Immunorecognition of Leukemic Stem Cells by NK cells: The role of HDAC Inhibitors in NKG2D ligand-mediated anti-tumor Responses in Acute Myeloid Leukemia.

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Abbreviations

ABC	ATP-binding cassette
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
CD	Cluster of differentiation
CFU	Colony forming unit
CR	Complete remission
DC	Dendritic cell
DLI	Donor lymphocyte infusion
ELISA	Enzyme-linked immunosorbent assay
FAB	French american british
FACS	Fluorescence activated cell scan
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GvHD	Graft-versus-Host disease
GvL	Graft-versus-Leukemia
HAT	Histone acteyltransferase
HDAC	Histone deacetylase
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
i.f.	intrafemural
IFN	Interferon
IL	Interleukin
i.p.	intraperitoneal
ITAM	Immunoreceptor tyrosine based activatory motif
ITD	Internal tandem duplication
i.v.	intravenous
KIR	Killercell immunoglobulin like receptor
LSC	Leukemic stem cells
MDS	Myelodysplastic syndrome
MIC	MHC class I chain related molecule
mRNA	messenger RNA
NCR	Natural cytotoxicity receptor
NK	Natural Killer cell
NOD/SCID	Nonobese/Severe combined immunodeficiency
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
SCF	Stem cell factor
SL-IC	SCID leukemia initiating cells
TLR	Toll like receptors
TPO	Thrombopoietin
TRM	Treatment related mortality
ULBP	UL-16 binding protein
VA	Valproic acid
WHO	World health organization

I Summary

The diagnosis of acute myeloid leukemia (AML) is associated to a poor long-term outcome due to frequent relapse despite intensive chemotherapy, radiation and hematopoietic stem cell transplantation (HSCT) as well as continuous advances in treatment modalities. Relapses might be caused by leukemic stem cells (LSC). According to a recently emerging concept, LSC display many features of normal hematopoietic stem cells (HSC) like quiescence and self renewal capacity and therefore are poorly accessible for conventional therapies which primarily reach the rapidly proliferating cells. Additionally, LSC are apparently able to escape from immunorecognition and thereby sustain the disease. NK cells, as the main innate immune effectors against tumor cells, are able to recognize and kill malignant cells when triggered by cell surface expression of a multitude of activating ligands. The best-described receptor-ligand pair in humans is NKG2D and its ligands, ULBP and MICA/B. Furthermore, NCR is an important family of activating receptors on NK cells, whose ligands are not yet known. The regulation of NK cells is completed by several inhibitory receptors (KIR) specific for different HLA class I molecules on potential target cells. While preceding work in our lab was describing the interaction between NK cells and leukemic blasts of AML, there is no information available on the recognition of LSC by NK cells.

In this study we aimed to elucidate the interaction of NK cells with LSC of AML. The cell surface expression of ligands for activating and inhibitory NK cell receptors on LSC was in focus of these studies. Moreover, we applied a pharmacological approach to treat the patient-derived primary AML leukemic cells and examined the consequences for cell surface expression of NK cell-specific ligands. By employing hematopoietic colony forming assays, cytotoxicity assays as well as *in vivo* NOD/SCID xenotransplantation we aimed to functionally assess the implications of the upregulation of activating ligands for NK cell immunorecognition of LSC.

In initial experiments, we demonstrated that activating ligands for the NKG2D receptor and NCR receptors on NK cells are absent or only weakly expressed on the surface of patient derived AML blasts. This expression could be increased by pharmacological means applying bryostatin-1, a modulator of PKC activity. Upregulation of cell surface expression of NKG2D ligands on AML blasts led to

increased immunorecognition by NK cells in cytotoxicity assays. Subsequently, we demonstrated that similarly to total blasts, LSC of AML as judged by the phenotype $CD45^{dim}CD34^{+}CD38^{-}$, did not express ULBP and MICA/B on their surface. To pharmacologically increase their expression, we employed the HDAC inhibitor valproic acid (VA), a drug acting through epigenetic modification of gene expression and having long-term records in different clinical applications. This treatment with VA proved to be of importance for the immunorecognition by NK cells. In the functional assays we employed NK cells selected for the KIR-HLA class I mismatch in order to circumvent inhibitory signals inactivating the NK cells. Serial replating colony forming unit (CFU) assays with LSC after treatment with VA and after coincubation with KIR-HLA mismatched NK cells demonstrated an efficient reduction in colony formation capacity upon this synergistic treatment. The cytotoxicity assays with VA-treated LSC as targets and KIR-HLA mismatched NK cells as effectors revealed interindividual differences among patient samples, reflecting a complex regulation of NK cell activation and immunorecognition. Altogether, a direct interaction of NK cells and LSC could be demonstrated *in vitro*.

In the *in vivo* setting, by transplantation of AML cells intrafemurally into NOD/SCID mice with consecutive treatment of VA and HLA-mismatched NK cells, we were able to achieve a stable engraftment of human AML in the mouse bone marrow. However, the combined treatment with VA and NK cells was not influencing the content of malignant cells as compared to untreated mice. The ongoing studies aim at optimization of AML treatment with NK cell-based immunotherapy in the preclinical NOD/SCID transplantation model.

Taken together, these results showed the potential of VA as an applicable anti-neoplastic drug to enhance immunorecognition of LSC of AML by NK cells, mediated by increased cell surface expression of activating ligands. The functional consequences of an enhanced immunorecognition by NK cells in abolishing the colony forming capacity of patient derived LSC are promising beneficial effects for innovative AML treatments in future.

II Introduction

1 : Acute myeloid Leukemia (AML)

1.1 Disease

Acute myeloid leukemia (AML) is a severe malignant disease of the hematopoietic system. The loss of the ability to differentiate and proliferate normally leads to a clonal disorder of hematopoietic progenitor cells. With a prevalence of 3.8 cases per 100.000 it is the most common acute leukemia, and the prevalence rises to 17.9 cases per 100.000 in adults aged 65 years and older[1]. The overall 5-year general survival rate is 21.2 % and the frequency is 12.000 newly diagnosed patients per year in the USA, demonstrating together with a bad prognosis even after intensive therapy with radiation and chemotherapy in combination with hematopoietic stem cell transplantation the need for additional research.

1.2 Risk factors, pathogenesis

Data from the National Cancer Institute indicates, that the male gender is a risk factor, with three men affected for every two women. Further risk factors proven are exposure to chemical compounds which influence the DNA stability. Benzene is suspected to cause AML, characterized by particular chromosomal aberrations (trisomy of chomosome 8[2], translocations between chromosome 8 and 21[3]) and frequently belonging to the AML subtype (FAB) M2[4]. The most common source of benzene exposure is cigarette smoking. Consequently smoking increases the risk to develop AML 1.2 to 2.3 times[4].

Iatrogenically induced AML can occur after cytotoxic chemotherapy, mainly used to treat solid tumors. Alkylating agents can cause AML 5-10 years after exposure. These therapy-induced AML are characterized by deletions or monosomies of chromosome 5 and/or 7[5]. After the therapeutic use of inhibitors of topoisomerase, like doxorubicine or ethopside, AML can be detected mainly with abnormalities involving the long arm of chromosome 11 and translocations between chromosome 15 and 17, t(15;17), as well as between chromosome 8 and 21, t(8;21)[5].

Similar to chemical agents influencing stability of DNA, ionizing radiation is also able to induce leukemia. The main sources of radiation demonstrated are that of atomic bombs in Japan[6] and that of nuclear tests in the USA as well as radioactive fallout after failures in nuclear power plants[7]. Interestingly, excessive flying (more than 5000h) was shown to increase the risk of AML 5.1 times, supposedly due to cosmic radiation[8].

The fact that agents or conditions influencing the stability of DNA are carcinogenic has implications for understanding the pathogenesis of leukemia. This disease is caused by cytogenetic lesions which also determine the therapeutic response. Often the underlying structural changes in DNA are associated with distinct AML subtypes und have major influence on outcome and therapy. The most common targets of translocations in AML are genes coding for DNA-binding transcription factors and components of regulatory transcription complexes[9]. The translocation results in the generation of fusion proteins which are interfering with the functional wild type proteins. The most prominent and frequent example is AML1-ETO, caused by a translocation t(8;21). This fusion is found in approximately 40 percent of all AML FAB M2 without being restricted to this subtype[10].

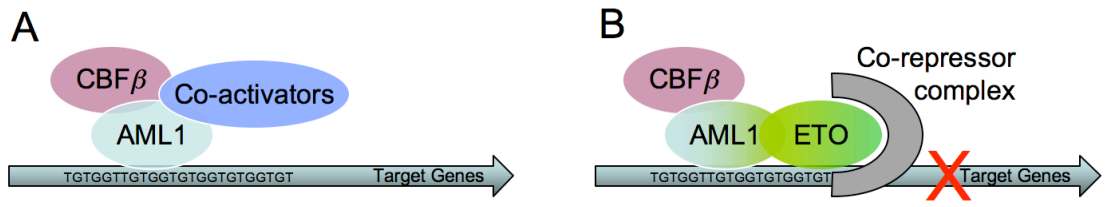

Figure II.1: AML1-ETO fusion protein and its effect on transcription, modified from [10]
(A) The transcription factor AML1 is forming a complex with CBFβ and other co-activators to activate gene expression. (B) The fusion protein AML1-ETO is recruiting a co-repressor complex to the core enhancer sequence inhibiting the expression of genes essential for normal development.

AML1 is, as well as ETO, a transcription factor, which in normal cells forms a heterodimeric transcription-factor complex together with CBFβ. After recruitment

of further co-activators it activates gene expression by binding to the TGTGGT core enhancer sequence in the transcriptional regulatory regions of AML1-regulated target genes (see figure **II.1 A**).

As a result of the fusion of the N-terminal part of AML1 with the C-terminal part of ETO in t(8:21), AML1 is still able to mediate the formation of an activating complex with CBFβ, but the fusion partner ETO is recruiting a nuclear co-repressor complex (see figure **II.1 B**). This is leading to a dominant repression of transcription of AML1 regulated genes. Since these are hematopoiesis-specific genes essential for normal development of the hematopoietic system, the fusion leads to a block in differentiation.

Another example for translocations, providing explanations as to the pathophysiology of disease and its treatment, is t(15;17). The resulting chimeric protein PML-RARα is the target of all-*trans*-retinoic-acid [11]. A list with the most commonly found genetic modifications and their associations to AML subtypes is given in table **II.1**.

Table II.1: Genetic modifications in AML

	Common aberration	Genes	Morphological association	Incidence
Translocations / Inversions	t(8;21)(q22;q22)	RUNX1; RUNX1T1	M2	6%
	inv(16)(p32q22) or t(16;16)(q13;q22)	CBFB; MYH11	M4Eo	7%
	t(15;17)(q22;q11-21)	PML; RARA	M3	7%
	t(9;11)(q22;q23)	MLL; AF9	M5	2%
	t(6;11)(q27;q23)	MLL, AF6	M4, M5	~1%
	inv(3)(q21q26) or t(3;3)(q21;q26)	EVI1; RPN1	M1, M4, M6	~1%
	t(6;9)(q23;q34)	DEK, NUP214	M2, M4	~1%
Chromosomal imbalances	+8		M2, M4, M5	9%
	-7/7q-		no preference	7%
	-5/5q-		no preference	7%
	-17/17q-	TP53	no preference	5%
	-20/20q-		no preference	3%
	9q-		no preference	3%
	+22		M4, M4Eo	3%
	+21		no preference	2%
	+13		M0, M1	2%
	+11	MLL1	M1, M2	2%
	complex karyotype			10%
	normal karyotype			40%

A frequently found mutation in patients with AML is the internal tandem duplication of FLT3 (FLT ITDs)[12]. FLT3 is a tyrosine kinase receptor III which is together with its ligand FL an important signaling molecule for normal hematopoiesis and immune development. Disruption of a repressor sequence in the receptor by internal tandem duplications, mostly in exons 14 and 15, or by mutations in the juxtamembrane domain is leading to a constitutive activation and thereby to development of AML[13]. With 30 – 35 % of AML patients carrying a mutation in the gene for FLT3, it is the most frequent genetic abnormality in AML conferring a poor prognosis especially in patients aged 60 and older and is representing a promising target for pharmaceutical intervention[14].

A model for the genetic events necessary for leukemogenic transformation of hematopoietic progenitor cells was introduced by Gilliland et al. They postulate two types of genetic damage. The first type (class I mutations) results in constitutive activation of oncogenes like RAS or cell surface tyrosine kinase receptors like FLT3 and c-KIT[15,16]. These mutations cause a survival or proliferative advantage of the affected hematopoietic cell, leading to a clonal expansion. The second type of lesions (class II mutations) lead to a block in myeloid differentiation and are caused by mutations and overexpression of HOX genes or formation of fusion genes like t(8;21) or inv(16). According to a two-hit-model, class I mutations or class II mutations alone are not able to cause leukemia in mouse models[16].

However, this two-hit-model describing structural aberrations of DNA does not integrate the influence of epigenetic modifications of DNA, like hypermethylation and other ways of gene silencing.

In addition, individual predisposition of patients to develop leukemia might be determined by the genetic variation of enzymes employed in detoxification of carcinogens. An example is NAD(P)H quinone oxyreductase 1 (NQO1) known to be involved in detoxification of benzene[17]. About 20 % of europeans and white americans are heterozygote for a variant form of NQO1 with a decreased protein activity due to a single amino acid change. This decrease was demonstrated to be associated with an increased risk to develop acute leukemia after benzene exposure[18]. Other factors influencing the detoxification and thereby being associated to an increased risk to develop leukemia are members of the cytochrome P450 family[19].

1.3 Pathophysiology, diagnosis, classification

The clinical features of AML are mainly linked to bone marrow failure due to infiltration of the bone marrow by leukemic blasts and repression of normal hematopoiesis. This leads to an ineffective generation of mature erythrocytes, monocytes, neutrophils and platelets. The inhibition is not only caused by pure steric replacement of healthy bone marrow progenitors but also by soluble factors like cytokines secreted by malignant cells[20]. The most important sign of bone marrow failure is infection, mainly by endogenous aerobic gram-positive and gram-negative bacteria and fungi like Candida and Aspergillus[21]. A lack of red blood cells leads to anemia and fatigue, thrombocytopenia to bleedings. A massive load of leukemic cells in the peripheral blood can cause hemostasis, which results in bleeding and bruising.

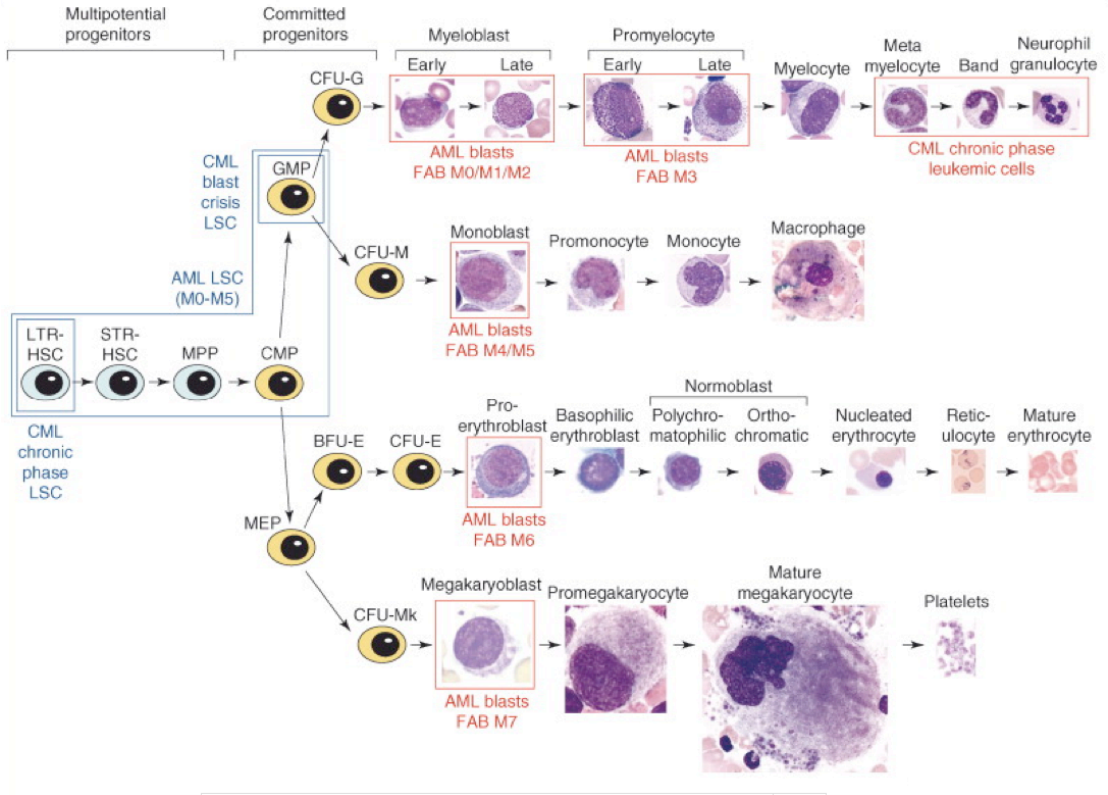

Figure II.2: Normal myeloid hematopoietic development and relationship to AML, from[22].

Some subtypes of AML can be linked to distinct clinical presentation. Infiltration of the gingiva, skin, meninges and soft tissue can be characteristic for the monocytic subtypes (AML M4/5). Patients with a translocation t(8;21) have a tendency to develop *chloroma*, a granulocytic mass of leukemic cells in soft tissues, breast, uterus, ovary, cranial or spinal dura, gastrointestinal tract, lung, mediastinum, prostate, bone and other organs.

Characteristic for AML is the accumulation of blasts resulting from a block in differentiation in different stages of development (see figure **II.2**). A classification based on cytochemistry and cytomorphology is given by the French American British (FAB) system[23] and defines AML subtypes M0 to M7. Following the FAB system, a diagnosis is confirmed when the marrow contains more than 30 % blasts. The more recent classification by the WHO is additionally based on cytogenetic characteristics. The minimal blast content in bone marrow is 20 %, the infiltrating blasts must be shown to be of myelocytic origin by expression of CD33 or CD13 on at least 20 % of the blasts[24]. A table with the FAB classification is shown in table **II.2**.

1.4 Therapy: cytotoxic agents, stem cell transplantation, GvL effect

Generally, treatment of AML is consisting of induction therapy, aiming at inducing a complete remission (CR), and postremission therapy with the goal of sustaining the remission and preventing relapse. CR is defined as absence of detectable dysplasia or extramedullary leukemia and the reduction of blasts in the bone marrow to less than 5 % as well as presence of regenerated blood lineages with an increase in hemoglobin (>11 mg/dl without EPO-therapy), peripheral neutrophils (>1.5 * 10^9/l) and platelets (>100 * 10^9/l). This is achieved in young patients (<60 years) by cytotoxic therapy with a combination of anthracycline and cytarabine. The most commonly applied protocol is 45-60 ml/m^2 of anthracycline for 3 days and 100 mg/m^2 of cytarabine over 7 days, resulting in complete remission rates of 65 % - 75 % in patients aged 18-60 years[25]. Adjustments of therapy are necessary for distinct cytogenetic abnormalities, existence of mutations or different leukemic subtypes. Elderly patients have a worse response to induction therapy and therefore a less favorable outcome. Different protocols are under continuous evaluation, but in general dose escalation to

increase the rate of CR is followed by increased toxicity and treatment related mortality (TRM).

Table II.2: Classification of AML

Subtype	FAB Type	Frequency	Morphology	MP	SE	NSE	Immune Markers	Cytogenetic abnormalities
Acute myeloblastic leukemia (AML) with minimal differentiation	M0	2 %	No azurophilic granules	-	-	-	MY7 (CD13) MY9 (CD33)	-
AML without maturation	M1	19 %	Few azurophil granules or Auer rods	+/-	+/-	-	MY7 (CD13) MY9 (CD33) HLA-DR	del(5), del(7), +8
AML with maturation	M2	24 %	Some maturation beyond promyelocytes, Auer rods	++	++	-	MY7 (CD13) MY9 (CD33) HLA-DR MY10 (CD34)	t(8;21), t(6;9)
Acute promyelocytic leukemia (APL)	M3	10 %	Hypergranular promyelocytes, Auer rods	+++	+++	-	MY7 (CD13) MY9 (CD33)	t(15;17)
Acute myelomonocytic leukemia (AMML)	M4	30 %	≥ 20 % monocytes, monocytoid cells in blood	++	++	++	MY4 (CD14) MY7 (CD13) MY9 (CD33) MO1 (CD11b)	inv(16), del(16), t(16;16), t(4;11)
Acute monocytic leukemia	M5	10 %	Monoblastic (M5A), Promonocytic (M5B)	-/+	-	+++	MY4 (CD14) HLA-DR MO1 (CD11b)	t(9;11), t(10;11)
Acute erythroleukemia	M6	4 %	Predominance of erythroblasts, dyserythropoiesis	-	-	-	Glycophorin A MY9 (CD33)	-
Acute megakaryocytic leukemia	M7	1 %	Punctio sicca,biopsy with blasts and dysplastia	-	-	-	-gpIIb/IIIa (CD41a) HLA-DR MY9 (CD33) MY10 (CD34)	-

Abbreviations: MP, myeloperoxidase; SE, specific esterase (chloracetate); NSE, nonspecific esterase (naphtylbutyrate). Frequency in adults.

Different strategies for postremission therapy are existing and are evaluated depending on the kind of AML, the age of the patient, response to the induction treatment and, if hematopoietic stem cell transplantation is considered, the existence of a stem cell donor. A standard postremission therapy is 3-4 courses of high dose cytarabine (cumulative dose 54-72 g/m^2) and results in survival rates of 60-75 %[26].

However, the overall rate of postremission disease-free survival in AML remains poor, usually 50 % at 5 years.

Hematopoietic stem cell transplantation (HSCT) is a promising tool to sustain CR and reduce relapse in selected patients and represents the most efficient antileukemic treatment[27]. Elderly patients rarely profit from HSCT mainly due to high TRM caused by the conditioning regimen.

HSCT can either be autologous (re-implantation of the patients own stem cells) or allogeneic (the use of stem cells from a human leukocyte antigen (HLA) –matched related or unrelated donor). A third source recently used with increasing frequency is umbilical-cord blood stem cells. Whereas autologous stem cell transplantations simply replace hematopoiesis after intensive chemotherapeutic treatment, allogeneic stem cells are able to induce an immunological response against tumor cells, termed graft-versus-leukemia effect (GvL) and are therefore the preferred treatment in leukemia. This beneficial effect is accompanied by the risk of graft-versus-host disease (GvHD), a serious side effect of HSCT. To prevent GvHD the donor of stem cells is matched with respect to the recipient`s HLA-loci HLA-A, HLA-B, HLA-DR. A completely matched donor is considered to be ideal- but bears the risk of missing GvL effect. Mismatches between donor and recipient come along with an increased risk of GvHD, but show a more favorable outcome in terms of lower rates of relapse. Advances in understanding the mechanisms of GvL and GvHD are allowing a calculated mismatch with beneficial effects on the outcome for the patient.

1.5 Cancer stem cells, leukemic stem cells

A recent concept in cancer biology aims at explaining the frequent relapse of malignant diseases and other underlying phenomena in cancer. It could be proven for several tumor entities that tumor cells are not an uniform bulk of malignant cells but – in analogy to the hematopoietic system – are hierarchically organized. Following this theory, cancer is consisting of more developed, short lived cells, which are replenished by cancer stem cells, thus mimicking any tissue repair. The first cancers shown to consist of stem cells and differentiated bulk tumor cells are AML[28]

(leukemic stem cells, LSC), breast cancer[29] and brain tumor[30]. Recently stem cells of colon cancer[31] and pancreatic cancer [32] could be isolated.

In AML it could be shown that only a minor population among the bulk of leukemic cells is able to cause leukemia when transplanted in NOD/SCID mouse models (termed SCID leukemia initiating cells SL-IC)[33] and is able to form colonies in semisolid culturing systems (methylcellulose)[34]. The phenotype of these LSC is a subject of ongoing research, reflecting the fact that single LSC-specific markers are not yet known. Candidate markers are CD33[35], CD123 (IL-3 receptor chain α)[36] and others, but the most reliable and best described markers are CD34 and CD38. In normal bone marrow and cord blood, expression of CD34 and absence of expression of CD38 defines the earliest hematopoietic stem cell. In analogy it could be shown that LSC are found in the $CD34^+CD38^-$ fraction of leukemic blasts[37]. Intracellularly the phosphatase and inhibitor of proliferation PTEN (phosphatase and tensin homologue) has been postulated to distinguish normal and leukemic stem cells[38] [39].

The importance of human LSC could be demonstrated in transplantation experiments, where different amount of cells with different maturation status were transplanted in a xenograft model into immunocompromised mice, which then were monitored for the development of human leukemia. Hereby it could be shown that the $CD34^+CD38^-$ population of leukemic cells have the highest capacity of leukemia initiation. This fraction represents from 0.1 % to 1 % of the whole AML population[40]. Shortcomings of this phenotypical description is the existence of AML patients, whose blasts are not expressing CD34 at all. There the LSC must be defined by different cell surface markers[41].

By virtue of their stem cell characteristics like quiescence and self-renewing capacity LSC are believed to be more resistant against cytotoxic treatment. Furthermore LSC are due to an increased expression of the ATP-binding cassette (ABC) family of drug transporters less accessible for chemotherapeutic agents[42]. Chemotherapy and radiation induces a decrease in leukemic cell burden by mainly targeting mature AML cells, but LSC remain unaffected and ultimately relapse is observed (figure **II.3 A**). Thus LSC are believed to be responsible for relapse and therefore represent a target of novel therapeutic concepts (see figure **II.3 B**).

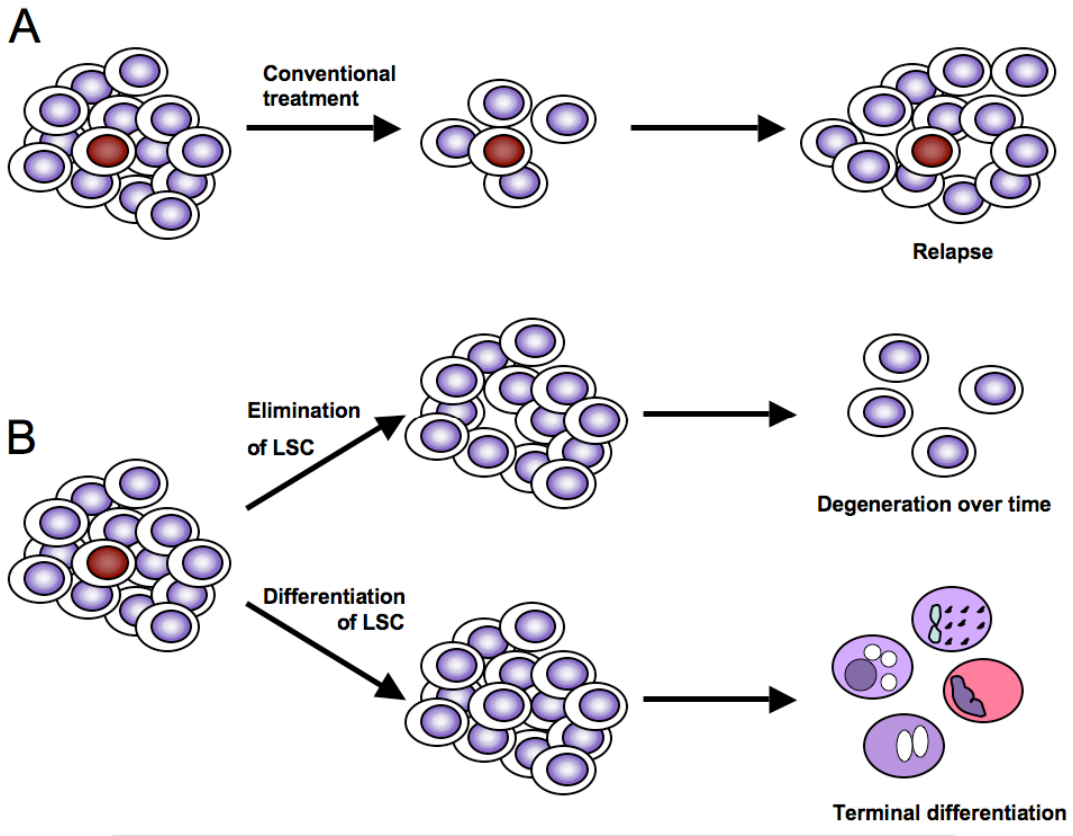

Figure II.3: Leukemic stem cells and their importance for the maintenance of AML. Modified from[43]. (A) Total blasts of AML are responding to cytoreductive therapy by apoptosis and necrosis, leading to a massive reduction in leukemic blast burden. LSC remain intact and eventually cause relapse. (B) Treating LSC by direct elimination or differentiation is a promising method to effectively treat leukemia.

2 : Natural killer (NK) cells

2.1 Introduction

NK cells are, due to their ability to lyse target cells and secrete immunoregulatory cytokines, essential components of the innate immune system, comprising about 10-20 % of all circulating blood lymphocytes[44]. Initially they were discovered by their ability to reject allogeneic bone marrow in lethally irradiated mice ("hybrid resistance")[45,46], later they could be shown to mediate tumor cytotoxicity in an HLA-unrestricted manner[47]. In contrast to T cells of the adaptive immune system, NK cells can readily display effector functions upon encountering infected or transformed cells, thus were labeled as "naturally active"[48].

2.2 Functional and phenotypic features, development

Mature NK cells are characterized phenotypically by expression of CD56 and absence of CD3. Furthermore, resting NK cells can be subclassified into two subsets, one of which showing a low expression of CD56, but a high expression of CD16 ($CD56^{dim}CD16^{bright}$ NK) and comprising about 90 % of total NK cells, the remaining 10 % are of the $CD56^{bright}CD16^{dim}$ phenotype[49]. The first subset shows cytotoxic activity, the latter is active in secreting immunomodulatory cytokines.

As all leukocytes, NK cells are derived from $CD34^+$ hematopoietic stem cells undergoing maturation in the bone marrow. This maturation can be separated into two phases. Initially, the primary stimulus for maturation of NK cell progenitors ($CD34^+$ Lin^-) is induced by early acting cytokines like Flt-3 ligand (FL) and c-kit ligand (SCF) and leads to the expression of the IL-15-receptor ($CD34^+$ $IL\text{-}15R^+$). Subsequently IL-15 promotes the further development of mature NK cells[50]. After maturation NK cells can be found in bone marrow, peripheral blood, lymph nodes and spleen[51]. There they protect the host against infectious and malignant threats in a direct way by secretion of cytokines and target cell lysis via granzymes and FAS ligand as well as indirectly by interaction with local dendritic cells. After activation and direct interaction of NK cell and target cell - or physiologically more important after stimulation of NK cells by dendritic cells - they can exert their immunologic functions. Toll like receptors (TLR) and their pathogen-associated ligands seem to be crucial for NK cell activation by infectious pathogens, either through direct stimulation of NK cells[52] or by stimulation of dendritic cells with consecutive NK cell stimulation[53-55]. After activation NK cells play an important role in linking the innate with the adaptive immune system[56].

2.3 Regulation of NK cells: receptors and signaling

In contrast to T- and B cells NK cells do not require gene rearrangement for antigen recognition. They express their own repertoire of receptors that regulate the activation of NK cells by balance of activating and inhibitory signals[57]. In general, receptors can be acting as either activating or inhibitory receptors (see table **II.3**). Based on their molecular structure, receptors on NK cells can be classified as

killer immunoglobulin-like receptors (KIR), C-type-lectin receptors or others. They can depend either on HLA class I as a ligand or be HLA class I independent. The net signal generated by activating and inhibitory receptors is determinating whether the target cell – NK cell interaction is resulting in lysis or not[58].

2.4 Inhibitory KIR receptors on NK cells

Inhibitory receptors mainly engage HLA class I molecules on the surface of their target cells as their ligands and initiate inhibitory signals. The main group of receptors exerting inhibitory signals in NK cells are receptors belonging to the KIR group. This is a highly polygenic and polymorphic family of receptors. There are 16 different KIR genes known, located on chromosome 19q13.4[59], but not all of them have to be present in every individual. Individuals vary in terms of the number of KIR genes that they have between 6 and 16[60]. Beside of the polygeneity KIR genes are polymorphic and are clonally expressed with variation of the frequency of expression in different individuals. For example, one KIR receptor might be present on 50 % of NK cells of one person and on 5 % of NK cells of the other[61]. The regulation of expression of KIR in NK cells is complex. Presence of HLA class I expressing cells in proximity to the developing NK cell in the bone marrow as well as epigenetic modifications of KIR promoters might play important roles[62]. Finally this is leading to a clonal expression of different repertoires of NK cells in one individuum. These are differently inhibited by the HLA class I molecules expressed on all nucleated cells depending on their individual receptor repertoire[63,64].

Activating receptors include some types of KIR, C-type-lectins and natural cytotoxicity receptors (NCR), but other activating co-receptors have been described. They mainly initiate their stimulating signals by ITAM (Immunoreceptor tyrosine based activatory motif) in the intracellular part of the receptor. This signal is transmitted via several tyrosine kinases to phospholipase C which in the end leads via a Ca^{++} signal to activation of transcription factors like NF-AT and NK-κB.

Table II.3: Human Activating and Inhibitory NK Cell Receptors and their corresponding ligands

Type	Activating Receptor	Ligand	Inhibitory Receptor	Ligand
Killer immunoglobulin receptors	KIR2DS1	Group 2 HLA-C	KIR2DL1 (CD158a)	Group 2 HLA-C
	KIR2DS2	Group 1 HLA-C	KIR2DL2 (CD158b)	Group 1 HLA-C
	KIR2DL4	HLA-G	KIR2DL3 (CD158b)	Group 1 HLA-C
	KIR2DS4	Unknown	KIR3DL1	HLA-Bw4
	KIR2DS5	Unknown	KIR3DL2	HLA-A3, -A11
	KIR3DS1	Unknown	KIR3DL7	Unknown
C-type lectin receptors	CD94/NKG2C	HLA-E	CD94/NKG2A/B	HLA-E
	CD94/NKG2E/H	Unknown		
	NKG2D	MICA/B, ULBP 1-3		
Natural Cytotoxicity Receptors	NKp46	Unknown		
	NKp44			
	NKp30			

2.5 Activating NKG2D receptor and its ligands ULBP and MIC

One well described and probably the most important receptor-ligand-pair in the context of tumor recognition is NKG2D[65]. This is a 42 kDa type II lectin-like protein expressed on all NK cells, γδ T cells and the CD8 expressing subset of αβ T cells[66]. NKG2D is expressed constitutively and its levels can be upregulated on human NK cells by IL-15, IL-12 and IFN-α[67].

After binding its ligands, NKG2D is delivering the activating signal into the cell via phosphorylation of the adapter molecule DAP10[68]. Further signaling events are the recruitment of PI3K, ZAP70, SLP76, PLC-γ2 and Rac as well as Ca^{++}-release [69] (see figure **II.4 A**).

The ligands for human NKG2D are the HLA class I chain-related antigens MICA and MICB[70] and the UL16 binding proteins ULBP[71]. The expression of both is induced upon cellular stress. This includes stress after viral infection as well as malignant transformation. Due to the efficient lysis of ligand-expressing cells by NK cells, expression NKG2D ligands seem to be a strong and effective mechanism to control and eradicate transformed cells[65].

MICA and MICB are glycosylated proteins with 18-30 % of homology to HLA-A, -B and -C. They consist of α1, α2 and α3 regions and do not require β2-microglobulin or peptide binding for stability on the surface and protein folding (see figure **II.4 A**). Even though a low level of expression of transcripts for MIC can be found in many cell types, mainly virally and bacterially infected cells as well as stressed cells display the ligands at the surface[72]- demonstrating that surface expression is at least in part regulated on a post-transcriptional level.

Additional modification of surface expression of MIC is achieved by shedding. Some epithelial tumors were shown to shed MIC from their surface[73]. The resulting soluble form of MIC after shedding causes downregulation of NKG2D on NK cells by endocytosis. Consequently, low levels of both receptor and its ligands provide two ways of evasion of tumor recognition by NK cells.

ULBPs are – similar to MIC - members of the HLA class I family and act as ligands for NKG2D/DAP10. They are consisting of α1 and α2 subdomains, glycosylphosphatidylinositol (GPI)- linked (see figure **II.4 A**) and mapped on chromosome 6p21[71]. As with MIC the presence of mRNA transcripts does not correlate with surface expression. There are indications that expression is partly regulated in response to DNA damage[74].

Experimental blocking of both ULBP and MIC by antibodies preventing ligand - NKG2D receptor interaction as well as blocking NKG2D receptors on NK cells are strongly abrogating NK cell mediated lysis. This is indicating that NKG2D together with its ligands ULBP and MICA/B are important regulators of immunosurveillance.

2.6 Natural cytotoxicity receptors (NCR) on NK cells

Natural cytotoxicity receptors (NCR) are a family of activating receptors exclusively expressed on NK cells. Known members are NKp30, NKp44 and NKp46 (the number is indicating the molecular weight in kDa of the respective receptors), all of them belonging to the immunoglobulin superfamily with up to now unidentified ligands[75,76]. Whereas NKp30 and NKp46 are expressed on resting and activated NK cells, NKp44 can only be found to be expressed after IL-2 stimulation[77].

The expression of NCRs differs in the intensity among individuals. Some persons homogenously express NCR at high density (NCR^{bright}), others have subsets of NCR^{bright} and NCR^{dim} NK cells. Clones with different expression levels of NCR differ in their cytolytic activity[78].

NKp46 and NKp30 are associated with CD3ζ in their cytoplasmatic domain, p30 is additionally recruiting FcεRIγ[79]. NCRp44 in turn is using DAP12 as adapter molecule[77] (see figure **II.4 B**). NCR are major determinants of NK cell mediated immunoreactivity against tumors. This could be shown by the block of lysis of several types of tumor cells by experimentally blocking NCR[78]. Yet the cellular ligands of NCR could not be identified. Experimental data suggest that viral hemagglutinins serve as possible ligands[80], but a characterization and description of specific cellular ligands for the different NCR is still missing. Thus antibodies staining ligands for NCR on putative target cells for NK cells are not existing.

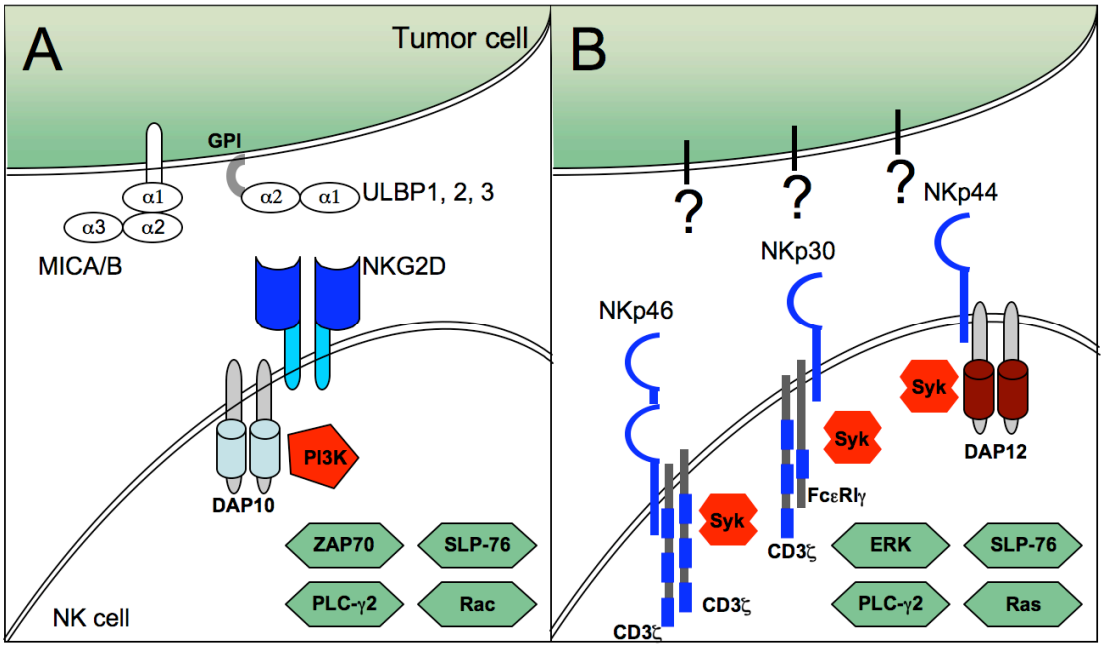

Figure II.4: Signaling in NK cells. (A) is showing NKG2D together with its ligands and intracellular signaling events, in (B) NCR and their downstream signaling is depicted.

Beside signals initiated by triggering activating receptors, a number of cytokines are able to stimulate NK cells via their corresponding receptors. Known activating cytokines are IFN-α, IL-2, IL-12, IL-15 and IL-18[81]. The costimulatory role of cytokines is presumably the induction of specific molecules in both NK cells as well as target cells to support cell adhesion and mediate cytolysis leading to a more active state of NK cells and a broader spectrum of their targets[51].

2.7 Effector functions, mechanisms of cytotoxicity

The complicated mechanisms of activation of NK cells are allowing the postulation of four scenarios in the interaction of NK cells and their prospective target cells. In absence of ligands for activating receptors engagement of only few inhibitory receptors by HLA-ligands is maintaining the NK cell in a resting state (figure **II.5 A**). Conversely few ligands for activating receptors in absence of stimulation of inhibitory receptors are sufficient to lead to the lysis of target cells (figure **II.5 B**). In cases where both stimulating and inhibitory ligands are expressed on target cells, the balance of the recruitment of both activating and inhibitory receptors defines whether the NK cell is activated or not (figure **II.5 C + D**)[57].

Once activated, NK cells exert their cytotoxic functions by secretion of lysosome-like vesicles containing perforin, serine esterases like granzyme and sulfated proteoglycanes. Perforin is capable of pore formation on the target cell, leading to a osmotic lysis[82]. Granzymes, which are protected by proteoglycans from protease inhibitors-mediated inactivation[83], are inducing apoptosis[84].

Recruitment of other cells by secretion of chemokines and cytokines like TNF-α and INF-γ is linking the innate and acquired immune systems.

Independently of chemokines and granzymes NK cells can induce apoptosis in their target cells by virtue of expression of FAS-ligand[85] and TRAIL[86]. FAS-ligand is intracellularly expressed in resting NK cells at significant levels, and becomes upregulated on the cell surface upon activation[87].

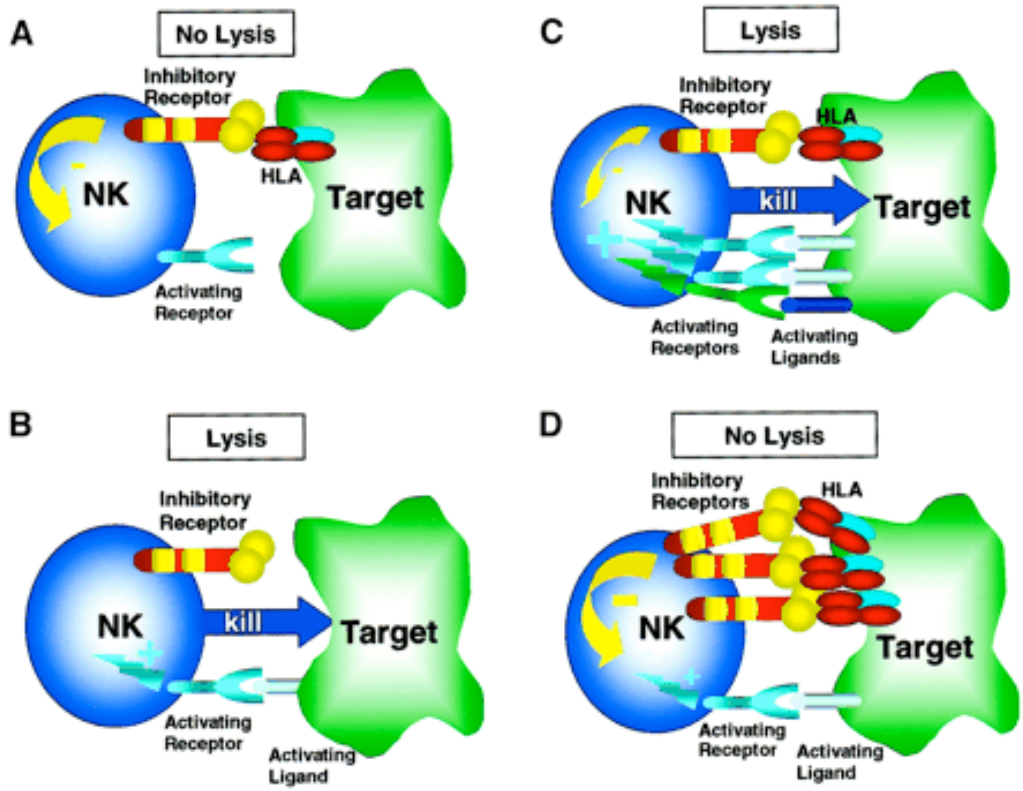

Figure II.5: Scenarios of activation of NK cells. NK cells become activated and lyse their target cell if only activating receptors (B) or more activating receptors than inhibitory receptors (C) are engaged. Stimulation of inhibitory receptors only (A) or in higher numbers than activating receptors (D) leads to inhibition of NK cells.

2.8 NK cells and AML

NK cells play an important role in the immunosurveillance and killing of leukemic cells[88]. The activity of NK cells against leukemic blasts is determined by two major features. The presence of activating ligands on leukemic blasts like ULBP and MICA/B for NKG2D and ligands for NCR is a major determinant for NK-leukemia recognition. Inhibition of NK cells mediated through KIR by expression of the respective HLA-ligands on the tumor cell is another feature having an important influence on the immunosurveillance. Leukemic cells of AML were shown to have low to absent expression of activating ligands for NK cell receptors, thereby evading

immunorecognition[89,90]. This low surface density of ULBP and MICA/B is caused by both repression of expression[91] and proteolytic shedding[92,93]. The possibility to induce the upregulation of cell surface expression of these ligands is therefore holding out prospects to enable NK cells to recognize and lyse leukemic blasts.

Additionally, NK cells of leukemic patients are shown to be functionally impaired by displaying a skewed receptor repertoire and consequently a lower cytotoxic activity[89,94]. Furthermore, NK cells are efficiently inhibited by the engagement of inhibitory KIR by HLA ligands which are present at high levels on potential leukemic targets. Circumventing this inhibition by selecting NK cells with a mismatch of the KIR repertoire and the HLA expression of the tumor cells is leading to increased anti-tumor-activity of these NK cells[92]. In the clinical setting, hematopoietic stem cell transplantations mismatched with regard to the HLA-class I haplotype of the recipient and KIR repertoire of donor were evaluated and proved to beneficially influence the transplantation outcome by reducing the incidence of relapse[95]. This improvement is at least in part influenced by the graft-versus-leukemia effect of NK cells. When stem cell grafts were depleted from NK cells, the engraftment was impaired and the incidence of relapse was higher than in non-depleted grafts. Furthermore, presence of NK cells in PB after stem cell transplantation in AML was shown to maintain the disease in remission, whereas loss of leukemia-reactive NK cells is associated with relapse[96].

3 : Epigenetic gene regulation

3.1 Introduction

The chromatin of eukaryotic cells is a complex structure composed of DNA, histones and non-histone proteins[97]. Nucleosomes as the components of DNA are subunits of chromatin consisting of approximately 146 bp DNA wrapped around one histone complex composed of 2 copies of the four histones, H2A, H3A, H3 and H4.

Acetylation and deacetylation of histones are important epigenetic determinants of transcriptional regulation of eukaryotic cells, as first postulated in 1964[98]. The acetylation status is determined by histone acetyltransferases (HAT) and histone deacetylases (HDAC). HAT are adding acetyl groups to lysine residues on histone proteins and other proteins. HDAC are removing these acetyl groups. Acetylation of histones promotes a more relaxed, active chromatin structure[99]. Further epigenetic mechanisms influencing gene expression are histone methylation on CpG islands as well as ubiquitination[100]. There is now abundant evidence that remodeling the chromatin proteins is influencing the epigenetic regulation of gene expression and thereby representing a promising tool in anti-cancer therapy[101].

3.2 Histone deacetylases (HDAC)

In humans HDAC has been identified in 18 different forms, classified based on the homology to yeast[102] and subgrouped into 4 classes, each of them with different localization, expression and specificity. Class I HDAC are primarily localized in the nucleus, are ubiquitously expressed and require Zn^{++} for their enzymatic activity. Class II HDAC are cytoplasmatic proteins that migrate between cytoplasm and nucleus and are Zn^{++}-dependent, similar to class I HDAC. They are expressed in a tissue-specific manner[103]. Class III HDAC are NAD^{+}-dependent. Class IV HDAC share the catalytic core region of class I and II HDAC.

Beside histones as substrates, HDAC have a variety of non-histone substrates, emphasized by phylogenetic analyses that HDAC preceded the evolution of histone proteins[104] (see table **II.4**). The activity of these targets may be either enhanced or repressed upon acetylation, depending on the protein. Non-histone protein targets are involved in many biological processes influencing proliferation, differentiation and cell death suggesting that inhibitors of HDAC could have multiple mechanisms of inducing cell death and growth arrest [105].

Table II.4: Nonhistone proteine substrates of HDAC

Function	Proteins
DNA binding transcriptional factors	p53, c-Myc, AML1, BCL-6, E2F1, E2F2, E2F3, GATA-1, GATA-2, GATA-3, GATA-4, Ying Yang 1 (YY1), NF-kB, (RalA/p65), MEF2, CREB, HIF-1, BETA2, POP-1, IRF-2, IRF-7, SRY, EKLF
Steroid receptors	Androgen receptor, estrogen receptor a, glucocorticoid receptor
Transcription coregulators	Rb, DEK, MSL-3, HMGI(Y)/HMGA1, CtBP2, PGC-1α
Signaling mediators	STAT3, Smad7, β-catenin, IRS-1
DNA repair enzymes	Ku70, WRN, TDG, NEIL2, FEN1
Nuclear import	RCH1, importin-α7
Chaperone protein	HSP90
Structural protein	α-tubulin
Inflammation regulator	HMGB-1
Viral proteins	E1A, L-HDAg, S-HDAg, T antigen, HIV Tat

3.3 HDAC inhibitors

Inhibitors of HDAC (HDACi) are structurally different molecules with partly selective inhibition of the different HDAC classes present in the cell.

They have been shown to selectively alter gene expression, influencing 7-10 % of genes in leukemic cell lines[106,107]. The pattern of alterations of gene expression is similar for different HDACi but differs for the various cells. HDACi induce about the same number of genes as they repress [108].

The direct effects of HDACi are an inhibition of cell growth of transformed cells by cell cycle arrest[109] and cell death by inducing the intrinsic and extrinsic apoptotic pathway as well as mitotic cell death[110]. Furthermore HDACi are able to inhibit angiogenesis by inhibition of hypoxia inducible factors 1 and 2 (HIF-1, HIF-2)[111]. Normal cells show a relative resistance to HDACi-induced modification[112], making inhibition of HDAC an interesting target of novel pharmacological treatment modalities for cancer therapy.

Indeed several studies on tumor bearing animals as well as clinical studies have been exploring the anti-tumor effect of HDACi[113,114] (see table **II.5**).

Table II.5: Inhibitors of HDAC and their therapeutical use

Class	Compound	HDAC target	Potency	Stage of Development
Hydroxamate	SAHA (vorinostat)	Class I, II	μM	FDA approved for CTCL
	LBH589	Class I, II	nM	Phase I
	PXD101	Class I, II	μM	Phase II
	ITF2357	Class I, II	nM	Phase I
	PCI-24781	Class I, II	Not available	Phase I
				Phase II
Cyclic peptide	FK228	HDAC 1, 2	nM	Phase II
Benzamide	MS-275	HDAC 1, 2, 3	μM	Phase II
	MGCD0103	Class I	Not available	Phase II
Aliphatic acid	Phenylbutyrate	Classes I, IIa	μM	Phase II
	Valproic acid	Class I	mM	Phase II
	AN-9	Not available	μM	Phase II
	Baceca	Class I	Not available	Phase II
	Savicol	Not available	Not available	Phase II

3.4 Valproic acid (VA)

The aliphatic acid valproic acid (VA) was first synthesized in 1884 by Bruton and was used as inergic solvent for organic compounds until its anti-epileptic effect was discovered by Pierre Eymard in 1962[115]. Its clinical use is dating back to 1973. By blocking the neurotransmitter γ-aminobutyric acid (GABA) in the brain it is in clinical use as an anticonvulsant therapy as well as a mood stabilizer in treatment of epilepsy and bipolar disorders. Additional indications are migraine and schizophrenia. Recently, it was demonstrated that VA also has an effect as an inhibitor for HDAC and induces differentiation and apoptosis in a variety of malignant cells *in vitro*. Due to the neurological experience over decades and the low frequency and severity of side effects it is considered a promising drug for the epigenetic modification in neoplastic diseases.

3.5 Epigenetic modifications in AML

Beside of the irreversible structural genomic aberrations in AML which have been recognized as pathophysiological determinants of the disease (see table **II.1**), there is increasing evidence that epigenetic modifications are contributing to a loss of normal hematopoietic function as well [99]. Methylation of promoters could be shown to induce aberrant gene expression in several hematological neoplasias[116]. Histone deacetylation was proven to be involved in several steps of leukemic transformation and progression. The fusion gene AML1/ETO is inducing histone deacetylation and transcriptional repression as a result of repressed histone acetylation (see chapter **I 1.2**)[117]. Prominent example for the impact of epigenetic silencing by histone deacetylation is the acute promyelocytic leukemia (APL) with the translocation t(15;17). Due to the genetic alteration of the alpha subunit of the retinoic acid (RA) receptor (RARA), a transcriptional repressor complex (N-CoR/Sin3/HDAC) is recruited to RA target genes[118]. A therapeutically high dose of all-*trans*-retinoic-acid is releasing the repressor complex and causing transcriptional reactivation of RA target genes, leading to myeloid differentiation and disease remission of APL[119].

A pathogenetic role for chromatin remodeling in myeloid leukemia could be shown for Evi-1, a gene on chromosome 3q26 which is overexpressed in AML and myelodysplastic sydrom (MDS). Recruitment of C-terminal binding protein by Evi-1 is activating HDAC1 which in turn is transcriptionally downregulating Smad[120]. Inhibiting HDAC with trichostatinA reduces the repression induced by Evi-1, indicating that chromatin remodeling by histone deacetylation is an important mechanism causing this silencing.

By showing that inhibition of HDAC does not only lead to cellular differentiation and block of proliferation, but also induces the expression of ligands for NK cells, mainly ULBP and MICA/B[91,121], it is tempting to consider the therapeutic use of HDAC inhibition applied on LSC to render them accessible for NK cell mediated recognition and killing.

III Research objectives

Acute myeloid leukemia (AML) is a severe malignant hematologic disease. Even with advances in understanding pathophysiology and pathogenesis and novel treatment options AML remains virtually an incurable disease. According to an emerging concept in cancer biology, the existence of leukemic stem cells (LSC) plays a central role in disease initiation and progression. LSC are tumor cells that are similar to hematopoietic stem cells in terms of a quiescent cell state, making them inaccessible for conventional cytotoxic therapy. Since LSC are believed to be responsible for treatment failure and relapse, the development of novel therapies focusing on this small leukemic cell subpopulation is of crucial importance.

Natural killer (NK) cells are a major component of antitumor immune defense. NK cells recognize and kill malignant cells by virtue of their activating receptors, such as NKG2D and the natural cytotoxicity receptors NCR, on their cell surface. These are interacting with specific ligands on the putative target cell, such as ULBP and MICA/B as well as the ligands for NCR. The ligands can be upregulated on tumor cells upon cellular stress, nevertheless many tumor cells have developed mechanisms to either suppress expression of these ligands or shed them from their surface, and thus are evading the immunorecognition.

Preceding studies in our laboratory have shown that leukemic blasts in AML are expressing activating ligands for NK cells at a very low level. Furthermore, NK cells from AML patients are functionally impaired in their cytotoxic activity. Pharmacologically it has been possible to increase the expression of activating ligands on AML blasts, resulting in an increased immunorecognition and killing. The NK cell subsets mismatched with respect to inhibitory receptors (KIR) and patient's HLA class I have been demonstrated as the most efficient effectors. These experiments have lead to the novel question whether LSC can be recognized by NK cells and whether this interaction can be influenced pharmacologically. So far, there are no published reports on the interaction of NK cells and LSC and it is not known whether NK cells have the potential to act as a directed immunotherapy against LSC.

The first goal of this work was to characterize the expression of ligands for activating receptors on LSC. Since the homogenous cell populations of leukemic cell lines do not contain *bona fide* LSC, it was necessary to work with patient-derived primary cells and define the LSC population according to the phenotypic characteristics.

In the second part of this work, we used a pharmacological approach to influence the expression of activating NK cell ligands on LSC with the perspective of increasing the tumor recognition by NK cells. The goal of these experiments was to use compounds which reverse the epigenetic silencing mechanisms and achieve an upregulation of expression of activating ligands on LSC.

Subsequently, in the third part of this work the functional consequences of increased expression of activating ligands on LSC were determined. Since stem cells have the capability of forming colonies in semisolid cultures as well as engrafting in the bone marrow microenvironment in a NOD/SCID xenotransplantation model, the goal was to examine the susceptibility of LSC to NK cell cytotoxicity *in vitro* and *in vivo*.

Understanding the interactions of LSC and NK cells might prove to be beneficial for therapeutic applications, either complementary to current protocols or as an alternative for the so far disappointing therapies. In the scope of developing novel immunotherapeutic strategies to target LSC of AML, the effect of epigenetic modification of gene expression by VA in combination with KIR-HLA mismatched NK cells should be evaluated.

IV Material and Methods

1 AML Patients

All control and clinical samples were obtained with informed consent from the University Hospitals of Basel (Switzerland) and Warsaw (Poland). Only patients with newly diagnosed untreated AML, from which enough material could be obtained were enrolled in the study. The diagnosis of the AML subtypes FAB M1 – M7 was based on morphologic, cytogenetic and immunophenotypic criteria. Subsequently, patients were selected for high blast content in the peripheral blood, expression of CD34 on the blasts, preferentially the existence of a HLA-mismatch regarding single KIR NK cell clones and the efficiency of engraftment upon NOD/SCID xenotransplantation. Table **IV.1** is showing the characteristics of 12 AML patients, which fulfilled most criteria and were included in our study.

Table IV.1: Characteristics of AML patients.

PUN	Age, sex	AML subtype (FAB)	Blast content % of PBMC	$CD34^+$ expression % of blasts	Mismatch with NK cells
1	37, f	M2	95	0.5	KIR a
2	73, m	M4	81	43	KIR e
3	35, f	M2	47	95	KIR a, e
4	71, f	M0-1	85	76	no typing
5	65, f	M2	92	80	KIR e
6	31, f	M4	93	82	KIR e
7	46, f	M5	98	52	KIR a
8	21, m	M2	95	45	KIR b
9	41, m	M5	74	16	KIR a, e
10	76, f	M2	67	94	KIR a, e
11	41, f	M1	97	47	no mismatch
12	59, m	M2	71	95	KIR a

2 Cell culture of primary AML cells and HL-60 cell line

Mononuclear cells from peripheral blood (PBMC) of AML patients were prepared using density-gradient centrifugation (Ficoll Histopaque, Sigma-Aldrich, St Louis, MO) and red blood cell lysis (RBC lysis buffer, Spitalpharmazie Basel, Universitätsspital Basel). Cells were cryopreserved in iscove`s modified dulbecco`s medium (IMDM, Invitrogen, Carlsbad, CA), dimethyl sulfoxide (DMSO, 10 %, Sigma-Aldrich) and fetal calf serum (FCS, 20%, Invitrogen) in liquid nitrogen. Upon use PBMC were cultured in X-Vivo 10 medium (Lonza, Basel, Switzerland) supplemented with the following growth

factors: interleukin-3 (IL-3, 20 ng/ml, Novartis, Basel, Switzerland), IL-6 (20 ng/ml, Novartis), stem cell factor (SCF, 100 ng/ml, Novartis), Flt-3 Ligand (FL, 100 ng/ml, Novartis), granulocyte - colony stimulating factor (G-CSF, 20 ng/ml, Roche, Basel, Switzerland) granulocyte macrophage – colony stimulating factor (GM-CSF, 20 ng/ml, Sandoz, Basel, Switzerland), thrombopoietin (TPO, 50 ng/ml, Roche) and 20 % BIT9500 (BSA, insulin, transferrin, Stem cell technologies, Vancouver, Canada). HL-60 were cultured in IMDM, 10% FCS supplemented with the growth factors FL, SCF and GM-SCF and interferon-γ (IFN-γ, 100U/ml, Roche). 1-2 x 10^6 cells were cultured in 2 ml in a 24 well plate. After 2 days, cells were counted, washed in PBS (Invitrogen) and analyzed for the cell surface expression of ligands for NKG2D and NCR by FACS, the colony-forming ability in methylcellulose and in cytotoxicity assays (see below).

3 Differentiating drugs

When appropriate, cells were treated with bryostatin-1 (Sigma-Aldrich) at a concentration of 10nM or valproic acid (VA, Orfiril, Desitin Pharma, Liestal, Switzerland) at 1mM.

4 Fluorescence Activated Cell Scan (FACS)

For the phenotypical analysis of cells by FACS, cells were stained with primary labeled antibodies against CD45, CD34 and CD38 linked to fluoresceinisothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin-chlorophyll protein complex (PerCP) (all purchased from BD PharMingen, Franklin Lakes, NJ). NKG2D ligands were stained with the following unlabelled mouse antibodies: ULBP 1 (10 μg/ml, clone M295, IgG1), ULBP 2 (10 μg/ml, clone M311, IgG1), ULBP 3 (10 μg/ml, clone M551, IgG1) and MICA/B (10 μg/ml, clone M673, IgG1, all kind gifts from David Cosman, Amgen Washington Inc, Seattle, WA), in a second step detected with a FITC-labelled goat-anti-mouse antibody (IgG, 1:200 dilution, Jackson ImmunoResearch, West Grove, PA). Incubation was done in FACS-PBS (PBS supplemented with 0.5 % FCS, 0.02 % NaN_3) for 20 minutes on ice in the dark. After staining cells were washed twice with FACS-PBS and resuspended in FACS-PBS containing 0.5 μg/ml propidiumiodide (0.5 μg/ml, PI, Sigma-Aldrich) to exclude dead cells and analyzed with a CyAn ADP Flow Cytometer (Dako Cytomation, Glostrup, Danmark) using Summit software (Dako Cytomation). Analysis was done using FlowJo software (Tree Star, Stanford, CA).

The expression level of ligands for NKG2D was defined as the mean fluorescence intensity ratio (MFR) of values obtained with specific mAbs divided by values given by the secondary or control mAbs.

Since the ligands for the NCR receptors NKp30, p44 and p46 on NK cells are unknown, antibodies for measuring the cell surface expression of these ligands are not existing. To allow the staining of putative ligands, dimeric complexes of the soluble receptor molecules (sNCR) tagged with BirA1.4 and anti-BirA1.4-antibodies were generated. Supernatants containing sNKp30, sNKp44 or sNKp46 and anti-BirA1.4 mouse IgG were kindly provided by G. De Libero, Laboratory of Experimental Immunology, University Hospital Basel, Switzerland. Dimer formation was carried out in the supernatants containing the sNCR with 180 ng of anti-Bir antibody per 50 µl of supernatant, based on titration. Binding of cells by dimers was revealed using a secondary FITC-conjugated goat-anti-mouse antibody (IgG, Jackson ImmunoResearch). The NCR ligand expression was quantified as the mean fluorescence intensity ratio MFR of values given by the dimers + secondary goat-anti-mouse antibody divided by values given by anti-BirA1.4 antibody + secondary.

5 FACS sorting

Sorting of AML leukemic stem cells was performed on a FACSVantage SE (Becton Dickinson, Franklin Lakes, NJ) in the cell sorting facility of the University Hospital Basel. Up to 10^8 PBMC were stained in 300 µl final volume with 50 µl of each antibody for 20 minutes on ice with CD45-FITC, CD34-PE, CD38-APC (all BD PharMingen). Before sorting cells were filtered through a cell strainer cap and sorted into 1 ml X-Vivo 10 medium containing 20 % FCS. Up to 10^6 cells were collected from each population. Sorted cells were cultured under medium conditions mentioned above in a density of 1-2 * 10^5 cells in 200 µl in 96 well plates. Sorted cells were used for CFU assays, preparation of RNA and for cytotoxicity assays.

6 RNA preparation

Cells were washed in ice cold PBS and counted. Cells were spun 12.000 rpm for 8 minutes and pellet was resuspended in 1 ml Trizol (Invitrogen) and 200 µl chloroform (Merck, Darmstadt, Germany) was added after 5 minutes of vigorous mixing. Centrifugation at 12.000 rpm for 15 minutes separated an aqueous upper phase containing the RNA from an

organic lower phase containing proteins and DNA. The aqueous phase was collected, 1 µg of glycogen (Invitrogen) was added to increase efficiency of the RNA precipitation conducted with 500 µl isopropanol (Merck) and centrifuged (12.000rpm, 15 minutes). The pellet was washed with 75 % ethanol and centrifuged (7500 rpm, 5 minutes). After removing the supernatant, the pellet was allowed to air-dry in the flow of a sterile bench and resuspended in 50 µl DEPC-H_2O. Concentration of RNA was measured using NanoDrop (Fisher Scientific, Wilmington, DE) and stored at -70 °C.

7 Reverse transcription real-time PCR

Reverse transcription was performed using Omniscript RT kit (Qiagen, Hilden, Germany) according to the protocol. 1 µg of RNA was used for 20 µl reaction volume. 1-2 µl of the cDNA was used for the subsequent real-time PCR reaction using TaqMan MGB primer sets (Applied Biosystems, Foster City, CA) for ULBP 1 (Hs00360941_ml), ULBP 2 (Hs00607609_ml) and ULBP 3 (Hs_00225909) with hypoxanthine phosphoribosyl transferase (HPRT, Hs00355752_ml) as standard. The Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems) and analyzed as usual.

8 Serial replating colony forming unit (CFU) assay

To assay leukemic cells directly after sorting or after incubation with valproic acid for their stem cell characteristics, CFU assays were performed. $1 * 10^5$ sorted cells were suspended in 600 µl IMDM medium supplemented with FCS (15%), human plasma from donors of the blood group AB^+ (AB^+ serum, 15%, Blutspendezentrum Basel), β-mercaptoethanol (48 µM, Sigma-Aldrich), L-glutamine (20 µM, Invitrogen), bovine serum albumin (BSA, 1 %, Fraction V, Roche Diagnostics, Mannheim, Germany), erythropoietin (3 U/ml, Epo Eprex 4000, Janssen-Cilag, Baar, Switzerland), IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), GM-CSF (20 ng/ml), SCF (100 ng/ml), FL (100 ng/ml) and mixed with 600 µl of methylcellulose preparation (Fluka AG, Buchs, Switzerland) to a final volume of 1200 µl and plated in a dish with 3 cm diameter. All experiments were performed in duplicates. After 14 days the colonies were counted under the microscope and the average number of colonies in the duplicates were calculated. Afterwards cells from primary (1°) cultures were washed out of the methylcellulose with IMDM and re-plated using

the same procedure into the secondary (2°) cultures. For this purpose, the 1° duplicates were pooled and again separated into 2° duplicates.

9 Cytotoxicity assay

To analyze direct interaction of NK cells and AML leukemic stem cells, a standard chromium release assay was performed. Target leukemic cells or HL-60 cell line were loaded with 250 μCurie of $Na_2{}^{51}CrO_4$ (Amersham, Little Chalfont, UK) for 2 hours in 37 °C in a total volume of 300 μl and co-cultured at 37 °C for 4 hours with NK cells in IMDM supplemented with FCS (10 %) in different effector : target-ratios (E:T ratio) ranging from 20 to 0.5. Target cell maximal chromium release upon lysis of cells with 1 % triton-X (Sigma-Aldrich) and spontaneous release were determined to calculate the percentage of specific killing. Experiments were set up in triplicates and contained ^{51}Cr-labelled 10^3 target cells in 200 μl final volume. After 4 hours 40 μl of supernatant was transferred onto Luma scintillation plates (Perkin Elmer, Waltham, MA) and allowed to air-dry. Chromium release was assessed using a TopCount NXT gamma-counter (Packard Perkin Elmer). Results are expressed as percentage of specific ^{51}Cr-release and calculated as follows: [(cpm experimental release – cpm spontaneous release) / cpm target maximal release – cpm spontaneous release)] * 100.

10 Single KIR NK cell clone isolation and culture

NK cells were obtained from the peripheral blood of healthy donors. NK cells were purified from PBMC using magnetic-activated cell sorting (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, 1-3 * 10^7 viable cells after thawing were incubated for 10 min in 300 μl PBS buffer containing 0.5 % BSA and 2mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) with 100 μl of antibody cocktail against multiple lineage epitopes not expressed on NK cells (CD3, CD4, CD14, CD15, CD19, CD36, CD123, glycophorin A, Miltenyi Biotech). To purify NK cells by the negative selection step, antibodies coupled to magnetic beads were used and non-NK cells were retained in a column (LS separation column, Miltenyi). Purified NK cells were collected in the flow through. The purity of NK cell preparation was > 95 % with less than 0.5 % contaminating T cells.

Subsequently 2 * 10^5 NK cells were cultured by stimulation on 2 * 10^6 irradiated (30 Gy) allogenic PBMC as feeders in IMDM medium containing human AB^+ serum (5 %),

IL2 (100 U/ml, Novartis) as well as nonessential aminoacids, L-glutamine, penicillin/streptomycine and sodium pyruvate (all Gibco) together with phytohaemagglutinin (PHA, 2 μg/ml, Murex Biotech, Datford, England) in a total volume of 2 ml. About 10 days later cells were expanded by transferring them to 6 well plates. At 2-3 weeks, an approximately 600-1000 fold expansion of NK cell numbers was achieved.

To isolate single KIR NK cell clones, NK cells were FACS sorted 14 days after stimulation. Remaining T cells were excluded by gating on $CD3^-$ cells. To sort for single KIR a NK cells, anti-KIR a- mAb labelled with FITC (CD158a, BD Pharmingen), was incubated for 20 min on ice with NK cells together with a mixture of anti-KIR b-PE and anti-KIR e-PE (all purchased from BD Pharmingen). Single KIR b NK and single KIR e NK were obtained accordingly. After sorting, single KIR NK cells were restimulated on allogenic feeders as described above and used on days 14 to 30 after restimulation.

11 TM-β1 antibody production and purification

To enhance engraftment of human cells in the mouse xenotransplantation model, remaining lymphocytes and macrophages in NOD/SCID mice after irradiation were depleted by murine IL-2 receptor beta chain (CD122) blockade using a rat-anti-mouse antibody against muCD122. The rat hybridoma cell line TM-β1 producing a rat monoclonal antibody recognizing the murine IL-2 receptor beta chain was kindly provided by Dr. Tanaka, Tokyo, Japan and grown in IMDM supplemented with 3% ultra-low IgG FCS (Invitrogen). Supernatant was collected, filtered through a 45 μm filter and subsequently purified over a protein G sepharose column (Protein G Sepharose 4 Fast Flow, GE Healthcare, Uppsala, Sweden, generously performed by Sebastiano Sansano, Laboratory of Experimental Immunology, University Hospital Basel, Switzerland). After dialysis against PBS (Float-A-Lyzer®, MWCO 10kDa, SpectrumLabs, Rancho Dominguez, CA), the concentration of TM-β1 antibody was measured by ELISA and antibody was stored at -70 °C until use.

12 ELISA for TM-β1

To measure the concentration of TM-β1 antibody, enzyme-linked immunosorbent assay (ELISA) was performed. 96 well plates (Nunc-Immuno Maxi-Sorp™, Nunc Roskilde, Danmark) were coated at 4 °C overnight with goat-anti-rat IgG (100 μl/well, 3 μg/ml, R 5130, Sigma-Aldrich). After blocking with 100 μl of a 2 % BSA solution in PBS for 1 hour at room

temperature, purified and dialyzed TM-β1 was applied in dilutions of 1:1000 to 1:10000 (1h, RT). Anti-muCD122 clone TM-β1 (Cat. 14-1222-82, eBioscience, San Diego, CA) served as standard in concentrations of 10 mg/ml to 500 mg/ml. Antibodies were revealed for 1 hour at RT with a rabbit-anti-rat IgG conjugated to peroxidase (100 µl of 1:10000 dilution, A5795, Sigma-Aldrich). Substrate reagent pack (Dy999, R&D systems, Minneapolis, MN) was used before reading in an ELISA reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) at 450 nm weavelength.

13 NOD/SCID intrafemural xenotransplantation model of human leukemia

NOD/LtSz-scid/scid (NOD/SCID) mice (The Jackson Laboratory, Bor Harbor, ME) were bred and maintained under specific pathogen-free conditions in the local animal facility of the University Hospital Basel. During the experiment mice were kept on acidified drinking water supplemented with trimethoprim (26.7 µg/ml)/sulfamethoxazol (133.3 µg/ml, Nopil, Mepha Pharma AG, Aesch, Switzerland).

For AML transplantation mice were sub-lethally irradiated with 375 cGy (^{60}Co source, 2cGy/min) and injected with 180 µg purified TM-β1-antibody i.p. produced as described above 24 hours prior to injection of human AML cells. Intrafemural transplantations were performed under inhalation anaesthesia with isoflurane (Attane ad us. vet., Minrad INC, Buffalo, NY). The left knee of anaesthesized mice was desinfected, bended and a hole was drilled with a 26 gauge needle (Sterican®, B. Braun Melsungen AG, Melsungen, Germany) into the femur via the distal end of the bone. The needle was removed and a syringe with a 29 gauge needle (BD Micro-fine™, Becton Dickinson) was inserted to inject the 20 µl cell-suspension containing 1 * 10^7 human AML cells. Postoperative analgesia was provided with 1 µg buprenorphine s.c. (Temgesic, Essex Chemie AG, Luzern, Switzerland). Mice were monitored for pain and symptoms of disease 3 times weekly. 4 weeks post transplantation mice were grouped into 4 treatment groups with no treatment, valproic acid (400 mg/kg, corresponding to 100 µl i.p.), NK cells (5 * 10^6 IL-2-activated single KIR HLA-mismatched NK cells 14-25 days after restimulation i.v.) and a combination of valproic acid and NK cells. Treatment was performed 3 times in the 5th week after initial transplantation. Mice were sacrificed 1 week after the last treatment and bone marrow of the injected left femur and non-injected bones as well as mouse PB were separately analyzed by FACS for tumor load with human AML, expressed as percentage of huCD45$^+$CD33$^+$ cells of PI negative cells.

V Results

1 Expression of ligands for NK cells on the AML cell line HL-60 and primary AML cells and its functional consequences.

1.1 Differentiating drugs upregulate cell surface expression of ligands for NKG2D in HL-60 cells.

Epigenetic alterations have been implicated in the pathogenesis of acute myeloid leukemia by causing transcriptional silencing of genes encoding the regulators of cell growth and differentiation. In contrast to oncogenic changes caused by gene fusions - mutations and insertions/deletions - epigenetic changes in principle are reversible and therefore accessible for pharmacologic intervention.

Since a large number of genes are affected by epigenetic modifiers, defining target molecules relevant for therapeutic effects has been difficult. In our study, we focused on the expression of ligands for NK cells as a response to anti-neoplastic drugs promoting cell differentiation through epigenetic mechanisms.

With the intention to assess treatment options at the level of epigenetic modifications, we tested in the initial phase of this study a number of differentiating agents, acting through different post-transcriptional mechanisms. These included 5-aza-2`deoxycytidine (AZA), a hypermethylating agent, trichostatin A (TSA), a HDAC inhibitor, 1-alpha,25-dihydroxy-vitamin D3 (VitD), binding to nuclear receptors and thereby influencing transcription, bryostatin-1, a protein kinase C activator and all-*trans*-retinoic-acid (ATRA), causing a disassembly of the transcriptional co-repressor-complex with HDAC activity. We used the AML cell line HL-60 (FAB M 2/3) as a model for human leukemia and selected for the most active drug. Upon treatment, we monitored cells for the expression of ligands for NK cells, ULBP1, 2 and 3 by FACS as possible mediators for immunorecognition and killing. As bryostatin-1 in combination with various growth factors and interferon-γ turned out to be the most potent inducer of cell surface expression of NK cell ligands on tumor cells, we focused on bryostatin-1 and used it in the initial experiments with both the HL-60 cell line and primary AML blasts.

Figure **V.1 A** shows an example of an upregulation of the NK cell ligands ULBP1, 2 and 3 upon treatment of HL-60 cells with bryostatin-1. Since NK cells express

multiple inhibitory receptors for HLA class I leading to a tolerance, we simultaneously measured the expression of HLA class I as a consequence of treatment. Interferon-γ is known to induce expression of HLA genes. In three independent experiments with HL-60 cells treated with bryostatin-1, we observed an upregulation of cell surface ligands, most prominent for ULBP1. ULBP2 and 3 showed a less strong reaction to bryostatin-1. HLA class I, a possible opponent of ULBP for the activation of NK cells, was only slightly upregulated to the same extend as the surface expression of ULBP2 and 3. Figure **V.1 B** shows the increase of the cell surface expression of ULBP1, 2, 3 and HLA class I as a mean of three independent experiments.

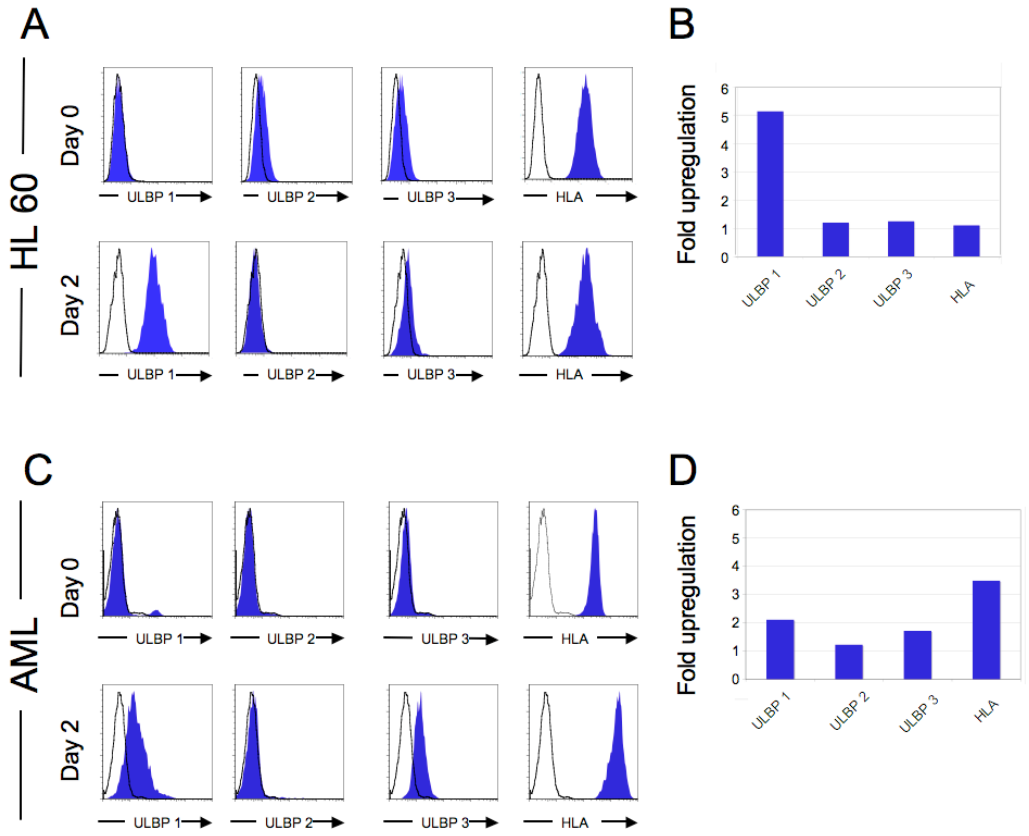

Figure V.1
Cell surface expression of ULBP on HL-60 cell line and primary AML cells is absent and upregulated following treatment with bryostatin-1.
FACS staining of ULBP1, 2, 3 and HLA class I + secondary FITC-labeled goat-anti-mouse IgG (blue area in histograms) or secondary FITC-labeled goat-anti-mouse IgG alone (thin black line) on HL-60 (A) and AML cells (C) before (Day 0) and after 2 days of incubation with bryostatin-1 at 10 nM (Day 2). Representative example out of n=3 for HL-60, n=3 for primary AML cells.
Fold upregulation measured as MFR after 2 days of treatment with bryostatin-1 as compared to day 0 for ULBP1, 2, 3 and HLA class I on HL-60 (B) and AML (D) cells. Average of 3 independent experiments for (B) and (D).

To confirm the effect of bryostatin-1 in a more physiological setting, we studied the effect of bryostatin-1 on the regulation of ligands for NK cells using primary cells from AML patients. As published before, primary AML blasts judged by the $CD45^{dim}$-phenotype do not express any known ligands for NKG2D (figure **V.1 C**, upper row). Bryostatin-1 caused an increase in the expression of ULBP molecules, as seen with HL-60 cells (figure **V.1 C,** lower row). Due to the heterogeneity of AML in individual patients, increased ULBP expression was more variable in between different samples than with the HL-60 cell line. Furthermore, the strong increase in ULBP1 compared to ULBP2 and 3 observed in HL-60 cells is not applying in primary AML cells. Stronger than in cell lines, primary blasts react to bryostatin-1 with an increased expression of HLA class I. Figure **V.1 D** shows the average of upregulation of ULBP ligands and HLA class I from n=3 independent experiments with primary AML cells. In agreement with the differentiating potential of bryostatin-1, AML cells acquired the cell surface expression of the myeloid marker CD14 (not shown). It might be noted that primary AML cells show the individual capacity to be cultured and their survival in culture differs strongly even over the short period of 2 days - leaving the possibility of a selection bias for those primary samples which can be maintained in culture.

1.2 Expression of ligands for the Natural Cytotoxicity Receptors NKp30, NKp44 and NKp46 is increased after treatment with bryostatin-1.

NK cells also use other activating receptor-ligand interactions to recognize tumor cells beside the NKG2D - ULBP/MICA/B system. One further family of activating receptors are natural cytotoxicity receptors (NCR), representing a group of the receptors NKp30, NKp44 and NKp46. We asked whether ligands for NCR are also upregulated by the differentiating effect of bryostatin-1. Cellular ligands for NCR have not yet been described, even though viral hemagglutinins seem to bind NKp44 and NKp46. To enable the measurement of ligand expression on putative target cells, the group of Gennaro de Libero (Laboratory of Experimental Immunology, University Hospital Basel, Switzerland) created recombinant proteins composed of the extracellular domains of the NCR receptors coupled to a Bir1.4 tag. Two of these recombinant molecules form a homodimer with an anti-Bir1.4 antibody, allowing the

detection and quantification of the yet unknown NCR ligands. In a collaborative study together with the Laboratory of Experimental Immunology, we used these dimers to stain HL-60 cells before and after treatment with bryostatin-1 in combination with growth factors and interferon-γ. HL-60 cells do express a basic level of NCR ligands (figure **V.2 A**, upper row), which can be upregulated up to threefold upon treatment (figure **V.2 A**, lower row). Figure **V.2 B** shows the average upregulation of n=2 independent experiments. These results indicate that several NK cell activating ligands can be upregulated upon treatment of AML cell lines with the differentiating drugs, such as bryostatin-1.

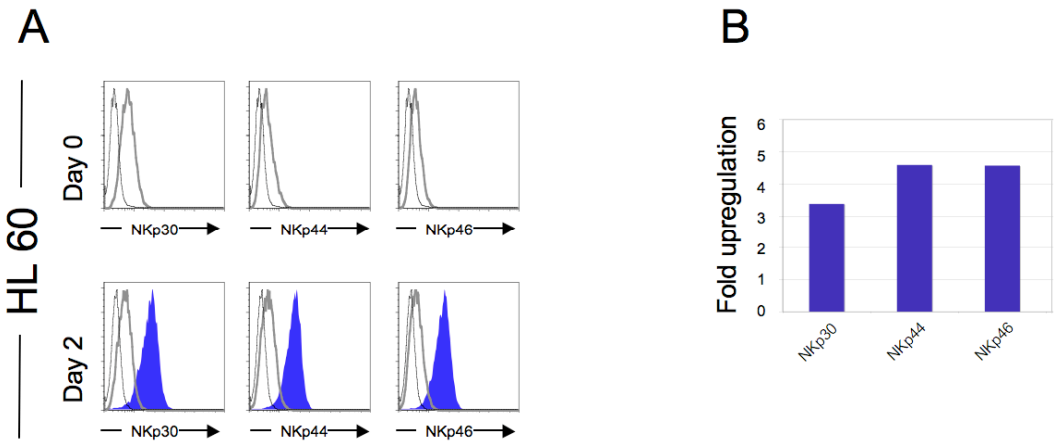

Figure V.2
Ligands for NCR on HL-60 cells are upregulated upon treatment with bryostatin-1. FACS staining of ligands for NCR with dimers of soluble NCR NKp30, NKp44 or NKp46 bound to anti-BirA1.4 + secondary goat-anti-mouse FITC (grey thick line in histograms) or anti-Bir1.4 + secondary FITC-labeled goat-anti-mouse IgG alone (thin black line) on HL-60 cells before (Day 0) and after 2 days in medium + growth factors (thick grey line, Day 2) and after 2 days treatment with bryostatin-1 at 10 nM (blue area, Day 2) (A). Representative example out of n=2.
Fold upregulation measured as MFR after 2 days of treatment with bryostatin-1 as compared to day 0 for NKp30, NKp44, NKp46 and HLA I on HL-60 cells. Average of 2 independent experiments (B).

1.3 Upregulation of cell surface expression of ligands for NK cells enhances the immunorecognition.

Due to the induced increase in cell surface expression of ligands for NK cell receptors on AML cells, we wanted to determine the impact of the drugs on the immunorecognition of tumor cells by NK cells. To this end we treated cells with bryostatin-1 for 2 days followed by a cytotoxicity assay with NK cells.

Figure **V.3 A** shows the specific lysis of HL-60 cells which increases from approximately 40 % to 70 % (at E:T ratio of 8:1) in response to treatment with bryostatin-1. This lysis can be reduced to 55 % by the use of ULBP-blocking antibodies. This is demonstrating a direct effect of ULBP on NK cell-tumor cell interaction. The contribution of the different NKG2D ligands ULBP1, 2, 3 and MICA/B is experimentally adressed by blocking with different combinations of antibodies against individual ligands as well as the NKG2D receptor (figure **V.3 B**). The remaining killing after blockage of either NKG2D (+anti-NKG2D) or all known ligands for NKG2D (+anti-MICA/B +anti-ULBP mAb) is indicating the contribution of further activating ligand-receptor pairs.

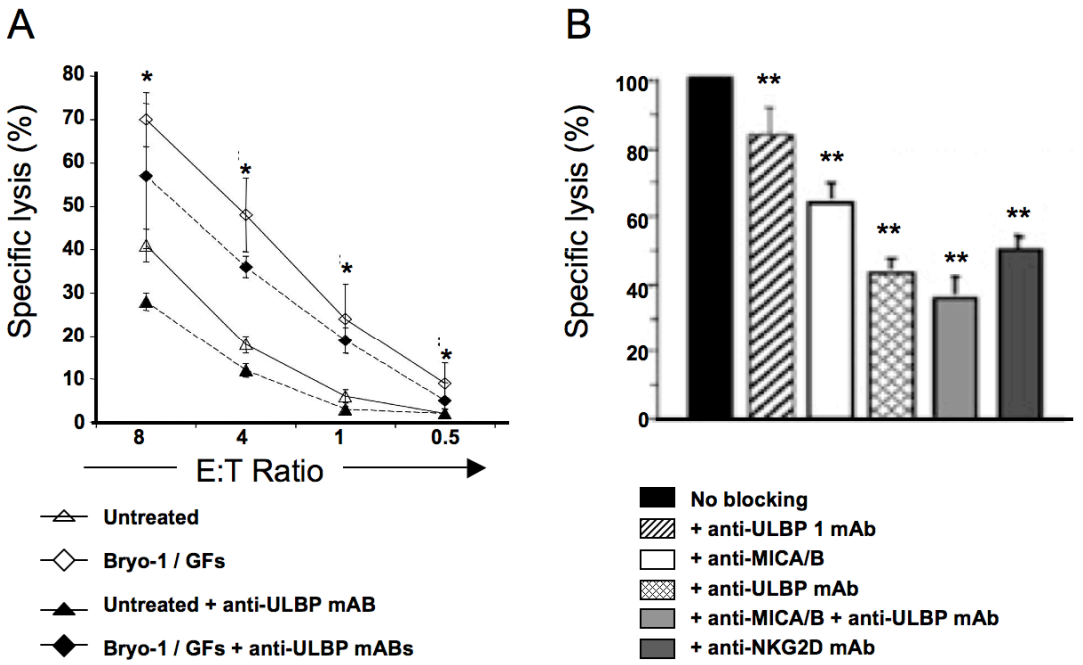

Figure V.3

Increased NK cell-mediated killing of HL-60 cells following treatment with bryostatin-1.
HL-60 cells were stimulated with bryostatin-1 for 2 days and used together with unstimulated control cells in the cytotoxicity assay as targets for primary NK cells at the indicated effector to target ratio (E:T ratio). Blocking antibodies against ULBP1, 2 and 3 were preincubated with the HL-60 cells prior to the cytotoxicity assay (A). Effect of blocking antibodies on NK cell mediated lysis of HL-60 cells after treatment with bryostatin-1. Anti-ULBP and anti-MICA/B mAbs were preincubated with HL-60 cells and anti-NKG2D mAbs with NK cells prior the cytotoxicity assay. Lysis in the absence of blocking mAbs was defined as 100 % (B). *P<0.05 and **P<0.001, significant differences as compared to lysis without blocking mAbs obtained at 3 NK cell : target cell ratios of 8:1, 4:1 and 1:1. Mean ± S.E.M. of three independent experiments is presented.

These results demonstrate that the upregulation of cell surface expression of activating ligands for NK cells by differentiation inducing drugs has functional consequences on the immunorecognition and killing of cells of the leukemic cell lines HL-60.

2 Leukemic stem cells (LSC) of AML and their interaction with NK cells.

2.1 Defining LSC of AML based on their phenotype.

The increased susceptibility to NK cells described above has been observed with a total population of leukemic blasts. Our next goal has been to address the distinct leukemic subpopulation of AML leukemic stem cells (LSC), which are – in contrast to blasts – difficult to target clinically with conventional cytoreductive therapy. Based on the most widely accepted phenotypical characterization, LSC are present among cells expressing the marker CD34. LSC of AML do not express CD38 on their cell surface but acquire this marker during maturation. Since CD34 and CD38 represent also markers of healthy hematopoietic stem cells (HSC), we used in addition the staining with anti-CD45 antibodies to distinguish $CD45^{bright}$ normal from $CD45^{dim}$ leukemic cells. LSC can therefore be defined according to the $CD45^{dim}CD34^{+}CD38^{-}$ phenotype.

Figure **V.4** shows the representative examples of three different AML patients of the same FAB subtype (AML M2) characterized by their size and granularity (FSC, SSC, 1st column), their blast content in comparison to healthy blood cells (CD45, 2nd column) ranging from 47 to 95 % as well as their phenotypical expression pattern of CD34 and CD38 (3rd column). Remarkable is the difference in expression of CD34, ranging from virtually its absence (AML 1, upper row) to presence on nearly all leukemic blasts (AML 3). Due to a lack of established other markers for LSC, it was necessary to select for AML patients expressing CD34 on their blasts. For comparison, the expression of CD34 and CD38 on normal hematopoietic stem cells of healthy bone marrow is shown (figure **V.4**, bottom row).

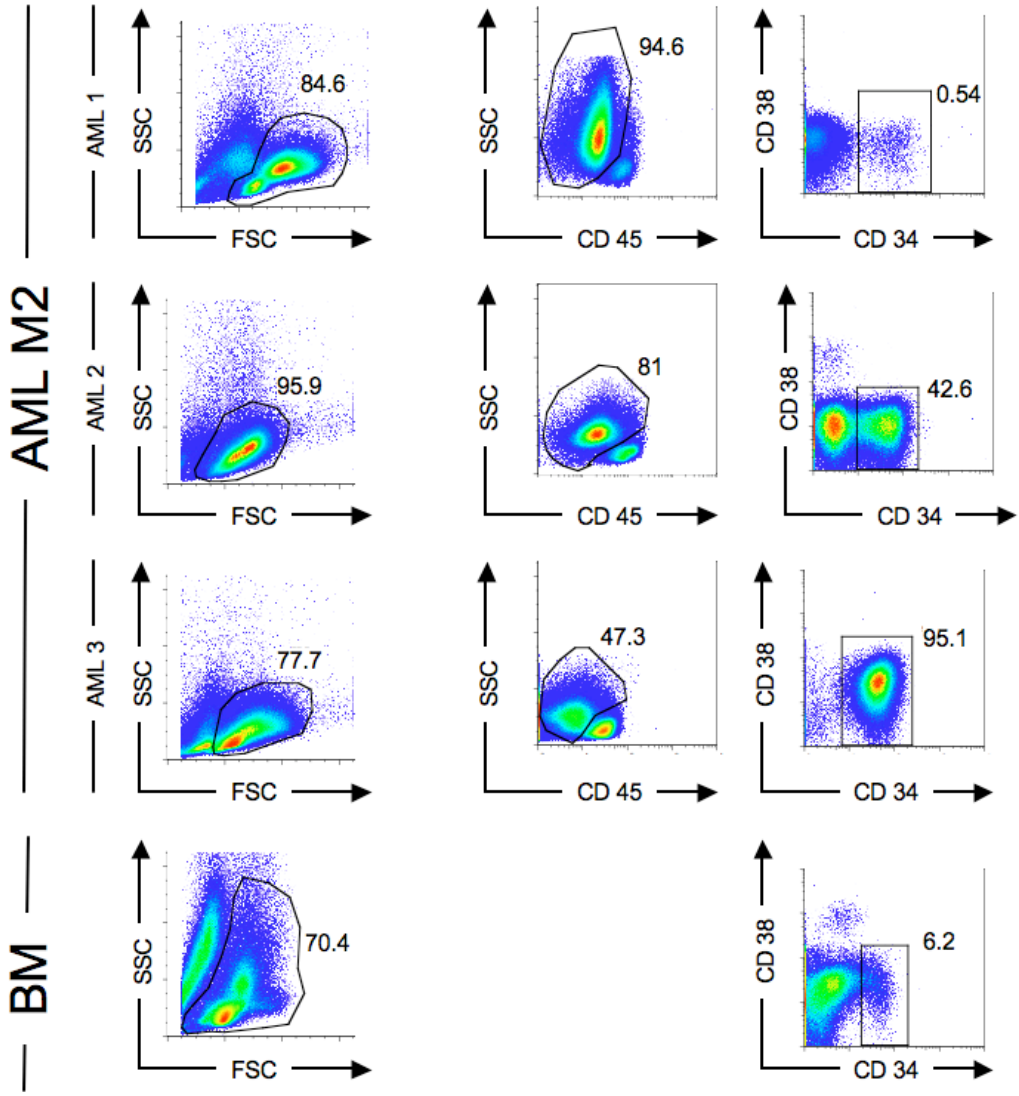

Figure V.4

Defining the phenotype of leukemic stem cells in primary AML.

AML leukemic stem cells (LSC) were phenotypically defined based on FSC, SSC and cell surface expression of $CD45^{dim}$ and $CD34^{+}CD38^{-}$. Three different AML patients of the same subtype (FAB M2) with different content of CD34-expressing cells are exemplarily shown. Normal hematopoietic stem cells from healthy bone marrow (BM) as judged by expression of $CD34^{+}38^{-}$ are shown as a control. Numbers refer to the percentages of sequentially gated cell populations.

2.2 Absence of NKG2D ligands on LSC of AML and on HSC.

To evaluate the possibilities of LSC recognition by NK cells, we were measuring the expression of ligands for the NKG2D receptor, ULBP1, 2, 3 and MICA/B on LSC. Throughout the different FAB subtypes of AML, LSC as judged by $CD45^{dim}CD34^{+}CD38^{-}$ showed no expression of any of these ligands (figure **V.5 A**). Absence of activating ligands may represent one of the reasons for the survival of

LSC and relapse of AML after treatment. Similar to LSC, HSC from healthy bone marrow do not express ULBP or MIC as well (figure **V.5 B**). HSC serve as an important control for treatment of LSC. Due to their physiologic indispensability, side effects of any treatment must spare this vital population, but the phenotypic and functional similarities of HSC to LSC may render them vulnerable to targeted LSC treatments.

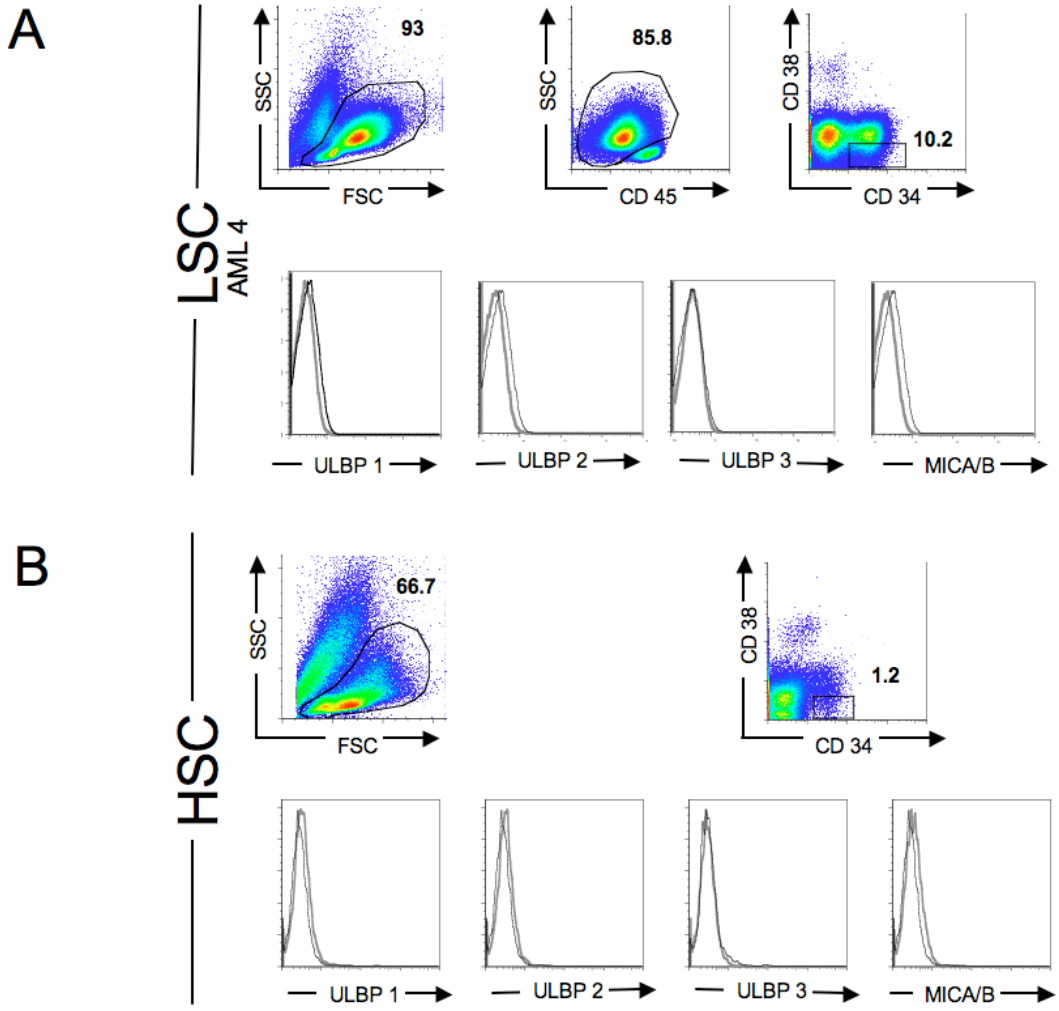

Figure V.5
Absence of cell surface expression of ULBP and MICA/B on primary AML leukemic stem cells. FACS staining for ULBP1, 2, and 3 as well as MICA/B on LSC with FITC labeled goat-anti-mouse Ab (thick grey line) or FITC labeled goat-anti-mouse Ab alone (thin black line) on LSC as judged by the $CD45^{dim}$ and $CD34^{+}CD38^{-}$-phenotype (A) and on normal hematopoietic stem cells from bone marrow (HSC) (B). Representative examples of AML (n=6) and healthy BM (n=4) are shown.

2.3 Transcripts of NKG2D ligands are detectable in LSC of AML and in HSC.

To investigate further the regulation of expression of NKG2D ligands, we sorted LSC and HSC according to CD34 and CD38 markers (see chapter **V.2.5**) and evaluated the mRNA transcripts for ULBP ligands in the early and more differentiated subpopulations by real-time PCR. LSC showed a very low to absent transcription of ULBP2 in both $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ cells (figure **V.6**). ULBP1 and ULBP3 were weakly transcribed with a Δ-CT of 10 and 6.7, respectively. HSC in turn had higher levels of ULBP1 and 2 transcripts, resembling the level of HPRT-1 reference transcripts (Δ-CT of 1.5 and 0.7 respectively), but an only weak expression of ULBP3 mRNA (Δ-CT of 8.1). Combined with the measurement of cell surface expression by FACS, these findings are in line with the observation that NKG2D ligands are at least in part posttranscriptionaly regulated and cell surface expression is not directly reflecting the mRNA transcript levels.

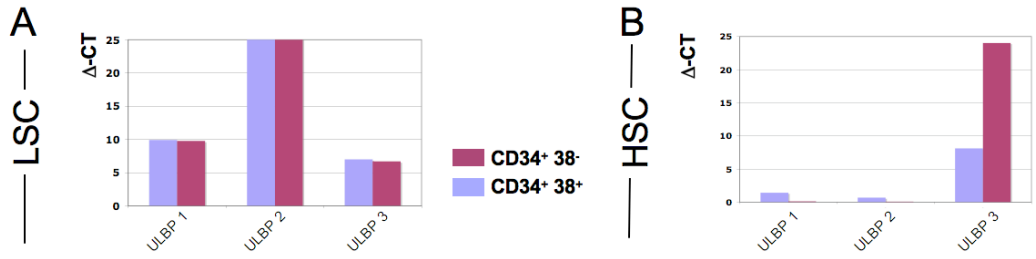

Figure V.6
mRNA transcripts for ULBP in LSC and HSC.
Quantitative PCR for the mRNA of ULBP1, 2 and 3 was performed with RNA isolated from untreated LSC (A) and HSC (B) directly after FACS sorting of the populations $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$ as indicated. Results are expressed as difference (Δ-CT) to HPRT-1 reference gene.

2.4 Treatment of LSC with the HDAC inhibitor valproic acid (VA) is increasing the expression of NKG2D ligands.

LSC, constituting a minor cell population in AML, represent a promising candidate for a targeted immunotherapy, which may have an impact on disease outcome by

influencing cells not responding to conventional treatment. Applying a differentiating treatment to LSC in order to render them accessible for NK cell mediated killing is an encouraging option. Bryostatin-1, although the most efficient *in vitro*, would not serve as a drug in the applied setting due to an only very limited clinical experience with this compound. Therefore we were applying valproic acid (VA), a drug that is acting as HDAC inhibitor and has a long-term record of clinical use and detailed knowledge about tolerance and side effects. VA has been clinically used over decades in treatment of neurological disorders, but it also promotes the myeloid differentiation of leukemic cells.

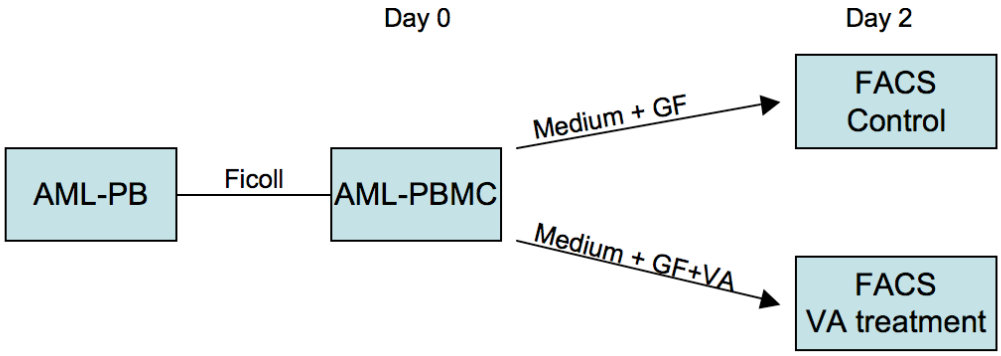

Figure V.7
Scheme of suspension culture of AML leukemic stem cells.
Total AML blasts of PB were cultured for 2 days in X-Vivo Medium containing growth factors (GF) in absence and presence of 1 mM valproic acid (VA). Subsequently cells were analyzed by FACS for cell surface expression of the NK cell ligands ULBP and MICA/B on leukemic stem cells ($CD45^{dim}$, $CD34^{+}CD38^{-}$).

Figure **V.7** shows the experimental setup of the differentiating treatment of leukemic blasts with VA. The expression of ligands for NKG2D was explored on the LSC population of PBMC from AML patients directly after thawing ("Day 0") and after incubation for 2 days in a suspension culture containing a mix of growth factors to provide elementary survival factors together with VA or without VA (control). As shown in figure **V.8**, VA is able to induce an upregulation of cell surface expression of ULBP and MICA/B. The shifts in the histograms (blue area) are indicating increased expression of the ligands, as compared to expression after culturing AML cells in medium supplemented with growth factors alone (grey thick line).

Shown are 4 examples of individual AML patients out of 8 analyzed. Reflecting the heterogeneity of individual AML patients, differences in the extend of upregulation as well as the predominant ligand responding with the strongest increase in expression can be observed.

As depicted in figure **V.8 B**, ULBP1 showed the most prominent increase of a 3.4 fold upregulation compared to the expression on control untreated LSC. ULBP3 and MICA/B were upregulated moderately with a 2.7 and 2.3 fold higher expression, respectively. The weakest upregulation of cell surface expression in response to VA treatment was measured for ULBP2.

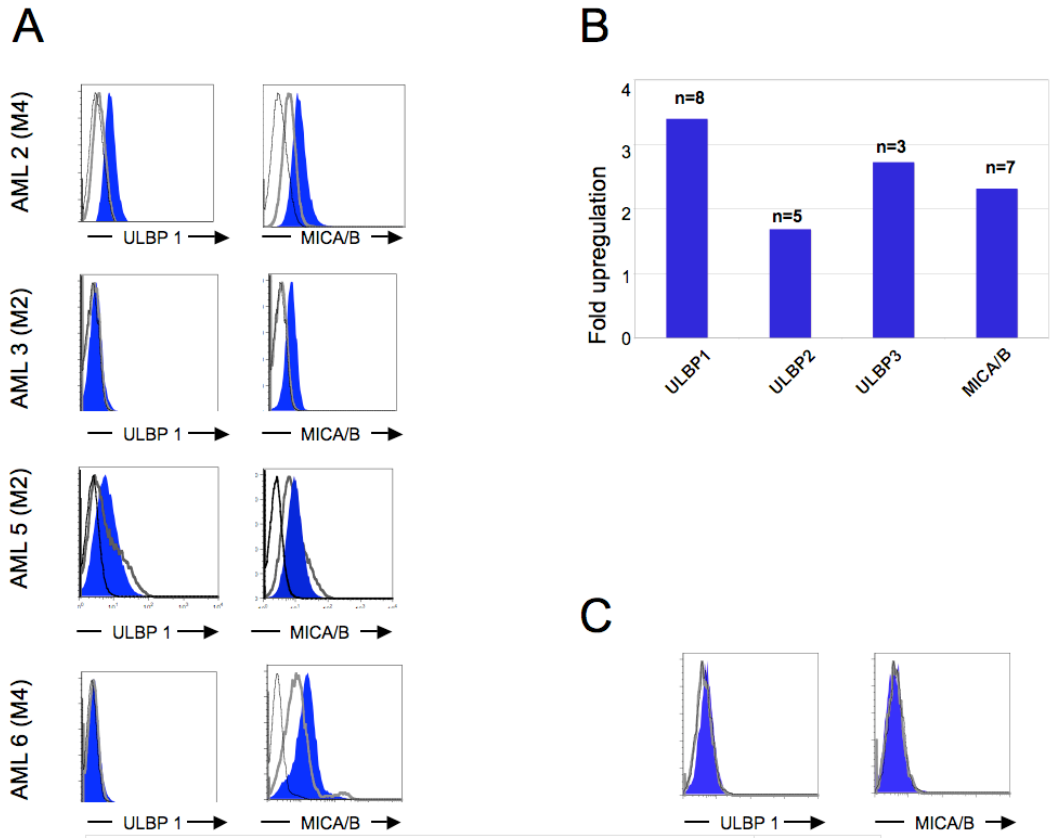

Figure V.8
Valproic acid upregulates cell surface expression of ULBP and MICA/B on LSC.
(A) FACS staining of secondary FITC-labeled goat-anti-mouse IgG alone (thin black line) and ULBP1 and MICA/B + secondary FITC-labeled goat-anti-mouse IgG on control LSC cultured in medium + GF alone (thick grey line) and after 2 days incubation with valproic acid at 1 mM (blue area in histogram). 4 representative examples out of n=8 are shown. (B) Average of upregulation of 4 different NKG2D ligands. (C) Normal HSC cultured in medium + GF (thick grey line) and VA-treated (blue area in histogram). Isotype: thin black line. Representative example out of n=4.

This result is demonstrating the potentially beneficial effect of VA on LSC in inducing an increased expression of ligands activating the NK cells. Interestingly,

normal HSC do not show any reaction to treatment with VA, since cell surface ligands remain in the same low expression level as without treatment (figure **V.8 C**). Hence, since VA is not acting on healthy HSC that need to be spared while treating leukemia, this drug is a candidate for a clinical application to induce an enhanced LSC-NK cell interaction.

2.5 Isolation of LSC of AML.

Phenotypical analysis of cell surface molecules expressed on LSC can be performed with total blasts by adequate gating during FACS analysis. However, to analyze the functional properties of LSC, it is necessary to isolate AML LSC out of the heterogeneous cell population. For this purpose, we established a FACS sorting protocol which enabled circumventing a number of obstacles such as fragility of LSC and a heterogeneity of expression levels of CD38 present in continuously increasing amount on early and more differentiated LSC and progenitors during differentiation.

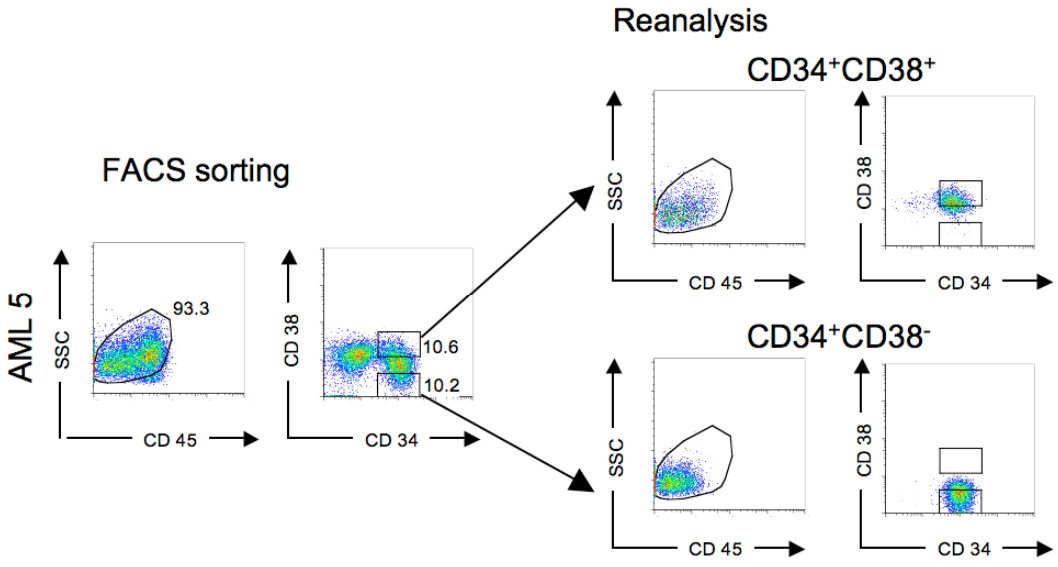

Figure V.9
FACS sorting of leukemic stem cells of AML.
FACS sorting of primary human AML leukemic stem cells by gating on leukemic blasts ($CD45^{dim}$) according to expression of CD34 and CD38. Reanalysis after sorting shows the separation of early leukemic stem cells ($CD34^{+}CD38^{-}$) and more differentiated leukemic progenitor cells ($CD34^{+}CD38^{+}$). Shown is one representative example. Numbers refer to the percentages of sequentially gated cell populations.

By choosing the appropriate fluorescent dye with a signal strong enough to enable the FACS sorter to distinguish and separate $CD38^+$ and $CD38^-$ cells as well as by increasing the amount of antibody used to stain the AML cells we could overcome the obstacles. Figure **V.9** shows a representative example of a FACS sorting procedure to separate early and more differentiated AML leukemic stem and progenitor cells and the reanalysis confirming successful sorting into two distinct and pure subpopulations. Up to 10^6 cells of each sorted subpopulation were recovered.

2.6 Generation of single KIR NK cell lines.

Inhibition of NK cells by the engagement of their inhibitory cell surface receptors is a major determinant of immunosurveillance. Since expression of HLA class I on tumor cells is frequently blocking NK cells in their activity, we wanted to circumvent this problem by searching for NK cells whose inhibitory receptors do not find their respective ligands on the patient's cell. Clonal expression pattern of KIR genes makes it possible to select NK cells with single expression of only one type of inhibitory receptor of the KIR family. KIR negative NK cells in turn are anergic and do not show any cytotoxic activity. Mismatching between NK cells and primary patients` cells is possible if AML cells lack the HLA allele coding for a ligand that would be engaging the specific single KIR NK cell. Not all patients are suitable for mismatching with respect to single KIR NK cells, since cells of those patients who do express HLA ligands for all three major inhibitory KIRs on NK cells do not enable for an HLA-KIR mismatch.

As shown in figure **V.10**, using a purified and expanded NK cell culture we excluded the remaining T cells and NKT cells by gating out CD3 expressing cells and further gated on the single KIR expressing NK cells. After sorting, single KIR clones need to be increased in quantity by restimulation cultures as described in chapter **IV 10** and can be used to test their cytotoxic activity on days 10 to 25 after restimulation.

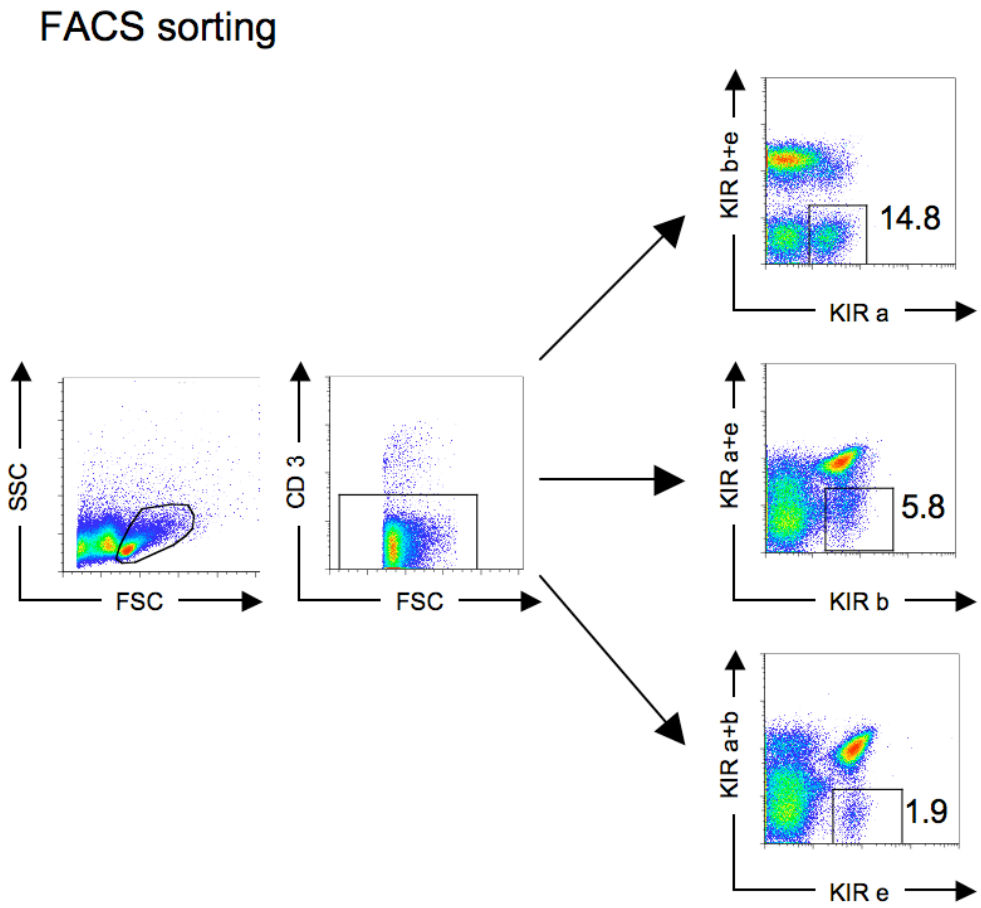

Figure V.10
FACS sorting of NK cells into single KIR NK cell clones.
FACS sorting of purified and expanded primary NK cells according to expression of KIR a, b and e. Single KIR clones are obtained by staining for the respective KIR with a FITC labeled mAb and a mixture of PE labeled Ab recognizing the two other KIR. Shown is one representative example. Numbers refer to the percentages of single KIR NK cell populations.

3 Functional consequences of interaction of LSC of AML and single KIR NK cells

3.1 Serial replating colony forming unit (CFU) assays show diminished colony forming capacity of LSC after treatment with VA and single KIR NK cells.

Increased expression of NK cell ligands on the cell surface of tumor cells is relevant for facilitating the immunorecognition by NK cells and their cytotoxic activity. This could be proven for leukemic cell lines and primary cells (see chapter **V 1.3**). We have also shown that activating ligands can be increased on LSC, but due to the

complex regulation pattern of NK cell activation, a direct proof of the functional consequences for LSC has not been provided yet. In case of LSC, monitoring the colony formation capacity by hematopoietic cells gives a good information about their functionality and possible interactions with NK cells. To do so we designed an experimental setup using serial replating colony forming unit (CFU) assays in semisolid methylcellulose culture media.

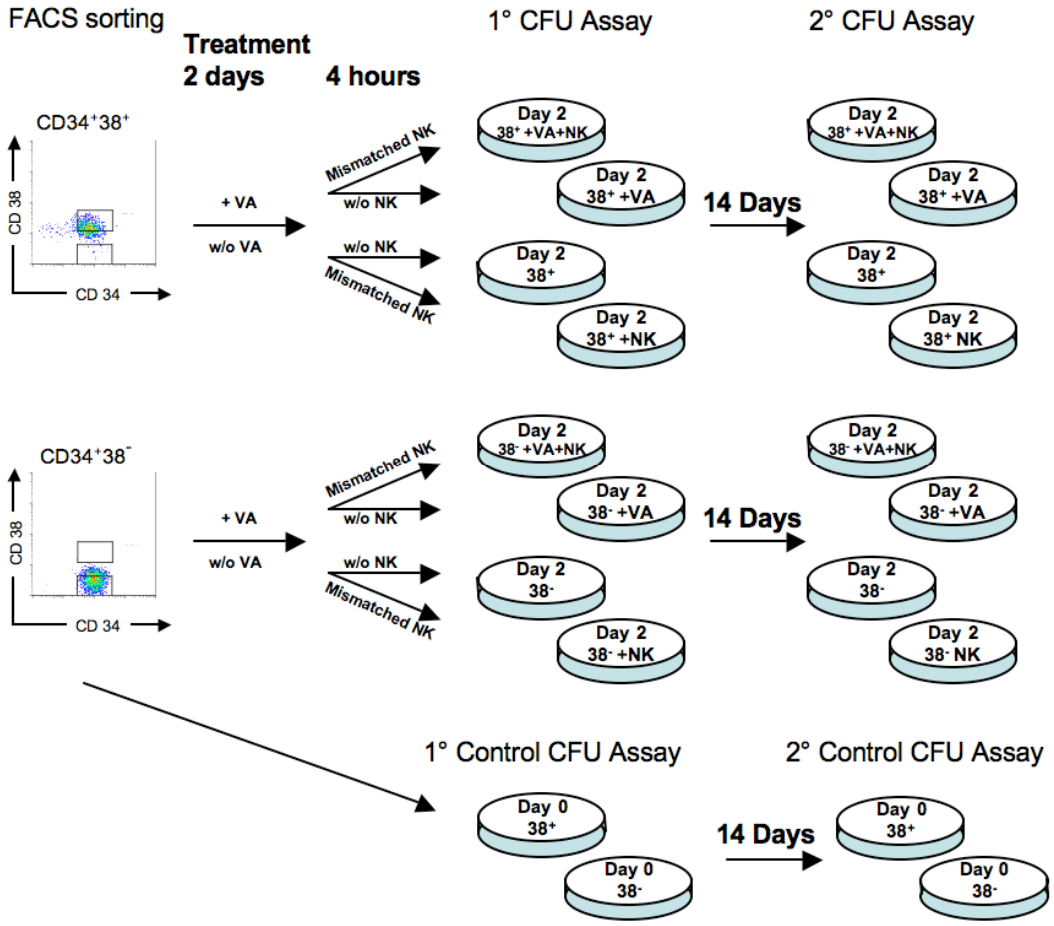

Figure V.11

Scheme of treatment of LSC of AML with valproic acid for serial replating colony forming unit (CFU) assay.

Sorting of early AML leukemic stem cells ($CD45^{dim}CD34^{+}CD38^{-}$) and more differentiated leukemic progenitor cells ($CD45^{dim}CD34^{+}CD38^{+}$) was performed. Cells were either plated in methylcellulose supplemented with growth factors directly on the day of sorting (Day 0) or cultured for 2 days in a suspension culture with medium supplemented with growth factors with or without (w/o) valproic acid (VA). After 2 days cells were counted, cultured for another 4 hours in medium or cocultured with HLA-mismatched NK cells (E:T ratio 5:1) and subsequently plated in methylcellulose (1° CFU). Each methylcellulose culture was incubated for 14 days. Colonies were counted and subsequently replated in a second set of methylcellulose cultures (2° CFU).

As illustrated in figure **V.11**, after sorting of PBMC from AML patients into early and differentiated leukemic stem and progenitor cells ($CD45^{dim}CD34^{+}CD38^{+/-}$), cells were plated directly into methylcellulose supplemented with growth factors (see **IV 8**), referred to as control CFU assays "Day 0". Duplicates for both $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ cells were performed with $1 * 10^5$ cells per plate. In parallel, the same populations of cells were cultured in suspension cultures supplemented with growth factors (see chapter **IV 2**) with or without valproic acid. After 2 days cells were washed and $1 * 10^5$ cells were cultured alone or with mismatched single KIR NK cells for 4 hours in the E:T ratio of 5:1 ($5 * 10^5$ NK cells). Subsequently the sorted leukemic cells were plated together with effector NK cells into methylcellulose in duplicates referred to as 1° CFU assays. After 14 days of incubation, colonies were counted, documented by photography and cells were replated in a second round of methylcellulose (2° CFU assay) with another 14 days of incubation before again scoring the colony formation capacity.

With this experimental setup we were able to serially replate human primary leukemia-colonies in 1° and 2° methylcellulose cultures. NK cells plated alone do not give any colonies (not shown), indicating that LSC are the only source of colonies in the CFU assays.

Figure **V.12 A** is showing the CFU results obtained with $CD45^{dim}CD34^{+}CD38^{-}$ and $CD45^{dim}CD34^{+}CD38^{+}$ cells from 3 AML patients (AML 2, AML 5 and AML 6). The CFU numbers in untreated samples (figure **V.12 A**, left column, Day "0") differed in between the individual patients but there was a tendency to a stronger colony forming capacity in the initial CFU assay (1° CFU) and after replating (2° CFU) for the early LSC as compared to the more differentiated progenitors in all the three examples performed. The $CD34^{+}CD38^{+}$ cells seem to have a limited self renewing capacity as determined by only few or absent colonies in the 2° CFU.

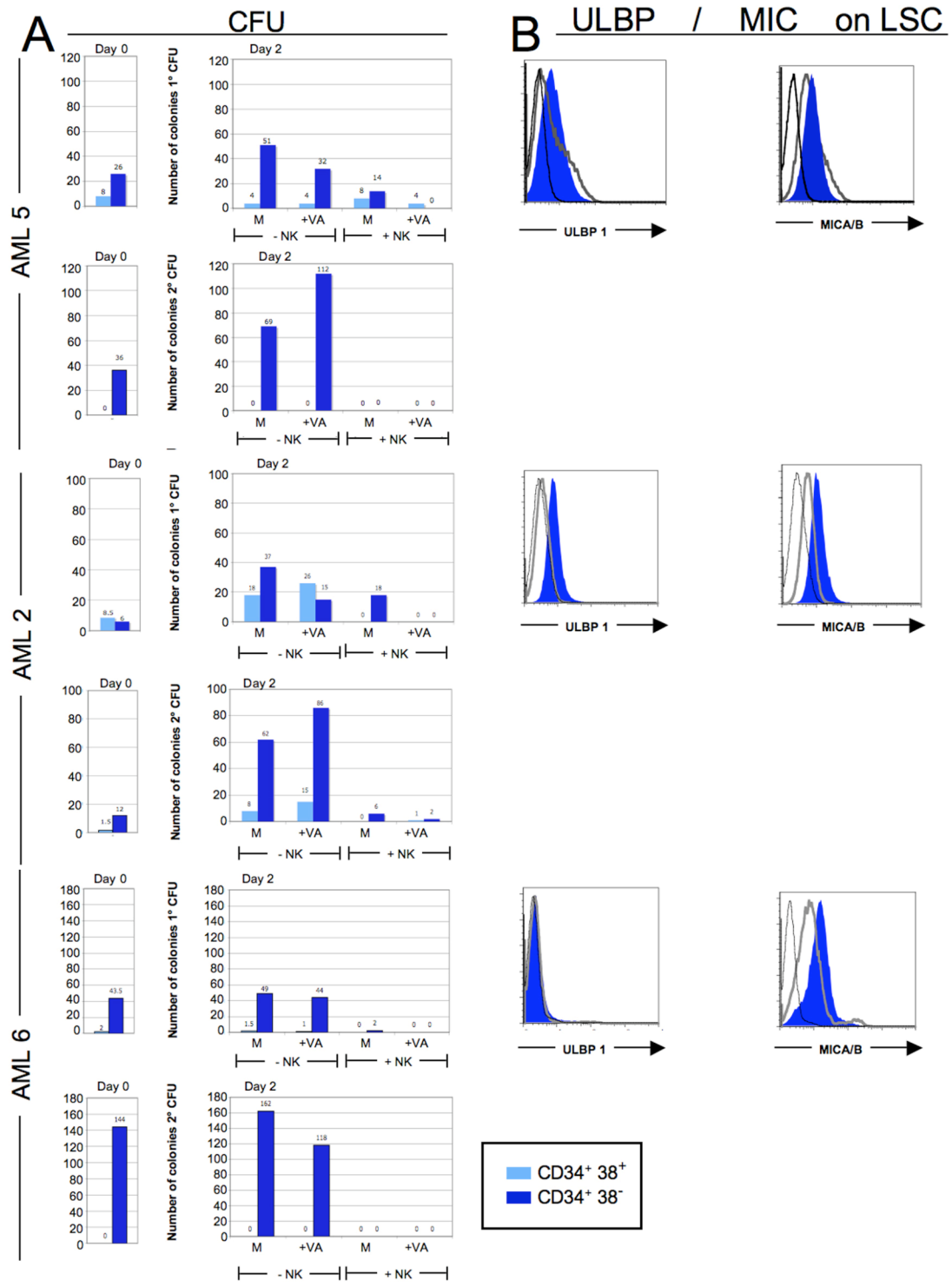

Figure V.12

Serial replating CFU assays of AML LSC after treatment with VA and HLA-mismatched single KIR NK cells.

After sorting of early ($CD45^{dim}CD34^{+}CD38^{-}$, dark blue columns) and more differentiated ($CD45^{dim}CD34^{+}CD38^{+}$, light blue columns) LSC of three AML patients (AML 5, 2 and 6), 1 * 10^5 cells were plated in methylcellulose directly after sorting (day 0), the rest were incubated in medium supplemented with GF with or without VA. After 2 days cells were cocultured with HLA-mismatched NK cells for 4 hours and subsequently plated in methylcellulose. (A) shows the number of colonies of the primary culture (1° CFU) and after replating (2° CFU) for early and differentiated LSC. In B, histograms of upregulation of ULBP1 and MICA/B as ligands for NK cells by treatment with VA for 2 days (solid area) compared to culturing in medium + GF (thick grey line) and secondary Ab alone (thin black line) on early LSC ($CD45^{dim}CD34^{+}CD38^{-}$) of the respective patients are shown.

Upon suspension culture, without as well as with treatment with VA, cells having the early LSC phenotype showed the much stronger colony forming and self renewing capacity than their more differentiated counterparts (figure **V.12 A**, 2nd column). VA treatment itself seemed to increase the self renewing capacity of early LSC as judged by higher CFU numbers in the second round of replating (2° CFU, AML 5 and AML 2) as compared to 1° CFU. Treatment with HLA-mismatched single KIR NK cells had an important influence of LSC, reducing the numbers of colonies formed in the first round of cultures dramatically and virtually abolishing their replating in methylcellulose. This is indicating a strong effect of NK cells on LSC, presumably due to immunorecognition and cytotoxicity. A combined treatment with VA to induce NK cell ligand expression on LSC and sequentially with HLA-mismatched single KIR NK cells inhibited colony formation already in the initial 1° CFU cultures which was was even more evident in the 2° CFU assays.

Figure **V.12 B** is showing the upregulation of NKG2D ligands of the respective patient's early LSC. In case of every patient included in this experiment, either ULBP1 or MICA/B or both ligands were upregulated in response to VA.

These results are demonstrating the eradication of LSC by NK cells on the basis of the functional assay of CFU cultures.

3.2 Control serial replating CFU assays show no effect of NK cells and VA on HSC and demonstrates importance of KIR-HLA-mismatch.

To validate the results obtained by treating and culturing leukemic stem and progenitor cells, the same experimental procedure was performed with normal HSC derived from bone marrow of healthy donors. This control has been performed to evaluate the potential side effects of VA and of mismatched single KIR NK cells, which might be detrimental to the residual normal HSC and normal hematopoiesis. $CD34^+CD38^-$ (HSC) and $CD34^+CD38^+$ progenitor cells were sorted from the bone marrow of 2 healthy donors (BM 1 and BM 2) and plated to measure 1° and 2° CFU capacities.

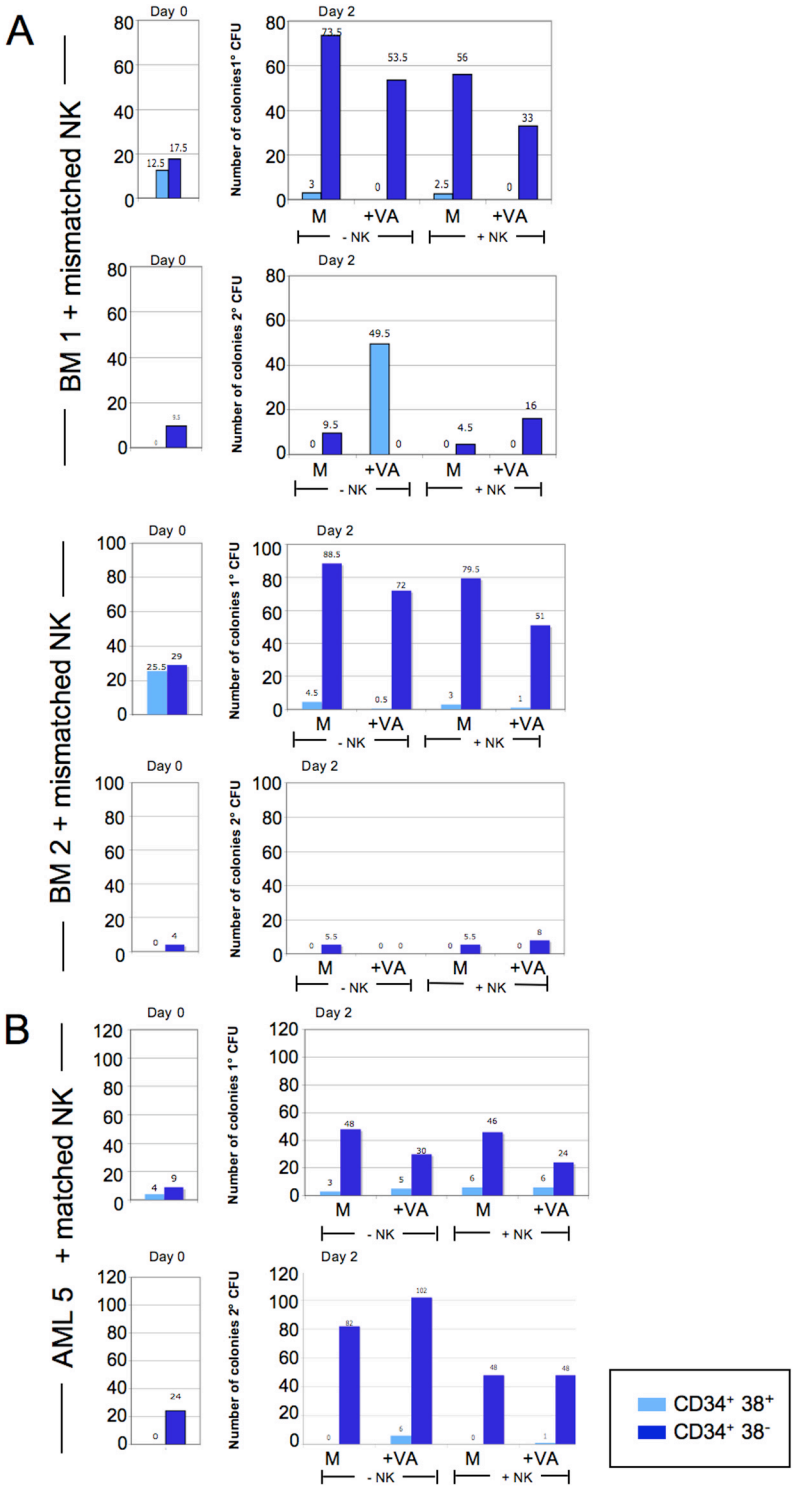

Figure V.13

Control serial replating CFU assay with normal HSC after treatment with VA and HLA-mismatched single KIR NK cells and AML-LSC with VA and HLA-matched NK cells.

Control experiments with normal hematopoietic stem cells sorted from healthy BM and treated with VA and mismatched NK cells shows no effect of VA and NK cells on HSC (A) . Treatment of AML leukemic stem cells with VA and matched NK cells had no effect on the colony forming capability as well, indicating that the efficiency of an HLA-mismatch of NK cell and LSC is triggering immunorecognition (B) .

Cells from BM 1 and BM 2 donor showed the self-renewing capacity judged by replating only in the early HSC subpopulation (figure **V.13 A**). Treatment with VA was not deteriorating the colony formation capacity in either the initial round of CFU assay (1° CFU) as well as after replating (2° CFU). Single KIR NK cells selected for an HLA mismatch reduced, but did not abolish neither colony forming capacity (1° CFU) nor replating capacity (2° CFU).

Treating AML leukemic stem cells with VA and HLA-matched single KIR NK cells has underlined the importance of circumventing inhibition of NK cells by HLA ligands. In comparison to *mis*matched NK cell treatment of AML leukemic stem cells (AML 5, figure **V.12 A**), matched NK cells did not alter the plating (1° CFU) and replating (2° CFU) characteristics (AML 5, figure **V.13 B**).

3.3 VA induces differences in morphology of colonies in CFU assay.

Colonies formed by early LSC of AML in CFU assays displayed a morphology distinct to colonies found in cultures of normal HSC. Leukemic clones were more uniform, bigger in size, less compact and showed a more diffuse growth (figure **V.14**, AML 5 and AML 6 Day 0). Colonies of healthy HSC were heterogeneous in form, showing differentiation into different lineages and had an altogether smaller size (figure **V.14**, BM 1). Treatment by VA did not change the morphologic aspect in HSC cultures (Day 2, +VA) and even treatment with HLA-mismatched single KIR NK cells had no influence not only on the number of colonies (figure **V.13**) but also on their morphology (figure **V.14**). In CFU assays with LSC of AML, treatment with NK cells abolished colony-forming capacity and therefore did not allow the assessment of morphology of colonies. VA alone was changing the morphology of AML CFU into small, compact colonies showing no diffuse growth into the surrounding area (figure **V.14**, AML 5 and AML 6, Day 2, VA).

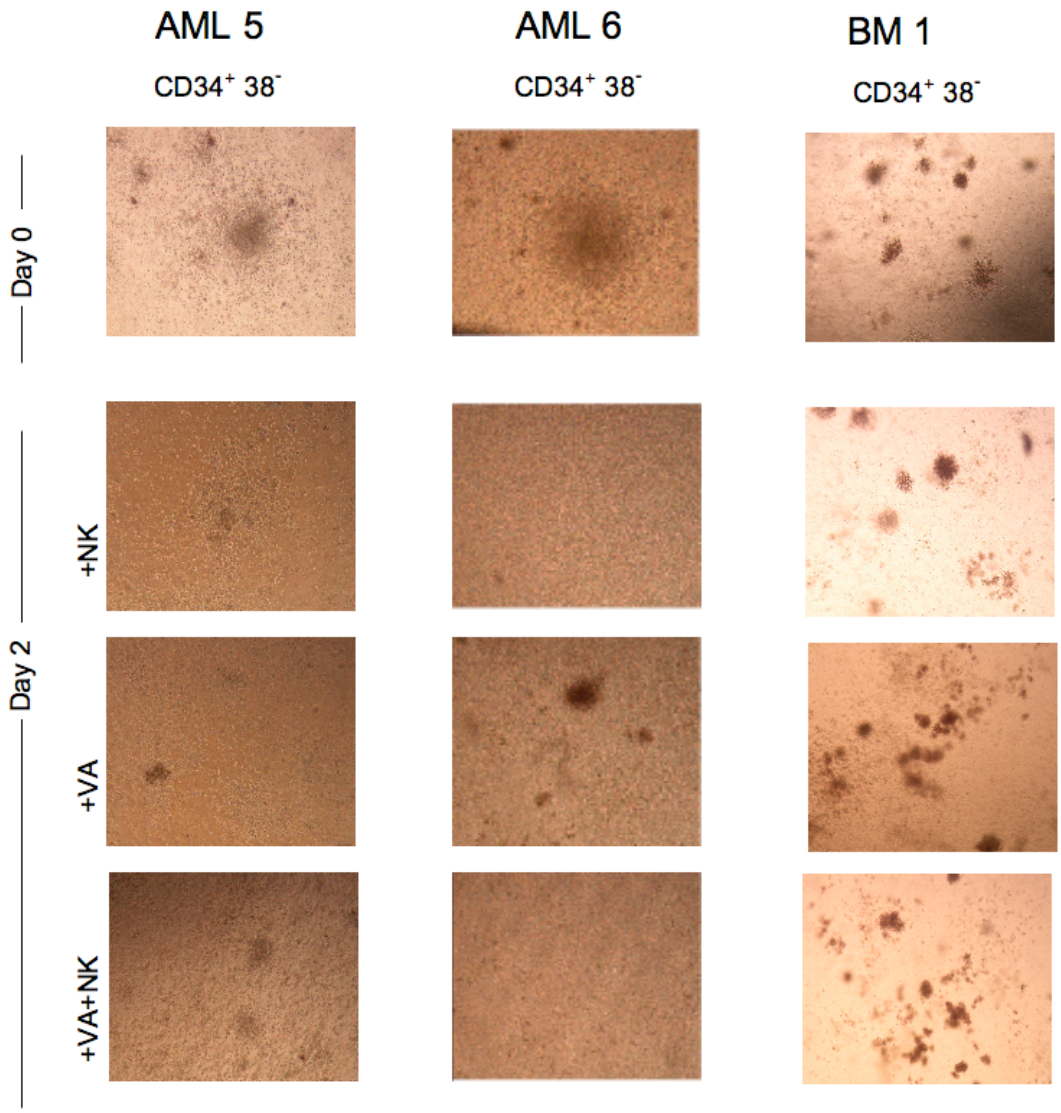

Figure V.14
Pictures of colonies in serial replating CFU assay
Pictures of colonies in CFU assays 14 days after plating $CD34^{+}CD38^{-}$ LSC after treatment with VA and mismatched NK cells. AML-LSC showed different morphologies of the colonies than normal HSC with bigger but fewer colonies. Treatment with NK cells lead to an abolishment of the colony forming capacity of AML-LSC, but not normal HSC in 1° CFU cultures. Magnification : x 40.

3.4 Cell surface expression of NKG2D ligands induced by VA increases susceptibilityof AML blasts to NK cell killing.

The complexity of NK cell-tumor cell interaction with multiple activating and inhibitory signals integrated into a final NK cell response makes it difficult to predict the final outcome of a change introduced by one single interaction of one receptor-ligand pair. In analogy to the functional cytotoxicity assay described in chapter **V 1.3** we wanted to address the question whether the upregulation of cell

surface ligands for NKG2D on total leukemic blasts induced by VA would lead to an enhanced immunorecognition and antitumor activity by NK cells.

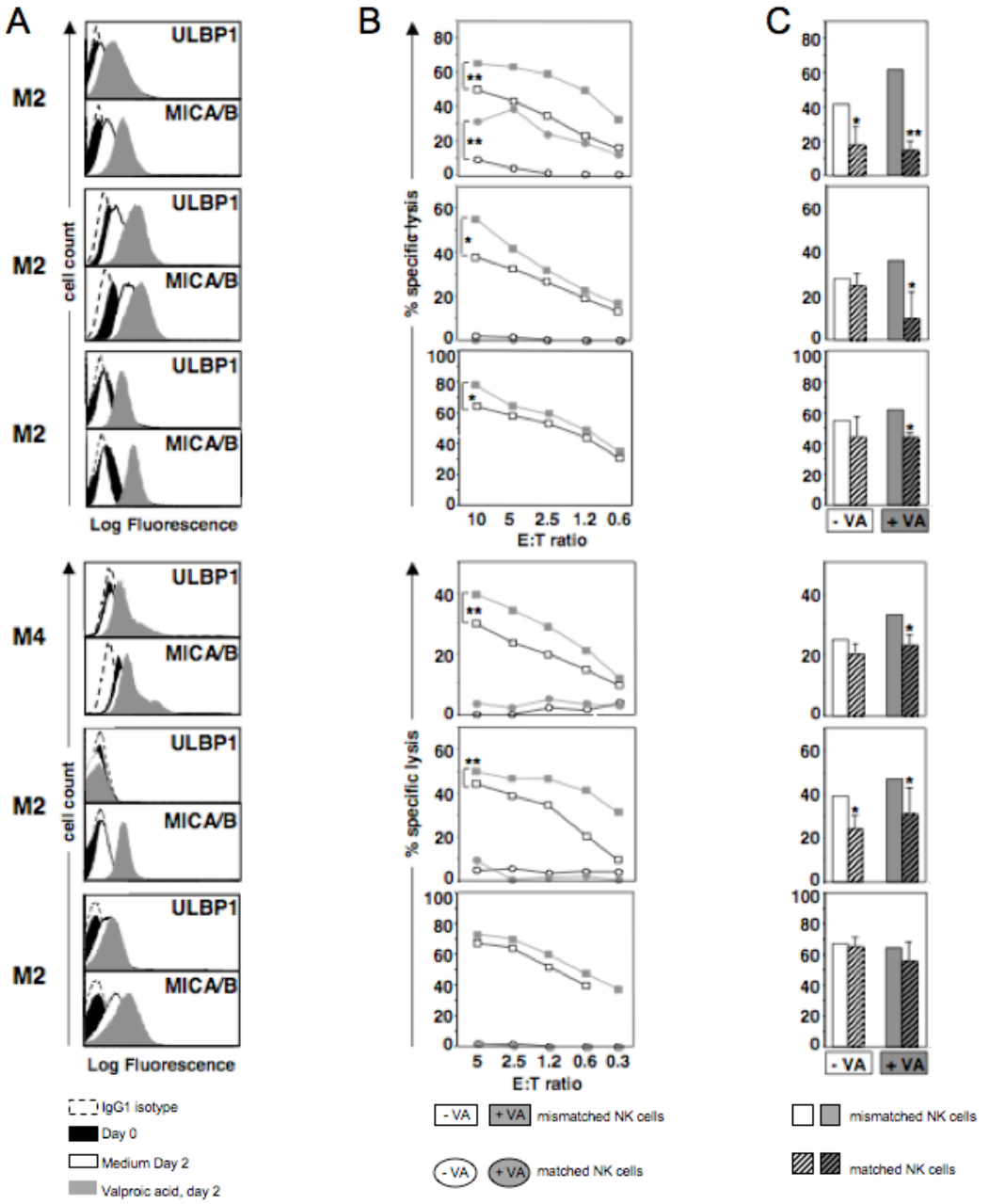

Figure V.15

Increased susceptibility of leukemic blasts to NK cells following treatment with VA.

(A) FACS analysis of ULBP1 and MICA/B expression levels by leukemic blasts from 6 AML patients untreated (black area), and after 2 days treatment with medium supplemented with growth factors alone (black line) or VA (grey area); broken line, isotype-specific mAb staining. (B) Specific lysis of cells treated with medium alone (open symbols) and VA (filled symbols) by HLA-mismatched (squares) and matched NK cells (circles). *P <0.05; **P<0.01; significant difference between cytolysis of VA-treated and VA-untreated AML blasts by NK cells. (C) Reduction of specific lysis of AML cells by α-NKG2D blocking mAbs. HLA-mismatched NK cells were preincubated with blocking mAbs and used as effectors against AML cells cultured in medium without VA (open bars) and with VA (grey bars). The effect of α-NKG2D mAbs is the average of results obtained at 3 NK cell:target cell ratios of 10:1 (or 5:1), 2.5:1 and 1.2:1. Lysis after use of α-NKG2D mAbs is shown as stripped bars. No blocking was observed with control IgG1 Abs (not shown). *P <0.05; **P<0.01; significant difference between lysis of VA-untreated and VA-treated AML blasts in the presence of α-NKG2D mAbs.

Total unfractionated blasts from 6 AML patients were treated with VA. After monitoring the effect on cell surface expression of NK cell ligands by FACS, cells were cocultured for 4 hours with HLA-mismatched NK cells and analyzed in a ^{51}Cr-release cytotoxicity assay. The two types of controls were VA-untreated cells, as well as VA-treated cells but exposed to HLA-matched NK cells. The result obtained with 6 individual examples showed increased expression of ULBP1 and MICA/B (figure **V.15**, 1st column) as well as the positive effect on immunorecognition and killing by NK cells (figure **V.15**, 2nd column). The difference between the killing without treatment with VA and killing of VA-treated targets is demonstrating the specific effect of this pharmacologic treatment. In all these examples, HLA-matched NK cells showed a remarkably lower impact on tumor lysis (figure **V.15**, 2nd column). To confirm that increased killing is caused by increased NKG2D-ligand interaction, blocking experiments with anti-NKG2D antibodies were performed (figure **V.15**, right column).

Addition of blocking anti-NKG2D antibodies partly abolished the cytolysis, and the blocking effect was even stronger with VA-treated than VA-untreated cells, indicating the contribution of NKG2D-ligand interactions to VA-modulated lysis of AML cells. These data demonstrates a role of HDAC inhibitors in enhancing the recognition and cytolysis of AML blasts by alloreactive NK cells.

3.5 Functional consequences of treatment with VA on immunorecognition of LSC by NK cells.

To estimate whether alloreactive NK cells are able to interact with LSC and to evaluate possible approaches to increase such interaction, we performed cytotoxicity assays with sorted AML LSC and HLA-mismatched NK cells as effectors.

Therefore we were sorting early and differentiated LSC from PBMC of AML patients and cultured them for 2 days with or without VA. The cells after pharmacologic treatment were used as targets and coincubated with HLA-mismatched NK cells. Figure **V.16** is showing the specific lysis of LSC ($CD34^+CD38^-$, left column, light grey) and leukemic progenitor cells ($CD34^+CD38^+$, middle column, dark grey) from 3 independent patients (AML 5, 6 and 10). Cells of all patients showed an increased cell surface expression

of ligands for NKG2D after treatment with VA, possibly enabling NK cells for an increased recognition. All 3 patients demonstrated an immunorecognition of LSC and leukemic progenitor cells by HLA-mismatched NK cells as judged by specific cytolysis over all E:T ratios, providing a proof that NK cells are able to identify and lyse LSC.

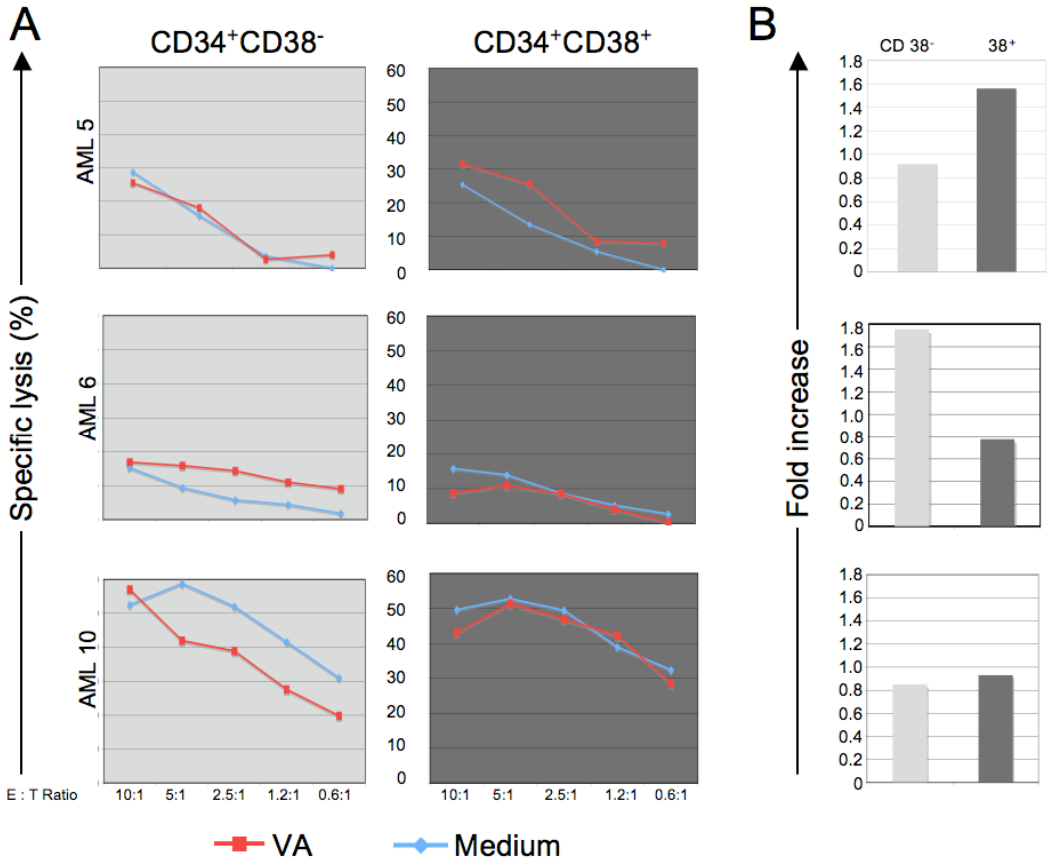

Figure V.16
Specific lysis of LSC after VA treatment
(A) Cytotoxicity assay of early ($CD34^+CD38^-$, left column, light grey) and more differentiated ($CD34^+CD38^+$, middle column, dark grey) LSC of 3 AML patients after sorting. Untreated (medium alone, blue line) and cells treated for 2 days with VA (red line) were exposed to HLA-mismatched NK cells. The specific lysis in different E:T ratios from 10:1 to 0.6:1 are shown. (B) Fold increase of specific lysis induced by treatment with VA in average of all E:T ratios. Triplicates of all reactions have been performed.

Patient AML 5 (figure **V.16** 1st row) showed no increase in the specific lysis of VA-treated LSC in comparison to untreated LSC over the different E:T ratios. However, the more differentiated leukemic progenitor cells were subjected to an enhanced specific lysis after treatment, with an 1.6 fold increase. The specific lysis

against cells of patient AML 6 showed an altogether poor performance with less than 20 % specific lysis (figure **V.16** 2nd row). The recognition of early LSC population was increased due to VA treatment with an 1.7-fold increase whereas the immunorecognition of more differentiated progenitor AML cells was not affected. Cells of the third patient (figure **V.16** 3rd row) were strongly responding to HLA-mismatched NK cells as effectors and showing a high specific lysis of LSC even without VA-treatment (medium). Surprisingly, VA treatment of LSC was accompanied by a decreased lysis by NK cells despite an upregulation of activating NKG2D-ligands.

Treatment with VA is not effective in all of the cases in increasing the immunorecognition and thereby specific lysis. Nevertheless, an interaction of LSC with HLA-mismatched NK cells could be proven by demonstrating a consecutive specific lysis of LSC and leukemic progenitor cells. A lack of an effect of the upregulation of cell surface expression of activating ligands for NK cells on the immunorecognition is indicating that recognition of target cells by NK cells is an complicated process regulated by multiple factors.

4 *In vivo* assay of the effect of VA and KIR-HLA-mismatched NK cells on LSC

4.1 NOD/SCID transplantation of human leukemia and treatment in mice serves as disease model *in vivo*.

To assess the ability of the combined treatment of VA and HLA-mismatched NK cells to target leukemic cells in vivo, we established a model of AML transplantation into NOD/SCID mice by intrafemural (i.f.) injections. Patients cells were chosen by the criteria of a profound engraftment in mouse bone marrow in preceding test transplantations (see table **V.1**), existence of an HLA class I mismatch in respect to single KIR NK cells, an effect of VA on the upregulation of cell surface expression of NKG2D ligands and availability of sufficient quantity of cells to perform transplantation in a group of at least 25 mice.

Table **V.1** is showing the result of tests for the efficiency of engraftment after i.f. transplantation using primary patients mononuclear cells of peripheral blood

(PBMC) of 13 individual AML patients. For these tests, except of irradiation no additional preparatory treatment was applied to mice, especially no TM-β1 Ab. The engraftment is varying from 0 % to 65 % of human cells in the mouse bone marrow 4 weeks after transplantation. Migration of transplanted cells from the injected to noninjected bone or peripheral blood is low in general. In summary, the level of engraftment is not dependent on either blast content, CD34 expression of blasts or FAB subtype of AML. The 2 individuals chosen according to the criteria mentioned above for the following NOD/SCID transplantation and treatment experiment are marked with asterisks.

Table V.1: Test of engraftment of AML in NOD/SCID mice

			% of human blasts in:		
AML	blasts in PB	$CD34^+$ of blasts	Injected bone	Noninjected bone	PB
AML 8 *	95 %	45 %	65.5	13	1.4
# 1	83 %	0 %	51	0.1	0
# 2	81 %	1 %	50	2.2	0
AML 7 *	98 %	52 %	41	0.5	0
# 3	85 %	100 %	18.5	12	2
# 4	77 %	37 %	12	0.5	0.3
# 5	65 %	3 %	8	0	0
# 6	87 %	0 %	2	0	0
# 7	96 %	52 %	0.5	0	0
# 8	91 %	1 %	0.2	0	0
# 9	95 %	0 %	0	0	0
# 10	97 %	20 %	0	0	0
# 11	92 5	2 %	0	0	0

For the transplantation series, mice were pretreated with irradiation and TM-β1 Ab, a rat antibody against mouse IL-2 receptor β-chain designated to deplete residual lymphocytes and macrophages and thus enhance subsequent engraftment of human cells. 24 hours after the preparatory treatment, mice were transplanted intrafemurally with $1 * 10^7$ unfracturated total primary AML blasts. At 4 weeks mice were grouped into 4 treatment groups receiving no treatment, treatment of VA

(400 mg/kg = 10 mg/mouse) i.p., HLA-mismatched single KIR NK cells (5 * 10^5 cells) i.v. or a combination of NK cells and VA. Treatment was repeated two more times with a total amount of 1.5 * 10^7 NK cells and 30 mg VA per mouse given in one week. One week after the last treatment, bone marrow of the transplanted femur and nontransplanted bones were further assessed for human AML blast content by FACS (see figure **V.17**).

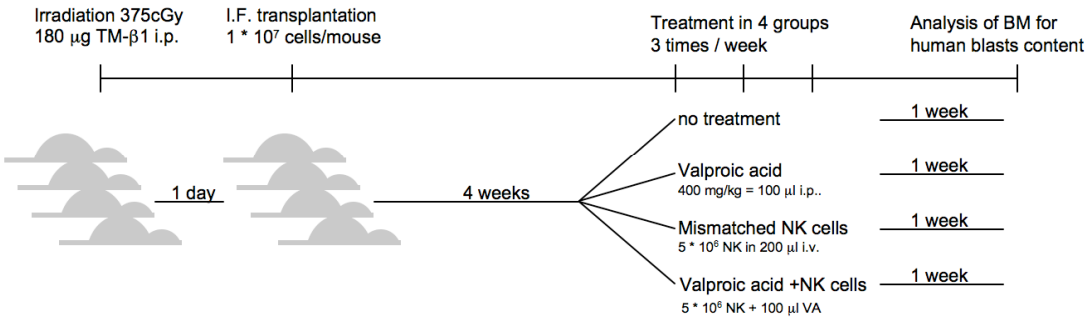

Figure V.17
Scheme of intrafemural transplantation of human AML into NOD/SCID mice.
24 hours after irradiation (375 cGy) and i.p. injection of 180 µg TM-β1 mAb, NOD/SCID mice were intrafemuraly transplanted with 1 * 10^7 human AML cells. Subsequently mice were grouped in 4 treatment groups receiving no treatment, 400 mg/kg valproic acid (VA), corresponding to 100 µl VA i.p., 5 * 10^6 mismatched NK cells i.v. or a combination of VA and NK cells in the same dose than VA or NK cells alone. Treatment was performed 3 times with one treatment free day in between. One week after the end of treatment mice were sacrificed and bone marrow of the injected femur and noninjected bones and PB was analyzed for content of human blasts as judged by expression of human CD45 and CD33 (percentages of viable PI negative cells).

4.2 NK cells and VA fail to influence AML in mice with high blast load.

Figure **V.18** shows the engraftment of human AML of two individual AML patients (AML 7, AML 8). FACS pictures (left columns) indicate the method of assessing the level of engraftment as judged by viable cells (PI negative) carrying the human myeloid markers CD45 and CD33. Depicted are the percentages of human cells in the mouse bone marrow of the individual mice (light blue bars) grouped to the 4 treatment groups and an average of each treatment group (dark blue bar). Mice receiving an intrafemural injection of human unfractionated AML blasts after the preparatory regimen of irradiation and TM-β1 antibody showed an engraftment of

human AML of 15-50 % (“untreated”). On average, cells of patient AML 7 constituted 35 % of the mouse bone marrow, whereas patient AML 8 showed an engraftment of 53 %, both in mice not receiving either VA or NK cells.

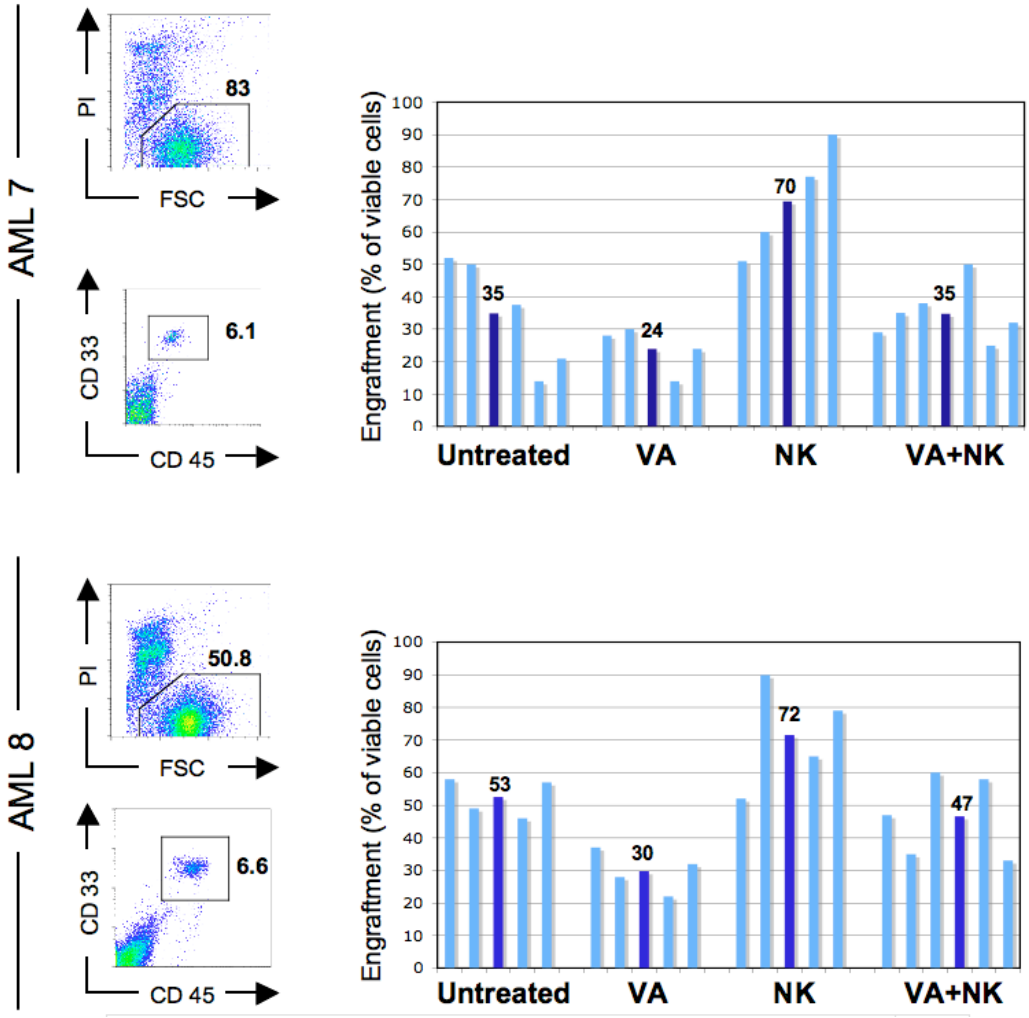

Figure V.18

I.F. Xenotransplantation model for human AML into NOD/SCID mice

NOD/SCID mice transplanted intrafemurally with human AML were grouped 4 weeks after transplantation into 4 treatment groups and received no treatment, VA and/or HLA-mismatched NK cells as indicated. Percentages of human cells on total mouse bone marrow was evaluated by FACS as judged by myeloid human cells (CD45, CD33) of viable, PI negative cells (left column). 2 sets of unfractionated AML blasts from 2 patients (AML 7 and AML 8) were performed. Shown are the percentages of engraftment of the individual mice (light blue bar) and average of engraftment of each treatment group (dark blue bar). Two sets of transplantation experiments with 2 different AML samples were performed and are shown.

Treatment of the mice 4 weeks after transplantation with VA was reducing the blast content in the mouse bone marrow ranging between 24 and 30 % with patient AML 7

and AML 8, respectively as compared to untreated mice. Surprisingly, the treatment with HLA-mismatched human NK cells led to an increased engraftment of human cells in the NOD/SCID mice with both patient's samples (70 % and 72 %, respectively). The combined treatment of VA together with mismatched NK cells (figure **V.18**, "VA+NK") was reducing the extend of human blasts in the mouse bone marrow to the level of those of untreated mice (35 % and 47 %, respectively).

These results show a stable engraftment of human AML blasts after intrafemural transplantation demonstrating the prospects of this method to be performed in search for novel options in the treatment of leukemia. It is underlining as well the need to challenge and adapt *in vitro* findings to the *in vivo* situation.

VI Discussion

1 NKG2D ligands on LSC of AML.

Ligands for NK cell receptors represent a new focus of medical research. Expression of the activating ligands is a possible mechanism facilitating interactions between tumor cells and the immune system, thus promoting immunosurveillance. Accordingly, downregulation of these cell surface ligands or their receptors is a newly recognized immune escape mechanism developed by tumors to circumvent their detection and thereby destruction. Understanding these mechanisms and investigating the regulation and expression of NK cell ligands with the aim to influence their expression is subject of ongoing studies and is of therapeutic relevance.

The expression of ULBP and MICA/B as the most prominent representatives of activating NK cell receptor ligands in AML is proven to be low to absent, reflecting the poor reaction of the body`s immune system to fight the tumor[89]. The reason of an absence of expression of ULBP and MICA/B is at least partially the cleavage of cell surface ligands [122,123]. This is, in turn leading to an additional protection of the tumor cell: immunorecognition is hampered because NKG2D receptor on NK cells becomes internalized by the soluble form of ULBP circulating in plasma[124].

Leukemic cells of AML can be targeted by the classical cytoreductive therapies of irradiation and cytotoxic agents. This induction therapy is leading to a complete remission in 65 - 75 % of patients within the first two cycles of chemotherapy[24]. The high incidence of relapse and finally resistance to therapy is indicating a remaining cell population of AML that is not affected by conventional treatment and able to survive even the postremission therapy. To target this remaining cell population by immunotherapeutic approaches is highly tempting.

By defining the population of AML cells that is the most resistant to treatment and responsible for adverse outcome of the disease, it would be desirable to focus the immunotherapeutic approaches onto this subpopulation. The mere calculation of cell numbers is indicating a bigger chance that this theoretical approach can be efficient. The blast load is often very high – in the range of 1-10 * 10^{11} of malignant cells. Even after cytoreductive chemotherapy the remaining number of leukemic cells can be

estimated as about 1 * 10^9. Considering an estimated total number of residual NK cells as 1-10 * 10^8, the ratio between effector immune cells and tumor cells is highly unfavorable (E : T ratio 0.0001 – 1 : 1).

Consequently, absence of activating ligands for NK cells on leukemic cells is explaining the limited efficiency of HSCT for the treatment of AML. The findings presented here that cell surface expression of ligands for the activating NK cell receptors NKG2D, ULBP and MICA/B (figure **V.1**) and for NCR (figure **V.2**) is low to absent is in line with the observation that AML cells are able to evade immunorecognition. Accordingly, pharmacologically induced upregulation of these ligands by bryostatin-1 is leading to an increased immunorecognition and cytolytic activity of NK cells (figure **V.3**).

Extending the investigations to the leukemic subpopulation of LSC is demonstrating the absence of ligands as on total blasts as well (figure **V.5**). However, a targeted therapy with the intention to increase the expression of activating ligands on LSC with the HDAC inhibitor VA is possible (figure **V.8**). This increased cell surface expression of ligands for NK cells has the functional consequences of enhanced immunorecognition, which can be proven by serial replating CFU assays in methylcellulose (figure **V.12**).

Interestingly, cytotoxicity assays are demonstrating an interaction of KIR-HLA mismatched NK cells and LSC of AML even without treatment of VA (figure **V.16**). Obviously, other receptor-ligand pairs than the ones examined here are playing important roles in immunorecognition as well. Furthermore, an increased cell surface expression of the ligands for NK cells on LSC is not necessary leading to enhanced immunorecognition (figure **V.16**), underlining the complexity of the activation mechanisms of NK cells.

Not only ULBP and MICA/B ligands, but also other activating ligands such as molecules recognized by NCR on NK cells are apparently not expressed in a sufficient level to overcome inactivity of NK cells as well. Identification of the ligands for the NCR NKp30, NKp44 and NKp46 on NK cells would increase the possibilities to characterize interactions and intervention possibilities.

The unequivocal phenotypic definition of LSC remains difficult. Due to a lack of established markers which are identifying and distinguishing normal HSC and LSC, the most widely used marker for hematopoietic and leukemic progenitor cells is CD34. This is a glycoprotein, expressed on normal HSC and down-regulated during

maturation[125]. Due to their common origin it is not surprising that leukemic stem cells share the CD34-expression with normal HSC[37]. Since CD34 expression does not enable to distinguish normal from leukemic stem cells and since AML cells in some individuals are negative for CD34, additional markers for early stemness in AML are searched for[35,126,127]. Up to now there is no alternative reliable marker than CD34 for the phenotypic description of AML LSC. Choosing this marker for experiments with primary AML cells is associated with a selection of patients who express CD34 at least on subpopulations of their blasts. Only functional assays like xenotransplantations of whole blasts where no phenotypical characterization of the LSC subpopulation is necessary can circumvent this selection bias. Another selection bias is associated with the difficulty that not all primary AML blasts allow *in vitro* culturing. A cytokine mixture in the culture medium supplying essential growth and survival signals necessary to keep primary cells viable in cell suspension culture might influence any cellular process as well. This selection is skewing interpretation and generalization of data acquired in experiments with primary patient material. Despite the experimental shortcomings, the information provided by studies with primary human cells is extremely valuable and superior in many aspects to the data obtained with cell lines.

2 Immunorecognition of LSC by NK cells.

The induction of cell surface expression of ligands for the receptor NKG2D on NK cells by valproic acid described here is tempting as it may lead the way to enhance immunorecognition. LSC are important determinants of an adverse outcome in AML patients, thereby are representing the main cell population to be treated. Methodically, immunorecognition and killing can be demonstrated by a direct interaction of tumor cell and NK cell in a cytotoxicity assay, and further functionally by analyzing colony forming ability in CFU assays. In this study, both methods were applied to monitor the LSC behavior and demonstrated an increased immunorecognition of AML LSC by NK cells, thus providing a promising outlook for scenarios closer to the clinical situation. By applying the experimental setup to the *in vivo* setting it would be possible to test an immunorecognition of LSC under

in vivo conditions, where localization and interactions with the stem cell niche within bone marrow microenvironment might exert important regulatory effects. *In vivo* experiments with cells of two AML patients in the NOD/SCID xenotransplantation model did not recapitulate the beneficial effects of NK cells and VA seen *in vitro*. Considering the multifactorial circumstances in mice it is impossible to determine a single reason of the observed ineffectiveness. One possible explanation is the quantity of tumor cells that needs to be eradicated by NK cells to show any measurable effect. By administering $1 * 10^7$ cells localized into the injected bone and $5 * 10^6$ NK cells given 3 times i.v. and distributed throughout of the whole mouse body, the NK cell- tumor cells proportions might be not sufficient to markedly influence the leukemic burden. Investigations into the distribution of human NK cells in a mouse body after i.v. injection was providing evidence for the presence of human NK cells in the mouse bone marrow. However, 24 hours after injection, only 0.03 % of the mouse bone marrow was estimated to be of human NK origin[128]. Therefore the quantity of NK cells and thereby the ratio between NK cell and target cell might be too low to efficiently lyse leukemic cells and have an influence on the course of the disease. A modified experimental protocol is therefore foreseen and it is introducing a chemotherapeutic treatment step in mice before the actual NK cell administration. This is done with the intention to reduce tumor burden and shift effector-target ratio to more advantageous numbers. It is expected that chemotherapeutic treatment with arabinosyl-cytosin[129] will preferentially destroy mature leukemic blasts and leave the LSC relatively intact, thus will bring the experimental system closer to a clinical setting of AML treatment in human.

3 Effect of VA on normal and leukemic cells.

HDAC inhibitors in general and valproic acid in particular are in the focus for an innovative therapy for tumor disease since recently. Experimental data on the effect of epigenetic modification of gene expression by HDAC inhibitors on tumor cells demonstrate an activity in inducing cell cycle arrest, causing cell death and apoptosis of transformed cells[109,110]. An anti-tumor effect could also be shown *in vivo* in tumor

bearing mice[113,114]. Focusing on the blasts of AML, several groups could show that HDAC inhibitors are able to differentiate AML tumor cells to mature blasts and induce apoptosis *ex vivo*[130]. Applied in the human setting, it could be shown that VA improves the clinical parameters of AML patients by reducing the blast cell quantity and leads to a peripheral hypergranulocytosis as a result of restored normal hematopoiesis in the bone marrow[131] as well as dominance of normal hematopoiesis over the malignant clone[132]. These effects can not only be explained by induction of differentiation and apoptosis, but other mechanisms of action are postulated[131]. For solid tumors as well as for leukemia, an increase of cell surface expression of ligands for activating NK cell receptors upon treatment with VA could be demonstrated[92,121]. This upregulation was causing increased immunorecognition and killing, giving a possible explanation for additional effects of valproic acid beside of induction of apoptosis and differentiation. The induction of cell surface expression of ligands for NK cells would offer an interesting additional treatment option complementing immunotherapies like HSCT and donor lymphocyte infusions (DLI) in AML patients.

Following investigations of the effect of VA on tumor cells, the effect on HSC with respect to differentiation, proliferation and influence on the self-renewal capacity was examined. It was shown that inhibiting HDAC by VA is increasing both proliferation and self-renewal of normal HSC *in vitro*[131]. The cell cycle progression was accelerated. The molecular basis of this finding was shown to be due to a down-regulation of $p21^{cip\text{-}1/waf\text{-}1}$, a cyclin-dependent kinase (CDK) inhibitor, as well as inhibition of the protein kinase GSK3β which in turn is activating Wnt signaling[131]. Our own investigations into the consequences of treatment with VA on the expression of ULBP and MICA/B as ligands for NK cells revealed that normal hematopoietic stem cells do not show a significant increase in cell surface expression of these molecules. Clinically, the long term experience with VA as anti-epileptic drug without adverse effects noted on the hematopoietic system is providing additional strong arguments against a deleterious side effect of this drug.

Whether HDAC inhibitors and VA in particular have an effect on AML LSC has not yet been described. The effect of VA on progenitor cells of AML was reported to be in favor of quantitative proliferation with colonies bigger than the control in methylcellulose assays[133]. An increase in cell count is in line with our finding that VA

is increasing the number of colonies (figure **V.12**). However the concern stated by the authors[133] that VA could stimulate leukemic progression seems not to be justified in a combined treatment protocol with VA and KIR-HLA mismatched NK cells. Furthermore, to estimate an existing hazard for leukemic progression, self-renewal is of bigger importance than mere number of cells or colonies. Maturation of leukemic progenitor cells or stem cells is most likely going along with an increase in leukemic blast number, which is not sustained owing to a depletion in self-renewing LSC. This aspect is supported by our finding that in primary AML the more differentiated $CD34^+CD38^+$ stem cells have a higher capability to form colonies in the first plating in CFU assays, but not in replating (2° CFU) (figure **V.12**). These cells seem to be terminally differentiated and supposedly less harmful in a course of future relapses.

4 Clinical consequences for AML treatment.

AML therapy is up to now a frustrating endeavor due to high incidence of relapse and short survival times. Hence additional treatment options need to be introduced. Multiple clinical trials are evaluating novel strategies to replace or support conventional therapies, which are burdensome for the patient but nevertheless without showing an overwhelming long-term success. Treatment with VA is one of the pharmacological intervention under evaluation. The novel strategy with HLA-mismatched NK cells together with VA presented here is combining two different approaches. The synergy between them make them an interesting tool in anti-leukemic therapy. Both strategies individually as well as in combination seem to be of little harm[134] – thereby in contrast to the burdensome conventional treatment. The interesting finding *in vitro* presented here that VA is helping NK cells to interact and recognize tumor cells is indicating an explanation for the phenomenon that VA as a solely agent without NK cells was of limited benefit in preliminary clinical trials even though good experimental data *in vitro* for induction of apoptosis and differentiation are existing. HCST and consecutively GvL effect as well as NK DLI are leading to a cure of AML disease only in limited cases. Apparently small subpopulations of AML are able to escape immunorecognition even after HSCT

or NK DLI[134]. A selected mismatch according to the HLA-class I allotype of the patient`s target cell with the donor`s single KIR effector NK cells as NK cell DLI could be an additional treatment option in AML patients, overcoming the inhibition of effectors by inhibitory KIR receptors.

NK cells provide an array of further advantages with respect to HSCT treatment of AML. Beside of the cytotoxic effect on tumor cells exerted by NK cells, the engraftment efficiency of stem cells transplanted with a graft containing NK cells is proven to be higher. It is postulated that this effect is caused by a lytic activity on the recipient`s T cells. This is even permitting a reduced toxicity conditioning regimen before transplantation[95]. Furthermore, based on the ablation of dendritic cells (DC) of the recipient, which are the major trigger for graft-versus-host disease (GvHD), allogeneic NK cells are protecting the host from suffering GvHD[135].

The combination of both VA and HLA mismatched NK cells has hereby shown *in vitro* to enable the beneficial interaction and is advocating the addition of valproic acid as a not harmful, but beneficial drug into the existing NK cell experimental therapeutic strategies.

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III Publications

Papers

Luhm J, Langenkamp U, Hensel J, Frohn C, Brand JM, Hennig H, Rink L, Koritke P, Wittkopf N, Williams DL, Mueller A: Beta-(1-->3)-D-glucan modulates DNA binding of nuclear factors kappaB, AT and IL-6 leading to an anti-inflammatory shift of the IL-1beta/IL-1 receptor antagonist ratio. BMC Immunol, Mar 22;7:5, 2006.

Rohner A, Langenkamp U, Siegler U, Kalberer CP, Wodnar-Filipowicz A: Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemia cells to natural killer cell-mediated lysis. Leuk Res, Oct; 31(10):1393-402, 2007.

Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, Hofsteenge J, Gratwohl A, Tichelli A, Paluszewska M, Wiktor-Jedrzejczak W, Kalberer CP, Wodnar-Filipowicz A: NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. Blood, Feb 1;111(3):1428-36 2008.

Langenkamp U, Siegler U, Kalberer CP, Gratwohl A, Wodnar-Filipowicz A: Colony-forming AML leukemic stem cells are susceptible to immunorecognition by NK cell lines with single KIR-HLA class I specificity. Manuscript in preparation, 2008

Conference abstracts

Langenkamp U, Rohner A, Siegler U, Kalberer CP and Wodnar-Filipowicz A: Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of AML cells to NK cell-mediated lysis. Annual Meeting of the European Group for Blood and Marrow Transplantation, Hamburg 2006, oral presentation.

Baeriswyl V, Langenkamp U, Wodnar-Filipowicz A and Kalberer CP: Lentiviral delivery of siRNA abbrogates NKG2D receptor functions in human NK cells. Swiss Society for Allergology and Immunology Zürich 2006.

Kalberer CP, Langenkamp U, Himmelreich H, Diermayr S, Siegler U and Wodnar-Filipowicz A: Allorecognition of AML blasts and stem/progenitor cells by NK cell lines with single KIR-HLA class I specificities. Natural Killer Cell Symposium 2008, Bad Herrenalb 2008.

Langenkamp U, Siegler U, Diermayr S, Gratwohl A, Kalberer CP, Wodnar-Filipowicz A: Specific targeting of colony-forming AML leukemic stem cells by combined treatment with HDAC inhibitors and alloreactive NK cells. Annual Meeting of the European Group for Blood and Marrow Transplantation, Florence 2008, oral presentation.

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