend zeigt unsere Studie, dass das NSG-abgeleitete PDX Modell ein verlässliches Instrument ist. Die Expansion der AML-Zellen im Mausmodell erlaubt es uns, ihren Phänotyp über einen weiterreichenden Zeitraum zu

erforschen und machte es darüber hinaus möglich, ein tieferes Verständnis der zugrundeliegenden Mechanismen zu erlangen.

7. Literature

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Website, consulted 29.09.2022 Syngeneic Mouse Models | Charles River (criver.com)

8. Erklärung zum Eigenanteil

Die Arbeit wurde in dem Universitätklinikum Tübingen, Abteilung Innere Medizin 2, unter der Betreuung von Prof. Dr. Claudia Lengerke durchgeführt.

Die Konzeption der Studie erfolgte durch Dr. Anna Stanger, in Zusammenarbeit mit Marlon Arnone.

Sämtliche Versuche wurden, nach Einarbeitung durch das Labormitglied Marlon Arnone, von mir eigenständig und teilweise in Zusammenarbeit mit Marlon Arnone durchgeführt.

Die statistische Auswertung erfolgte eigenständig durch mich nach Beratung durch das Institut für Biometrie.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 06.11.2023

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