Tumor Recognition by Natural Killer Cells in Acute Myeloid Leukemia and after Hematopoietic Stem Cell Transplantation

Inauguraldissertation

zur
Erlangung der Doktorwürde
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Pegah Nowbakht
aus Riehen (BS)

Basel, 2005

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. sc. tech. Beat Ernst, Fakultätsvertreter
Prof. Dr. phil. Peter Erb, Externer Korreferent
Prof. Dr. phil. Aleksandra Wodnar-Filipowicz, Korreferentin und Dissertationsleiterin

Basel, den 11.01.05

Prof. Dr. sc. techn. Hans-Jakob Wirz,
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

Table of Contents

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APC	Allophycocyanin
APCs	Antigen presenting cells
BM	Bone marrow
BSA	Bovine serum albumin
CD40L	CD40 ligand
CFU-GM	colony-forming-units for granulocytes and monocytes
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
DC	Dendritic cell
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FAB	French–American–British
FACS	Flowcytometry (fluorescence activated cell sorting)
FcγRIII	Fcγ receptor III
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Flt3 ligand
G-CSF	Granulocyte colony stimulating factor
GFs	Growth factors
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glysosylphosphatidylinositol
gtαms	Goat anti-mouse
gtαrat	goat anti-rat
GvH	Graft-versus-host
GvHD	Graft-versus-host-disease
GvL	Graft-versus-leukaemia
h/hu	human
HA	Hemagglutinin
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
iDC	immature dendritic cell
IFN	Interferon

IL	Interleukin
IL-2Rαβγ	heterotrimeric IL-2 receptor
IL-2Rβγ	heterodimeric IL-2 receptor
IMDM	Iscove's modified Dulbecco's medium
ITAM	Immunoreceptor tyrosine based activation motive
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulinlike receptors
LFA-1	Lymphocyte-function associated antigen-1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-acivated cell sorting
M-CSF	Macrophage colony-stimulating factor
mDC	mature dendritic cell
MDS	Myelodysplastic syndrome
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC class I-related chain
MIP	Macrophage inflammatory protein
MNC	Mononuclear cell
mPB	mobilized PB
NCR	Natural cytotoxicity receptors
NK	Natural killer
PB	Peripheral blood
PE	Phycoerythrin
PerCP	Peridin chlorophyll protein
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
Rae1	Retinoic acid early inducible 1 proteins
RT	Reverse transcription
s	soluble
SCF	Stem cell factor
TCR	T cell receptor
TNF	Tumor necrosis factor
Tpo	Thrombopoietin
ULBP	UL16 binding protein
VLA-4	Very late after activation antigen-4

I. Summary

Natural killer (NK) cell-mediated cytolytic activity against tumors requires the engagement of activating NK cell receptors by the tumor-associated ligands. Here, we studied the role of NKG2D and natural cytotoxicity receptors (NCRs) in the recognition of human leukemia.

Hematopoietic stem cell transplantation (HSCT) is a common therapy in acute myeloid leukemia (AML) and newly developing NK cells are important for engraftment and antitumor immunity and thus for treatment outcome. Here, we studied the recovery and functional activity of NK cells after allogeneic HSCT.

Analysis of ULBP1, ULBP2 and ULBP3, the recently identified ligands for NKG2D, and of the yet not defined ligands for NKp30, NKp44 and NKp46 in healthy hematopoietic cells demonstrated ligand expression by peripheral blood (PB) derived B cells, monocytes, granulocytes and platelets. We show that upregulation of cell surface ligands occurs during myeloid development with ligand-negative bone marrow (BM)-derived $CD34^+$ progenitor cells acquiring ligand expression upon myeloid maturation in vivo and in vitro. ULBP1 and putative ligands for NKp30, NKp44 and NKp46 were further elevated by stimulation with interferon (IFN)-γ.

In acute myeloid leukemia (AML), leukemic blasts from about 80% of patients expressed very low levels of NKG2D- and NCR-specific ligands. Treatment with differentiation-promoting myeloid growth factors, flt3 ligand (FL), stem cell factor (SCF) and granulocyte macrophage colony-stimulating factor (GM-CSF) together with IFN-γ upregulated cell surface levels of ULBP1 and putative NCR ligands on AML blasts, conferring an increased sensitivity to NK cell-mediated lysis.

We conclude that the ligand-negative/low phenotype in AML is a consequence of cell maturation arrest upon malignant transformation and that defective expression of ligands for the activating NKG2D and NCR receptors may be an underlying cause for compromised leukemia recognition by NK cells. In addition, NK cells were significantly decreased in AML patients, but they expressed NKG2D and the NCRs at normal high

levels, providing a further argument for a dominant role of activating ligands and not their respective receptors in immune escape in AML.

Analysis of NK cells after allogeneic HSCT revealed a rapid reconstitution of NK cells, which reached normal levels as soon as 1 month after HSCT. However, there was a skewing of NK cell subpopulations, with a prevalence of IFN-γ producing $CD56^{bright}CD16^{dim/-}$ NK cells and a corresponding reduction in the highly cytotoxic $CD56^{dim}CD16^{bright}$ subset. Expression of the triggering receptor NKp46 in NK cells from transplanted patients was high. Our results indicate that fast recovering NK cells may have important implications in the prevention of leukemic relapses after allogeneic HSCT.

Altogether these data indicate that low expression of ligands for activating NK cell receptors on leukemic blasts results in poor immunogenicity of tumor cells. Moreover, in vivo upregulation of those ligands on target cells by appropriate compounds might improve recognition of blasts by NK cells, including the early developing allogeneic NK cells after HSCT, and thus reduce leukemic relapses.

II. Introduction

1. Natural Killer Cells

NK cells are effector cells of the peripheral immune system. They comprise about 5-10% of PB lymphocytes and can be distinguished from other cell types by the expression of surface markers CD56 and CD16, and the absence of CD3. NK cells are also found in peripheral tissues including the liver, peritoneal cavity and placenta[1,2].

NK cells derive from hematopoietic stem cells. Since thymectomized or splenectomized mice and humans[3-6] have normal numbers of functional NK cells, neither spleen nor thymus appears to be essential for the generation of NK cells. Present consensus states that BM is the main site for NK cell generation. Interleukin (IL)-15 was found to be the crucial factor for the development of human and murine NK cells[7-10]. FL or SCF increase the frequency of NK cell precursors through upregulation of expression of the IL-15 receptor complex[7,11]. FL or IL-15 deficient mice have markedly reduced numbers of NK cells[9,12].

NK cells are part of the innate immune system and as such, they are able to kill cancer and virus-infected cells without need for prior antigen stimulation[1]. Activation of NK cells results in the release of cytotoxic mediators as well as in the production of several cytokines and chemokines, including IFN-γ, tumor necrosis factor (TNF)-α, TNF-β, GM-CSF, macrophage inflammatory protein (MIP)-1α, MIP-1β and I-309[13,14]. NK cells are highly responsive to many cytokines, including IL-2, IL-12, IL-15 and IFNs which rapidly increase their proliferative, secretory, cytolytic and anti-tumor functions[15].

1.1. NK Cell Subsets

Based on their cell-surface density of CD56, human NK cells can be divided into two subsets, $CD56^{bright}$ and $CD56^{dim}$ cells (see Figure II-1). Each subset displays distinct phenotypic and functional properties[16]. The majority (≈90%) of human NK cells are $CD56^{dim}$ and express high levels of Fcγ receptor III (FcγRIII, CD16), whereas ≈10% of NK cells are $CD56^{bright}CD16^{dim}$ or $CD56^{bright}CD16^{negative}$.

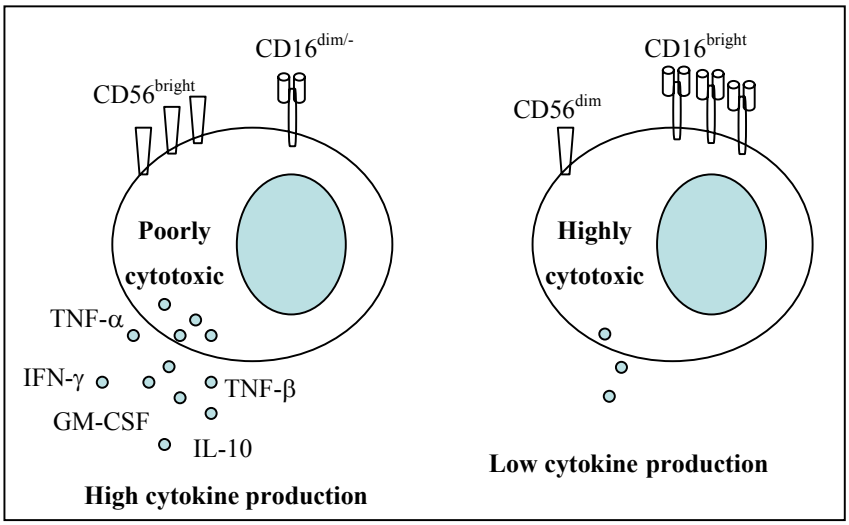

Figure II-1. NK Cell Subsets

The CD56 antigen is an isoform of the human neural-cell adhesion molecule with unknown function for human NK cells[17], although early studies suggested that this molecule might mediate interactions between NK cells and target cells[18,19]. Thus, there is no known direct functional significance of high – or low - level of CD56 expression. So far, no murine homologue of CD56 has been found. Consequently, it is not known, whether mice have NK cell subsets analogous to $CD56^{bright}$ and $CD56^{dim}$ cells.

CD16 is the low-affinity FcγRIII on the surface of NK cells. It binds to the Fc portion of antibodies. CD16 signals through associated subunits containing an immunoreceptor

tyrosine based activation motive (ITAM). Binding of CD16 to antibody-coated, opsonized, targets directly results in antibody-dependent cellular cytotoxicity (ADCC)[20].

Early studies of resting $CD56^{dim}$ NK cells revealed that these cells are naturally more cytotoxic than $CD56^{bright}$ NK cells[21], although after activation with IL-2 or IL-12 in vitro or following low dose therapy with IL-2, $CD56^{bright}$ and $CD56^{dim}$ cells have similar levels of cytotoxicity[22-24]. Freshly isolated $CD56^{bright}$ human NK cells are the primary source of NK cell derived immunoregulatory cytokines, including IFN-γ, TNF-β, IL-10, IL-13 and GM-CSF, whereas the $CD56^{dim}$ NK cell subset produces consistently negligible amounts of these cytokines following stimulation with recombinant monokines in vitro[25].

All NK cells express a functional heterodimeric IL-2 receptor (IL-2Rβγ) with intermediate affinity for IL-2. In 1990, it was shown that the $CD56^{bright}$ NK cells constitutively express the high-affinity heterotrimeric IL-2R (IL-2Rαβγ)[22,23]. This subset has a high proliferative response to low doses of IL-2 alone and can be expanded in vitro and in vivo in response to picomolar concentrations of IL-2[26,27]. By contrast, resting $CD56^{dim}$ NK cells express IL-2Rβγ only and show almost no proliferation in response to high doses of IL-2 in vitro[22,27].

1.2. Target Recognition

A role for NK cells in rejection of tumors and virus infected cells in vivo has been proposed shortly after their discovery as a unique lymphocyte subset[28,29]. From studies performed to date two general mechanisms have been implicated for NK cells in target cell recognition: missing self recognition, and recognition of ligands for activating NK cell receptors. Unlike T and B lymphocytes, NK cells do not rearrange genes encoding receptors for antigen recognition, but they have the ability to recognize target cells through inhibitory and stimulatory receptors expressed on the cell surface of NK cells. The balance between activating and inhibiting signalling determines the triggering status of the NK cells.

1.2.1 Missing Self Recognition

Initially, Karre et al proposed that NK cells discriminate target cells based on the levels of target cell self-MHC (major histocompatibility complex) class I expression. By comparing the activity of NK cells against wild type and class I-low tumor cell lines it was shown that NK cells preferentially attack cells with reduced or abolished MHC class I expression[30]. Resistance to NK cell-mediated cytotoxicity of class I-low tumors could be restored by reintroduction of class I molecules[31,32]. In further support of this model, three families of inhibitory, MHC class I-recognizing, receptors expressed by NK cells were subsequently discovered. The first inhibitory receptor identified was Ly49 in mice[33-35]. Subsequently, killer cell immunoglobulinlike receptors (KIRs; also collectively termed CD158) were discovered in humans[36-39] and CD94/NKG2A in both mice and humans[40-43].

1.2.1.1. Inhibitory Receptors

A key aspect of the immune system is the ability to respond to target molecules alien to the organism while sparing the organism itself. Equally critical is the capacity to limit and ultimately terminate a response, inactivating or eliminating the relevant pathways when they are no longer required. Loss of inhibitory signalling is often associated with autoreactivity and uncontrolled inflammatory responses[44].

In human NK cells two families of inhibitory receptors, KIR and CD94/NKG2A recognizing MHC class I ligands prevent from autoreactivity. The cytoplasmic domains of all inhibitory NK cell receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) with the consensus sequence I/VxYxxL/V[45,46]. These domains recruit intracellular tyrosine phosphatases SHP-1 or SHP-2 that mediate the inhibition of cytotoxicity and cytokine release[45,47-49].

KIRs are encoded by about 12 polymorphic genes and recognize polymorphic epitopes on human leukocyte antigen classes HLA-A, HLA-B and HLA-C. KIRs are expressed on subsets of NK cells and also memory T cells.

The CD94/NKG2A receptor, conserved in rodents and primates, is a disulfide-bonded heterodimer. It is typically expressed in about 50% of the NK cells and a subset of memory CD8+ T cells. CD94 lacks cytoplasmic signalling domain while NKG2A contains two ITIMs[49,50]. This heterodimeric receptor recognizes tetramers of the human non-classical MHC molecule HLA-E and the homologous mouse $Qa1^b$ molecule. While KIR, and also murine Ly49, directly binds to intact MHC class I molecules, CD94/NKG2A binds to a peptide derived from the signal sequence of classical MHC class I molecules that is presented in the groove of HLA-E and $Qa1^b$[51,52]. In the absence of these peptides, HLA-E and $Qa1^b$ are retained in the cytoplasm of the host cells and degraded. Expression of several class I alleles is needed to get maximal protection through CD94/NKG2A[53], indicating that the signal sequence-derived peptide is in limited supply. NK cells, relying entirely on CD94/NKG2A for inhibition, are well suited to detect a reduction in the overall synthesis of MHC class I antigen.

The various MHC specific inhibitory receptor genes are expressed by overlapping subsets of NK cells such that an individual cell expresses a few types of inhibitory receptors. This leads to a complex combinatorial repertoire of NK specificities for MHC class I molecules. Expression of the inhibitory receptor repertoire among NK clones is stochastic. The only rule appears to be that every NK cell has at least one inhibitory receptor specific for a self-MHC class I molecule in order to avoid autoreactivity.

1.2.2. Activating Receptors

Beside inhibitory receptors NK cells express a wide panel of stimulatory receptors (listed in Table II.1). The biological roles of many of these receptors are not well understood, primarily because many of the ligands have not been identified and genetic loss-of-function studies have not yet been carried out in most cases. The main triggering receptors identified to date are the NCRs and the NKG2D receptor (see Figure II-2). Their activation results in direct killing of target cells and can even override negative signals generated by engagement of inhibitory MHC class I receptors[13,54-56]. In addition, CD2[57], CD16[58], CD69[59] and DNAM-1[60] have been shown to trigger NK cell-mediated

killing in redirected cytotoxicity assays. Another activating NK cell receptor is NKp80, which belongs to the type II C-lectin-like receptor family. NKp80 acts as a coreceptor and functions synergistically with NCRs. The NKp80-mediated recognition of target cells has been documented in killing of phytohemagglutinin (PHA) transformed blasts, while lysis of various tumor cell lines could not be inhibited by masking NKp80 with mAbs[61].

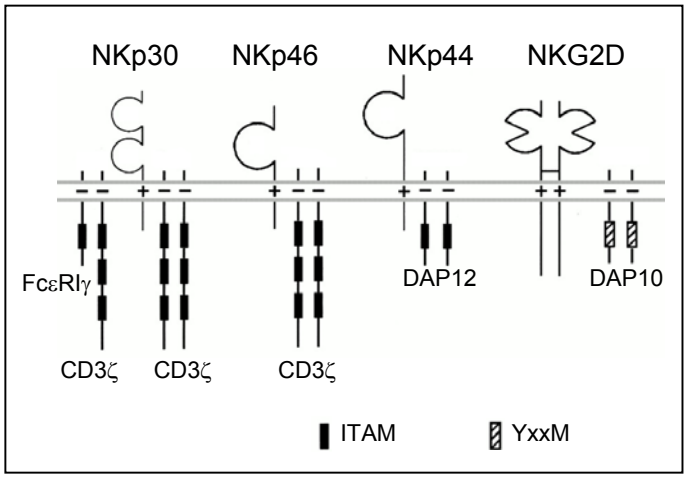

Figure II-2. Activating NK Cell Receptors

Some members of the KIR and NKG2 receptor family also have activation properties. NKG2C and NKG2E are stimulatory members of the CD94/NKG2 family[40,62,63]. They recognize (similarly to their inhibitory counterparts) the Qa1 molecule in mice[62] and the HLA-E molecule in humans[64]. KIR2DS and KIR3DS are stimulatory members of the KIR family[65-67]. There is evidence that the activating receptors bind the self-MHC class I with lower affinity as compared to the inhibitory receptors[68]. Thus autoimmunity could be prevented by a balance towards negative NK cell regulation. Similar to the inhibitory receptors, the MHC class I-specific stimulatory receptors are expressed in a variegated and predominantly stochastic fashion by subsets of NK cells[69].

Commonly, activating receptors lack the ITIM motif in their cytoplasmic domains. Instead, they have charged residues in their transmembrane domains that are necessary for association with adaptor signalling proteins, which have short extracellular domains

and are not likely to participate in ligand binding. Instead, the intracellular domains of the adaptor proteins have docking sites for downstream stimulatory signalling molecules. Most adaptors (FcεRγI, CD3ζ and DAP12) contain ITAMs in their cytoplasmic domains, which allow them to associate with ZAP70 and/or syk-family kinases[70,71]. DAP10, which so far only associates with NKG2D[72,73], has a YxxM motif in its transmembrane domain, which allows recruitment of phosphatidylinositol 3-kinase (PI3K) (see Figures II-3,4)[72,74].

Table II-1. Stimulatory Receptors on Human NK Cells

	Ligands	Adaptors	Expression pattern
Stimulatory receptors recognizing MHC class I molecules			
NKG2C, NKG2E	HLA-E	DAP12	variegated
KIR2DS	HLA-C	DAP12	variegated
KIR3DS	HLA molecule	DAP12	variegated
Stimulatory receptors recognizing non-MHC class I molecules			
NKG2D	MICA, MICB, ULBP1, 2, 3, RAET1E, RAET1G	DAP10	All NK cells, all CD8+ αβ T cells, ca. 50% of NKT cells, γδ T-cell subset
NKp46	viral hemagglutinin	CD3ζ, FcεRγI	All NK cells
NKp44	viral hemagglutinin	DAP12	All NK cells after IL-2 stimulation
NKp30	unknown	CD3ζ, FcεRγI	All NK cells
CD16	IgGs	CD3ζ, FcεRγI	Most NK cells

1.2.2.1 Natural Cytotoxicity Receptors (NCRs)

So far three different NCRs (see Figure II-2) have been cloned, namely NKp46, NKp44 and NKp30[54,75,76]. Molecular cloning of NCRs confirmed that they are structurally distinct from each other[76]. The NCRs belong to the immunoglobulin superfamily[77]. While NKp46 and NKp30 are expressed uniquely on both non-activated and activated NK cells,

NKp44 is present on IL-2 activated NK cells only and on a minor subset of T cell receptor (TCR)-γδ+ T cells[75].

NCR surface density varies among individuals. Thus, NK cells from some donors homogeneously express the NCRs at high density (NCR^{bright}), whereas in other individuals two subsets of NK cells carrying either high or low (NCR^{dim}) receptor densities were detected[76,78]. Importantly, NCR^{dim} clones display a low cytolytic activity compared to that of NCR^{bright} clones isolated from the same or a different individual[78,79].

NKp46, the first NCR identified, is a 46kDa glycoprotein with a protein backbone of approximately 33kDa. Its transmembrane domain contains a positively charged arginine, which interacts with an aspartic acid residue in the transmembrane domain of the adaptor molecule CD3ζ[54,80,81]. Triggering of NKp46 results in Ca^{2+} mobilization, cytolytic activity and cytokine production[54]. It was later suggested that the NKp46 was the major lysis receptor in NK cells, since anti-NKp46 monoclonal antibody (mAb) blocked the lysis of a large panel of tumor cells[78]. Involvement of NKp46 was also demonstrated through the lysis of autologous Epstein-Barr virus (EBV)-transformed cell lines, which are protected from NK cell mediated killing by the expression of self HLA class I molecules. Masking of HLA class I induces efficient killing of autologous EBV-transformed cell lines by NK cells. Additional mAb-mediated masking of NKp46 results in significant (50-60%) inhibition of lysis thus implying that NKp46-specific ligands are also expressed in normal cells[78]. This result also shows that the triggering function of NKp46 is negatively regulated by the interaction between inhibitory receptors and HLA class I molecules.

NKp46 homologue has been cloned in mouse[81] and rat[82] displaying around 60% identity with the human NKp46. The human receptor was also found to induce killing of YAC-1 cells, murine lymphoma cell line, indicating that its ligand may be conserved in humans and mice[78,80].

NKp44 is a 44kDa glycoprotein present on IL-2 stimulated NK cells, suggesting that it may contribute to the increased efficiency of activated NK cells to elicit tumor cell lysis[1,75]. NKp44 contains a charged lysine in its transmembrane region that participates in association with the ITAM bearing DAP12 adaptor molecule[75]. mAb mediated masking of NKp44 resulted in partial inhibition of cytolytic activity against certain tumor target

cells[83]. This inhibition was greatly increased by additional masking of NKp46. Therefore NKp44 appears to function as a NCR selectively expressed by activated NK cells, which may cooperate with NKp46. Surface density of NKp44 correlates in most instances to that of NKp46 (NKp46/p44^{bright} and NKp46/p44^{dim} clones)[83].

NKp30 is a 30kDa glycoprotein that associates with the adaptor molecules CD3ζand FcεRIγ through a positively charged amino acid in the transmembrane region[76]. Its surface expression correlates to that of NKp46. NKp30 was shown to co-operate with NKp46 and NKp44 in the induction of cytotoxicity against a variety of target cells. It was shown to represent the major receptor responsible for killing of those tumor target cells which were largely NKp46/p44 insensitive[76].

Importantly, no cellular ligands for NCRs have been described, yet. NKp46 and NKp44 but not NKp30 can recognize viral hemagglutinins (HA). The binding of the receptors to HA involves sialic acid residues on the NCRs. The activating signalling resulting from the interaction of HA with NKp46 and NKp44 could overcome inhibitory signalling mediated by MHC class I molecules expressed on the target cells and resulted in killing of influenza virus infected cells[56,84].

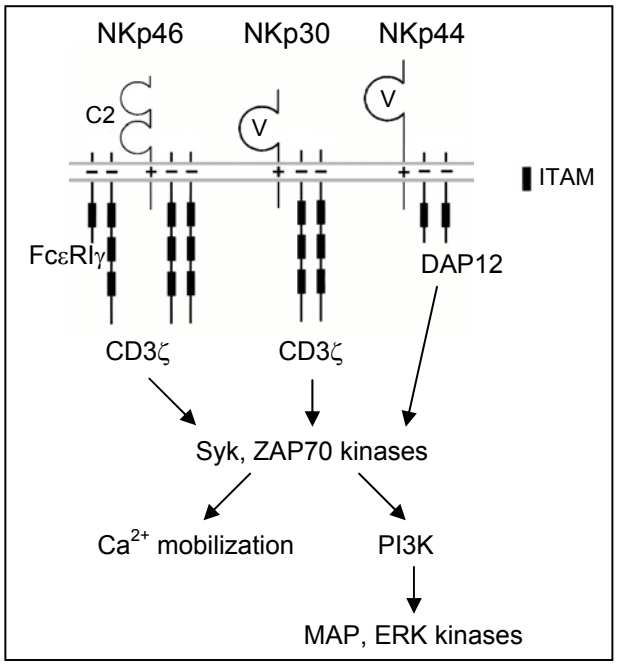

Figure II-3. NCRs, Signalling Cascade

1.2.2.2. NKG2D Receptor

NKG2D was first identified in 1991 by the discovery of the respective cDNA expressed by human NK cells[40]. Further characterization of this receptor was hampered by technical difficulties in expressing NKG2D on the cell surface by transfection. So its role was ignored up to 1999 when it was found that NKG2D needs association to the adaptor molecule DAP10 for its surface expression (see Figure II-4) and when NKG2D was identified as an activating receptor for an MHC class I like molecule, MIC A[55,72].

NKG2D is a type II C-lectin-like protein encoded by a gene located next to the NKG2A, C, E genes within the NK gene complex on human chromosome 12p12-p13 and mouse chromosome 6. Due to this chromosomal localization NKG2D was originally classified as a member of the NKG2 family. However, it has only 20% sequence homology with the highly conserved NKG2 isoforms and does not associate with CD94 but forms a homodimeric structure[72].

NKG2D is constitutively expressed on all human and mouse NK cells[73]. It can be upregulated on human cells by IL-15, IL-12 and IFN-α[85]. NKG2D is also detectable on all human $\gamma\delta$ T cells and CD8+ $\alpha\beta$ T cells[55], and is upregulated on CD8+ T cells by IL-15[86]. In mouse, NKG2D surface expression has also been detected on macrophages stimulated with LPS, IFN-γ or IFN-α/β[73,87].

NKG2D couples through the positively charged arginine in its transmembrane domain with the opposite charged aspartic acid residue in the transmembrane region of DAP10. Upon cross-linking of NKG2D the cytoplasmic YxxM motif of DAP10 is tyrosine phosphorylated and can recruit the p85 subunit of PI3K, resulting in the activation of Akt. DAP10 has also been shown to bind the adaptor protein Grb2[72,74]. Signalling via PI3K plays a pivotal role in the pathway that triggers cytotoxicity of NK cells. Pharmacological inhibition of PI3K blocked killing by preventing mobilization of perforin and granzyme B to the cell-cell interface between the NK cell line NK92 and Raji target cells[88]. Rejection of NKG2D ligand-expressing murine RMA or RMA-S lymphoma cell lines required functional perforin but not IFN-γ, indicating that degranulation of pore-forming cytotoxic granules, rather than release of IFN-γ from NK cells and CD8+ T cells, is the main effector mechanism for tumor rejection[89].

Engagement of NKG2D also leads to calcium mobilization as well as the activation of JAK2, STAT5 and the ERK MAP kinases (see Figure II-4). Consequently, binding of cognate NKG2D ligands results in enhanced survival, proliferation and cytotoxicity, as well as cytokine and chemokine production (IFN-γ, GM-CSF, TNF-α and TNF-β, MIP-1α and β, and I-309)[85,90]. NKG2D engagement complements NCRs in mediating NK-dependent lysis of tumor cells[91]. Similarly, cooperation between NKG2D and activating KIRs has been shown for both cytotoxicity and IFN-γ secretion[92]. It is thus possible that on human NK cells, NKG2D may serve both as a primary receptor whose engagement triggers cytotoxicity, and also as a co-stimulation molecule, which cooperates with other activating receptors (e.g. activating KIR or NCR) for cytokine secretion. Indeed on cytomegalovirus (CMV)-specific CD28-CD8+ αβ T cells and γδ T cells, NKG2D acts as a co-stimulatory molecule for TCR-dependent signals[93-95].

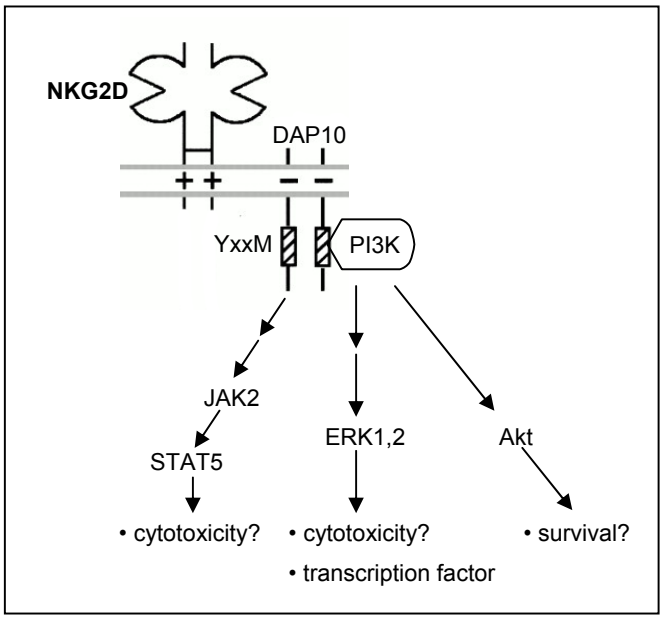

Figure II-4. NKG2D, Signalling Cascade

1.2.3. NKG2D Ligands

NKG2D recognizes several families of cellular ligands, all of which are distantly related to MHC class I molecules. NKG2D ligands are upregulated on tumor cells, virally infected cells and 'stressed' cells (see Figure II-5). Blockade of the NKG2D receptor-ligand interaction results in reduced NK mediated killing of NKG2D ligand-positive tumor cell lines, suggesting that the expression of NKG2D ligands on target cells potently induces NK cell cytotoxicity[87,91,96]. Depending on the levels of NKG2D ligands, the stimulatory signal can override inhibitory signals provided by the same target cell[13,55,96,97]. However, the stimulatory signal transmitted by NKG2D is not entirely refractory to inhibitory signals[91].

Many human tumors of epithelial origin and most mouse tumor cell lines of diverse origin express ligands for NKG2D[98,99]. The fact that so many NKG2D ligands exist in both human and mouse, and that NKG2D is expressed not only on NK cells but also on T cells and macrophages, suggests that the NKG2D receptor likely plays an important role in innate and adaptive immune responses to pathogens and tumors.

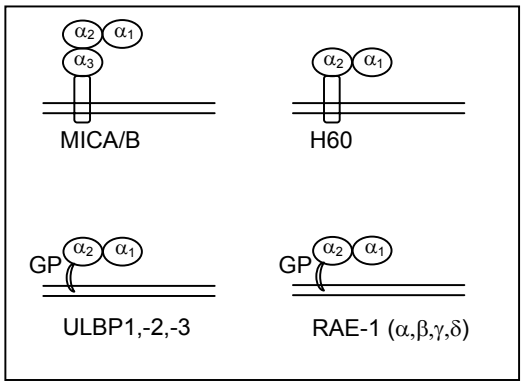

Figure II-5. Human and Murine NKG2D Ligands

Expression of MICA/B and ULBP1, -2 and -3 is induced upon human cytomegalovirus (hCMV) infection. However, only MICA and ULBP3 reach the cell surface, while MICB, ULBP1 and ULBP2 are retained within the infected cells. This intracellular retention is caused by UL16, a protein encoded by hCMV. UL16 accumulates in the

endoplasmic reticulum and cis-Golgi apparatus, specifically binds MICB, ULBP1 and ULBP2, and causes their retention within infected cells. In cells expressing the NKG2D ligands, cotransfection with UL16 encoding cDNA markedly reduces cell surface levels of MICB, ULBP1 and ULBP2, and decreases susceptibility to NKG2D mediated cytotoxicity[100-102].

1.2.3.1. MICA and MICB

The first identified human NKG2D ligands were the MHC class I-related chain A and B (MICA/B), which were shown to bind to NKG2D expressed on NK cells, CD8+ T cells and γδ+ T cells. MICA/B expressing tumor cells were specifically killed by NK cells, demonstrating that NKG2D was a functional receptor for MICs[55]. The human MICA and MICB genes map in the MHC class I region, close to the HLA-B locus[103]. They code for heavily glycosylated proteins with low homology (18-30%) to HLA-A, -B and –C, and consist of an α1, α2 and α3 region (see Figure II-5). Considerable polymorphism exists for the MICA and MICB genes, and 54 alleles of MICA and 16 alleles of MICB have been defined[103] with substitutions throughout the α1, α2 and α3 regions. In contrast to MHC class I molecules, MICs do not require β2-microglobulin or peptide binding for protein folding and stable surface expression[104,105].

Transcripts of MICs are found in low abundance in many cells, but surface expression of MIC protein is rare in adult tissue. MICs become upregulated in many epithelial tumor cells[98], in cells infected with hCMV[94], in bacterially infected cells[106] and in stressed cells[104]. A low level of MICA/B expression is maintained on the epithelial cells lining the gastrointestinal surfaces, which may be due to interactions of these cells with various environmental stress factors[104]. MIC genes are under the control of promotor elements similar to those of the HSP70 genes. Indeed, heat shocking of epithelial lines at 42°C for 2 hours was shown to increase expression of MIC transcripts and proteins[104]. Since cell culture at 42°C is not of physiological relevance, the mechanisms leading to MIC upregulation in transformed cells remain unknown. Although NKG2D receptors are

conserved among mouse, rat and human, no homologues of MIC ligands have been identified in mice.

Groh et al. found that epithelial tumor cells are able to shed MICs. Binding of soluble MICs to NKG2D results in endocytosis and degradation of the receptor. This downregulation of NKG2D expression on NK cells, CD8+ T cells and γδ T cells leads to decreased cytolytic activity against target cells[107]. Thus, MIC shedding serves as a mechanism of epithelial tumors to escape recognition by NK and T cells.

1.2.3.2. ULBP1, ULBP2 and ULBP3

The UL16 binding proteins (ULBPs) were initially identified based on the ability of some members (ULBP1 and ULBP2) to interact with UL16, a protein encoded by hCMV. In parallel, it was shown that soluble ULBPs bind to NKG2D/DAP10 heterocomplexes and that anti-NKG2D mAb completely blocked binding of ULBPs to primary NK cells[13]. ULBP1, ULBP2 and ULBP3 are 55-60% identical among each other. They are distantly related to members of the extended MHC class I family, with whom they share 23-26% amino acid sequence identity. ULBPs possess α1 and α2 domains but differ from traditional MHC class I molecules in that they lack an α3 domain and do not associate with β2-microglobulin (see Figure II-5). ULBPs are glysosylphosphatidylinositol (GPI)-linked rather than transmembrane proteins. Furthermore, unlike most members of the extended MHC class I family, including the MICs, which map to chromosome 6p21, genes for ULBPs are localized outside the MHC region on chromosome 6q25[13]. ULBPs lack many of the conserved amino acids that are known to be important for peptide binding by classical MHC class I molecules[108] and are therefore unlikely to present peptide antigens.

ULBP messages are expressed in a wide range of tissues, including heart, lung, testis, thymus, PB and BM. But the protein expression doesn't always correlate with the presence of mRNA. In addition, comparison of ULBP message levels in several matched normal and tumor tissue samples showed no consistent differences[13,109]. How ULBP

protein expression is controlled under physiological and pathological conditions requires further studies.

Expression of ULBPs on NK cell resistant, MHC class I positive lymphoblastoid B cell line Daudi, transfected with β2-microglobulin, rendered the Daudi cells sensitive to NK cell lysis. The killing of Daudi cells was mediated through the binding of ULBPs to the NKG2D receptor on NK cells, since Fab fragments of both anti-ULBP and anti-NKG2D mAbs completely blocked killing[13]. Together, these findings show that engagement of NKG2D with ULBPs generates an activating signal that can override a negative signal transduced by inhibitory receptors for MHC class I and trigger NK cytotoxicity. This is in line with the fact that NK cells were not only shown to kill cells with downregulated MHC class I, such as some tumor and virus-infected cells, but also cells with unaltered expression of MHC class I[110-113].

Stimulation of NK cells with soluble ULBPs leads to production of several cytokines and chemokines, including GM-CSF, TNF-α, TNF-β and IFN-γ, playing an important role in antiviral immunity[13,14].

Recently, other members of the ULBP family have been cloned, RAET1E (ULBP4) and RAET1G. In contrast to ULBP1,-2 and -3, RAET1E and RAET1G are transmembrane proteins with cytoplasmatic tails. RAET1G but not RAET1E binds to hCMV protein UL16. Surface expression of RAET1G and RAET1E results in NKG2D dependent killing of target cells by human NK cells[114,115].

1.2.3.3. Murine NKG2D Ligands

Two families of ligands, Rae1 and H60, for the mouse NKG2D receptor have been cloned[73,116]. The retinoic acid early inducible 1 proteins (Rae1) are encoded by a family of five very closely related genes (*Raet1a-e*)[117]. H60 was initially identified as a dominant minor histocompatibility antigen in the response of C57BL/6 mice against BALB.B cells[118]. H60 and Rae1 proteins are distantly related to class I molecules. Like ULBPs, the RAE-1 proteins are GPI-linked proteins, whereas H60 is a transmembrane protein, similar to RAET1E and RAET1G. The basic structure of RAE-1 and H60

proteins, consisting of only α1 and α2 domains, is similar to the one of the ULBPs (see Figure II-5). However, murine NKG2D ligands share very low sequence identity with the ULBPs and MICs, indicating that they represent functional rather than true evolutionary homologues.

Most interestingly, the Rae1 proteins are, like MICs, not expressed by most normal cells, but are upregulated by many tumor cells of diverse origin[73,99,119]. H60 is expressed by some tumor cells from BALB/c mice but is also expressed at low levels by activated lymphoblasts and at high levels by BALB/c thymocytes[73].

1.3. NK Cells in Leukaemia

Deficient HLA class I expression has been described in leukaemic cells[120-122] making them candidate targets for NK cells. However, this phenomenon is not ubiquitously observed in leukaemia.

In chronic myeloid leukaemia (CML) NK cell number and NK cell function has been shown to decrease progressively during the spontaneous course of the disease[123]. Both NK cell number and function recover upon IFN-α treatment[124]. Moreover, activated autologous NK cells were shown to suppress growth of primitive CML progenitors in long-term culture[125].

In AML analysis of NK cell function demonstrated impaired NK cell activity in 16 out of 18 patients. The impaired cytotoxic function correlated with a low NCR surface density (NCR^{dim}). The remaining two patients were characterized by NK cells having an NCR^{bright} phenotype, but were unable to kill autologous blasts in vitro. Importantly, the leukaemic blasts from these two patients were also resistant to lysis mediated by normal NCR^{bright} allogeneic NK cells, indicating that expression of NCR ligands in those blasts was low or absent[79]. NK cell activity in AML was further shown to correlate positively with the relapse-free survival of patients in complete remission[126].

In leukaemia patients treated with haplotype mismatched stem cell transplantation, donor derived NK cells have been reported to exert an antileukaemic effect. This graft-versus-leukaemia (GvL) effect, which so far had only been attributed to the function of CD8+ T

cells, was due to KIR epitope-mismatch in the graft-versus-host (GvH) direction. Missing expression of KIR ligands on mismatched cells of the recipient triggered NK cell alloreactivity against the leukemic blasts. Despite the high number of alloreactive NK cells, there was no evidence of graft-versus-host-disease (GvHD). And interestingly, the alloreactive response persisted only for a period of four months, after which time it was no longer detectable[127]. The clinical outcome of transplantation was more favourable in the patients with alloreactive NK cells: in this group, the probability of five-year 'event-free' survival was 60%, compared with 5% in the patients who did not receive alloreactive NK cells[128].

Altogether these data suggest that NK cells play an important role in the control and clearance of leukaemic cells.

2. Monocytes

Monocytes are generated in the BM. They derive from a progenitor cell, the CFU-GM, which also gives rise to granulocytic cells. The maturation process of monocytic lineage in vivo starts at a monoblast stage, passes through the promonocyte stage and subsequently ends up as monocytes[151]. IL-3, GM-CSF and macrophage colony-stimulating factor (M-CSF) stimulate in vivo generation of monocytes[152]. In vitro, hematopoietic progenitor cells cultured with GM-CSF induce CFU-GM to differentiate towards granulocytes, while addition of FL and SCF shifts differentiation from granulocytic to monocytic lineage[153,154]**.** Newly formed monocytes leave the BM within 24 hours and migrate to the PB. Circulating monocytes adhere to endothelial cells of the capillary vessels and are able to migrate into various tissues[155]. This adherence and migration involves surface proteins, lymphocyte-function associated antigen-1 (LFA-1), CD11 and very late after activation antigen-4 (VLA-4), belonging to the intergrin superfamily of adhesion molecules[156]. These integrins interact with selectins on endothelial cells. Monocytes which migrated into tissues differentiate into macrophages. According to their localization macrophages have distinct names (e.g microglia in central nervous system and Kupffer cells in the liver). Some mononuclear phagocytes may

differentiate into dendritic cells. Since proliferation of macrophages is very limited, renewal of the macrophage population depends on the influx of monocytes and their local proliferation and differentiation[157,158].

2.1. Monocyte Function

Monocytes are members of the mononuclear phagocyte system. During inflammation monocytopoiesis increases[159,160] resulting in elevated monocyte numbers. Furthermore, inflammatory mediators, IL-1, IL-4, IFN-γ and TNF-α upregulate expression of selectins on endothelial cells promoting migration of monocytes into tissues. The same cytokines modulate expression of the adhesion molecule integrin on monocytes[161]. At the site of inflammation monocytes are involved in the phagocytosis of opsonized microorganisms or immune complexes via surface Fcγ receptors (CD64, CD32) and complement receptors (CD11b, CD11c). The microorganisms are synergistically killed by reactive oxygen and nitrogen metabolites and through several hydrolytic enzymes (acid phosphatase, esterase, lysozyme and galactosidase)[162-164]. Importantly, monocytes stimulate T cells by antigen presentation and thus, are involved in the recognition and activation phases of adaptive immune responses[165,166].

Monocytes secrete a large number of bioactive products which play an important role in inflammatory, proliferative and immune responses, including growth factors (GM-CSF, G-CSF, M-CSF, IL-1) and antiproliferating factors (IFNs, TNF)[166].

Lipopolysaccharide (LPS) or endotoxin is a predominant integral structural component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation. LPS binds to the CD14 glycoprotein that is expressed on the surface of monocytes. The binding of LPS induces production of TNF-α, IL-1, -6, -8 and -10[167-169]. Other than LPS, one of the most efficient stimuli for cytokine production in vitro is the direct cell-cell contact of monocytes with activated lymphocytes[170,171]. Mature T cells express CD40 ligand (CD40L) and it has been demonstrated that interaction of CD40L with CD40, a member of the TNF-receptor family, on monocytes induces the production of several cytokines[172-174]. Furthermore, incubation of monocytes with

CD40L-transfected cells results in tumoricidal activity against a human melanoma cell line. CD40 expression on monocytes can be upregulated by IFN-γ, IL-3 and GM-CSF [174]. Functional interactions between monocytes and NK cells have been described. Monocytes have a proliferative effect in vitro on cultured NK cells. This effect is in part mediated by soluble factors but is enhanced through direct cell-cell contact[175]. In addition release of IL-12, TNF-α, IL-15 or IL-1β by activated monocytes induces production of IFN-γ by cocultured NK cells[176,177]. Short-term cultures of NK cells with monocytes increased cytotoxic potential of NK cells, whereas long-term cultures resulted in decrease of NK cytotoxicity[178,179], suggesting time dependent functional interactions between the two cell populations.

2.2. Dendritic Cell and NK Cell Interactions

Dendritic cells (DCs) are components of the innate immune system. They circulate through the blood and non-lymphoid peripheral tissues, where they can become resident cells[180]. Immature DCs (iDCs) recognize pathogens through cell surface receptors, including Toll-like receptors[181]. After uptake of antigen DCs mature and migrate to lymph nodes. Mature DCs (mDCs) are efficient antigen presenting cells (APCs) which mediate T cell priming[180].

A predominant role of DCs in NK cell activation has been described in mice and humans. Both immature and bacterially activated human monocyte-derived DCs have been shown to induce cytokine secretion and cytotoxicity by NK cells[182,183]. Moreover, infected DCs are much stronger inducers of NK cell activation and proliferation than uninfected DCs[184]. Conversely, in vitro crosstalk between NK cells and DCs can either promote cytokine release by DCs and their further maturation or results in the killing of DCs by autologous NK cells. At low NK to DC ratios (1 to 5) the interaction dramatically amplifies DC responses, whereas at high ratios (5 to 1) DCs are lysed by NK cells[185,186].

In mice mature DCs are a potential source of both IL-15 and IL-12 representing one possible mechanism how DCs can promote NK cell survival, differentiation and

activation[187,188]. However, in vitro studies have shown that certain types of interaction between NK cells and DCs requires direct cell-to-cell contact[183,185].
Interestingly, it was described that after HSCT alloreactive NK cells kill host-type DCs. This prevents antigen presentation of host antigens to graft T cells and thus allows to avoid the initiation of GvH reactions. Thus, alloreactive NK cells do not mediate GvH disease but rather prevent it by killing of host DCs[128].

3. Acute Myeloid Leukaemia (AML)

AML is characterized by an increase in the number of myeloid cells in the BM and an arrest in their maturation, frequently resulting in hematopoietic insufficiency (granulocytopenia, thrombocytopenia, or anemia), with or without leukocytosis. The presence of more than 30% leukaemic blasts in a BM aspirate is required for a definitive diagnosis of acute leukaemia. Symptoms that are typical include fatigue, hemorrhage or infections and fever due to decrease in red cells, platelets or white cells, respectively.

3.1 Characterization and Classification

AML is a heterogeneous disease caused by a variety of pathogenic mechanisms. At a morphologic level, this heterogeneity is manifested by variability in the degree of commitment and differentiation of the myeloid cell lineage (see Figure II-6). This variability has been used to define specific morphologic subgroups. The most commonly used method of classification is that developed by the French–American–British (FAB) group (see Table II-2), which divides AML into nine distinct subtypes that differ with respect to the particular myeloid lineage involved and the degree of leukemic-cell differentiation. This distinction is based on the morphologic appearance of the blasts and their reactivity with histochemical stains. In addition, immunologic methods have been incorporated into the diagnostic criteria for some FAB subgroups, but genetic alterations

are not always included in the FAB classification. Currently, cytogenetic or direct molecular genetic methods have become an essential part of the routine diagnostic workup of patients with AML to identify subgroups with distinct clinical features and therapeutic responses. This combination of morphologic, immunologic, and genetically based diagnostic approaches not only makes it possible to modify the therapy according to the sensitivity of biologically defined subtypes, but also provides unique markers to monitor a patient's response to therapy[129].

AML often results from alterations in transcriptional cascades (e.g. transcription factor AML1-CBFβ) that are normally involved in regulating cell fate. Other mechanisms, that are less frequent, involve alterations of growth factor-signalling pathways including structural mutations of the receptor for granulocyte colony stimulating factor (G-CSF)[130]. Cellular transformation is a multistep process and the abnormalities discussed above are insufficient by themselves to lead to leukaemia. Only about 50% of all AML cases involve chromosomal rearrangements, whereas in the remaining cases the underlying molecular genetic abnormalities remain to be identified.

Table II-2. AML Classification

FAB Subtype	Common Name
M0	Acute myeloblastic leukemia with minimal differentiation
M1	Acute myeloblastic leukemia without maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Erythroleukemia
M7	Acute megakaryocytic leukemia

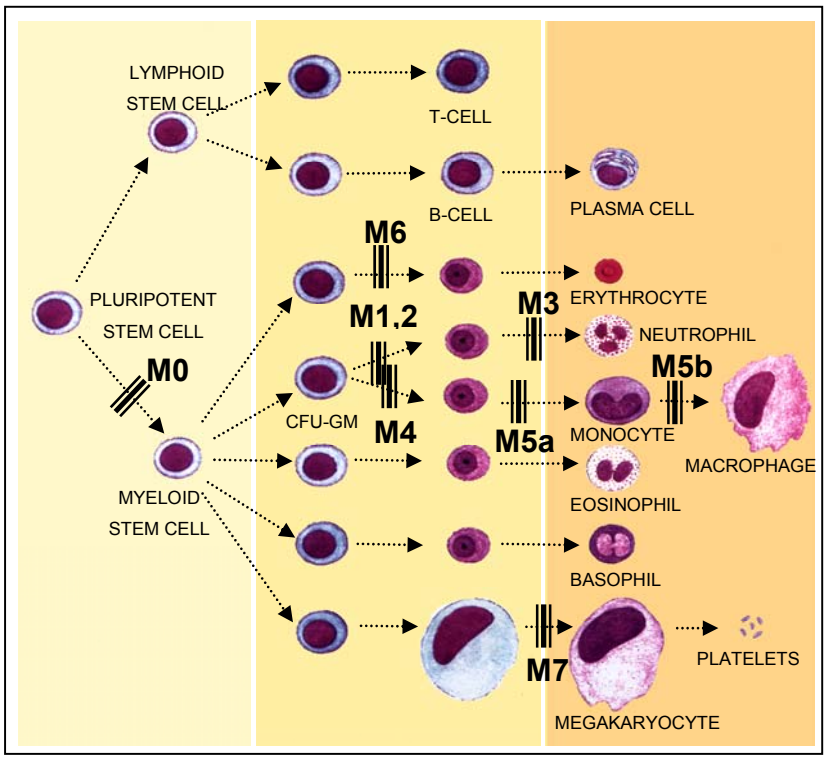

Figure II-6. AML, Differentiation Arrest

The majority of patients have no risk factors or exposures that could account for the development of the disease and thus are considered to have primary AML. Secondary AML may develop in patients with a hematologic disorder (e.g., severe congenital neutropenia) or an inherited disease (e.g., Bloom's syndrome and Fanconi's anemia), in patients who have had myelodysplastic syndrome (MDS) for at least three months, or in those who have been exposed to leukemogenic agents, often as a component of therapy for an unrelated neoplasm. Thus, AML can be expected to develop in three to ten percent of patients who receive alkylating agents as part of their therapy for Hodgkin's disease, non-Hodgkin's lymphoma, ovarian cancer, breast cancer, or multiple myeloma[131]. The risk of this complication is the highest at five to ten years after the start of chemotherapy. The prognosis for these patients is considerably worse than that for patients with primary AML[132,133].

Treatment with topoisomerase II inhibitors, such as epipodophyllotoxins, can also result in therapy-induced AML[134]. In contrast to alkylating agent-induced secondary AML, it develops after a relatively short latency period of two to three years.

3.1. AML Treatment

The primary objective in treating patients with AML is to induce remission and thereafter prevent relapse. Remission is conventionally defined morphologically by the presence of less than five percent blasts in BM together with the recovery of peripheral-blood counts. More than 75% of patients with AML are older than 60 years. In this age group there is an uneven distribution of unfavourable prognostic factors (e.g., cytogenetic abnormalities, features of drug resistance, or a history of MDS)[135,136]. In addition, older patients poorly tolerate an intensive chemotherapy and often have intercurrent medical conditions that are exacerbated by cancer chemotherapy or its sequelae. Withholding induction chemotherapy generally results in low survival rates and a poor quality of life[137]. There is some evidence that the use of low-dose maintenance chemotherapy for several months after the induction of remission reduces the probability of relapse[135]. AML treatment is conventionally divided into induction and postinduction of remission.

3.1.1. Induction of Remission

Induction chemotherapy is administered to all newly diagnosed AML patients except those with myelodysplasia or secondary AML. With the use of daunorubicin and cytarabine or their analogues, complete remission can be routinely induced in 70 to 80% of patients who are 60 years of age or younger and in approximately 50% of older patients. Up to 25% of patients die of severe bacterial and fungal infections during and after induction therapy. Administration of growth factors, such as G-CSF and GM-CSF, stimulates the production and activation of granulocytes and monocytes and promotes their mobilization. This results in a reduced duration of neutropenia and translates into

fewer days of antibiotic[138] or antifungal therapy[139]. None of the studies found that this approach reduced the number of documented infections. Although AML blasts generally express functional G-CSF and GM-CSF receptors on their surface[140], the fear that treatment with G-CSF or GM-CSF could provoke the growth of leukaemic cells in patients has not been confirmed.

3.1.2. Allogeneic Stem Cell Transplantation

Once remission is induced, further intensive treatment of patients with AML is essential to prevent relapse. Three options are available for younger patients: allogeneic HSCT from an HLA matched related or unrelated donor, autologous HSCT and chemotherapy only without following HSCT.

HSCT was conceived as a treatment to replace the entire hematopoietic system of the patient[141,142]. The first successful human HSCTs were carried out about 30 years ago. Since then it has become a routine treatment of choice for malignant and non-malignant disorders of hematopoiesis as well as for solid tumors[142,143]. BM is no longer the only source of stem cells. The use of mobilized PB (mPB), into which hematopietic stem cells migrated after administration of cytokines, predominates now[144,145], and cord blood is used increasingly.

Allogeneic HSCT from an HLA-matched sibling can cure 50 to 60% of recipients[146,147]. The risk of relapse among patients in first complete remission who receive an HLA-matched transplant from a sibling is generally less than 20%. The reduced relapse rate is the result not only of the use of myeloablative chemotherapy prior to HSCT, but also of the allogeneic effect mediated by the graft against residual leukemia in the host (GvL). However, this favorable effect is partially offset by the toxicity of GvHD, which is caused by alloreactive cytotoxic CD8+ effector T cells of the donor attacking the tissues of the recipient, in particular skin, intestines and liver. The Matching of donors and recipients for MHC class I and II molecules is important to prevent GvHD, and is necessary for the recipient to recover a functional adaptive immune system. After transplantation, the recipient becomes a stable chimera, in which hematopoietic cells are

of donor HLA type, but all other cells are of recipient HLA type. The preferred donor for any patient undergoing HSCT is and HLA-identical sibling. Upon depletion of T cells to reduce GvHD the incidence of both graft rejection and disease relapse were increased, indicating that alloreactive T cells also play a favourable role in graft acceptance and anti-tumor responses[141,148-150].

III. Research Objectives

NK cells are important effectors of innate immunity because of their ability to lyse tumor cells and virus-infected cells without need for prior antigen stimulation[1]. The activity of NK cells is regulated by a balanced system of inhibiting and activating signals, which enable the NK cells to distinguish between healthy cells and abnormal target cells. Those signals are transferred through inhibitory receptors, KIR and CD94/NKG2A[30], and the activating NK cell receptors, NKG2D and NCRs[54,55]. MIC and ULBP molecules have been identified as human ligands for NKG2D. They are expressed on epithelial tumor cells and several tumor cell lines, making them susceptible for NK mediated lysis[13,55]. Endogenous ligands for the NCRs, NKp30, NKp44 and NKp46, have not yet been discovered, although NK-dependent lysis of many tested tumor cell lines and primary tumor cells is NCR dependent[76,80,83].

AML is a heterogeneous hematopoietic disease resulting from malignant transformation and developmental arrest of myeloid progenitor cells at an immature differentiation stage. HSCT is an important therapeutic option in AML[135,142]. Early after HSCT, immune function is determined by both mature immunocompetent cells transferred with the allogeneic graft and by immune populations that arise from transplanted stem cells. Donor derived NK cells after haplotype mismatched HSCT were recently shown to exert an important antileukemic effect due to KIR epitope-mismatch in graft-versus-host direction[127]. Furthermore, NK cell activity in AML was shown to correlate positively with relapse free survival of patients in complete remission[126].

These previous studies show the important role of NK cells in the recognition and eradication of tumor cells including leukemic blasts. However, rapid disease progression in AML and the high incidence of relapses following treatment with chemotherapy or allogeneic HSCT suggest that leukemic blasts can escape recognition by NK cells. Lack of NK cell recognition can be due to decreased surface levels of activating NK cell receptors and impaired NK cell function. Another reason for the poor immunogenicity of

leukemic blasts could be the absence of triggering ligands on malignant cells, reflecting in vivo selection and possible tumor escape from NK mediated lysis. This thesis characterizes the properties of NK cells at the onset of AML and following HSCT with the goal to understand their role in leukaemia surveillance by the immune system.

To address the question of poor immunogenicity of leukemic blasts, the first goal of this project was to analyze NK cells from AML patients for the expression of the activating receptors, NKG2D and NCRs, whereas leukemic blasts were examined for the expression of the specific ligands for these receptors. Since cellular NCR ligands are still unknown, complexes of recombinant NCRs were used as binding reagents recognizing the putative respective ligands.

It may be hypothesized that high levels of triggering ligands on malignant cells might improve their recognition by NK cells, however, the regulation of those ligands remains poorly understood. Thus, the next goal of this project was to investigate conditions allowing to upregulate the surface expression of NKG2D and NCR ligands. The susceptibility of primary AML blasts to NK cell cytotoxicity was monitored in order to establish the optimal condition for tumor cell recognition by NK cells.

After HSCT, developing NK cells are likely to function as potent effectors capable to eradicate residual leukemic blast. Nevertheless, relapses remain a serious post-transplant complication[189,190]. To examine whether NK cells arising from donor stem cells function normally, the third goal of this thesis focussed on the phenotypic and functional characterization of NK cells in AML patients undergoing HSCT.

Findings of these studies may improve the understanding of the interactions between NK cells and the leukemic blasts as well as the mechanisms of tumor escape from NK cell recognition. In addition, new strategies of increasing susceptibility of tumor cells to NK cell mediated cytotoxicity may evolve.

IV. Materials and Methods

1. Flowcytometry (FACS)

Fluorescence marked cells were measured using FACS Calibur® and analysis was performed with CellQuest software (both from Becton Dickinson, San Jose, CA).

1.1 FACS Analysis of NKG2D Ligands on PB and BM Derived Subpopulations

To analyse lymphocytes, monocytes and granulocytes staining was performed in 100µL aliquots of heparinized PB followed by lysis of erythrocytes with FACS Lysing Solution (Becton Dickinson). Cells were stained at room temperature for 15 minutes with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated mAb against human CD3, CD19, CD33, CD56 or isotype control antibodies (all from BD PharMingen, San Jose, CA). To analyse erythrocytes PB was diluted 1:1000 with PBS and 100µL aliquots were stained at 4°C for 20 minutes with PE-conjugated mAb against human glycophorin-A (BD PharMingen) without lysing. To analyse platelets PB was spinned serially for 10 minutes at 700 rpm and 2000 rpm. 100 µL aliquots were stained with FITC-conjugated mAb against human CD61 (BD PharMingen) without lysing.

Staining with unlabelled mAb anti-ULBP1 (M295; 10µg/mL), -ULBP2 (M311; 20µg/mL) and -ULBP3 (M550; 10µg/mL; each a generous gift from D. Cosman, Immunex, WA) was detected with FITC-conjugated goat anti-mouse (gtαms) antibody (diluted 1:300; Jackson ImmunoResearch, West Grove, PA). Normal mouse serum (Jackson ImmunoResearch) was used 1:10 to saturate free binding sites of secondary antibodies before cells were subsequently incubated with directly labelled lineage specific antibodies. For MIC analysis hybridoma supernatant containing rat mAb specific

for MICA/B proteins (1:5; kind gift from M. Colonna, Basel Institute for Immunology, Switzerland) was used. Binding of anti-MICA/B mAb was revealed by secondary FITC-labelled goat anti-rat (gtαrat) IgG (diluted 1:100; Jackson ImmunoResearch). NKG2D ligand expression was quantified as the mean fluorescence intensity (MFI) ratio of values obtained with specific mAbs divided by values given by secondary gtαms IgG and secondary gtαrat IgG alone.

For leukemic patients peridin chlorophyll protein (PerCP)- conjugated anti-CD45 mAb (BD PharMingen) was added to distinguish blasts from residual mononuclear cells (MNC). To analyze ULBP expression on BM MNCs PE-conjugated anti-CD33, PerCP-conjugated anti-CD14 and APC-conjugated anti-CD34 mAb (all BD PharMingen) were used.

1.2 FACS Analysis of NCR Dimer Binding

To measure putative NCR ligands, dimeric complexes of sNKp30 (10μg/mL), sNKp44 (5μg/mL) and sNKp46 (5μg/mL) were used as staining reagents (kindly provided by G. De Libero, University Hospital Basel, Switzerland) and the binding was revealed using FITC-conjugated gtαms IgG (Jackson ImmunoResearch). Putative NCR ligand expression was quantified as the MFI ratio of values obtained with specific mAbs divided by values given by secondary gtαms IgG alone (for PB and BM derived cells) or control anti-BirA1.4 mouse IgG plus FITC-labelled gtαms (for tumor cell lines). Directly labelled lineage specific mAbs were added after blocking free binding sites of the secondary antibody by normal mouse serum (Jackson ImmunoResearch), as above

1.3 FACS Analysis of in vitro Generated Dendritic Cells

To detect DCs generated in vitro cells were stained with PE-labelled anti-CD1a, PerCP-conjugated anti-CD14 and APC-labelled anti-HLA-DR mAb or isotype control

antibodies (Becton Dickinson). Maturity of DCs was tested using unlabelled mAb against human CD80, CD83 or CD86 and secondary FITC-conjugated gtαms IgG (Jackson ImmunoResearch). Directly labelled DC-specific mAbs were added after blocking free binding sites of the secondary antibody by normal mouse serum (Jackson ImmunoResearch), as above. NKG2D ligand expression was measured using unlabelled anti-ULBP1 (M295) and –ULBP3 (M550) mAbs and anti-MICA/B rat antibody (hybridoma supernatant; M. Colonna, Basel Institute for Immunology), as above.

1.4 FACS Analysis of NK Cells

For the analysis of NK cells heparinized PB was stained with APC-labelled anti-CD56 mAb, PE-conjugated anti-HLA-DR, anti-CD16 or anti-CD161 mAb, FITC-labelled anti-CD69 mAb and PerCP-conjugated anti-CD3 mAb or isotype control mAbs (all from Becton Dickinson).
Staining with unlabelled anti-NKG2D mAb (at 10 μg/mL, M585; kindly provided by D. Cosman, Immunex) and anti-NKp46 mAb (diluted 1:5, 9E2; kind gift from M. Colonna, Basel Institute for Immunology) was detected with FITC-conjugated gtαms IgG (Jackson ImmunoResearch). Receptor expression was quantified as the MFI ratio of values obtained with specific mAbs divided by values given by secondary gtαms IgG alone. Directly labelled lineage specific mAbs were added after blocking free binding sites of the secondary antibody by normal mouse serum (Jackson ImmunoResearch), as above.

2. Purification of Cell Populations

To isolate specific populations from BM and PB samples, MNCs were separated by Histopaque (Sigma, St. Louis, MO) density-gradient centrifugation and the subsequent erythrocyte lysis (lysis buffer from Kantonsspital Basel, Switzerland). The cells were cryopreserved in liquid nitrogen until use. Frozen samples were thawed and incubated per

1 x 10^7 cells per mL overnight at 4°C in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS; both from Invitrogen, Carlsbad, CA) and DNase (500 U/mL; Sigma). For isolation process cells were resuspended in MACS buffer (PBS, containing 0.5% bovine serum albumin and 2mM EDTA) after washing.

2.1 Cell Purification by Magnetic-Activated Cell Sorting (MACS)

To purify $CD34^+$ progenitor cells up to 1 x 10^8 viable MNCs from BM or mobilized PB were incubated per 300 µL MACS buffer for 30 minutes with 100 µL magnetic labelled anti-CD34 mAb and 100 µL FcR blocking reagent (both from CD34 Progenitor Cell Isolation Kit, Miltenyi, Bergish Gladbach, Germany) at 4°C. After washing up to 10^8 MNCs were resuspended per 1 mL MACS buffer and separated through magnetic columns (Miltenyi).

To purify $CD14^+$ monocytes PB derived viable MNCs were incubated per 10^7 cells in 80 µL MACS buffer with 20 µL magnetic labelled anti-CD14 mAb (Miltenyi) for 15 minutes at 4°C. After washing cells were resuspended per 10^8 MNCs in 500 µL MACS buffer and separated through magnetic columns (Miltenyi).

$CD56^+CD3^-$ NK cells were purified using MACS NK cell isolation kit II (Miltenyi). Per 10^7 PB derived viable MNCs were incubated in 40 µL MACS buffer with 10 µL of a biotin labelled lineage specific antibody cocktail (against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and glycophorin A; Miltenyi) for 10 minutes at 4°C. Next, 30 µL MACS buffer and 20 µL magnetic labelled anti-biotin mAb were added per 10^7 MNCs and incubated for 15 minutes at 4°C. After washing up to 10^8 MNCs were resuspended in 1 mL MACS buffer and separated through magnetic columns (Miltenyi). Purified NK cells were collected in the flow through.

When labelled cells corresponded up to 10^7 cells, MNCs were separated using a magnetic MS column (Miltenyi). When labelled cells corresponded up to 10 cells, MNCs were separated using an magnetic LS column (Miltenyi). MS and LS columns were first rinsed

with 500 µL and 3 mL MACS buffer, respectively, before adding MNCs through a MACS pre-separation filter (Miltenyi) on the column. Columns were washed 3 times with MACS buffer. For negative NK cell purification cells were collected in the flow through. For the monocyte and progenitor cell purification cells were eluted from the column using MACS buffer.
Cell purity was analyzed by FACS, as described above and purified cells were put into cultures, as described below.

2.2 Cell Purification by FACSorting

To separate $CD16^{bright}$ and $CD16^{dim/-}$ NK cell subsets MNCs from PB of healthy donors and patients after HSCT NK cells were first purified by MACS, as described above. Subsequently, NK cells were incubated per 10^8 cells in 150 µL MACS buffer with FITC-labelled anti-CD56, PE-labelled anti-CD16 and APC-labelled anti-CD3 mAb (10 µL each; BD PharMingen) for 20 minutes in ice and darkness. After washing cells were filtered (Polystyrene Tube with Cell Strainer Cap, Falcon, Becton Dickinson) and NK cell subsets were separated using a FACS. Isolated NK cells were restimulated prior to test them for INF-γ production, as described below.
To purify CD3+ T cells, CD19+ B cells and CD14+ monocytes PB derived MNCs were incubated per 10^8 cells in 150 µL MACS buffer with FITC-labelled anti-CD3 or PE-labelled anti-CD3 and/or FITC-labelled anti-CD14 and/or PE-labelled anti-CD19 mAb (10 µL each; BD PharMingen) for 20 minutes in ice and darkness. After washing cells were filtered (Polystyrene Tube with Cell Strainer Cap, Falcon, Becton Dickinson) and separated by FACSVantage SE (Becton Dickinson). Sorted cells were used for RNA isolation, as described below.

3. Cell Cultures

Cells were cultured in IMDM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen).

3.1 Progenitor Cell Cultures

To maintain $CD34^+$ progenitors in vitro 8 x 10^5 cells were cultured in 24-well plates in 1 mL medium containing 10% FCS (from Invitrogen), 0.5% bovine serum albumin (BSA; Dade-Behring, Düdingen Schweiz), transferrin (Calbiochem, La Jolla, CA), flt3 ligand (FL, 50 ng/mL) and thrombopoietin (Tpo, 50 ng/mL; both kindly provided by Amgen Inc, Thousand Oaks, CA). Killing of $CD34^+$ progenitors using autologous NK cells was assessed after 3 days of in vitro culture.

3.2 Myeloid Cell Cultures

To induce myeloid differentiation 0.5 to 1 x 10^6 $CD34^+$ cells were cultured in 6-well plates in 3 ml medium containing 10% FCS (Invitrogen), 0.5% BSA (Dade-Behring) and transferrin (Calbiochem) in the presence of FL at 100 ng/ml, SCF at 100 ng/ml and GM-CSF at 20 ng/ml (all kind gifts of Amgen Inc). After 1 week generated myeloid cells were analyzed by FACS using mAbs against CD34 and CD33, as described above.
To maintain $CD14^+$ in culture, 3 x 10^6 cells were kept in 3 ml IMDM with FL, SCF and GM-CSF, as above.
$CD14^+$ monocytes from healthy donors and MNCs from AML patients containing > 80% blasts were cultured in the same condition used for myeloid differentiation.
To induce NKG2D and NCR ligand upregulation monocytes and primary AML blasts were further incubated with interferon-γ (100 U/ml; PeproTech, London, UK), IL-1b (20 U/ml; kind gift of Sandoz, Basel, Switzerland), TNF-α (50 ng/mL; kindly provided by M.

Fuchs, Sandoz), IFN-α (100 U/mL; Sigma) and LPS (10 ng/ml; from S. abortus equi NG420, kind gift of R. Landmann, Kantonsspital Basel, Switzerland) for 3 to 4 days, as indicated. Ligand upregulation was monitored by FACS, as described above.

3.3 Dendritic Cell Cultures

To generate DCs 1 x 10^6 in vitro derived myeloid cells were incubated with 10% FCS (Invitrogen), GM-CSF (20ng/mL; kind gift from Amgen) and IL-4 (20ng/mL; kindly provided by M Zurrini, Novartis, Basel, Switzerland) for 3 days. Maturation was induced using CD40 ligand (500ng/mL; kindly provided by S. Lyman, Immunex). Development and maturation of DCs and NKG2D ligand expression was analyzed by FACS as described above.

3.4 NK Cell Cultures

2 x 10^6 purified NK cells were cultured in 3 mL medium supplemented with 5% human AB^+ serum (Blutspendezentrum, Basel, Switzerland), IL-2 (100 U/mL kind gift E. Andersen, Novartis), nonessential aminoacids (1:100), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL kanamycin (all from GIBCO Life Technologies, Grand Island, NY). After 2-4 days expression of activating ligands was analyzed by FACS and cytotoxic assays were performed, as described.

For restimulation, 3 x 10^5 $CD56^+CD3^-$ NK cells were seeded onto 2 x 10^6 irradiated (30 Gy) allogeneic MNCs in 24-well plates containing 2 ml medium, as above, supplemented with PHA (1 μg/mL; Murex Biotech, Dartford, UK). When NK cell subsets were restimulated 6 x 10^4 and up to 2 x 10^4 NK cells were seeded onto 1 x 10^6 and 4 x 10^5 irradiated feeder cells, respectively.

After 1 week proliferating NK cells were transferred into 6-well plates. Between day 12 and 28 expression of activating ligands was analyzed by FACS, as described above, and restimulated NK cells were used in killing assays and IFN-γ release assays as described below.

4. mRNA Expression of ULBP1, ULBP2 and ULBP3

Expression of ULBP mRNA was analyzed in PB-derived $CD19^+$ B cells, $CD3^+$ T cells and $CD14^+$ monocytes isolated by FACS sorting (>98% purity), as described above.

4.1 Isolation of mRNA from Purified PB Subpopulations and RT-PCR

1 x 10^6 to 5 x 10^6 cells were used for the extraction of total cellular RNA. Cells were washed in PBS and centrifuged at maximal speed (12'0000rpm) for 1-2 minutes. The cell pellet was resuspended in 1 mL of Trizol (GIBCO Life Technologies), vortexed vigorously and incubated for 15 minutes at room temperature. 200 µL of chloroform was added, mixture was vortexed vigorously and incubated for 10 minutes at room temperature. To separate the lipohpilic and the aqueous phase the mixture was centrifuged at maximal speed. The upper hydrophilic phase was carefully transferred into a new tube and 500 µL (1:1 ratio) of isopropanol (Merck, Darmstadt, Germany) was added. After short vortexing tubes were incubated for 10 minutes at room temperature. The mRNA was pelleted by centrifugation at maximal speed for 15 minutes. The supernatant was discarded and mRNA pellet was washed in 1 ml of 70% ethanol (Fluka Chemie, Buchs, Switzerland) and centrifuged at maximal speed for 15 minutes. The ethanol was completely removed and the pellet was left to dry for several minutes. RNA was dissolved in 50 µL DEPC (Sigma) solution and stored at -70°C for further use in reverse transcription (RT)-PCR.

RT-PCR was performed in a total volume of 20 µL. 8 µL solution containing 1-2 µg RNA was mixed with 2 µL buffer (containing $MgCl_2$; PerkinElmer, Bostin, MA), 0.75 µL RNase inhibitor (40 U/µL; Promega, Madison, WI), 1 µL of random hexamers (50 µM GIBCO Life Technologies), 0.25 µL SuperScript™ II (200 U/µL; GIBCO Life Technologies) and 4 x 2 µL of dNTPs (10mM each; Promega). The RT reaction was incubated at 21°C for 12 minutes, followed by 45 minutes at 42°C and stopped at 95°C for 5 minutes. Resulting cDNA was stored at -20°C for further real-time PCR.

4.2 Real-Time PCR of ULBP mRNAs

For real-time PCR cDNA was amplified in duplicates using TaqMan MGB primer sets for ULBP1 (Hs00360941_m1), ULBP2 (Hs00607609_mH) and ULBP3 (Hs00225909_m1) and control primers for hypoxanthine phosphoribosyl transferase (HPRT) (Hs00355752_m1; all from Applied Biosystems, Foster City, CA). Amplification (40 cycles; 95°C 15 seconds, 60°C 1 minute) was monitored using the Taq Man® MGB probe labelled with 6-FAM dye and nonfluorescent quencher on the ABI Prism 7000 Sequence Detection System.

5. Cytotoxicity Assays

To analyze the killing of cell lines and primary cells, a calcein-acetyoxymethyl (calcein-AM)-based assay or a standard chromium release assay was used, respectively. During the loading process NK cells were prepared in 96-well round bottom plates (Becton Dickinson). Serial 1:2 dilutions were done starting from an effector to target ratio of 10 in 100 µL. Labelled target cells and blocking reagents were added to NK cells resulting in an endvolume of 200 µL. Target and effector cells were incubated for 4 hours at 37°C. Maximum Calcein or ^{51}Cr release was determined with target cells lysed in 1% Triton-X (Sigma). Percentage of cytotoxicity was calculated as follows: 100 x (experimental

release - spontaneous release) / (maximum release - spontaneous release). Experiments were performed in triplicates.

In blocking experiments anti-MHC class I mAb (10 μg/mL; W6/32, ATCC, Manassas, VA), control anti-MHC class II mAb (10 μg/mL; L243, hybridoma supernatant), anti-NKG2D mAb (20 μg/mL (M585, provided by D. Cosman, Immunex) and a mixture of sNKp30, sNKp44 and sNKp46 (at 5, 20 and 50 μg/mL) were used as indicated.

5.1 Calcein Release Assay to Measure Killing of Cell Lines

2 x 10^6 THP-1, HL60 or K562 cells were labelled for 30 minutes at 37°C in the dark using 10 μM calcein-AM (from Calbiochem) in 1 ml serumfree M199 medium (purchased from Invitrogen) containing 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO Life Technologies). After washing cells were incubated for another 30 minutes at 37°C and washed again to decrease the background signal and target cells were added to NK cells in M199 medium containing 5% FCS. Spontaneous and maximal calcein-AM release was determined in six wells each. 50 μL of supernatants were transferred into a flat bottom 96-well plate (Becton Dickinson) and assessed for calcein-AM release using a Spertramax Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, CA; excitation 485 nm, emission 530 nm).

5.2 Chromium Release Assays to Measure Killing of Primary Cells

2 x 10^6 monocytes, $CD34^+$ progenitors or primary blasts were labelled with 250 μCi $Na_2{}^{51}CrO_4$ (Amersham, Little Chalfont, UK) for 2 hours at 37°C. Cells were washed twice, incubated for 30 minutes at 37°C and washed again to decrease the background signal. Labelled target cells wer added to NK cells in IMDM containing 5% FCS and IL-2 (100 U/mL; kindly provided by E. Andersen, Novartis). Spontaneous and maximal

chromium release was determined in three wells each. 30 µL of supernatants were transferred to Luma® scintillation-plates (PerkinElmer), dried over night and assessed for chromium release using a TopCount NXT gamma-counter (Packard PerkinElmer).

6. IFN-γ Production by NK Cells

6.1 IFN-γ Release Detected by ELISA

IFN-γ release by healthy NK cells after cocultures with target cells in 5% FCS (Invitrogen) and IL-2 (100 U/mL; kindly provided by E. Andersen, Novartis) or IL-12 (10 U/mL; Roche, Nutley, NJ) was measured using enzyme-linked immunosorbent assay (ELISA). 1 x 10^5 THP-1 cells were put into 96-well round bottom plates (Becton Dickinson) and serial 1:2 dilutions were done starting from an effector to target ratio of 2 in 50 µL. 5 x 10^4 restimulated NK cells from healthy donors and blocking reagents were added to THP-1 cells resulting in an endvolume of 100 µL. As background control NK cells and THP-1 cells were cultured separately. As a positive control 5 x 10^4 NK cells were cultured in the presence of IL-12 (10 U/mL; Roche) and IL-18 (100 ng/mL; PeproTech). After 18 hours of incubation cultures were substituted with 100 µL cytokine containing medium without any blocking reagents. 42 hours after coculturing plates were centrifuged for 2 minutes at 1400 rpm and 140 µL of supernatants were assessed for IFN-γ release.

Flat bottom 96-well Maxisorp™ plates (NUNC, Roskilde, Denmark) were coated at 4°C overnight with mouse anti-huIFN-γ (43-11, kindly provided by Ch. Heusser, Novartis) at a concentration of 5 µg/mL in a volume of 50 µL per well. Plates were washed and blocked with RIA buffer containing 5 % BSA (Roche Diagnostics, Mannheim, Germany) and 0.2% Tween (Merck) for 1 hour. After washing 70 µL of culture supernatants or IFN-γ standard were added. Serial 1:2 dilutions of the IFN-γ standard (recombinant hu IFN-γ ; from BD PharMingen) were made starting with a final concentration of 30

ng/mL in BSA containing buffer. After incubating 90 minutes plates were washed and 50 µL biotinylated mouse anti-hu IFN-γ mAb (diluted 1:2000; 45-15, kindly provided by Ch. Heusser, Novartis) was added. Plates were incubated for 90 minutes, washed and 50 µL streptavidine-alkaline phosphatase (diluted 1:2000; Sigma) was added per well. After incubation of 1 hour plates were washed and 150 µL substrate, p-nitrophenyl phosphate (Sigma) diluted 1:100 in buffer containing 0.1g/L $MgCl_2$, was added to each well and incubated for 30 minutes. The enzymatic reaction was stopped by the addition of 50 µL NaOH solution (1M; Merck). Absorbance was analyzed at 405nm using an SpectraMAX190 ELISA reader (Molecular Devices).
In blocking experiments, anti-NKG2D mAb (M585; provided by D. Cosman, Immunex), sNKp30, sNKp44, sNKp46 or anti-BirA1.4 mAb were added, as indicated.

6.2 IFN-γ Production by Purified NK Cell Subsets Detected by FACS

PB derived $CD16^{dim/-}$ or $CD16^{bright}$ NK cell subsets were proliferated in vitro and assessed for IFN-γ production by MACS IFN-γ secretion assay (Miltenyi Biotec). 2 x 10^5 NK cells were incubated in 96-well plates (Becton Dickinson) in 200 µL IMDM containing 5% human AB+ serum, IL-12 (10 U/mL; Roche) and IL-18 (100 ng/mL; PeproTech). After 18 hours at 37°C cells were washed in ice-cold MACS-buffer (see above). Cells were incubated for 5 minutes in ice with an IFN-γ catch reagent (1:10; Miltenyi) in 90 µL IMDM containing 10% FCS. Subsequently, cells were transferred into 10 mL warm (37°C) IMDM containing 10% FCS. To prevent false positive results 9 x 10^5 murine BaF3 cells were added and cells were rotated for 45 minutes at 37°C to allow cytokine release. IFN-γ release was immediately stopped by transferring cells into ice for 5 minutes. After washing cells were resuspended in 78 µL ice-cold MACS-buffer and transferred into 96-well plates (Becton Dickinson). Captured IFN-γ was revealed by an APC-conjugated IFN-γ detection mAb (1:10; Miltenyi) and NK cells were marked with

FITC-conjugated anti-CD56 mAb and PE-labelled anti-CD16 mAb (both from BD PharMingen).
Cells were analyzed using FACS Calibur® and analysis was performed using CellQuest software (both from Becton Dickinson).

7. Primary Materials

All control and clinical samples were obtained with informed consent in compliance with the guidelines of the Ethical Committee of the University Hospital Basel, Switzerland.

7.1 Healthy Donors

To analyze NKG2D ligands in subpopulations of MNCs and activating receptors on NK cells compared to AML 22 PB samples and 7 BM samples of healthy donors were tested. To analyze NK cells phenotypically and functionally 13 PB samples and 6 BM samples of healthy donors were tested.

7.2 AML Patients

35 patients with AML were enrolled in the study. Twenty-eight patients had newly diagnosed untreated AML and 6 patients had a relapse of the disease (Table IV-1). The diagnosis and definition of AML subtypes M1-M7 was based on morphologic, cytogenetic and immunophenotypic criteria. The average blast content in PB was 52.8 ± 5.2%.

Table IV-1, Characteristics of AML patients

Patients	Age, y	AML subtype	Status	% Blasts per TNC
HI	67	M1	ND	23
JJ	37	M1	ND	11.2
SH	73	M1	ND	84.3
AG	53	M2	ND	73.3
CA	39	M2	ND	55.4
CL	67	M2	ND	73.5
LE	44	M2	relapse	65.4
PL	67	M2	ND	nd
SR	74	M2	relapse	58.9
BH	46	M2	ND	3.1
SC	69	M2	ND	88
VP (patient B)	39	M3	ND	93.7
BA	76	M4	ND	85
DC	52	M4	ND	37.7
EA (patient E)	68	M4	ND	76.5
SJ	43	M4	relapse	26.5
ZaC (patient D)	47	M4?	ND	36.5
BaT	57	pTx, M5b	relapse	92
CC (patient C)	43	M5	ND	93.5
DS	28	M5b	ND	37.4
DR	31	M5b	relapse	88.5
GG	22	M5	ND	8
SE	74	M5	ND	-
MU (patient A)	46	M5	ND	37.2
Akt	-	M7	-	53.5
TL	35	M7	ND	17.4
WG	55	MD	ND	65.3
ZeC	50	MD	ND	32.9
OE	71	MD	ND	0.7
Z	54	MD	ND	2.4
BeT	68	2nd AML	ND	78.2
MI	66	2nd AML	relapse	82.6
RM	86	2nd AML	ND	55.5
KA	66	unknown	ND	42.5
RA	83	unknown	ND	63.1

TNC, total nucleated cells; ND, newly diagnosed; blasts of (Patients A-E) were used for in vitro killing assays; p, post.

7.3 Patients after Hematopoietic Stem Cell Transplantation (HSCT)

28 patients who underwent an allogeneic HSCT were enrolled in this study (Table IV-2). High intensity conditioning consisted of cyclophosphamide at 60 mg/kg, followed by total body irradiation at 6 x 2 Gy, preceded in high-risk patients by additional etoposide

at 30 mg/kg. Prevention of GvHD in all patients was based on cyclosporine A (150-300 mg/d) in a short course of methotrexate. 6 patients were part of a double-transplantation program. Median time to reach the neutrophil count of more than 500 per μL was 14 days. On day 30, clinically evident acute GvHD grade was observed in 8 patients and grade II or III in 14 patients. On day +180, 19 patients were in complete remission, including 1 patient who had rejected the graft and achieved autologous reconstitution. 4 patients died of a GvHD, 3 of infections, 1 of graft rejection and 1 of leukaemia relapse.

Table IV-2, Characteristics of Patients Undergoing HSCT

					GvHD		
UPN	**Age, y**	**Diagnosis**	**No. of HSCTs**	**Engr day**	**Day 30**	**Day 90**	**Status d +180**
603	32	CML	1	16	0	0	CR
605	32	CML	1	14	II	I	CR
671	16	B-ALL	1	8	I	0	CR
727	23	B-ALL	1	14	II	NA	† of GvHD
730	17	AML	2	10	II	II	† of infection
767	44	AML	2	11	0	NA	† of relapse
804	35	CML	1	20	II	0	Rejection/CR
816	38	MM	1	12	II	0	CR
818	42	SAA	1	14	I	IV	† of GvHD
820	40	AML	1	17	II	0	CR
822	45	CML	1	11	I	II	CR
831	50	CML	2	19	I	II	† of infection
832	44	AML	1	14	0	I	CR
834	27	CML	1	18	0	I	CR
845	17	SAA	1	11	0	0	CR
852	35	CML	1	12	I	0	CR
854	24	CML	1	13	I	I	CR
860	19	AML	2	13	II	I	CR
865	44	AML	2	11	II	II	CR
872	36	AML	2	13	II	I	CR
881	16	MDS	1	17	0	0	† of rejection
886	22	MDS	1	15	II	I	CR
891	25	CML	1	13	III	NA	† of infection
893	23	AML	1	15	II	III	† of GvHD
903	22	SAA	1	15	I	I	CR
904	42	CML	1	19	I	I	CR
906	51	AML	1	13	III	NA	† of GvHD
908	22	B-ALL	1	17	II	III	CR

UPN indicates unique patient number; Engr day, engraftment day indicates absolute neutrophil count > 500/μL for 3 consecutive days; CML, chronic myeloid leukemia; CR, complete remission; B-ALL, acute lymphatic leukemia of B-cell type; NA, not applicable; †, died; MM, multiple myeloma; SAA, severe aplastic anemia; MDS, myelodysplastic syndrome.

V. Results

1. Analysis of Expression and Regulation of Ligands for NKG2D and NCRs

Studies on the pattern of expression and the function of activating ligands represent the major part of this thesis. This is a novel field of research, since ligands for NKG2D receptor have been identified only recently[13,55] and ligands for NCRs remain still unknown. The purpose of our studies was to investigate differences in cell surface expression of activating ligands by normal PB cells and malignant leukemic blasts and to understand the role of these ligands for leukemia recognition by NK cells.

In the first part of our analyses, we performed a phenotypic analysis of normal and AML cells using mAbs specific against various NKG2D ligands and also novel reagents, based on recombinant NCRs, to detect the putative NCR ligands.

In the second part of our analyses, we investigated conditions allowing to modulate ligand levels and we examined the role of activating ligands in the interaction with NKG2D receptor and NCRs on NK cells.

1.1 ULBP Expression on PB Derived MNCs

1.1.1 ULPB Molecules Are Expressed on B cells, Monocytes, Granulocytes and Platelets

Previous studies addressing the ULBP expression by normal PB and BM MNCs detected ULBP transcripts[13,109], but expression of ULBP proteins by hematopoietic cells had not been described. In order to identify ULBP expressing cells, full blood samples from

healthy volunteers were labelled with mAbs specific for hematopoietic lineages and for ULBP1, ULBP2 and ULBP3 (Figure V-1).

Table V-1. Expression of ULBP Proteins by PB Cell Subpopulations

	ULBP1		**ULBP2**		**ULBP3**	
	MFI ratio		MFI ratio		MFI ratio	
	Mean ± SEM	(Range)	Mean ± SEM	(Range)	Mean ± SEM	(Range)
B cells n=19/10/19	21.7 ± 2.8	(7.3-57.6)	10.4 ± 1.7	(4.3-23.5)	17.2 ± 4.3	(6.3-90.6)
Monocytes n=22/12/22	3.4 ± 0.5	(1.1-9.0)	2.1 ± 0.3	(1.1-5.2)	2.4 ± 0.3	(1.0-6.1)
Granulocytes n=18/10/18	2.8 ± 0.6	(1.0-9.5)	1.9 ± 0.3	(1.0-3.8)	2.1 ± 0.3	(1.0-5.2)
Platelets n=3/3/3	2.0 ± 0.2	(1.8-2.5)	7.8 ± 1.6	(4.7-9.6)	3.7 ± 0.2	(3.4-4.1)

n indicates number of samples analyzed for ULBP1/ULBP2/ULBP3 expression

Within lymphoid populations, all three ULBPs were highly expressed by B cells. In NK cells and T cells, however, they were absent. Among myeloid lineages, erythrocytes did not express ULBPs, while platelets expressed ULBP2 at a MFI ratio of up to 9.6, as well as low levels of ULBP1 and ULBP3 (Table V-1). On monocytes and granulocytes ligand levels were highly variable among several tested healthy donors. ULBPs were not detectable or were very low (MFI ratio < 2.0) in monocytes from 8 out of 22 donors and granulocytes from 10 out of 18 donors, but well pronounced in the rest of the donors.
Ligand-positive monocytes always expressed all three ULBPs. ULBP1 was generally present at the highest levels with MFI ratios up to 9.5, and ULBP2 at lowest levels with MFI ratios of not more than 5.2 (Table V-1).

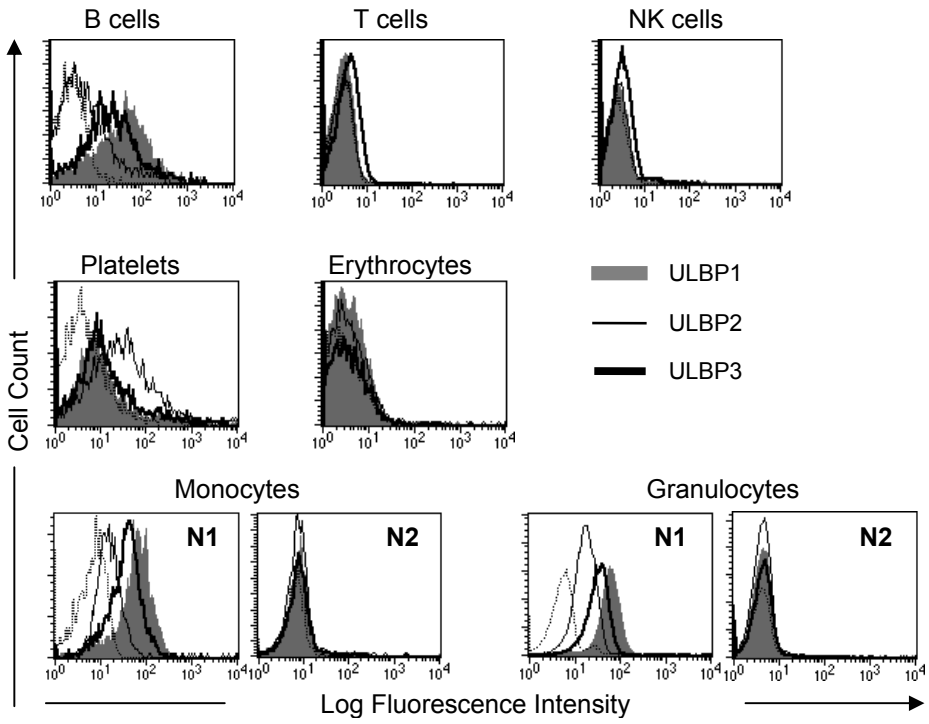

Figure V-1. ULBP molecules are expressed by PB cell subpopulations of normal donors. FACS analysis of PB cells from healthy donors after staining with mAbs for ULBP1 (shaded area), ULBP2 (thin solid line) or ULBP3 (thick solid line) or with secondary FITC-labelled goat anti-mouse IgG alone (dotted line). PB cell subpopulations, indicated above each histogram, were distinguished with mAbs specific for lineage markers. Analysis of monocytes and granulocytes in two healthy donors with ULBP-positive (N1) and ULBP-negative (N2) phenotype is shown.

1.1.2 mRNA Expression of ULBPs on B Cells and Monocytes

To confirm the data obtained with flowcytometry, mRNA from cell lysates of FACS sorter-purified B cells, T cells and ULBP-positive monocytes was isolated and presence of ULBP transcripts was analyzed by quantitative real-time RT-PCR. ULBP1 message was detectable in B cells and monocytes of all donors. ULBP2 and ULBP3 transcripts were detectable in B cells of some donors but were below detection level in monocytes. Some T cell preparations were positive for ULBP mRNAs, although they were always phenotypically ligand negative (Figure V-2). The presence of ULBP messages in T cells

was unlikely due to contaminations, because analyzed preparations were highly pure (>97%). The ULBP signal was not detectable with less than 32 amplification cycles, indicating very low abundance. HPRT was used as an internal control. These data confirm previous reports with hematopoietic cell lines[13,109] that levels of ULBP mRNA and protein does not always correlate.

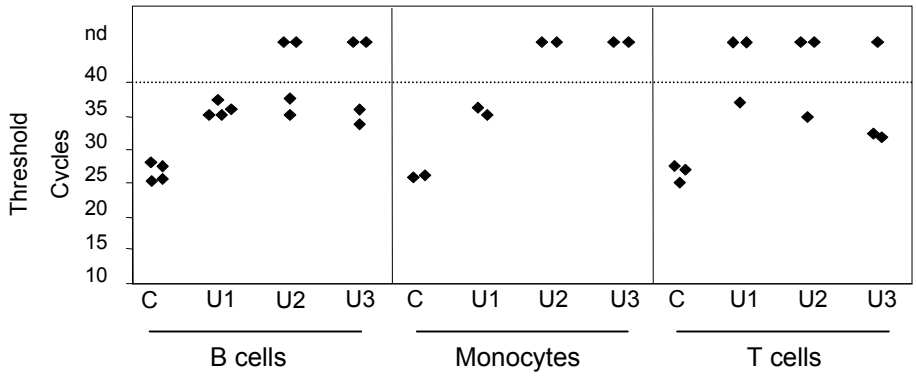

Figure V-2, mRNA Expression of ULBP1, ULBP2 and ULBP3. Real-time PCR analysis of ULBP1 (U1), ULBP2 (U2) and ULBP3 (U3) mRNA levels in B cells, monocytes and T cells purified from 2-4 donors, as indicated by the number of diamonds. The dotted line marks the upper limit of 40 amplification cycles. Threshold cycles; number of cycles at which the amount of PCR product passed the threshold of detection. C; control mRNA of HPRT. nd; not detectable

1.2 MIC Ligands Expressed on B Cells and Monocytes

Transcripts of MICs are found in low abundance in many cells, still surface expression of MIC protein is rare in adult healthy tissue. In contrast, MICs become upregulated in many epithelial tumor cells[98]. To analyze ULBP-positive monocytes and B cells as potential MIC expressing cells, blood from healthy donors was labelled with mAbs binding to MICA/B and with mAb binding to CD33 and CD19. Same as for ULBPs, there was a variegated expression of MICA/B on monocytes among healthy donors. MICA/B were not detectable or very low (MFI ratio < 2) on monocytes from 4 out of 8

donors but present in the remaining donors at MFI ratio of up to 8.7 (Table V-2). On B cells of all tested individuals MICA/B expression was highly pronounced with MFI ratio of up to 85.2.

These results show that MICA/B expression on B cells is markedly higher than on monocytes, which is in line with the data obtained for ULBPs.

Table V-2, Expression of MICA/B on Monocytes and B cells

	MFI ratio	
	Mean ± SEM	(Range)
Monocytes (n=8)	3.1 ± 0.9	(1.2 – 8.7)
B cells (n=7)	34.8 ± 8.6	(21.2 – 85.2)

1.3 NKG2D Ligands Are Absent on in vitro Derived Dendritic Cells

Since B cells and monocytes were identified as the major ligand expressing population in PB, it was of interest whether ligand expression represents a common feature of antigen presenting cells. Thus ligand expression was also analyzed on DCs. For this purpose in vitro generated CD14+ monocytes were differentiated into iDCs using GM-CSF and IL-4. After 3 days of incubation the culture contained 37% of CD1a+CD14- iDCs, which were lacking surface expression of ULBP1, ULBP3 and MICA/B (Figure V-3A). Addition of CD40L for 5 days resulted in 14.7% of mDCs displaying a $CD1a^{+}HLA\text{-}DR^{bright}$ phenotype expressing activation markers CD80, CD83 and CD86. ULBP1, ULBP3 and MICA/B were absent also on mDCs (Figure V-3B).

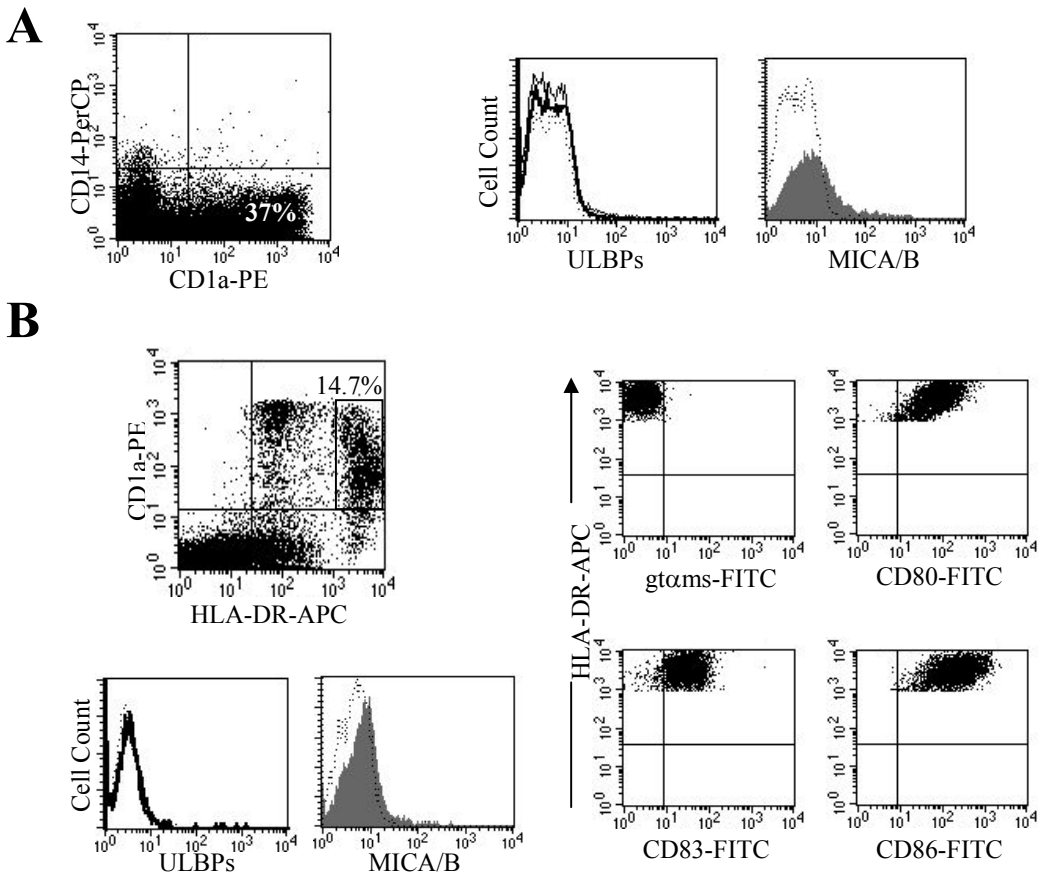

Figure V-3, Expression of Ligands on in vitro Generated DCs. FACS staining of ULBP1 (solid line), ULBP3 (thick solid line) and MICA/B (shaded area) or secondary FITC-labelled gtαms and gtαrat (dotted line) on (A) CD1a+CD14- iDCs and (B) CD80, CD83 and CD86 were stained on CD1a+HLA-DR^{bright} mDCs and detected by secondary FITC-labelled gtαms mAb.

This result shows that ULBP proteins are expressed on cell surface of B cells, platelets as well as monocytes and granulocytes from majority of donors. Except for platelets, which express ULBP2 at highest levels, the ligand which predominates in normal PB is ULBP1.

1.4 Validation of Soluble NCR Dimers

So far viral hemagglutinins were shown to bind to NKp46 and NKp44[56,84] but cellular ligands for NCRs have not yet been described. In order to identify activating cellular ligands the group or G. De Libero at the laboratory of Experimental Immunology (Department of Research, University Hospital Basle, Switzerland) generated dimers of recombinant proteins corresponding to extracellular domains of NKp30, NKp44 and NKp46. These soluble (s) NCRs were used in our study to identify potential ligand expressing cells.

Several cell lines were used to test the binding capacity of NCR dimers. Complexes of sNKp30, sNKp44 and sNKp46 bound to THP-1 (MFI ratios of 3.3±0.5, 4.3±0.5 and 5.3±0.9, respectively) and to HL60 (MFI ratios 2.7±0.2, 3.0±0.8 and 3.5±0.7, respectively), but not to K562-L cells (Figure V-4A). To confirm the specificity of the dimers, the ability of the sNCRs in inhibiting recognition of target cells was examined in killing assays and IFN-γ release assays. The sNCR cocktail strongly inhibited the killing of ligand expressing THP-1 and HL60 cells. In contrast, lysis of NCR ligand-negative K562-L cells remained unaffected by introducing sNCR dimers (Figure V-4B).

For assessing the IFN-γ production by NK cells due to ligand recognition, NK cells were cocultured with THP-1 or HL60 cells for 44 hours. Release of IFN-γ was measured in culture supernatants using ELISA. As a positive control, NK cells were stimulated with IL-12 and IL-18 in the absence of target cells and produced 617.5 ± 69.4 ng/mL IFN-γ (Table V-3).

NK cells, cocultured with THP-1 cells, produced up to 15.77 ng/mL and 79.9 ng/mL IFN-γ in response to IL-2 and IL-12, respectively (Table V-3, Figure V-5A). Incubation of NK cells with HL60 cells resulted in release of 1.85 ng/mL and 39.2 ng/mL IFN-γ in IL-2 and IL-12, respectively. Lower IFN-γ production after incubation with HL60 cells is in line with lower expression levels of NCR ligands on HL60 cells than on THP-1 cells. Note that NK cells alone released markedly less IFN-γ than after coculture with target cells (Table V-3).

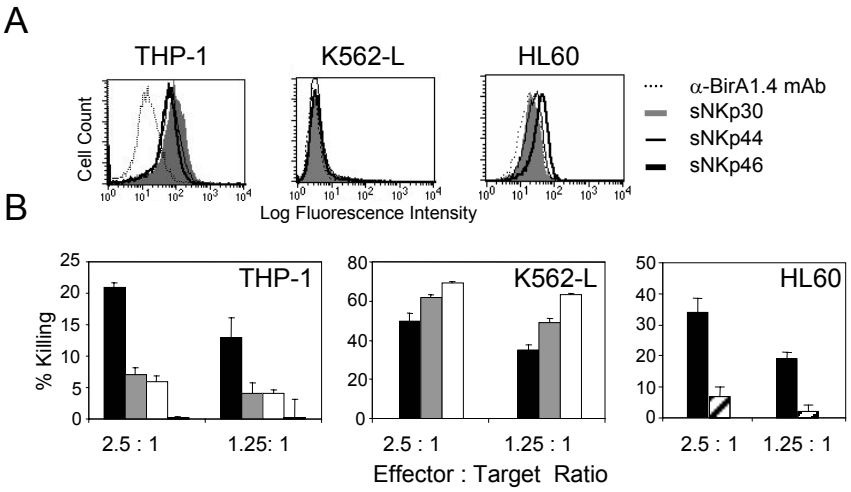

Figure V-4, Soluble NCR dimers recognize THP-1 and HL60 cells and inhibit their killing by NK cells. (A) FACS analysis of THP-1, K562-L and HL60 cells stained with sNKp30 (shaded area), sNKp44 (thin solid line), sNKp46 (thick solid line) and anti-BirA1.4 mAb (dotted line) all detected by secondary FITC-labelled goat anti-mouse IgG. (B) Inhibition of NK cell-mediated killing of THP-1 and HL60 cells by sNKp30, sNKp44 and sNKp46 dimers. A calcein release-based cytotoxicity assay was used to determine the lysis of THP-1, K562-L and HL60 cells by NK cells at the indicated effector:target ratios. The killing assay was performed in the absence of sNCRs (black bars) or in the presence of a mixture of sNKp30, sNKp44 and sNKp46 at 5 μg/ml (grey bars), 20 μg/ml (open bars) and 50 μg/ml (hatched bars; to low to depict in THP-1) of each dimer.

When sNKp30 and anti-NKG2D mAb were added to THP-1 cocultures, production of IFN-γ was reduced in a dose-dependent manner (Figure V-5B), while sNKp44 and sNKp46 were not interfering with the release of IFN-γ (data not shown). This suggests that primarily the ligands of NKG2D and NKp30 expressed by THP-1 cells are responsible for triggering the NK cell to release IFN-γ.

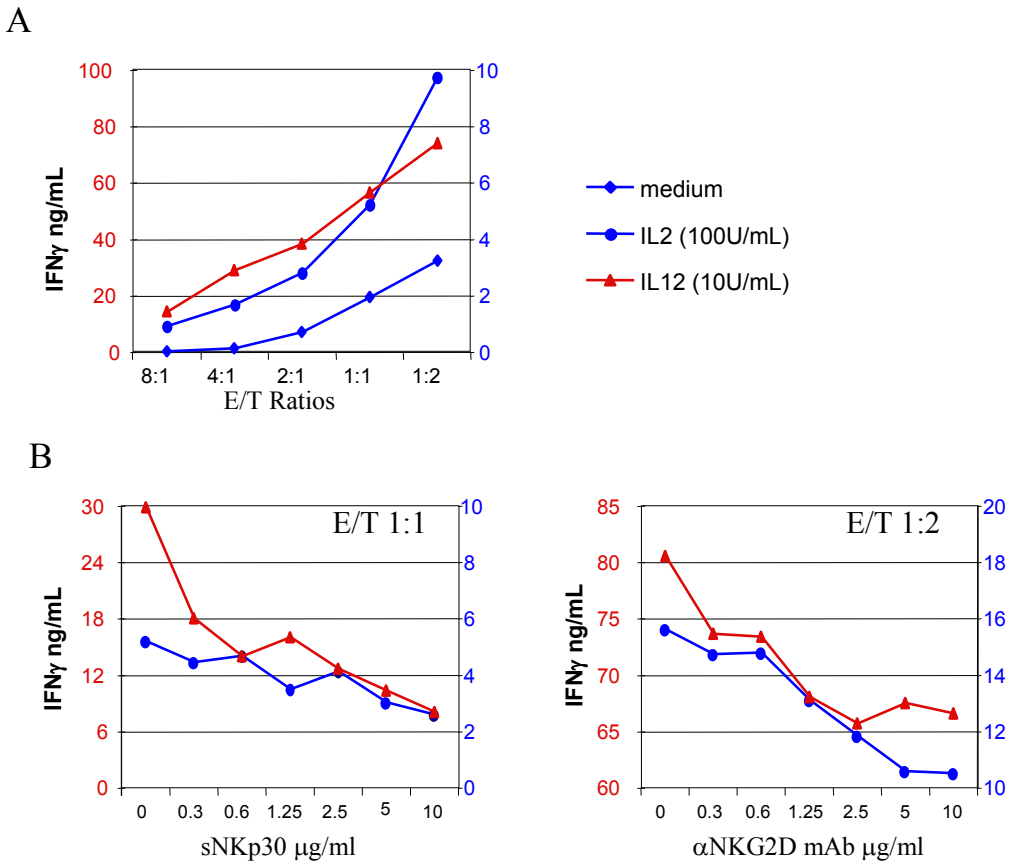

Figure V-5, IFN-γ Production of NK Cells upon Coculture with THP-1 Targets. NK cells were incubated in the presence of THP-1 cells in IMDM, 10% FCS (medium; blue diamond, blue y-axis), IL-2 (blue circle, blue y-axis) or IL-12 (red triangle, red y-axis) for 44 hours. IFN-γ release was measured in supernatants of cultures using ELISA. (A) Co-cultures were performed at different effector:target ratios (E/T). (B) Co-cultures were performed in IL-2 and IL-12 at effector:target ratio of 1:1 and 1:2, respectively. sNKp30 and anti-NKG2D mAb were added at different concentrations.

These findings show that expression of NCR ligands on target cells confers to them the ability to activate of NK cells resulting in lysis of targets and in production of IFN-γ. This NK cell stimulation can be inhibited by masking the interaction of activating NCRs with their ligands using sNCR dimers, confirming the functionality of the reagents.

Table V-3, IFN-γ Release by NK Cells in Cocultures with Target Cells

	NK cells + THP-1		NK cells + HL60		NK cells alone		
	IL-2	IL-12	IL-2	IL-12	IL-2	IL-12	IL-12, -18
	n=5	n=3	n=1	n=2	n=3	n=3	n=6
E/T	1:1	1:2	1:1	1:2			
IFN-γ	8.09 ± 1.98	59.9 ± 17.1	1.85	39.2	1.5 ± 0.12	5.97 ± 2.59	617.5±69.4
ng/mL	(5.18-15.77)	(25.8-79.9)	-	(38.2-40.2)	(1.3-1.7)	(0.92-9.5)	(370-900)

Cultures were performed in IL-2 (100U/mL), IL-12 (10U/mL) or in IL-12 and IL-18 (10U/mL and 100ng/mL, respectively). Numbers are showing mean values (ng/mL) ± SEM and (range). E/T indicates effector to target ratios.

1.5 Expression of NCR Ligands on B Cells and Monocytes

Using FACS, sNCR dimers were found to bind to monocytes and B cells of healthy donors. Similarly to ULBPs, not all individuals had ligand positive monocytes. Putative NCR ligands were not detectable or were very low (MFI ratio < 2.0) in monocytes from 4 out of 11 donors **(**Table V-4**)**.

These results show that similar to ULBPs putative ligands for NCRs are expressed on B cells and monocytes in PB of healthy donors and on tumor cell lines THP-1 and HL60.

Table V-4, Binding of sNCRs to B Cells and Monocytes in Healthy PB

	B cells		Monocytes	
	MFI Ratio		MFI Ratio	
	Mean ± SEM	Range	Mean ± SEM	Range
NKp30 Ligand n=7/8	25.1 ± 3.5	(11.8-43.1)	9.6 ± 5.8	(1.3-48.8)
NKp44 Ligand n=9/10	32.5 ± 7.6	(14.1-69.8)	6.0 ± 2.3	(1.5-19.1)
NKp46 Ligand n=12/13	26.2 ± 7.1	(5.0-60.0)	2.8 ± 0.7	(1.1-7.7)

n indicates number of B cell/monocyte samples analyzed

1.6 Expression of NCR and NKG2D Ligands Is Low on AML Blasts

To analyze ULBP and NCR ligand expression in AML, PB cells from 30 AML patients (Table IV-1) were stained with mAb against CD45 to distinguish $CD45^{dim}$ leukemic blasts from $CD45^{bright}$ residual normal cell populations (Figure V-6).

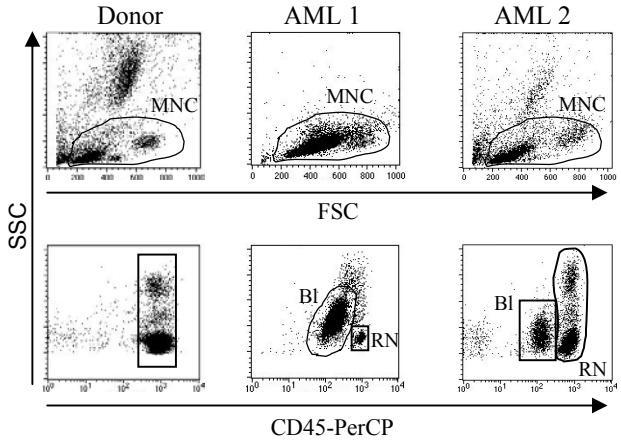

Figure V-6, Gating of AML Blasts in FACS Analysis. To distinguish AML blasts from residual normal cells, blood samples were stained with anti-CD45 mAb. Cell populations were gated according to forward (FCS), sideward (SSC) scatter and to CD45 expression level. FACS analysis of one healthy donor and two AML patients (AML1, AML2) are depicted. The left panel shows the gating of mononuclear cells (MNC). The right panel represents expression levels of CD45 on AML blasts (Bl) and on residual normal cells (RN) in AML patients.

$CD45^{bright}$ B cells of AML patients expressed NKG2D and NCR ligands at normal high levels (Table V-5 and -6) as was seen with B cells of healthy donors (Table V-1,-2 and -4). Furthermore, NKG2D and NCR ligands were found on $CD45^{bright}$ patients' monocytes and granulocytes at variable levels (Figure V-8, Table V-5), resembling the variability of ligand cell surface density observed in healthy donors (Table V-1,-2 and -4). However, ULBP-low phenotype of $CD45^{bright}$ cells was observed in only 6 patients of the cohort, while ULBP expression by $CD45^{dim}$ blasts was very low with a MFI ratio < 2.0 in 23 out

of 30 patients (Figure V-7). In addition, in 3 among the remaining 7 patients, only 6-37% of all blasts were ULBP positive.

Table V-5, Expression of ULBPs and Putative Ligands for NCRs on B Cells of AML Patients

	ULBP1 (n=27)	**ULBP2** (n=13)	**ULBP3** (n=27)	**NKp30L** (n=7)	**NKp44L** (n=9)	**NKp46L** (n=12)
MFI ratio	15.5 ± 1.6 (4.5-32.7)	7.6 ± 1.4 (3.8-20.8)	10.6 ± 1.2 (3.2-28.7)	19.3 ± 1.6 (14.7-28.4)	21.4 ± 4.0 (8.1-42.9)	22.0 ± 3.6 (9.7-45.1)

Numbers correspond to mean values ± SEM and (Range); L, ligand.

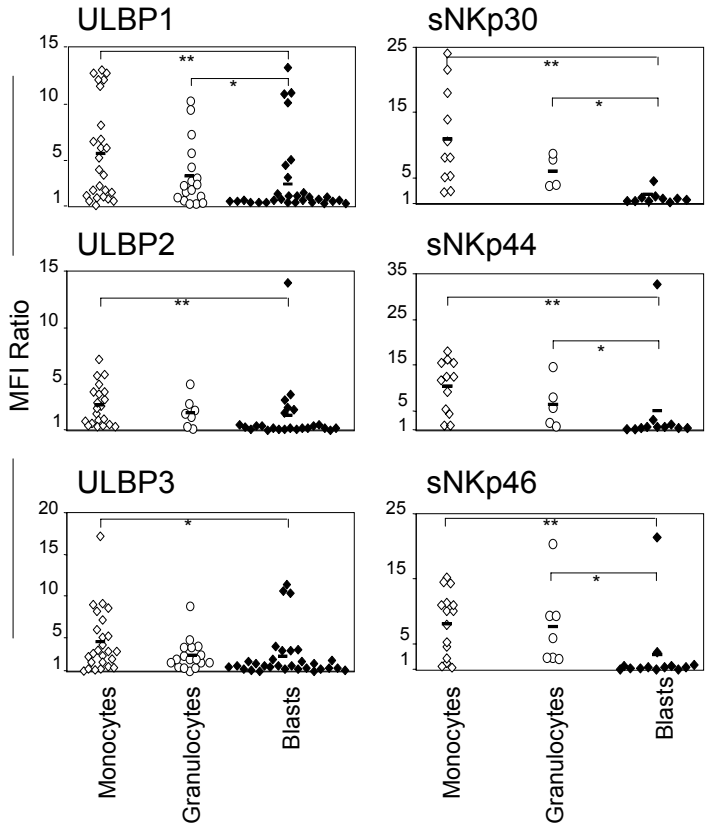

Figure V-7. Low ULBP and NCR ligand expression in AML Blasts. PB samples of AML patients were stained with mAbs against lineage-specific markers and anti-CD45 mAbs to distinguish $CD45^{dim}$ leukemic blasts from $CD45^{bright}$ residual normal monocytes and granulocytes. ULBP1, ULBP2 and ULBP3 (left panels) were measured with specific mAbs on monocytes (open diamonds), granulocytes (open circles) and blasts (black diamonds). Binding of sNKp30, sNKp44, and sNKp46 dimers (right panels) to monocytes (open diamonds), granulocytes (open circles) and blasts (black diamonds). Mean values are indicated as horizontal bars. * $p< 0.05$; ** $p< 0.005$.

MICA/B expression was absent on blasts of 6 out of 9 patients (Table V-6). Similarly to NKG2D ligands, $CD45^{dim}$ AML blasts displayed a very low sNKp30, sNKp44 and sNKp46 dimer-binding capacity (MFI ratio < 2.0) in 10 out of 12 analyzed patients (Figure V-7).

Table V-6, MIC Molecules in AML Patients

	$CD45^{dim}$ Blasts	**$CD45^{bright}$ B Cells**	**$CD45^{bright}$ Monocytes**
Average ± SEM	1.8 ± 0.3	33.6 ± 5.6	6.6 ± 2.6
(Range)	(1.0-3.6)	(15.3-64.0)	(2.0-26.8)

Values represent average mean fluorescence intensities of MIC proteins detected on blasts and residual MNCs of 9 analyzed AML patients.

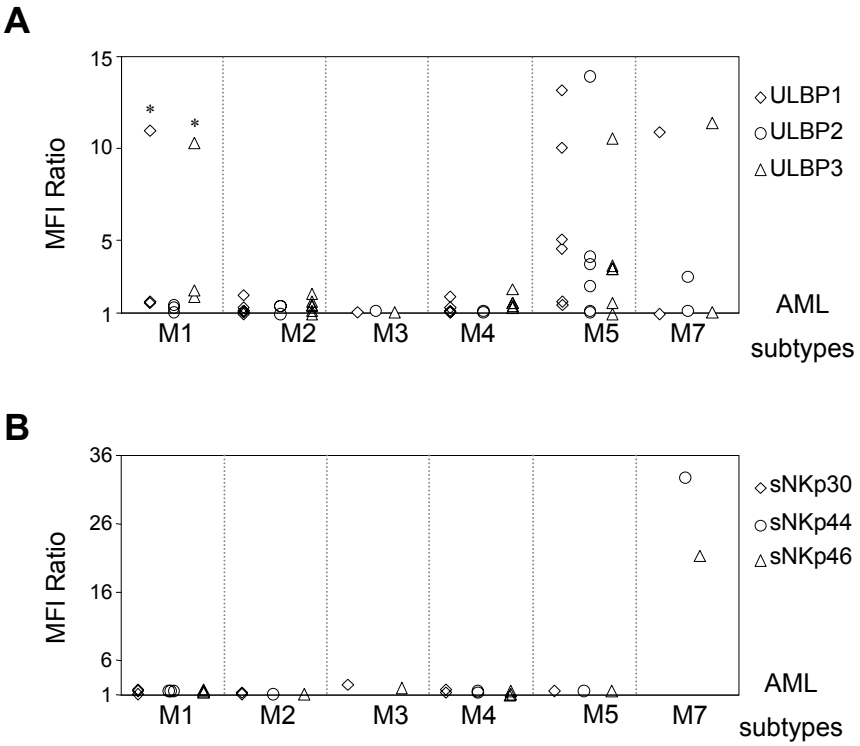

Figure V-8, Ligand Expression in AML Subtypes. ULBP and NCR ligand expression classified according to AML subtype. PB cells of patients with AML of subtypes M1-M7 were stained as described in the legend to Figure 3 and $CD45^{dim}$ leukemic blasts were analyzed for the expression levels of ULBP (A) and NCR ligands (B). *; only 20% of blasts were ULBP1 and ULBP3 positive.

These results demonstrated that AML blasts in the majority of patients display negative/low surface levels of ligands for NKG2D and NCRs. Analysis of ligand expression in terms of AML FAB classification revealed that 5 out of 6 patients with monocytic M5 and 1 out of 2 patients with megakaryocytic M7 had ULBP-positive blasts. Among other subtypes, only 1 patient with M1 had ULBP-positive blasts, which however constituted only 20% of his blast population (Figure V-8). This suggests that ligand expression by myeloid blasts is more frequent when leukemic transformation takes place at later stages of differentiation, although investigation with a larger group of patients is needed to conclude about the association of NKG2D ligand expression and the subtype of myeloid leukemia.

1.7 NKG2D and NCR Ligands Are Absent on BM Progenitors

To investigate further whether ligand levels may be related to the differentation stage of myeloid cells, NKG2D and NCR ligand expression by normal BM $CD34^+$ hematopoietic progenitors and precursors committed to the myeloid lineages were examined (Figure V-9A-B, Table V-7).

Table V-7, MIC Expression in Healthy BM

	$CD34^+CD33^-$	**$CD34^+CD33^+$**	**$CD34^-$ $CD33^+CD14^-$**	**$CD34^-$ $CD33^+CD14^+$**
Average ± SEM	1.6 ± 0.36	1.5 ± 0.20	3.8 ± 1.30	7.4 ± 1.41
(Range)	(1.1-2.3)	(1.2-1.9)	(1.7-6.2)	(6.0-10.3)

Values represent average mean fluorescence intensities of MIC proteins detected in 3 analyzed BM samples.

Early $CD34^+CD33^-$ progenitors and also $CD34^+CD33^+$ cells displayed very low surface levels of NKG2D and NCR-specific ligands (MFI ratio < 2.0). $CD34^-$ myeloid cells, both

$CD33^{+}CD14^{-}$ and mature $CD33^{+}CD14^{+}$ monocytes present in the BM, expressed the ULBPs, with a strong prevalence of ULBP1 compared to ULBP2 and ULBP3, and displayed MICA/B and all the NCR ligands on their surface. These results demonstrate that cell surface ULBPs, MICs and NCR ligands become expressed upon loss of the early hematopoietic marker CD34 and acquisition of the myeloid markers CD33 and CD14.

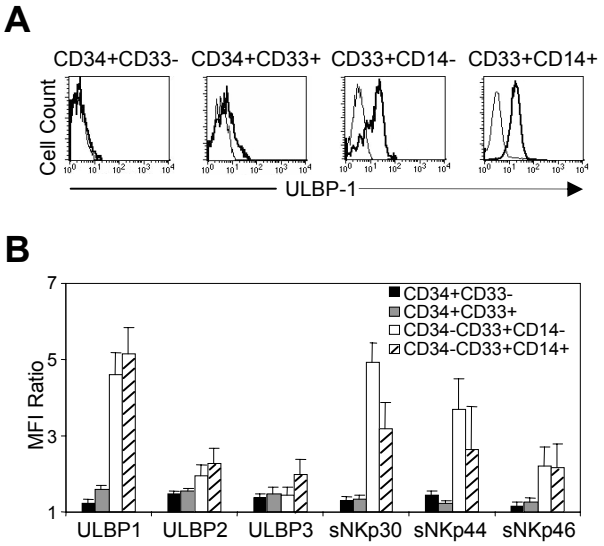

Figure V-9, ULBP and NCR ligand expression is low on $CD34^{+}$ cells and well pronounced on myeloid progenitors in normal BM. ULBP expression was determined by FACS in the indicated BM subpopulations. (A) BM staining with ULBP1-specific mAbs (thick line) or control secondary FITC-labelled goat anti-mouse IgG alone (thin line). (B) BM staining from 3 healthy donors with mAbs to ULBP1, ULBP2, ULBP3, as well as sNKp30, sNKp44 and sNKp46 dimers.

1.8 Progenitors and Monocytes Are Protected from Killing by Autologous NK Cells

To investigate whether expression of ligands for NKG2D and NCRs renders healthy cells susceptible to NK mediated killing, monocytes and autologous NK cells were purified by

magnetic labeling from PB of a healthy donor. Monocytes and NK cells were cultured separately for 2 days in the presence of myeloid growth factors and IL-2, respectively. Next, autologous killing was assessed by a chromium release assay. Killing of autologous monocytes was only 11% at an effector to target ratio of 10:1. When inhibitory signaling was blocked by anti-MHC class I mAbs killing was increased to 26%. Killing that was measured after adding control anti-MHC class II mAbs was always below lysis detected after adding anti-MHC class I mAbs (Figure V-10A).

$CD34^+$ progenitor cells purified from mobilized PB by magnetic labeling were cultured for 3 days in FL and Tpo. Killing of $CD34^+$ progenitor cells was assessed in a chromium release assay using autologous NK cells. Lysis of progenitors was not detectable at tested effector to target ratios ranging from 5:1 down to 0.6:1 (data not shown). Blocking of inhibitory signaling by anti-MHC class I mAbs induced 4% killing. However, addition of control anti-MHC class II mAbs induced the same cell lysis (Figure V-10B).

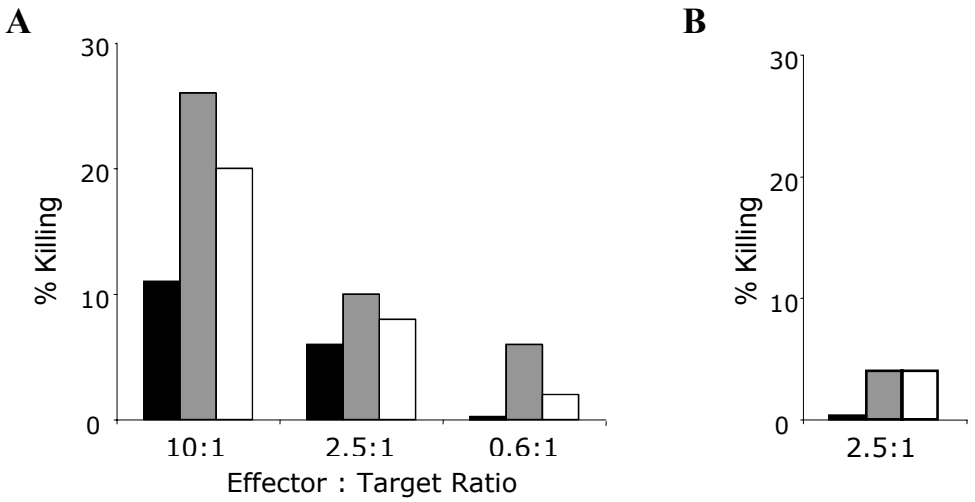

Figure V-10, Killing of Monocytes and CD34+ Progenitors by Autologous NK cells. (A) Monocytes cultured for 2 days in myeloid growth factors were analyzed at different effector to target ratios in cytotoxicity assay using autologous NK cells cultured for 2 days in IL-2. (B) CD34+ progenitors cultured for 3 days in FL and Tpo were assessed for killing at an effector to target ratio of 2.5:1 by autologous NK cells cultured for 2 days in IL-2. Killing was measured without addition of mAbs (black bars), with addition of anti-MHC class I mAb (10μg/mL; grey bars) or addition of control anti-MHC class II mAb (10μg/mL; open bars). Values represent one experiment carried out in triplicates.

Taken together these results show that ligand positive monocytes are protected from killing by autologous NK cells through engagement of inhibitory NK cell receptors. Blocking of inhibitory signaling with anti-MHC class I mAbs induced killing of monocytes, whereas killing of progenitors remained very low, indicating that cells lacking ligands for triggering NK cell ligands are not recognized as target cells.

1.9 In Vitro Upregulation of ULBP1 and NCR Ligands

1.9.1 ULBP1 and NCR Ligand Upregulation on Monocytes

To understand the variable ULBP and NCR ligand expression on monocytes and granulocytes from different individuals, ULBP-negative $CD14^+$ monocytes were purified from healthy donors by magnetic labeling. Isolated cells were maintained in culture by myeloid growth factors (GFs; FL, SCF and GM-CSF). Furthermore, various cytokines (IFN-α, IFN-γ, IL-1β and TNF-α) and LPS, all known to activate monocytes[161,167], were added to the cultures. Analysis of ULBP surface expression after 3 days of culture revealed that IFN-α, IL-1β, TNF-α and LPS used singly or in combination were not effective in inducing expression of ULBPs and putative NCR ligands (Table V-8).
However, ULBP1 was selectively expressed in monocytes cultured with myeloid growth factors. ULBP1 level was further increased when cells were additionally activated with IFN-γ(Figure V-10A). ULBP2 and ULBP3 were unaffected by any of these conditions (data not shown). Ligands for NCRs were also upregulated in response to myeloid growth factors and IFN-γ (Figure V-10A). The modulation of ULBP1 and NCR ligands with IFN-γ was dose-dependent and gradually increased within three days of incubation (Figure V-10B).
These experiments indicate that the presence of ligands for NKG2D and NCRs may correspond to the activation or maturation state of monocytes, since their expression can be induced by myeloid growth factors and IFN-γ. ULBP2 and ULBP3 seem to be regulated by a different pathway.

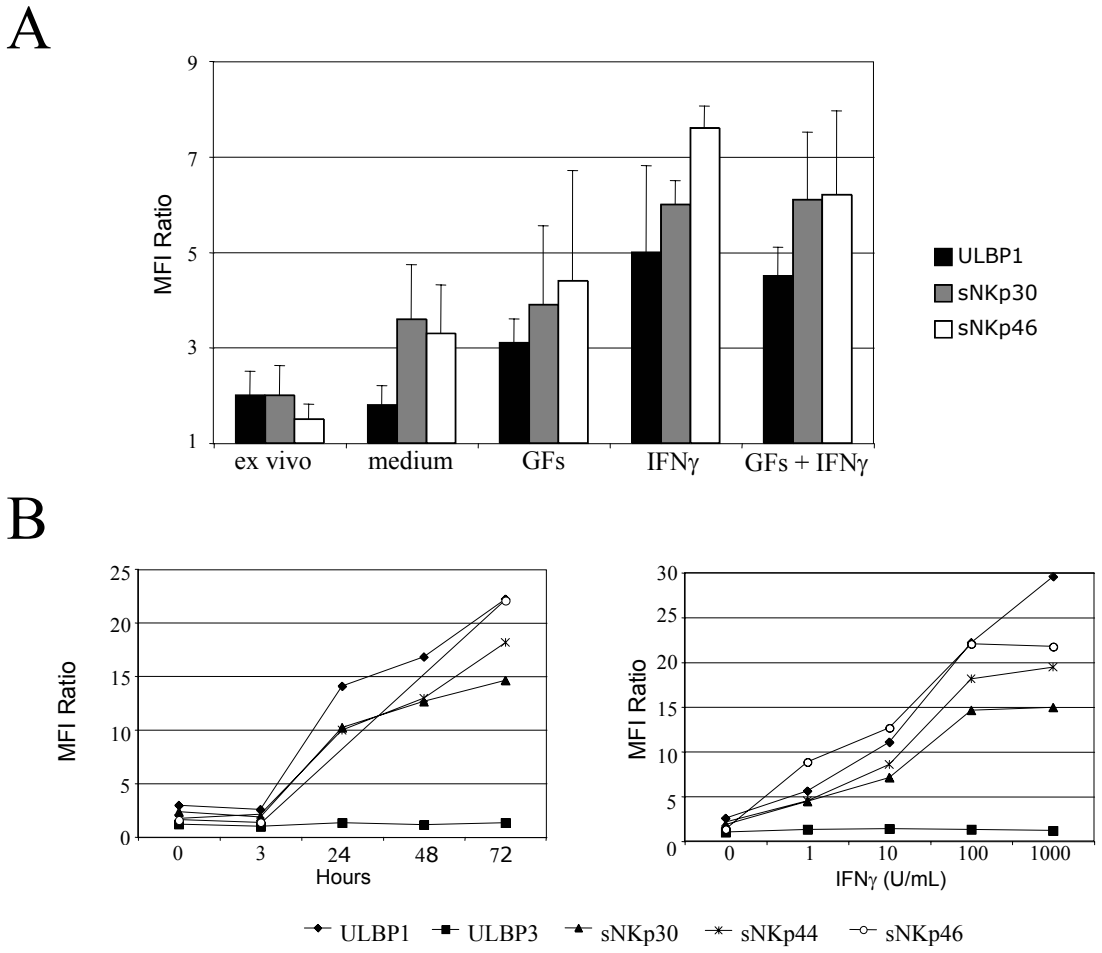

Figure V-10, Upregulation of ULBP1 and Putative NCR ligands in PB Derived CD14+ Monocytes. (A) FACS analysis of anti-ULBP1 mAb (black bars), sNKp30 (grey bars) and sNKp46 (open bars) surface binding by freshly isolated (ex vivo) $CD14^+$ monocytes. Monocytes were subsequently cultured for 3 days in IMDM, 10% FCS (medium) or with GFs (SCF+FL+GM-CSF;) and IFN-γ as indicated. Results represent mean values ± SEM of 3 independent experiments. (B) Myeloid CD33+ cells derived in vitro from CD34+ progenitors were incubated with IFN-γ before measuring anti-ULBP1 mAb (black diamond), anti-ULBP3 mAb (black quadrant), sNKp30 (black triangle), sNKp44 (asterix) and sNKp46 (white circle) surface binding by FACS. Left panel shows different time points of FACS analysis of cells cultured in 100 U/ml IFN-γ. Right panel shows FACS analysis of cells cultured for 72 hours in different concentrations of IFN-γ.

Table V-8, Expression of Ligands for NKG2D and NCRs on Monocytes after in vitro Culture

	ULBP1	ULBP2	ULBP3	sNKp30	sNKp44	sNKp46
medium	1.8*	1.1*	1.1	3.6±1.1**	3.3±1.0**	10.9±4.7**
GFs	3.1±0.5#	1.2	1.2	3.9±1.6**	4.4±2.3**	14.6±6.0**
GFs, IFN-γ	4.5±0.6#	1.1	1.1	5.7±1.2**	6.2±1.8**	11.1±5.0**
GFs, IFN-α	1.7*	1.1	1.1	3.9*	3.5*	9.8*
GFs, TNF-α	2.9	1.1	1.1	-	-	-
GFs, IFN-γ/α, TNF-α	2.4	1.2	1.1	-	-	-
GFs, IFN-γ, IL-1β, LPS	5.0*	-	-	-	-	-

GFs include FL, SCF and GM-CSF. If not otherwise indicated experiments were only done once. Numbers correspond to mean values obtained from * 2, ** 3 or # 4 experiments.

1.9.2 ULBP1 and NCR Ligands Are Upregulated upon Myelomonocytic Differentiation of BM Progenitors

To confirm that ligand expression is upregulated upon myelomonocytic differentiation, purified BM-derived $CD34^+$ cells were cultured in the presence of myeloid growth and differentiation factors, SCF, FL and GM-CSF. After 7 days, cultures consisted of about 84.5% ± 1.7% $CD34^-CD33^+$ cells containing 16.5% ± 2.8% CD14 expressing cells (Figure V-11A). Myeloid cells generated in vitro from the ligand-negative hematopoietic progenitors expressed ULBP1 and NCR ligands (Figure V-11B). The surface expression of ULBP1 and NKp30 ligand was further upregulated upon addition of IFN-γ, also used in combination with LPS and IL-1β̃ Expression of ULBP2 and ULBP3 was not enhanced under these conditions. These results show that ULBP1 and ligands for NKp30, NKp44 and NKp46 are induced by the myeloid growth and differentiation factors and in response to IFN-γ, whereas signals upregulating expression of ULBP2 and ULBP3 on $CD33^+$ cells remain unknown.

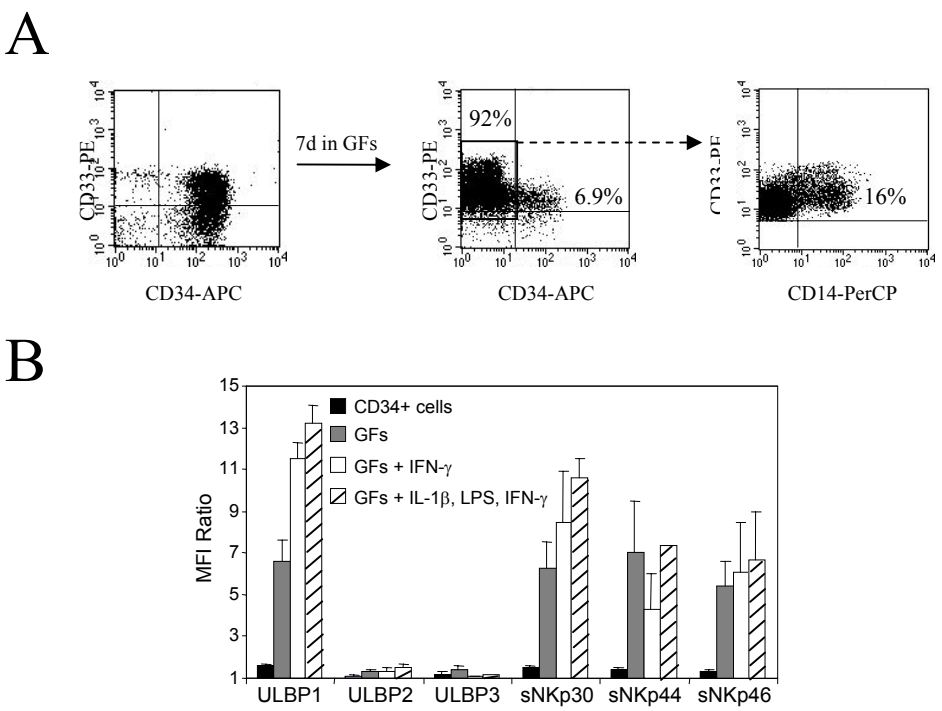

Figure V-11, ULBP and NCR ligand surface expression increases upon myeloid differentiation of normal BM progenitor cells in vitro. (A) Purified $CD33^{+/-}CD34^{+}$ BM progenitors were cultured with GFs (FL, SCF, GM-CSF) and analysed for CD34, CD33 and CD14 expression by FACS. Proportion of $CD14^{+}$ myeloid cells is displayed on $CD33^{+}$ gated cells. (B) FACS analysis of ULBP1, ULBP2 and ULBP3 surface expression and sNKp30, sNKp44 and sNKp46 binding by freshly isolated $CD34^{+}$ cells (black bars) and after 7 days of culture with GFs (SCF+FL+GM-CSF; grey bars), GFs together with IFN-γ (open bars) or together with IL-1β, LPS and IFN-γ (hatched bars) for an additional 3 days. Results represent the mean ± SEM of 3 BM samples.

1.9.3 Upregulation of Ligands for Activatory NK Cell Receptors on AML Blasts Increases NK Cell Mediated Killing

Next, it was investigated whether FL, SCF, GM-CSF and IFN-γ can upregulate the triggering ligands on AML blasts in vitro. Following 4 days in culture, ULBP1 and NCR ligand expression were increased on blasts of 2 out of 5 patients with initially low as well as high ligand levels (Figure V-12A). As already found with normal cells, ULBP2 and

ULBP3 remained unchanged on leukemic blasts. To test whether an increase in ULBP1 and NCR ligand levels raises the susceptibility of AML blasts to NK cell-mediated cytolysis chromium release assays were performed (Figure V-12B). The sensitivity to killing decreased following treatment with FL, SCF, GM-CSF and IFN-γ, most likely due to a protective effect of HLA class I molecules which were upregulated in response to IFN-γ two- to fourfold. However, when blasts were preincubated with antibodies preventing interactions of HLA class I ligands with the inhibitory receptors, the killing of blasts with upregulated ligand expression in response to growth factors and IFN-γ was markedly enhanced from 15% and 25% to about 30% and 45%, respectively.

To confirm inhibition of allogeneic NK cells through HLA class I molecules on the surface of blasts, HLA alleles of donors and patients patients were compared (Table V-9).

Table V-9, HLA Types of AML Patients and Allogeneic NK Cells Used in Killing Assays

Patient A	A3	A29	B38	B44	Cw16	
PBNK VII	A2	A32	B8	B40	Cw2	Cw7
Patient B	A2	A32	B41	B27	**Cw1**	Cw17
PBNK IX	A2		B40	B51	Cw2	**Cw3**
Patient C	A1	A26	B55	B57	**Cw3**	Cw6
PBNK X	A2	A29	B18	B44	**Cw7**	Cw16
Patient D	A2	A24	B7	B35	Not done	
PBNK VII	A2	A32	B8	B40	Cw2	Cw7
Patient E	A2	A3	B51	B60	**Cw3**	**Cw4**
PBNK IX	A2		B40	B51	**Cw2**	**Cw3**

Overlapping HLA molecules, that can inhibit NK cell activation, of AML patients and allogeneic NK cells are marked as bold. PBNK; PB derived NK cells.

Killing of blasts from patient A could not be increased by masking inhibitory signalling through MHC class I molecules. This was in line with the fact that cells from patient A do not engage the inhibitory KIRs present on the allogeneic NK cells used in the killing assay (Table V-9). Killing of blasts from patients B to E was markedly increased when engagement of inhibitory signals in NK cells was blocked by anti-MHC class I mAb.

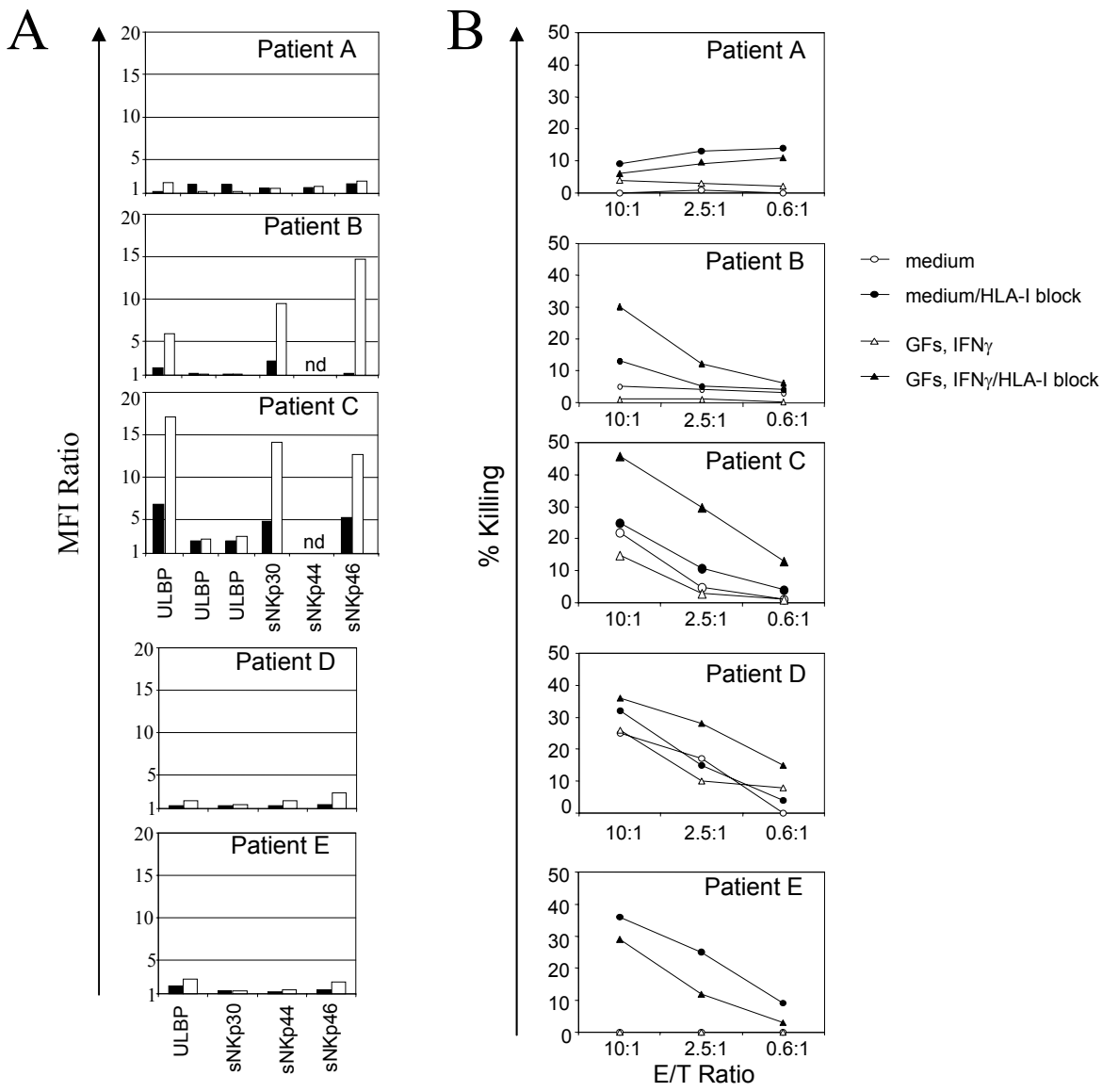

Figure V-12, Upregulation of ULBP and NCR ligands increases the susceptibility of AML blasts to NK cell-mediated cytotoxicity. (A) Upregulation of ULBP and NCR ligands on AML blasts from five patients. Blasts were cultured for 4 days in medium only (black bars) or with GFs (FL+SCF+GM-CSF) and IFN-γ (open bars) before FACS analysis. (B) Killing of blasts cultured for 4 days without (medium) or with GFs and IFN-γ was determined with or without blocking of HLA class I molecules with mAb (10μg/mL), as indicated. NK cells derived from healthy donors were used at the indicated effector:target ratios. nd, not done.

Analysis of HLA typing revealed that in patients B and C there was at least one match in HLA alleles compared to the allogeneic NK cells used in the killing assays. In patient E where masking of inhibitory signalling resulted in the most pronounced increase of cytotoxicity at least two HLA alleles matched compared to the healthy NK cells (Table V-9).

Interestingly, blasts from patient E were highly killed after blocking HLA class I molecules although ULBP1 and NCR ligands were only expressed at very low levels. This indicates that there might be other triggering ligands present, such as the very recently identified RAET1E and RAET1G[115].Killing of blasts from patients B and C was also tested with freshly isolated NK cells from healthy donors, cultured for 4 days in IL-2 (Figure V-13B). Importantly, blasts from patient B were highly killed without blocking of MHC class I molecules, although at least one HLA allele matched with the allogeneic NK cells used (PBNK X see Table V-9).

Cytotoxicity of NK cells was again improved when blasts from patient B had been treated with myeloid growth factors and IFN-γ. There was no increase in killing of growth factor and IFN-γ treated blasts from patient C, though. This might be explained by the fact that blast from patient C already expressed ligands ex vivo.

To analyse whether enhanced killing of blasts cultured with growth factors and IFN-γ was dependent on ligand upregulation, blocking with anti-NKG2D mAb was performed. Lysis of AML blasts from patient B and C was reduced from 30% to 23% and from 46% to 26%, respectively, when NKG2D was masked by specific mAb (Figure V-13A).

These results indicate that enhanced levels of ligands for the activating receptors NKG2D and NCR can increase the recognition of AML blasts by NK cells.

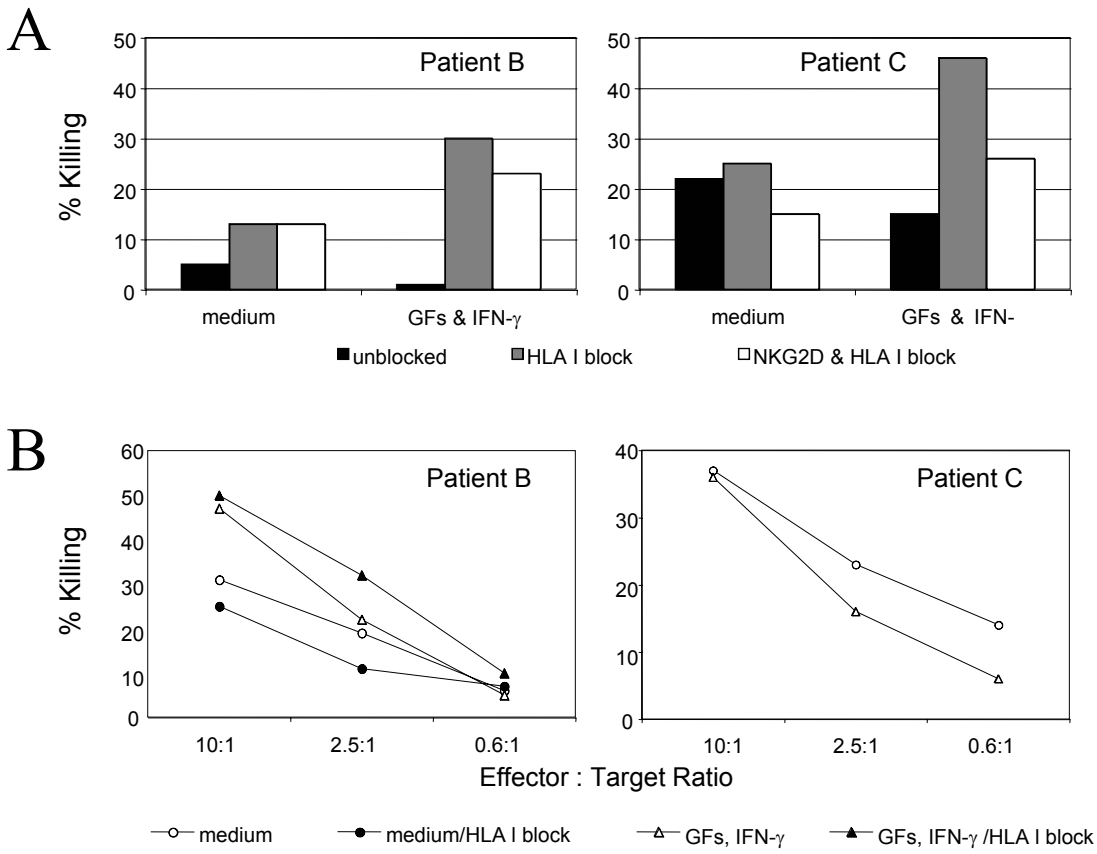

Figure V-13, Killing of AML Blasts after Ligand Upregulation is Partly NKG2D Dependent. Leukemic blasts of patients B/C were cultured for 4 days in medium only or with GFs (FL+SCF+GM-CSF) and IFN-γ. NK cell susceptibility was then assessed using (A) PHA/feeder cell/IL-2 activated healthy NK cells and (B) freshly isolated healthy NK cells cultured for 4 days in IL-2. To block inhibitory signals anti-MHC class I mAb (10 μg/mL) was added, as indicated. (A) Killing assays were performed at effector:target ratio of 10:1. Blocking with anti-NKG2D mAb (20 μg/mL) was performed, as indicated.

2. Characterization of NK Cells in AML Patients

The studies described above demonstrated that AML blasts from majority of patients at the onset or relapse of leukaemia display very low levels of ligands for the activatory NK cell receptors. This low ligand expression may be responsible for the poor

immunogenicity of leukemic blasts. Next, we investigated the expression of MHC class I ligands for inhibitory receptors and the number and phenotype of residual NK cells in AML.

2.1 Evaluation of PB Cell Subpopulations in AML Patients

To analyze the number of residual healthy hematopoietic lineages in AML patients whole blood of untreated patients at time of diagnosis or relapse was analyzed by FACS and compared to healthy donors. Distinct residual $CD45^{bright}$ cell populations were detected by specific lineage markers.

Percentage of $CD56^{+}CD3^{-}$ NK cells in total MNCs was decreased in AML in average 2.5 fold compared to healthy donors. Furthermore, in 11 out of 24 patients NK cells were below 1% of total nucleated cells (TNCs) with one patient lacking NK cells. In contrast, NK cells among 11 donors were only in one donor below 1% of TNCs (Table V-10).

Table V-10, PB Subpopulations in AML Patients

	% per MNC		% per TNC	
	Mean ± SEM	(Range)	Mean ± SEM	(Range)
NK cells				
Donors (n=11)	7.0 ± 1.5	(1.2-20.3)	2.6 ± 0.5	(0.3-6.7)
AML Patients (n=24)	2.8 ± 0.6*	(0-11.6)	2.0 ± 0.5	(0-8.8)
T cells				
Donors (n=11)	58.2 ± 3.1	(43.6-75.9)	21.7 ± 1.9	(11.6-34.2)
AML Patients (n=24)	27.3 ± 4.9**	(3.9-87.8)	18.3 ± 3.4	(2.8-57.6)
B cells				
Donors (n=15)	7.5 ± 0.7	(2.5-14.0)	2.9 ± 0.3	(1.3-4.9)
AML Patients (n=25)	6.2 ± 2.0	(0-43.8)	3.8 ± 1.1	(0-17.4)
Monocytes				
Donors (n=15)	18.9 ± 2.8	(4.2-44.0)	6.7 ± 0.8	(1.8-12.2)
AML Patients (n=25)	10.3 ± 3.0*	(0-56.5)	8.1 ± 2.5	(0-51.1)

* $p < 0.05$ compared with healthy donors; ** $p < 0.01$ compared with healthy donors.

$CD3^+CD56^-$ T cells and $CD33^{bright}$ monocytes in AML were also decreased 2.1 and 1.8 fold, respectively (Table V-10). Moreover, T cells in 10 among 24 AML patients were decreased below 10% of TNCs and monocytes in 7 out of 25 patients comprised less than 1% of TNCs.

$CD19^+$ B cells in MNCs of AML patients were present at normal levels. However, B cells in 9 out of 25 patients were decreased to levels below 1% of TNCs with one patient lacking B cells.

These results showed that there was a significant decrease in NK cells, T cells and monocytes of AML patients, with 18 out of 25 AML patients having deficient numbers of either NK cells, T cells, B cells or monocytes and with 10 out of 25 patients being affected in more than only one lineage.

2.2 Expression of MHC Class I Molecules on AML Blasts

To analyze MHC class I ligands in AML, PB cells from 22 AML patients were stained with mAbs specific against HLA-A,B,C and expression levels were tested on $CD45^{dim}$ leukemic blasts and $CD45^{bright}$ residual MNCs.

Healthy donors expressed HLA-A,B,C at a mean MFI ratio of 232.6 ± 19.9, whereas in AML patients MHC class I molecules were significantly decreased with MFI ratios of 115.1 ± 26.1 and 168.6 ± 27.0 in blasts and residual MNCs, respectively (Figure V-14).

These results indicate that leukemic blasts can escape through engagement of inhibitory KIRs from recognition by NK cells unless activating ligands are expressed at sufficient levels to overcome the inhibitory signalling. However, our results have shown that expression of ULBP ligands and putative ligands for NCRs on blasts is very low, suggesting that inhibiting signals generally prevail in AML blasts.

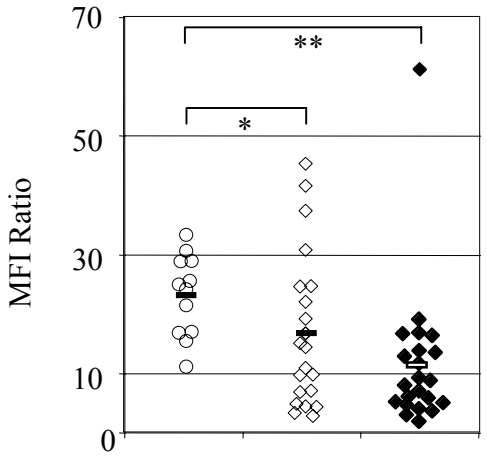

Figure V-14, HLA-A,B,C Expression in AML. PB samples of AML patients were stained with anti-HLA-A,B,C mAbs on $CD45^{dim}$ blasts (black diamonds) and on $CD45^{bright}$ residual MNCs (open diamonds). MFI ratios were compared to MHC class I expression levels on MNCs from healthy donors (open circles). Mean values are indicated as horizontal bars. * $p < 0.05$; ** $p < 0.005$.

2.3 Activating Receptors Are Expressed Normally on NK Cells from AML Patients

Expression levels of NKG2D and NKp46 receptors were measured on $CD56^{+}CD3^{-}$ NK cells within $CD45^{bright}$ residual healthy cells of fresh PB from AML patients using FACS. NKG2D expression on NK cells of AML patients was similar to receptor levels measured on NK cells of healthy donors with MFI ratios of up to 14.0 (Table V-11). Analysis of NKp46 receptor levels revealed no decrease on NK cells of AML patients, either, with MFI ratios of up to 18.2 (Table V-11).

NK cells were further analyzed by FACS for the expression of CD16, an activating Fcγ receptor, which is expressed on majority of NK cells[16]. In AML the proportion of $CD16^{+}$ NK cells was decreased to 63.4% ± 7.3% compared to healthy donors with 81.8% ± 3.6% $CD16^{+}$ NK cells (Table V-11). In 5 out of 22 AML patients only 0.1-9.7% of NK cells expressed CD16.

Table V-11, Expression of Activating Receptors on NK Cells

	NKG2D MFI Ratio		**NKp46** MFI Ratio		**CD16** % of NK cells	
	Mean ± SEM	(Range)	Mean ± SEM	(Range)	Mean ± SEM	(Range)
Donors (n=9/11/10)	6.8 ± 0.7	(4.2-10.9)	10.0 ± 0.9	(6.1-17.2)	81.8 ± 3.6	(51.7-92.6)
AML Patients (n=21/18/22)	6.8 ± 0.7	(2.7-14.0)	9.6 ± 1.1	(2.7-18.2)	63.4 ± 7.3	(0.1-92.7)

n indicates the number of PB samples analyzed for NKG2D/NKp46/CD16

These results show that the activating receptors NKG2D and NKp46 are expressed at normal high levels on NK cells of AML patients. However, the proportion of $CD16^+$ NK cells is decreased in 5 out of 22 AML patients. This decline may result in diminished NK-cell mediated target lysis, since $CD16^+$ NK cells are more cytotoxic than $CD16^-$ NK cells[21]. Cytokine production which is induced through stimulation of NKG2D and NCRs[54,85] and is a main function of $CD16^{dim/-}$ NK cells should not be affected[25].

3. Characterization of NK Cells in Patients after HSCT

The post-HSCT immunity is recognized as an important determinant of the clinical outcome. Recent progress in understanding the mechanism of NK cell cytotoxicity against tumor tissue has initiated interest in the anti-leukemic function of these cells. So far, little has been known about the reconstitution of NK cell compartment after HSCT. Therefore, our studies addressed the efficiency of NK cell development from donor-derived stem cells as well as phenotypic and functional properties of NK cells in the early period after allogeneic HSCT.

3.1 NK Cell Reconstitution in Transplanted Patients

To monitor the recovery of NK cells after allogeneic HSCT, the number of $CD56^+CD3^-$ cells was measured by FACS analysis of PB and BM samples on days 7, 14, 30, 90 and 180 after HSCT. NK cells were detectable already on day 7, although at very low numbers, and therafter their number increased rapidly (Table V-12). In PB, the frequency of $CD56^+CD3^-$ cells returned to normal at 3 months (3.09% ± 0.73% versus 3.31% ± 0.74% in healthy donors), although the absolute number at that time was still below normal (8.20 ± 1.83 versus 19.46 ± 4.35 x 10^4/mL). The recovery of NK cells in the BM was faster than in PB. These data show a rapid reconstitution of NK cells in patients after allogeneic HSCT.

Table V-12, NK Cell Content in PB and BM of Patients after Allogeneic HSCT

	PB NK Cells		BM NK Cells	
	% TNCs	**x 10^{-4}/mL**	**% TNCs**	**x 10^{-4}/mL**
Donors	3.31 ± 0.74	19.46 ± 4.35	0.76 ± 0.24	11.08 ± 3.01
Patients after HSCT				
Day 7 (n = 5/0)	1.98 ± 1.6	0.50 ± 0.34**	ND	ND
Day 14 (n = 16/8)	1.84 ± 0.44*	6.05 ± 1.82**	0.97 ± 0.30	8.53 ± 2.13
Day 30 (n = 16/14)	1.64 ± 0.22*	10.62 ± 2.30*	0.99 ± 0.30	8.87 ± 3.47
Day 90 (n = 15/12)	3.09 ± 0.73	8.20 ± 1.83*	1.18 ± 0.36	9.77 ± 2.66
Day 180 (n = 9/7)	3.08 ± 0.64	12.72 ± 2.15	1.35 ± 0.31	13.68 ± 5.00

All numbers are expressed as mean ± SEM; % TNCs is percent of total nucleated cells. n indicates number of PB/BM samples analyzed at each time point; ND, not done.

* $p < 0.05$ compared with healthy controls.

** $p < 0.005$ compared with healthy controls.

3.2 Phenotypic Analysis of NK Cells after Allogeneic HSCT

To characterize the NK cell population that develops after allogeneic HSCT, phenotypic analysis of cell-surface markers that define the maturation and activation stages of human NK cells was performed. Most remarkably, the $CD56^{dim}CD16^{bright}$ NK cells, which represent the predominant subset of mature cytotoxic NK cells in normal PB, were strongly reduced. Conversely, there was an accumulation of cells with a $CD56^{bright}CD16^{-/dim}$ phenotype (Figure V-15).

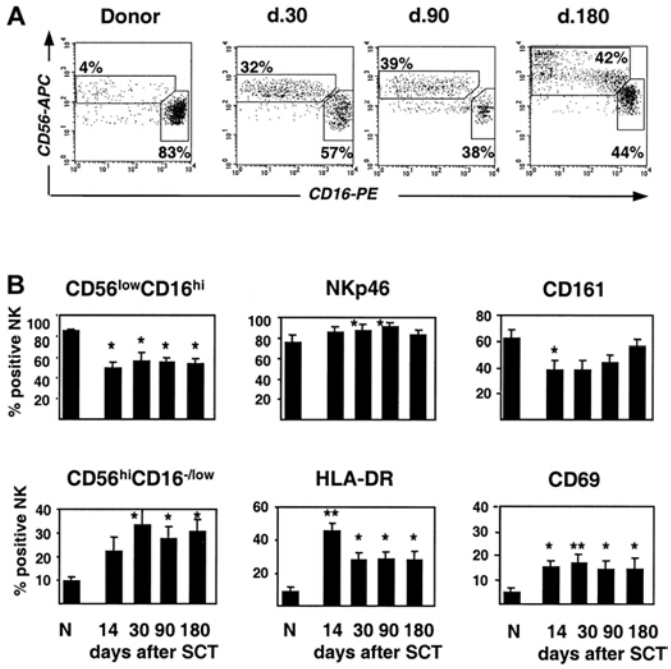

Figure V-15, Phenotypic Characteristics of NK Cells after HSCT. (A) FACS profile of $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}CD16^{bright}$ subpopulations of NK cells in a patient after HSCT compared with the healthy donor. (B) Percentage of $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}CD16^{bright}$ NK cell subpopulations and expression of NK cell markers Nkp46, CD161, HLA-DR and CD69 in patients after HSCT and healthy donors (N) is shown (mean ± SEM). Time after transplantation is indicated. *P < .05, **P < .005 compared with healthy donors.

The ratio of $CD56^{dim}CD16^{bright}$ versus $CD56^{bright}CD16^{-/dim}$ subsets after HSCT was 1.3 to 1.9 compared with 7.4 in control PB. This strong skewing persisted for as long as 6 months in all except for 2 patients. Of other analyzed markers, expression of CD161 present on both immature and mature NK cells[191] was reduced, whereas NKp46, the major NCR selectively expressed by NK cells[78], was at normal levels (Figure V-15B). Expression of activation markers CD69 and HLA-DR, was significantly increased on both $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}CD16^{bright}$ cell subsets. Thus, despite rapid numerical recovery of NK cells, the phenotypic profile reflects distinct abnormalities in NK cell differentiation after HSCT.

3.3 IFN-γ Production by NK Cells from Transplanted Patients

To examine the functional properties of the NK cell compartment during early recovery after HSCT, $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}CD16^{bright}$ NK cell subsets were purified from patients' PB on day 30. Due to low yields ($< 2x10^4$ $CD56^{bright}CD16^{-/dim}$ and $< 10^5$ $CD56^{dim}CD16^{bright}$ cells) isolated cells were subjected to proliferation in vitro by restimulation with feeder cells and PHA in IL-2 medium. Their ability to produce IFN-γ was examined in response to specific stimulation with IL-12 and IL-18.

Abundant IFN-γ production was seen with the $CD16^{-/dim}$ subset with 55% to 85% IFN-γ^+ NK cells, whereas stimulation of $CD16^{bright}$ cells resulted in 25% to 55% of IFN-γ^+ cells (Figure V-16).

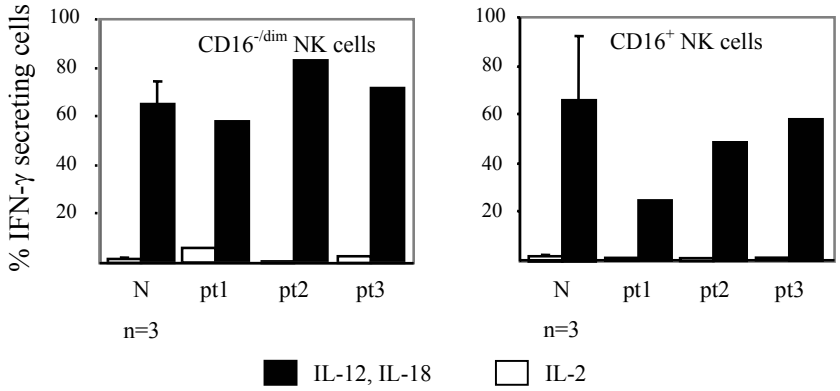

Figure V-16, IFN-γ Production by NK Cell Subsets Derived from Patients after HSCT. $CD16^{-/dim}$ and $CD16^{high}$ cells were purified, expanded, and stimulated with IL-2 (open bars) or IL-12 and IL-18 (black bars). Frequency of $INF\text{-}\gamma^{+}$ NK cells was determined by MACS IFN-γ secretion assay. Results obtained with healthy donors (N; mean values ± SEM) and 3 patients (pt) after HSCT are shown.

These results indicate that the $CD16^{-/dim}$ NK cells, which are present in increased proportions in the early posttransplantation period, have the ability to readily respond to the cytokine stimulus and produce IFN-γ comparable to NK cells from healthy donors.

VI. Discussion

1. Expression and Role of NK Activating Ligands in Normal PB Cells

Recently, MIC and ULBP molecules have been identified as activating ligands for NKG2D in humans[13,104]. MIC surface expression was described in epithelial tumors. The presence of surface MIC resulted in the killing of tumor cells by NKG2D expressing NK cells and T cells. Expression of ULBP molecules by several tested tumor cell lines lead to NK mediated cell lysis, too. So far, only little information has been published concerning NKG2D ligand expression in normal cells. Two groups found ULBP mRNAs in MNCs of healthy PB and BM[13,109] but ULBP and MIC protein expression was published to be absent in MNCs[13,104]. In contrast, in our study B cells, monocytes, granulocytes and platelets were identified as ULBP expressing cells. ULBP1 was always expressed at highest levels in B cells and monocytes. In platelets ULBP2 was the main ULBP ligand expressed. The ligands were absent on T cells, NK cells and erythrocytes. We also found MIC expression on monocytes and B cells. The controversy of our results with previous studies can be explained by our gating on distinct cell lineages. When analyzing ligand expression on total MNCs, as was done previously, ligand negative T cells, which are the main MNC population, hide potential ligand expression on B cells and monocytes.

Using real-time PCR we detected ULBP1, ULBP2 and ULBP3 transcripts in B cells, monocytes and T cells, confirming expression of these ligands by subpopulations in normal PB. However, transcript levels were low and did not correlate with the cell surface density of individual proteins. A discordance in expression of ULBPs at mRNA and protein levels was also seen with tumor cell lines[13], suggesting that ULBP expression is regulated at a level other than transcription.

Importantly, no cellular ligands have been discovered for the NCRs, yet. In order to identify cells expressing potential NCR ligands we used dimers of soluble recombinant

NCRs. Binding of these dimers demonstrated that, similar to NKG2D ligands, B cells and monocytes express potential NCR ligands.

We found that expression of NKG2D ligands and putative NCR ligands on monocytes and granulocytes varied between different healthy individuals. To explain this phenomenon donors with ligand negative monocytes were tested for hCMV infection, since ULBP1, ULBP2 and MICB are downregulated in hCMV infected cells[100-102]. However, there was no correlation between hCMV infection and ligand expression in monocytes (data not shown), indicating that differences in cell surface ligand levels are not CMV-related.

As we hypothesized that activation of myeloid cells may be a reason for ligand expression purified ligand negative monocytes were analyzed in vitro for conditions which may lead to ligand upregulation. So far, only very little was known about regulation of NKG2D ligands. It was shown that expression of MICA/B increases in response to various forms of cellular stress, including diseases such as tumor transformation and arthritis[86,98]. Heat-shock transcription elements present in the promotor regions of their genes are thought to be responsible for induction of MIC molecules, but have not been implicated in regulating the expression of ULBPs. Except for upregulation of ULBP3 in CMV-infected cells[100], signals regulating expression of ULBP proteins and the as-yet unknown NCR ligands have never been described.

We found that ULBP1 and putative ligands for NKp30, p44 and p46 were selectively upregulated in response to myeloid growth factors FL, SCF and GM-CSF. This upregulation was further enhanced by addition of IFN-γ. ULBP2 and ULBP3 were not affected by neither growth factors nor IFN-γ. Other inflammatory cytokines, such as IFN-α, IL-1β and TNF-α, and LPS which are known to stimulate monocytes were unable to elevate surface levels of ULBPs. These results showed that ULBP1, ULBP2 and ULBP3 are regulated by different pathways. The fact that ULBP molecules are expressed at different levels and that they don't share the same regulation mechanism implies different physiological roles for ULBPs. Different ligand functions may also explain the presence of so many cellular ligands, which bind with distinct affinities[13] to the same receptor.

The presence of triggering ligands on monocytes did confer susceptibility to attack by NK cells when HLA class I molecules specific for inhibitory KIR receptors were masked by mAbs. Only 11% of ligand positive monocytes used as targets for autologous NK cells were killed in vitro at an effector to target ratio of 10:1. Blocking of MHC class I ligands by mAbs increased the killing to 26%. Remarkably, ligand negative $CD34^+$ progenitors were fully resistant to cytolysis by autologous NK cells and remained protected also after blocking inhibitory MHC class I ligands. Thus, missing inhibitory signaling is not sufficient for NK cell recognition but engagement of activatory NK cell receptors are also needed. However, in monocytes level of triggering NKG2D and NCR ligands is apparantly too low to overcome protective inhibitory signaling.

The physiological relevance of ligand expression on B cells and myeloid cells is not clear. However, interaction between those cells and NKG2D expressing NK cells has already been demonstrated in previous studies. NK cells can interact with B cells resulting in maturation and immunoglobulin production by B cells[192] and conversely, secretion of cytokines by activated NK cells[193]. Interaction of monocytes with NK cells causes proliferation and production of cell type-specific cytokines[175,176]. Adhesion molecules, such as CD11a and CD54, as well as CD40 and CD40 ligand were implicated in these interactions and it remains to be seen whether ULBPs and NCR ligands expressed by normal blood cells function as co-stimulatory molecules for cells bearing cognate activating receptors.

When we analyzed in vitro generated DCs we found no surface expression of ULPB1, ULBP3 and MICA/B. However, immature and mature DCs were shown to activate NK cells dependent on NKp30 and to a lesser extent on NKp46[182], indicating that human DCs are expressing ligands for activating NK cell receptors. The detection of such ligands on DCs needs further investigations.

2. Expression and Role of NK Activating Ligands in AML

Acute leukemia is characterized by a rapid disease progression. The high incidence of relapses following treatment with high-dose chemotherapy or transplantation of allogeneic hematopoietic stem cells[189,190] suggests that leukemic blasts can escape recognition by the immune system. To explain the poor immunogenicity of malignant blood cells, we examined the expression of ligands for NK cell-activating receptors in human AML. We found that leukemic transformation is frequently associated with an absence or low cell surface density of ligands for NKG2D and NCRs, which may render the blasts insensitive to recognition by NK cells.

Staining of cells with mAbs specific for ULBP1, ULBP2, ULBP3 and MICA/B, and binding of soluble NCRs which reflects the distribution of putative NKp30, NKp44 and NKp46 ligands, demonstrated that ligand levels on leukemic blasts are very low in the majority of patients with AML. ULBPs were not detectable or low (MFI ratios of 1.0-2.0) in 23 out of 30 patients (77%), as were NCR ligands in 10 out of 12 patients (83%) and MICs in 6 out of 9 patients (67%). This confirms and extends a recent report of the ULBP-negative/low phenotype of primary leukemic blasts in 12/15 AML patients[194]. A low density of ligands triggering the NKG2D and NCR receptors in AML implies that interactions between activating receptors and their ligands may be insufficient to elicit strong cytolytic responses against the leukemic blasts.

Unlike leukemic cells, the residual normal monocytes, granulocytes and B cells in patients with AML expressed ULBPs and showed binding of soluble NCRs in comparable levels as in healthy donors.

Importantly, expression of ULBPs and NCR-specific ligands increases during hematopoietic cell differentiation. In healthy human BM, early $CD34^+$ progenitors are negative for ULBPs, MICA/B and NCR-specific ligands, whereas $CD34^-$ committed myeloid progenitors carrying CD33 and CD14 markers display the ligands on the cell surface. Consistent with this, growth factor-induced myeloid differentiation of $CD34^+$ cells in vitro is accompanied by the acquisition of the activating ligands. In AML, ligand

surface density appeared to correlate with the hematopoietic differentiation stage at which leukemic transformation took place, since ligand-positive blasts prevailed in AML patients with monocytic M5 and megakaryocytic M7 blasts which are more mature progenitors. ULBP-negative or ULBP-low blasts were found in AML of subtypes M1-M4 affecting early stages of hematopoiesis, confirming previous results in 15 M1-M4 AMLs[194]. In vitro culture of leukemic blasts from 2 out of 5 AML patients with myeloid growth factors was associated with upregulation of triggering ligands, arguing further that the ligand-low phenotype in AML is related to early maturation arrest during malignant transformation. Ligand expression could further be increased when cultures were supplemented with IFN-γ. Importantly, upregulation of the ligands on leukemic blasts correlated with elevated cytolysis of blasts by allogeneic NK cells from healthy donors. This lysis was in part dependent on the activation of NKG2D, since blocking of its interaction with cognate ligands by mAbs resulted in a decreased killing of up to 43%. These findings show the relevance of ligand expression on tumor cells for NK cell recognition.

Upregulation of ligands in response to IFN-γ is accompanied by elevated levels of MHC class I ligands. Thus, when we used allogeneic NK cells with at least one match in HLA alleles, recognition of blasts by NK cells was hampered through inhibitory KIRs and killing was only induced after blocking inhibitory signals.

Ruggeri et al recently showed that in haplotype mismatched HSCT allogeneic NK cells exert an antileukemic effect due to KIR mismatches between effectors and targets[127,128]. Growth factor and IFN-γ induced expression of triggering ligands on blast of patients may thus improve cytolysis of tumor cells by donor derived NK cells independently of HLA class I upregulation on blasts. Furthermore, identification of compounds that selectively elevate ligand levels on tumor cells without affecting MHC class I levels might be of interest for clinical use and warrants further investigation. Accordingly, clinical use of G-CSF concurrently with chemotherapy has improved long-term survival in AML[195] and one might speculate that the observed reduced incidence of relapses is related to the enhanced susceptibility of cytokine-treated blasts to killing by the immune effector cells.

Our results so far indicated that low expression of activating ligands may be responsible for the poor immunogenicity of leukemic blasts. Other reasons for insufficient tumor recognition by NK cells might be the presence of MHC class I molecules on blasts engaging inhibitory receptors on NK cells or diminished NK cell function in patients with AML. Indeed, we found that MHC class I ligands were present on leukemic blasts in comparable levels as in residual MNCs of AML patients. However, MHC class I expression was significantly reduced 2-fold on patients blasts compared with MNCs of healthy donors. These results indicate that due to reduced levels of inhibiting ligands, it might be possible to overcome the negative signal by inducing expression of ligands for activating NK cell receptors.

Analysis of NK cells from AML patients revealed a significant reduction of relative NK cell numbers in MNCs of AML patients, with 2.8 ± 0.6 % NK cells per MNCs, compared to 7.0 ± 1.5 % NK cells per MNCs in healthy donors. In addition, we also found a significant decrease in T cells and monocytes. These findings indicate an impaired immunity due to reduced numbers of effector cells responsible for tumor recognition.

NK cells from AML patients expressed the activating receptors NKG2D and NKp46 at normal high levels suggesting that recognition of ligand expressing target cells is not impaired. Our results differ from a previous report describing a NCR^{dim} phenotype of NK cells in AML[79]. This discrepancy is apparently due to the fact that we analyzed fresh NK cells in PB, whereas Costello et al cultured NK cells prior to detection of receptors. Thus, it is possible that NK cells from AML patients downmodulate surface NCRs after in vitro proliferation.

In 5 among 22 AML patients only 0.1 – 9.7 % of NK cells expressed the activating Fcγ receptor CD16, which is normally expressed on 90% of NK cells[16]. NK cells in these patients therefore might display a diminished cytolytic activity and recognition of opsonized target cells by NK cells is possibly reduced.

3. Characterization of NK Cell Compartment after HSCT

HSCT is a common treatment in AML and recent studies have shown an important GvL effect of donor derived NK cells in haplotype mismatched transplantations[127,128]. We investigated the recovery of NK cells after HSCT and their phenotypic and functional properties.

The reconstitution of NK cells after grafting was very rapid. As soon as 7 days after HSCT NK cells comprised 1.98 ± 1.6 % of PB total nucleated cells and reached normal levels of 3.09 ± 0.73 % at 3 months. However, absolute NK numbers are significantly decreased in patients undergoing HSCT and only return to normal levels (12.72 ± 2.15 x 10^{-4} cells per mL) at 6 months. Analysis of NK cells in BM of patients was first done 14 days after HSCT and revealed that NK cell levels of 0.97 ± 0.30 % were comparable to healthy donors, whose BM MNCs comprised 0.76 ± 0.24 % NK cells. According to a recent chimerism study, the major proportion of NK cells is donor derived[127] and, at this early time point, may represent a mixture of cells transferred with the graft and those developing from the newly engrafted hematopoietic progenitors.

Phenotypic analysis of NK cells from transplanted patients revealed that the NKp46 receptor is expressed normally. However, the activation markers CD69 and HLA-DR were significantly increased on NK cells from patients after HSCT. In contrast, CD161 which was shown to inhibit target lysis induced by engagement of NKp46 or CD16[196], was reduced. These findings indicate that developing NK cells in patients after transplantation are highly active.

Further phenotypic characterization of developing NK cells demonstrated a disproportional 5-fold increase in poorly cytotoxic $CD56^{bright}CD16^{dim/-}$ NK cells over highly cytotoxic $CD56^{dim}CD16^{bright}$ NK cell subset. This skewing of NK cell subsets persisted for 6 months and was in line with previous results[197]. Previously, it has been shown by our group that FL serum levels are highly elevated in patients treated by chemotherapy preceding HSCT[198,199]. Since NK cells generated from $CD34^{+}$ progenitors in vitro in response to FL are predominantly of the $CD56^{bright}CD16^{dim/-}$ type[11], high serum

FL might contribute to this skewed proportion of NK cell subsets in patients undergoing transplantation.

Our results suggest that $CD56^{bright}$ NK cells develop more rapidly in vivo and that $CD56^{dim}$ NK cells either require more prolonged exposure to maturation factors or arise from $CD56^{bright}$ cells. Actually, it is still discussed whether $CD56^{bright}$ NK cells represent an immature NK subset. The fact that $CD56^{bright}$ NK cells highly proliferate and acquire cytotoxic potential in response to IL-2 and IL-15, whereas $CD56^{dim}$ NK cells are already cytotoxic and have a low proliferative capacity in vitro, supports the hypothesis that $CD56^{dim}$ NK cells derive from the $CD56^{bright}$ subset. The recently discovered IL-21 has been shown to induce generation of NK cells with a $CD56^{dim}CD16^{+}$ phenotype from progenitors in vitro[200]. Thus, it remains unknown whether $CD56^{dim}$ NK cells develop from a unique precursor or whether additional soluble, such as IL-21, or cell-contact signals are required for the generation of characteristic $CD56^{dim}CD16^{bright}$ NK cells.

To test the capacity of developing NK cells from transplanted patients to produce IFN-γ, $CD56^{bright}CD16^{dim/-}$ NK cells were purified and expanded in vitro. IFN-γ production was measured in response to IL-12 and IL-18 and was normal compared with $CD56^{bright}CD16^{dim/-}$ NK cells from healthy donors. In contrast, lysis of MHC class I-deficient K562 cells by patient derived NK cells was reduced 3- to 4-fold, compared with NK cells from healthy donors[201]. It was suggested that this significant difference might be related to the reduced content of highly cytotoxic $CD16^{bright}$ subset within the NK-cell population.

Because recovery of T cells after myeloid ablation preceding HSCT is delayed and it may take years before T-cell immunity is restored[202,203], the rapid expansion of donor-derived NK cells has particular implications for the functional immune recovery in the early period after HSCT. Interactions between NK cells and also early recovering DCs[201] underlie the main immunologic responses important for induction of tolerance to prevent GvHD and for an antitumor effect to prevent relapse.

So far several escape mechanisms from NK cell recognition have been described; epithelial tumors have been demonstrated to secrete MIC ligands which bind to NKG2D receptors on NK cells and T cells thereby leading to their internalization resulting in reduced cytolytic activity of the effector cells. Furthermore, viral protein UL16 from hCMV is binding to ULBP1, ULBP2 and MICB and retaining the ligands intracellularly. In AML, we showed that triggering receptors on NK cells are not affected. However, our results indicate that the absence of surface ligands for activating NK cell receptors on leukemic blasts due to their insufficient maturation, provides tumortransformed cells with the advantage to evade NK cell recognition.

Together with our results demonstrating rapid recovering NK cells after HSCT, expressing normal levels of activating receptors, in vivo application of compounds, increasing surface expression of NKG2D and NCR ligands, together with the selection of donors for stem cells and NK cells with appropriate HLA class I mismatches suitable for tumor clearance, may be used as an immunotherapeutic strategy to reduce leukemic relapses.

VII. References

1. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376

2. Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood. 1990;76:2421-2438

3. Sihvola M, Hurme M. The development of NK cell activity in thymectomized bone marrow chimaeras. Immunology. 1984;53:17-22

4. Ramos SB, Garcia AB, Viana SR, Voltarelli JC, Falcao RP. Phenotypic and functional evaluation of natural killer cells in thymectomized children. Clin Immunol Immunopathol. 1996;81:277-281

5. Schwarz RE, Hiserodt JC. Effects of splenectomy on the development of tumor-specific immunity. J Surg Res. 1990;48:448-453

6. Passlick B, Izbicki JR, Waydhas C, Nast-Kolb D, Schweiberer L, Ziegler-Heitbrock HW. Posttraumatic splenectomy does not influence human peripheral blood mononuclear cell subsets. J Clin Lab Immunol. 1991;34:157-161

7. Mrozek E, Anderson P, Caligiuri MA. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood. 1996;87:2632-2640

8. Williams NS, Klem J, Puzanov IJ, Sivakumar PV, Schatzle JD, Bennett M, Kumar V. Natural killer cell differentiation: insights from knockout and transgenic mouse models and in vitro systems. Immunol Rev. 1998;165:47-61

9. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR, Brasel K, Morrissey PJ, Stocking K, Schuh JC, Joyce S, Peschon JJ. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med. 2000;191:771-780

10. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001;97:14-32

11. Yu H, Fehniger TA, Fuchshuber P, Thiel KS, Vivier E, Carson WE, Caligiuri MA. Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. Blood. 1998;92:3647-3657

12. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Teepe M, Lyman SD, Peschon JJ. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood. 2000;95:3489-3497

13. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 2001;14:123-133

14. Kubin M, Cassiano L, Chalupny J, Chin W, Cosman D, Fanslow W, Mullberg J, Rousseau AM, Ulrich D, Armitage R. ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. Eur J Immunol. 2001;31:1428-1437

15. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol. 2001;2:293-299

16. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol. 1986;136:4480-4486

17. Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J Exp Med. 1989;169:2233-2238

18. Nitta T, Yagita H, Sato K, Okumura K. Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction. J Exp Med. 1989;170:1757-1761

19. Suzuki N, Suzuki T, Engleman EG. Evidence for the involvement of CD56 molecules in alloantigen-specific recognition by human natural killer cells. J Exp Med. 1991;173:1451-1461

20. Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity. 1997;6:655-661

21. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol. 1989;143:3183-3191

22. Caligiuri MA, Zmuidzinas A, Manley TJ, Levine H, Smith KA, Ritz J. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. J Exp Med. 1990;171:1509-1526

23. Nagler A, Lanier LL, Phillips JH. Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. J Exp Med. 1990;171:1527-1533

24. Robertson MJ, Soiffer RJ, Wolf SF, Manley TJ, Donahue C, Young D, Herrmann SH, Ritz J. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. J Exp Med. 1992;175:779-788

25. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151

26. Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, Schow P, Ross ME, Klumpp TR, Soiffer RJ, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J Clin Invest. 1993;91:123-132

27. Baume DM, Robertson MJ, Levine H, Manley TJ, Schow PW, Ritz J. Differential responses to interleukin 2 define functionally distinct subsets of human natural killer cells. Eur J Immunol. 1992;22:1-6

28. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer. 1975;16:216-229

29. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975;16:230-239

30. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319:675-678

31. Ljunggren HG, Sturmhofel K, Wolpert E, Hammerling GJ, Karre K. Transfection of beta 2-microglobulin restores IFN-mediated protection from natural killer cell lysis in YAC-1 lymphoma variants. J Immunol. 1990;145:380-386

32. Franksson L, George E, Powis S, Butcher G, Howard J, Karre K. Tumorigenicity conferred to lymphoma mutant by major histocompatibility complex-encoded transporter gene. J Exp Med. 1993;177:201-205

33. Yokoyama WM, Jacobs LB, Kanagawa O, Shevach EM, Cohen DI. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. J Immunol. 1989;143:1379-1386

34. Chan PY, Takei F. Molecular cloning and characterization of a novel murine T cell surface antigen, YE1/48. J Immunol. 1989;142:1727-1736

35. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. Nature. 1992;358:66-70

36. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, Barbaresi M, Ciccone E, Moretta L. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. J Exp Med. 1993;178:597-604

37. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. Immunity. 1995;3:801-809

38. Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Malnati MS, Vitale M, Bottino C, Moretta L, Moretta A, Long EO. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. Immunity. 1995;2:439-449

39. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. Science. 1995;268:405-408

40. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. J Exp Med. 1991;173:1017-1020

41. Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol. 1996;157:4741-4745

42. Carretero M, Cantoni C, Bellon T, Bottino C, Biassoni R, Rodriguez A, Perez-Villar JJ, Moretta L, Moretta A, Lopez-Botet M. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. Eur J Immunol. 1997;27:563-567

43. Vance RE, Tanamachi DM, Hanke T, Raulet DH. Cloning of a mouse homolog of CD94 extends the family of C-type lectins on murine natural killer cells. Eur J Immunol. 1997;27:3236-3241

44. Clynes R, Maizes JS, Guinamard R, Ono M, Takai T, Ravetch JV. Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. J Exp Med. 1999;189:179-185

45. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, Kinet JP, Long EO. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. Immunity. 1996;4:77-85

46. Vivier E, Daeron M. Immunoreceptor tyrosine-based inhibition motifs. Immunol Today. 1997;18:286-291

47. Fry AM, Lanier LL, Weiss A. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. J Exp Med. 1996;184:295-300

48. Olcese L, Lang P, Vely F, Cambiaggi A, Marguet D, Blery M, Hippen KL, Biassoni R, Moretta A, Moretta L, Cambier JC, Vivier E. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. J Immunol. 1996;156:4531-4534

49. Le Drean E, Vely F, Olcese L, Cambiaggi A, Guia S, Krystal G, Gervois N, Moretta A, Jotereau F, Vivier E. Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. Eur J Immunol. 1998;28:264-276

50. Carretero M, Palmieri G, Llano M, Tullio V, Santoni A, Geraghty DE, Lopez-Botet M. Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. Eur J Immunol. 1998;28:1280-1291

51. Aldrich CJ, DeCloux A, Woods AS, Cotter RJ, Soloski MJ, Forman J. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. Cell. 1994;79:649-658

52. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur J Immunol. 1997;27:1164-1169

53. Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, Phillips JH, Lanier LL, Parham P. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity. 1997;7:739-751

54. Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, Moretta L, Moretta A. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med. 1997;186:1129-1136

55. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285:727-729.

56. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol. 2001;31:2680-2689.

57. Bolhuis RL, Roozemond RC, van de Griend RJ. Induction and blocking of cytolysis in CD2+, CD3- NK and CD2+, CD3+ cytotoxic T lymphocytes via CD2 50 KD sheep erythrocyte receptor. J Immunol. 1986;136:3939-3944

58. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. J Immunol. 1988;141:3478-3485

59. Moretta A, Poggi A, Pende D, Tripodi G, Orengo AM, Pella N, Augugliaro R, Bottino C, Ciccone E, Moretta L. CD69-mediated pathway of lymphocyte activation: anti-CD69 monoclonal antibodies trigger the cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing T cell receptor alpha/beta. J Exp Med. 1991;174:1393-1398

60. Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL, Phillips JH. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. Immunity. 1996;4:573-581

61. Vitale M, Falco M, Castriconi R, Parolini S, Zambello R, Semenzato G, Biassoni R, Bottino C, Moretta L, Moretta A. Identification of NKp80, a novel triggering molecule expressed by human NK cells. Eur J Immunol. 2001;31:233-242

62. Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. J Exp Med. 1999;190:1801-1812

63. Houchins JP, Lanier LL, Niemi EC, Phillips JH, Ryan JC. Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. J Immunol. 1997;158:3603-3609

64. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391:795-799

65. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, Moretta L. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med. 1995;182:875-884

66. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, Poggi A, Moretta A, Moretta L. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med. 1996;183:645-650

67. Bottino C, Sivori S, Vitale M, Cantoni C, Falco M, Pende D, Morelli L, Augugliaro R, Semenzato G, Biassoni R, Moretta L, Moretta A. A novel surface molecule homologous to the p58/p50 family of receptors is selectively expressed on a subset of human natural killer cells and induces both triggering of cell functions and proliferation. Eur J Immunol. 1996;26:1816-1824

68. Vales-Gomez M, Reyburn HT, Mandelboim M, Strominger JL. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. Immunity. 1998;9:337-344

69. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. Annu Rev Immunol. 2001;19:291-330

70. Olcese L, Cambiaggi A, Semenzato G, Bottino C, Moretta A, Vivier E. Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. J Immunol. 1997;158:5083-5086

71. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature. 1998;391:703-707

72. Wu J, Song Y, Bakker AB, Bauer S, Spies T, Lanier LL, Phillips JH. An activating immunoreceptor complex formed by NKG2D and DAP10. Science. 1999;285:730-732

73. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol. 2000;1:119-126.

74. Chang C, Dietrich J, Harpur AG, Lindquist JA, Haude A, Loke YW, King A, Colonna M, Trowsdale J, Wilson MJ. Cutting edge: KAP10, a novel transmembrane adapter protein genetically linked to DAP12 but with unique signaling properties. J Immunol. 1999;163:4651-4654

75. Cantoni C, Bottino C, Vitale M, Pessino A, Augugliaro R, Malaspina A, Parolini S, Moretta L, Moretta A, Biassoni R, Sivori S, Morelli L. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J Exp Med. 1999;189:787-796

76. Pende D, Parolini S, Pessino A, Sivori S, Augugliaro R, Morelli L, Marcenaro E, Accame L, Malaspina A, Biassoni R, Bottino C, Moretta L, Moretta A, Cantoni C, Vitale M. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190:1505-1516

77. Bottino C, Biassoni R, Millo R, Moretta L, Moretta A. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. Hum Immunol. 2000;61:1-6

78. Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, Moretta L, Moretta A. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol. 1999;29:1656-1666.

79. Costello RT, Sivori S, Marcenaro E, Lafage-Pochitaloff M, Mozziconacci MJ, Reviron D, Gastaut JA, Pende D, Olive D, Moretta A. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood. 2002;99:3661-3667.

80. Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med. 1998;188:953-960

81. Biassoni R, Pessino A, Bottino C, Pende D, Moretta L, Moretta A. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. Eur J Immunol. 1999;29:1014-1020

82. Falco M, Cantoni C, Bottino C, Moretta A, Biassoni R. Identification of the rat homologue of the human NKp46 triggering receptor. Immunol Lett. 1999;68:411-414

83. Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, Augugliaro R, Moretta L, Moretta A. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med. 1998;187:2065-2072

84. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Davis DM, Strominger JL, Yewdell JW, Porgador A. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. 2001;409:1055-1060.

85. Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J Immunol. 2002;168:671-679.

86. Roberts AI, Lee L, Schwarz E, Groh V, Spies T, Ebert EC, Jabri B. NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. J Immunol. 2001;167:5527-5530

87. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity. 2002;17:19-29

88. Jiang K, Zhong B, Gilvary DL, Corliss BC, Hong-Geller E, Wei S, Djeu JY. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat Immunol. 2000;1:419-425

89. Hayakawa Y, Kelly JM, Westwood JA, Darcy PK, Diefenbach A, Raulet D, Smyth MJ. Cutting edge: tumor rejection mediated by NKG2D receptor-ligand interaction is dependent upon perforin. J Immunol. 2002;169:5377-5381

90. Sutherland CL, Chalupny NJ, Cosman D. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. Immunol Rev. 2001;181:185-192

91. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A, Moretta L. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol. 2001;31:1076-1086.

92. Wu J, Cherwinski H, Spies T, Phillips JH, Lanier LL. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. J Exp Med. 2000;192:1059-1068

93. Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, Bukowski JF. MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function. Immunity. 2001;15:83-93

94. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol. 2001;2:255-260

95. Ugolini S, Vivier E. Multifaceted roles of MHC class I and MHC class I-like molecules in T cell activation. Nat Immunol. 2001;2:198-200

96. Masuda H, Saeki Y, Nomura M, Shida K, Matsumoto M, Ui M, Lanier LL, Seya T. High levels of RAE-1 isoforms on mouse tumor cell lines assessed by anti-"pan" RAE-1 antibody confer tumor susceptibility to NK cells. Biochem Biophys Res Commun. 2002;290:140-145

97. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A. 2001;98:11521-11526

98. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A. 1999;96:6879-6884.

99. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature. 2001;413:165-171.

100. Dunn C, Chalupny NJ, Sutherland CL, Dosch S, Sivakumar PV, Johnson DC, Cosman D. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med. 2003;197:1427-1439

101. Welte SA, Sinzger C, Lutz SZ, Singh-Jasuja H, Sampaio KL, Eknigk U, Rammensee HG, Steinle A. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol. 2003;33:194-203

102. Rolle A, Mousavi-Jazi M, Eriksson M, Odeberg J, Soderberg-Naucler C, Cosman D, Karre K, Cerboni C. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol. 2003;171:902-908

103. Bahram S. MIC genes: from genetics to biology. Adv Immunol. 2000;76:1-60

104. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A. 1996;93:12445-12450.

105. Li P, Willie ST, Bauer S, Morris DL, Spies T, Strong RK. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. Immunity. 1999;10:577-584

106. Tieng V, Le Bouguenec C, du Merle L, Bertheau P, Desreumaux P, Janin A, Charron D, Toubert A. Binding of Escherichia coli adhesin AfaE to CD55 triggers cell-surface expression of the MHC class I-related molecule MICA. Proc Natl Acad Sci U S A. 2002;99:2977-2982

107. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature. 2002;419:734-738.

108. Matsumura M, Fremont DH, Peterson PA, Wilson IA. Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science. 1992;257:927-934

109. Farag SS, George SL, Lee EJ, Baer M, Dodge RK, Becknell B, Fehniger TA, Silverman LR, Crawford J, Bloomfield CD, Larson RA, Schiffer CA, Caligiuri MA. Postremission therapy with low-dose interleukin 2 with or without intermediate pulse dose interleukin 2 therapy is well tolerated in elderly patients with acute myeloid leukemia: Cancer and Leukemia Group B study 9420. Clin Cancer Res. 2002;8:2812-2819

110. Leiden JM, Karpinski BA, Gottschalk L, Kornbluth J. Susceptibility to natural killer cell-mediated cytolysis is independent of the level of target cell class I HLA expression. J Immunol. 1989;142:2140-2147

111. Nishimura MI, Stroynowski I, Hood L, Ostrand-Rosenberg S. H-2Kb antigen expression has no effect on natural killer susceptibility and tumorigenicity of a murine hepatoma. J Immunol. 1988;141:4403-4409

112. Pena J, Alonso C, Solana R, Serrano R, Carracedo J, Ramirez R. Natural killer susceptibility is independent of HLA class I antigen expression on cell lines obtained from human solid tumors. Eur J Immunol. 1990;20:2445-2448

113. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. J Exp Med. 1993;178:1321-1336

114. Chalupny J. ULBP4 is a novel ligand for human NKG2D. Biochem Biophys Res Commun. 2003;305:129-135

115. Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J. Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. J Immunol. 2004;173:1078-1084

116. Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, Lanier LL. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity. 2000;12:721-727.

117. Nomura M, Zou Z, Joh T, Takihara Y, Matsuda Y, Shimada K. Genomic structures and characterization of Rae1 family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. J Biochem (Tokyo). 1996;120:987-995

118. Malarkannan S, Shih PP, Eden PA, Horng T, Zuberi AR, Christianson G, Roopenian D, Shastri N. The molecular and functional characterization of a dominant minor H antigen, H60. J Immunol. 1998;161:3501-3509

119. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC. Regulation of cutaneous malignancy by gammadelta T cells. Science. 2001;294:605-609

120. Urlacher A, Falkenrodt A, Tongio MM, Mayer S. HLA class I antigens on normal and leukemic cells (quantitative analysis). Tissue Antigens. 1987;29:237-245

121. Tongio MM, Falkenrodt A, Urlacher A, Mitsuishi Y, Bergerat JP, Boilletot A, Lutz P, Griveau AM, Troussard X, Mayer S. [Expression of class I and class II markers on populations of leukemic cells]. Pathol Biol (Paris). 1986;34:753-757

122. Elkins WL, Pickard A, Pierson GR. Deficient expression of class-I HLA in some cases of acute leukemia. Cancer Immunol Immunother. 1984;18:91-100

123. Pierson BA, Miller JS. CD56+bright and CD56+dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogenic natural killer cells, and exhibit decreased proliferation on a per cell basis. Blood. 1996;88:2279-2287

124. Pawelec G, Da Silva P, Max H, Kalbacher H, Schmidt H, Bruserud O, Zugel U, Baier W, Rehbein A, Pohla H. Relative roles of natural killer- and T cell-mediated anti-leukemia effects in chronic myelogenous leukemia patients treated with interferon-alpha. Leuk Lymphoma. 1995;18:471-478

125. Cervantes F, Pierson BA, McGlave PB, Verfaillie CM, Miller JS. Autologous activated natural killer cells suppress primitive chronic myelogenous leukemia progenitors in long-term culture. Blood. 1996;87:2476-2485

126. Tajima F, Kawatani T, Endo A, Kawasaki H. Natural killer cell activity and cytokine production as prognostic factors in adult acute leukemia. Leukemia. 1996;10:478-482

127. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, Urbani E, Negrin RS, Martelli MF, Velardi A. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood. 1999;94:333-339

128. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.

129. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341:1051-1062

130. Dong F, Brynes RK, Tidow N, Welte K, Lowenberg B, Touw IP. Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. N Engl J Med. 1995;333:487-493

131. van Leeuwen FE. Risk of acute myelogenous leukaemia and myelodysplasia following cancer treatment. Baillieres Clin Haematol. 1996;9:57-85

132. Kyle RA, Pierre RV, Bayrd ED. Multiple myeloma and acute myelomonocytic leukemia. N Engl J Med. 1970;283:1121-1125

133. Levine EG, Bloomfield CD. Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. Semin Oncol. 1992;19:47-84

134. Pui CH, Ribeiro RC, Hancock ML, Rivera GK, Evans WE, Raimondi SC, Head DR, Behm FG, Mahmoud MH, Sandlund JT, et al. Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. N Engl J Med. 1991;325:1682-1687

135. Lowenberg B, Suciu S, Archimbaud E, Haak H, Stryckmans P, de Cataldo R, Dekker AW, Berneman ZN, Thyss A, van der Lelie J, Sonneveld P, Visani G, Fillet G, Hayat M, Hagemeijer A, Solbu G, Zittoun R. Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy--the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. J Clin Oncol. 1998;16:872-881

136. Leith CP, Kopecky KJ, Godwin J, McConnell T, Slovak ML, Chen IM, Head DR, Appelbaum FR, Willman CL. Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. Blood. 1997;89:3323-3329

137. Lowenberg B, Zittoun R, Kerkhofs H, Jehn U, Abels J, Debusscher L, Cauchie C, Peetermans M, Solbu G, Suciu S, et al. On the value of intensive remission-induction chemotherapy in elderly patients of 65+ years with acute myeloid leukemia: a randomized phase III study of the European Organization for Research and Treatment of Cancer Leukemia Group. J Clin Oncol. 1989;7:1268-1274

138. Godwin JE, Kopecky KJ, Head DR, Willman CL, Leith CP, Hynes HE, Balcerzak SP, Appelbaum FR. A double-blind placebo-controlled trial of granulocyte

colony-stimulating factor in elderly patients with previously untreated acute myeloid leukemia: a Southwest oncology group study (9031). Blood. 1998;91:3607-3615

139. Heil G, Hoelzer D, Sanz MA, Lechner K, Liu Yin JA, Papa G, Noens L, Szer J, Ganser A, O'Brien C, Matcham J, Barge A. A randomized, double-blind, placebo-controlled, phase III study of filgrastim in remission induction and consolidation therapy for adults with de novo acute myeloid leukemia. The International Acute Myeloid Leukemia Study Group. Blood. 1997;90:4710-4718

140. Lowenberg B, Touw IP. Hematopoietic growth factors and their receptors in acute leukemia. Blood. 1993;81:281-292

141. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 1999;5:341-346

142. Little MT, Storb R. History of haematopoietic stem-cell transplantation. Nat Rev Cancer. 2002;2:231-238

143. van Rood JJ, Oudshoorn M. The quest for a bone marrow donor--optimal or maximal HLA matching? N Engl J Med. 1998;339:1238-1239

144. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood. 2001;98:2900-2908

145. Bensinger WI, Storb R. Allogeneic peripheral blood stem cell transplantation. Rev Clin Exp Hematol. 2001;5:67-86

146. Thomas ED, Buckner CD, Clift RA, Fefer A, Johnson FL, Neiman PE, Sale GE, Sanders JE, Singer JW, Shulman H, Storb R, Weiden PL. Marrow transplantation for acute nonlymphoblastic leukemia in first remission. N Engl J Med. 1979;301:597-599

147. Champlin RE, Ho WG, Gale RP, Winston D, Selch M, Mitsuyasu R, Lenarsky C, Elashoff R, Zighelboim J, Feig SA. Treatment of acute myelogenous leukemia. A prospective controlled trial of bone marrow transplantation versus consolidation chemotherapy. Ann Intern Med. 1985;102:285-291

148. Martin PJ, Hansen JA, Buckner CD, Sanders JE, Deeg HJ, Stewart P, Appelbaum FR, Clift R, Fefer A, Witherspoon RP, et al. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. Blood. 1985;66:664-672

149. Patterson J, Prentice HG, Brenner MK, Gilmore M, Janossy G, Ivory K, Skeggs D, Morgan H, Lord J, Blacklock HA, et al. Graft rejection following HLA matched T-lymphocyte depleted bone marrow transplantation. Br J Haematol. 1986;63:221-230

150. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringden O, Rozman C, Speck B, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75:555-562

151. Goud TJ, Schotte C, van Furth R. Identification and characterization of the monoblast in mononuclear phagocyte colonies grown in vitro. J Exp Med. 1975;142:1180-1199

152. Metcalf D. The colony stimulating factors: discovery, development, and clinical applications. In: Fortner JG RJ ed. Accomplishments in cancer research. Philadelphia: J.B. Lippincott Company; 1990

153. Gabbianelli M, Pelosi E, Montesoro E, Valtieri M, Luchetti L, Samoggia P, Vitelli L, Barberi T, Testa U, Lyman S, et al. Multi-level effects of flt3 ligand on human

hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood. 1995;86:1661-1670

154. Willems R, Henckaerts E, Lenjou M, Nijs G, Rodrigus I, Moulijn AC, Slegers H, Berneman ZN, Van Bockstaele DR. Establishment of serum-free pre-colony forming unit assays for differentiation of primitive hematopoietic progenitors: serum induces early macrophage differentiation and inhibits early erythroid differentiation of CD34++CD38- cells. Ann Hematol. 2001;80:17-25

155. van Furth R. Production and Migration of Monocytes and Kinetics of Macrophages. In: van Furth R ed. Mononuclear Phagocytes. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1992

156. Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA. The leukocyte integrins. Adv Immunol. 1989;46:149-182

157. Crofton RW, Diesselhoff-den Dulk MM, van Furth R. The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. J Exp Med. 1978;148:1-17

158. Blusse van Oud Alblas A, van der Linden-Schrever B, van Furth R. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intravenous administration of heat-killed bacillus Calmette-Guerin. J Exp Med. 1981;154:235-252

159. Shum DT, Galsworthy SB. Stimulation of monocyte production by an endogenous mediator induced by a component from Listeria monocytogenes. Immunology. 1982;46:343-351

160. van Waarde D, Hulsing-Hesselink E, Sandkuyl LA, van Furth R. Humoral regulation of monocytopoiesis during the early phase of an inflammatory reaction caused by particulate substances. Blood. 1977;50:141-154

161. Pober JS, Cotran RS. The role of endothelial cells in inflammation. Transplantation. 1990;50:537-544

162. Kuijpers T. Leukocyte Membrane Adhesion Proteins LFA-1, CR3 and p150,95: a Review of Functional and Regulatory Aspects. Res Immunol. 1989;140:461-486

163. Hibbs JB, Jr., Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science. 1987;235:473-476

164. Mellman I. Fc Receptor Function in Macrophages and Lymphocytes. In: van Furth R ed. Mononuclear Phagocytes. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1992

165. Nathan CF. Secretory products of macrophages. J Clin Invest. 1987;79:319-326

166. Naeim F. Pathology of Bone Marrow. In: Mitchell CW ed (ed second). Baltimore: Williams & Wilkins; 1998

167. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990;249:1431-1433

168. Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect. 2002;4:903-914

169. Foey AD, Feldmann M, Brennan FM. Route of monocyte differentiation determines their cytokine production profile: CD40 ligation induces interleukin 10 expression. Cytokine. 2000;12:1496-1505

170. Vey E, Zhang JH, Dayer JM. IFN-gamma and 1,25(OH)2D3 induce on THP-1 cells distinct patterns of cell surface antigen expression, cytokine production, and responsiveness to contact with activated T cells. J Immunol. 1992;149:2040-2046

171. Parry SL, Sebbag M, Feldmann M, Brennan FM. Contact with T cells modulates monocyte IL-10 production: role of T cell membrane TNF-alpha. J Immunol. 1997;158:3673-3681

172. Wagner DH, Jr., Stout RD, Suttles J. Role of the CD40-CD40 ligand interaction in CD4+ T cell contact-dependent activation of monocyte interleukin-1 synthesis. Eur J Immunol. 1994;24:3148-3154

173. Shu U, Kiniwa M, Wu CY, Maliszewski C, Vezzio N, Hakimi J, Gately M, Delespesse G. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. Eur J Immunol. 1995;25:1125-1128

174. Alderson MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC, Spriggs MK. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med. 1993;178:669-674

175. Miller JS, Oelkers S, Verfaillie C, McGlave P. Role of monocytes in the expansion of human activated natural killer cells. Blood. 1992;80:2221-2229

176. Carson WE, Ross ME, Baiocchi RA, Marien MJ, Boiani N, Grabstein K, Caligiuri MA. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. J Clin Invest. 1995;96:2578-2582

177. Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc Natl Acad Sci U S A. 1993;90:3725-3729

178. Chang ZL WT, Herberman RB. Immunoregulatory role of in vitro differentiated macrophages on human natural killer (NK)-cell activity. Cell Immunol. 1990;125:183

179. Bloom ET BJ, Kawakami K. Monocyte-mediated augmentation of human natural killer cell activity: Conditions, monocyte and effector cell characteristics. J Immunol. 1986;137:172

180. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252

181. Reis e Sousa C. Dendritic cells as sensors of infection. Immunity. 2001;14:495-498

182. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med. 2002;195:343-351

183. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med. 1999;5:405-411

184. Ferlazzo G, Morandi B, D'Agostino A, Meazza R, Melioli G, Moretta A, Moretta L. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. Eur J Immunol. 2003;33:306-313

185. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med. 2002;195:327-333

186. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med. 2002;195:335-341

187. Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, Aguila HL, Caligiuri MA. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood. 2002;100:3633-3638

188. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003;3:133-146

189. Brenner MK, Pinkel D. Cure of leukemia. Semin Hematol. 1999;36:73-83

190. Burnett AK. Current controversies: which patients with acute myeloid leukaemia should receive a bone marrow transplantation?--an adult treater's view. Br J Haematol. 2002;118:357-364.

191. Mori KL, Egashira M, Oshimi K. Differentiation stage of natural killer cell-lineage lymphoproliferative disorders based on phenotypic analysis. Br J Haematol. 2001;115:225-228

192. Gray JD, Horwitz DA. Activated human NK cells can stimulate resting B cells to secrete immunoglobulin. J Immunol. 1995;154:5656-5664

193. Carbone E, Ruggiero G, Terrazzano G, Palomba C, Manzo C, Fontana S, Spits H, Karre K, Zappacosta S. A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. J Exp Med. 1997;185:2053-2060

194. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, Steinle A. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood. 2003;102:1389-1396.

195. Lowenberg B, van Putten W, Theobald M, Gmur J, Verdonck L, Sonneveld P, Fey M, Schouten H, de Greef G, Ferrant A, Kovacsovics T, Gratwohl A, Daenen S, Huijgens P, Boogaerts M. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. N Engl J Med. 2003;349:743-752.

196. Poggi A, Costa P, Tomasello E, Moretta L. IL-12-induced up-regulation of NKRP1A expression in human NK cells and consequent NKRP1A-mediated down-regulation of NK cell activation. Eur J Immunol. 1998;28:1611-1616

197. Jacobs R, Stoll M, Stratmann G, Leo R, Link H, Schmidt RE. CD16-CD56+ natural killer cells after bone marrow transplantation. Blood. 1992;79:3239-3244

198. Wodnar-Filipowicz A, Lyman SD, Gratwohl A, Tichelli A, Speck B, Nissen C. Flt3 ligand level reflects hematopoietic progenitor cell function in aplastic anemia and chemotherapy-induced bone marrow aplasia. Blood. 1996;88:4493-4499

199. Chklovskaia E, Jansen W, Nissen C, Lyman SD, Rahner C, Landmann L, Wodnar-Filipowicz A. Mechanism of flt3 ligand expression in bone marrow failure:

translocation from intracellular stores to the surface of T lymphocytes after chemotherapy-induced suppression of hematopoiesis. Blood. 1999;93:2595-2604

200. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature. 2000;408:57-63

201. Chklovskaia E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: effects of endogenous flt3 ligand. Blood. 2004;103:3860-3868

202. Roux E, Dumont-Girard F, Starobinski M, Siegrist CA, Helg C, Chapuis B, Roosnek E. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood. 2000;96:2299-2303

203. Hakim FT, Gress RE. Reconstitution of thymic function after stem cell transplantation in humans. Curr Opin Hematol. 2002;9:490-496

Publications

Full Papers

Betz G, *Nowbakht P*, Imboden R, Imanidis G: Heparin penetration into and permeation through human skin from aqueous and liposomal formulations in vitro. Int J Pharm Oct 9; 228(1-2): 147-59, 2001

Chklovskaia E, *Nowbakht P*, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz: Reconstitution of dendritic and natural killer cell subsets after allogeneic stem cell transplantation: Effects of endogenous flt3 ligand and cyclosporin A. Blood May 15; 103(10): 3860-8, 2004

Nowbakht P, Ionescu M-CS, Rohner A, Kalberer CP, Rossy E, Mori L, Cosman D, De Libero G, Wodnar-Filipowicz A: Ligands for natural killer cell activating receptors are expressed upon maturation of normal myelomonocytic cells but are low in acute myeloid leukemias. Blood. Accepted for Submission.

Abstracts

Chklovskaia E, *Nowbakht P*, nissen C, Wodnar-Filipowicz A: Dendritic cell and natural killer cell reconstitution after stem cell transplantation. Oral presentation at International Society of Experimental Hematology, Tokyo, 2001.

Chklovskaia E, *Nowbakht P*, Nissen C, Gratwohl A, Wodnar-Filipowicz A: High endogenous flt3 ligand regulates lineage commitment of dendritic cells after stem cell transplantation and in aplastic anemia. Poster presentation at American Society of Hematology, Orlando, 2001.

Nowbakht P, Chklovskaia E, Nissen C, Gratwohl A, Wodnar-Filipowicz A: Endogenous flt3 ligand regulates lineage commitment of dendritic cells after stem cell transplantation and in severe aplastic anemia. Oral presentation at Swiss Society of Hematology, Geneva, 2002.

Nowbakht P, Rohner A, Kalberer CP, Cosman D and Wodnar-Filipowicz A: Ligands for the NKG2D receptor are upregulated during monocytic differentiation from hematopoietic progenitor cells but low on AML blasts. Poster presentation at German Austrian and Swiss Society of Hematology and Oncology, Basel, 2003.

Siegler U, Kalberer CP, *Nowbakht P*, Wodnar-Filipowicz A:
Analysis, expansion and genetic modification of cytotoxic natural killer cells from AML patients. Poster presentation at German Austrian and Swiss Society of Hematology and Oncology, Basel, 2003.

Rohner A, *Nowbakht P*, Kalberer CP, Buser A, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A: Monocytic differentiation of AML blasts is associated with upregulation of the ULBP ligand for NKG2D receptor. Oral presentation at Swiss Society of Hematology, Lausanne, 2004.

Kalberer CP, Siegler U, *Nowbakht P*, Rohner A, Wodnar-Filipowicz A: Role of NKG2D receptor-ligand interaction in antileukemic activity of human NK cells. Poster presentation in Annual meeting of the Society for Natural Immunity, Noordwijkerhout, The Netherlands, 2004.

Curriculum Vitae

Name	Pegah Nowbakht
Address	Ryffstrasse 11 4056 Basel – Switzerland tel. 0041 61 321 35 36 Pegah.Nowbakht@unibas.ch

Date of birth	24.05.1976 in Teheran
Citizenship	Swiss

Education

1983-1987	Primary School Erlensträsschen, Riehen
1987-1995	High School Gymnasium Bäumlihof, Basel
1995-2000	Studies of Pharmacy at the Faculty of Natural Sciences, University of Basel; Staatsexamen 2000
Jan. 2001-Dec. 2004	PhD. Thesis under supervision of Prof. Dr. A. Wodnar-Filipowicz in the laboratory of Experimental Hematology at the Department of Research, University Hospital Basel

Profession	Pharmacist

List of Academic Teachers

Prof. Dr .med. Jean-Pierre Barras

Prof. Dr .med. Jürgen Drewe

Prof. Dr. phil. Peter Erb

Prof. Dr. sc. tech. Beat Ernst

Prof. Dr. phil. Theodor W. Guentert

Prof. Dr. phil. Annetrudi Kress

Prof. Dr. phil. Hans Leuenberger

Prof. Dr. sc. nat. Peter C. Oelhafen

Prof. Dr. phil. Willi Schaffner

Dr. phil. André Scholer

Prof. Dr. phil. Urs Séquin

Prof. Dr. phil. Udo M. Spornitz

Prof. Dr. phil. Andreas D. Zuberbühler

Prof. Dr. Peter Philippsen

Acknowledgements

I would like to thank Prof. Dr. A. Wodnar-Filipowicz for giving me the opportunity to prepare my thesis in the laboratory of Experimental Hematology at the Department of Research of the University Hospital Basel. Her expert knowledge has been a great support for my work. Furthermore, I would like to thank her for the many opportunities to participate in international congresses.

I'm also thankful to Prof. Dr. G. De Libero for his sustained interest and interactive collaboration. His ideas and constructive criticism enhanced the progression of my project. Without his help the detection of putative NCR ligands would not have been possible.
In this regard I also wish to express my gratitude to Dr. Emmanuel Rossy who provided me with the very important soluble NCR dimers. His patience and encouragement were of great help in frustrating situations.

My thanks also go to Andreas Rohner for the work he did in testing patients' PB samples and for his ever-lasting good humor.

I also like to thank my colleagues for creating a helpful and friendly atmosphere. Most importantly I am thankful to Elena Chklovskaia, Silvia Sendelov, Uwe Siegler, Soon-Siong Teo, Christian Kalberer and Vreni Jäggin who introduced me into the world of cell cultures, FACS, quantitative PCR and killing assays.

My deepest thanks go to my parents and to Stefan. Their love, trust and patience were of great support in the long and difficult process of my PhD.