

Genetic Modification and Effector Functions of Natural Killer Cells in Acute Myeloid Leukemia

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I. SUMMARY

Acute myeloid leukemia (AML) is characterized by a poor long-term outcome in the majority of patients following conventional treatment with chemotherapy. Even after allogeneic or autologous hematopoietic stem cell transplantation (HSCT) the patients are prone to relapse, indicating that the leukemic blasts escape elimination by the immune system. Natural Killer (NK) cells have emerged as a major component of the innate immunosurveillance of AML and were identified to participate in the graft versus leukemia effect following allogeneic HSCT. The effector functions of NK cells are regulated by the balanced engagement of activating receptors and inhibitory receptors. Triggering of activating receptors by the corresponding ligands on target cells counteracts the signalling pathways of inhibitory receptors and thereby elicits target cell lysis. In AML the leukemic blasts were shown to express low amounts of ligands for the NK cell activating receptor NKG2D and the natural cytotoxicity receptors (NCRs), while the expression of HLA class I molecules, the ligands for inhibitory receptors, is mostly retained at normal levels. This predominance of inhibitory signalling together with a putative deficiency in the expression of NK cell activating receptors may result in an insufficient stimulation of cytolytic NK cell responses against leukemic blasts.

To investigate the mechanisms of impaired recognition and lysis of leukemic cells, we evaluated the phenotypic and functional properties of NK cells from AML patients (AML-NK cells). We examined the cytolytic activity against the autologous leukemic blasts *in vitro* and *in vivo* in the NOD/SCID transplantation mouse model, in order to exploit their potential in cellular immunotherapy of leukemia. Further we explored the feasibility to overexpress the NCR NKp46 in NK cells by lentivirus-mediated gene transfer. This approach was intended to test the hypothesis of shifting the receptor balance in AML-NK cells towards a status that favours NK cell activation and thereby increases the anti-tumor activity.

The results demonstrated a significant, about ten-fold, reduction in the content of NK cells from patients with newly diagnosed or relapsed AML as compared to healthy individuals (donor-NK cells). Nevertheless, AML-NK cells retained a high proliferative capacity and could be efficiently expanded *in vivo* in response to NK cell specific growth factors. Also, the expression pattern of NK cell receptors and activation markers by AML-NK cells did not differ from donor-NK cells. AML-NK cells were fully functional in terms of IFN- γ production in response to the activation with IL-12

and IL-18 and displayed a high cytolytic activity against the NK cell sensitive erythroleukemia cell line K562 *in vitro*. Also *in vivo*, the adoptive transfer of AML-NK cells to NOD/SCID mice engrafted with K562 cells lead to an efficient suppression of tumor formation. The cytolytic activity of AML-NK cells against autologous leukemic blasts *in vitro* was in general below 10% at the E:T ratio of 10:1. The antibody-mediated block of inhibitory interactions could enhance the killing responses to about 70%, indicating that AML-NK cells are able to recognize autologous blasts through activating receptors. Cytolytic activity of AML-NK cells was also seen in NOD/SCID mice engrafted with human leukemia. Adoptive NK cell transfer resulted in reduction of tumor load from 31% to an average of about 10% of human blasts in the BM of treated mice. This high *in vivo* activity of AML-NK cells might be due to an increased expression of the ligands for NKG2D and the NCRs.

Taken together, our results showed that AML-NK cells do not differ from healthy donor derived NK cells; they can be isolated and efficiently expanded to high cell numbers *in vitro* and display the same expression pattern of the major activating receptors. AML-NK cells have a normal cytokine producing ability, preserve their cytolytic activity throughout the process of *in vitro* expansion and display a strong anti-leukemic effect against autologous blasts *in vivo* in NOD/SCID mice repopulated with human leukemia. These results suggest that escape of AML blasts from the immunosurveillance by NK cells may be due to the reduction of the NK cell compartment and the predominance of signals elicited by the inhibitory receptors.

We used HIV-derived lentiviral vectors for the gene transfer of the GFP marker and the NKp46 receptor to NK cell lines, primary NK cells and NK cells generated *in vitro* from CD34+ hematopoietic progenitor cells. Both single-gene and bicistronic vectors expressing these transgenes were prepared. Through the FACS sorter based enrichment of transduced cells 100% transgenic lines and primary NK cell populations were generated with a transgene expression that remained stable during *in vitro* expansion. We demonstrated that GFP+ NK cells can be generated by the *in vitro* differentiation of lentiviral transduced CD34+ progenitors, representing a highly efficient approach to produce large amounts of modified NK cells.

However, a sustained expression of the exogenous NKp46 receptor was only achieved in NK cell lines. Except for a high pseudotransduction that resulted in the transient expression of NKp46, no stable expression of transgenic NKp46 was obtained in primary NK cells, neither in cells generated from the progenitors nor in peripheral blood-derived mature NK cells.

Summary

Moreover, exogenous NKp46 in NK cell lines and transiently transduced primary cells had no ability to trigger the cytokine release or cytotoxic responses, and further studies are required to achieve the overexpression of functional NKp46.

Our results demonstrated that lentiviral vectors are suitable to obtain genetically modified NK cell lines and primary NK cells. Transgenic NK cells can be expanded to high numbers without losing the transgene expression, thus indicating the possibility to use genetically modified and expanded patient-derived NK cells for the adoptive transfer in the immunotherapy of AML. So far, the lentivirus-based approach was successful with the GFP marker transgene, but requires further optimisation for transfer of the NKp46-encoding gene.

The over-expression of tumor-specific activatory receptors would be of importance in an immunotherapeutic approach to direct NK cell effector functions specifically towards the diseased cells, thereby contributing to a graft-versus-leukemia activity against residual malignant cells.

II. INTRODUCTION

1. Natural Killer Cells

1.1 General introduction

Natural Killer (NK) cells are effectors of the innate immune system capable to recognize and lyse tumor cells and virus infected cells. NK cells comprise 5-15% of all circulating peripheral blood mononucleated cells, and are found also in peripheral tissues, including the liver, peritoneal cavity and placenta. Resting NK cells circulate in the blood, but following activation by cytokines, are capable of extravasation and infiltration into pathogen-infected or malignant tissues. NK cells mediate spontaneous killing of various tumor cells following the triggering of NK cell surface receptors by their ligands on target cells. They also produce several cytokines such as IFN- γ , TNF- α , interleukin (IL)-1, GM-CSF and TGF- β , that induce inflammatory responses, modulate the proliferation and function of dendritic cells, monocytes and granulocytes and influence subsequent adaptive responses [2]. Moreover, NK cells produce or react on chemokines like CCL3, CX₃CL1, CXCL8 (IL-8) and CXCL22 that are released by immature DCs after antigen uptake or other cells like macrophages, endothelial cells or neutrophils. NK cells also express many co-stimulatory molecules that contribute to target cell recognition or enable the interaction with effector cells of the adaptive immune response [3]. The cytotoxic potential of NK cells is mainly mediated through perforin- and granzyme-dependent cell lysis and induction of apoptosis. In addition, NK cells mediate antibody dependent cellular cytotoxicity (ADCC) of targets through Fc γ RIII (CD16), the low affinity receptor for antibodies of the IgG subclass but also express fas ligand (CD178) and TRAIL and kill target cells by inducing apoptosis. Many cytokines such as IL-2, IL-12, IL-15, IL18 and IL-21 act on NK cells by affecting their proliferative, secretory and cytolytic activity.

Historically, NK cells were described in 1971 by the observation that lethally irradiated mice were capable of rejecting allogeneic or parental bone marrow (BM) cell allografts. This pattern of BM-rejection did not follow the „classical laws“ of transplantation, where the offspring is tolerant towards parental MHC determinants. By contrast, the F1 hybrid mice showed resistance against parental BM grafts, a phenomenon called „hybrid resistance“ [4]. The effectors were termed Natural Killer Cells, but the mechanisms underlying this specificity of BM rejection remained elusive. Studies in mice with severe combined immunodeficiency (SCID) and in vivo depletion studies demonstrated that NK cells alone could mediate the specificity of rejection [5]. One hypothesis put forward to explain the „hybrid resistance“ was that a subpopulation of remaining

host NK cells expressing receptors for „self“ but not for parental MHC class I molecules fail to recognize the BM-graft, thus resulting in a loss of inhibition of the lytic machinery. Consequently this model was termed the „missing-self theory“. Results from beta-2 microglobulin knock-out mice, numerous observations of an inverse correlation between MHC class I expression levels and susceptibility to NK cell lysis as well as the characterization of an inhibitory receptor that recognized MHC class I molecules supported that hypothesis [6].

However, MHC class I does not necessarily protect from lysis by NK cells, and inhibition by MHC class I is not always sufficient to prevent NK cytotoxicity. This is due to the fact that all NK cells do not only carry inhibitory receptors scanning for the „missing self“ MHC molecules, but also express a variety of receptors providing an activating signal upon engaging their ligands. Many observations point to the importance of activating receptors in the regulation of NK cell effector function. For example, NK cells can recognize and attack virus-infected and transformed cells that have down-regulated MHC class I molecules, but are tolerant to normal autologous cells. Conversely, some virus-infected cells that maintain expression of MHC class I at the cell surface can still be killed by autologous NK cells. This lead to the hypothesis that target cell recognition and the overall threshold of NK cell activation is regulated by a fine balance between inhibitory and activating signals.

1.2 Phenotypic and functional characterization of NK cell subsets

Two subsets of human NK cells are identified based on the cell surface density of the „NK cell marker“ CD56 together with CD16. The majority (~90%) of human NK cells express low levels (dim) of CD56 and high levels (bright) of CD16, whereas a minority (~10%) is CD56bright and CD16dim or CD16 negative [2]. These NK subsets are functionally distinct, with the immunoregulatory CD56bright cells producing abundant cytokines and the more cytotoxic CD56dim cells functioning as effectors of natural and antibody-dependent target cell lysis [7]. CD56bright cells are unique among resting PB NK cells in their constitutive expression of high affinity IL-2 receptor (IL-2R $\alpha\beta\gamma$) and intermediate affinity complexes (IL-2R $\beta\gamma$) with very low (picomolar) concentrations of IL-2 resulting in a substantial proliferation and IFN- γ production *in vitro*, but with only little increase in cytotoxicity [8]. In contrast, CD56dim cells lack the high affinity receptor but constitutively express the intermediate type and nanomolar concentrations of IL-2 or IL-15 are needed to augment the cytotoxicity while having little or no influence on the

proliferation of these cells [2, 9]. Although resting CD56dim NK cells are more cytotoxic against NK-sensitive targets (like K562 cell lines) than CD56bright cells, IL-2 or IL-12 activated CD56bright NK cells exhibit similar or enhanced cytotoxicity against NK targets compared to CD56dim cells [8]. In addition, CD56bright and CD56dim NK cell subsets show differences in their NK receptor repertoires. Resting CD56bright NK cells are large agranular cells and express high levels of the C-type lectin CD94/NKG2 family with only very small fractions expressing killer-cell immunoglobulin receptor (KIR)-family and ILT-2 receptors. Resting CD56dim NK cells, however, express CD16, KIRs and C-type lectin NK receptors at high surface density along with an abundance of cytoplasmic granules [8] [10].

PB NK cell subsets also have unique adhesion molecule and chemokine receptor expression profiles, suggesting that the subsets may traffic to different sites *in vivo*. For example, CD56bright cells constitutively express high levels of L-selectin (CD62L) and CCR7, two receptors implicated in the recruitment of lymphocytes to secondary lymphoid organs, while CD56dim cells lack these receptors [2].

1.3 NK cell development

Human and murine NK cells originate in the bone marrow from hematopoietic progenitor cells (HPCs) and require the bone marrow microenvironment for complete maturation. Bone marrow stroma-derived cytokines and growth factors, including stem cell factor (SCF) and flt-3 ligand (FL) in cooperation with IL-2, -7 and -15, are critical physiologic factors for NK cell development [11]. NK cells can be reproducibly generated *in vitro* from cord blood or bone marrow derived HPCs [Miller, 1994 #463][Mrozek, 1996 #176]. Murine models show that NK cell development is mainly driven by IL-2 and IL-15 since the disruption of genes encoding the receptor subunits IL-15R α , IL-2/15R β and γ_c or the signalling molecule Jak-3 completely abrogate NK cell development [12]. Accordingly, humans lacking the β -subunit or the γ_c -chain are deficient in NK cells [13, 14].

Human NK cell development can be divided into phases, which differ in cytokine responsiveness of progenitor cells. In a linear model of development (see Figure 1-1) hematopoietic stem cells respond in an initial phase to the stroma-derived growth factors SCF and FL leading to the commitment to the lymphoid lineage. These common lymphoid progenitors (CLP) further generate NK cell precursors (NKP), which finally differentiate to mature killer cells. An intermediate CD56-

negative NKP, characterized by the expression of the CD34 marker and the β -subunit of the IL-2/IL-15 receptor (IL-2/15R β), is responsive to IL-15 and marks the transition to a second phase, the NK cell maturation. This process is thought to include the generation of the receptor repertoire and the acquisition of self-tolerance and give rise to the functional subsets of NK cells with their different effector functions. NK cells first acquire the expression of CD161 (NKR-P1) and CD2 and are characterized as non-lytic immature CD56-negative NK cells (fail *in vitro* to lyse perforin-sensitive targets) that can produce IL-13 but do not secrete IFN γ . Subsequently, NK cells become cytolytic together with the expression of CD56, CD16, and the MHC-specific CD94-NKG2 complexes and the killer cell immunoglobulin-like receptors (KIRs; see 1.4.1) [15]. In a final phase of maturation NK cells leave the sites of development and enter the periphery. Here they build and maintain the steady-state NK cell pool of distinct subpopulations, which can be modified under diverse pathological conditions in a homeostatic process of proliferation and recirculation.

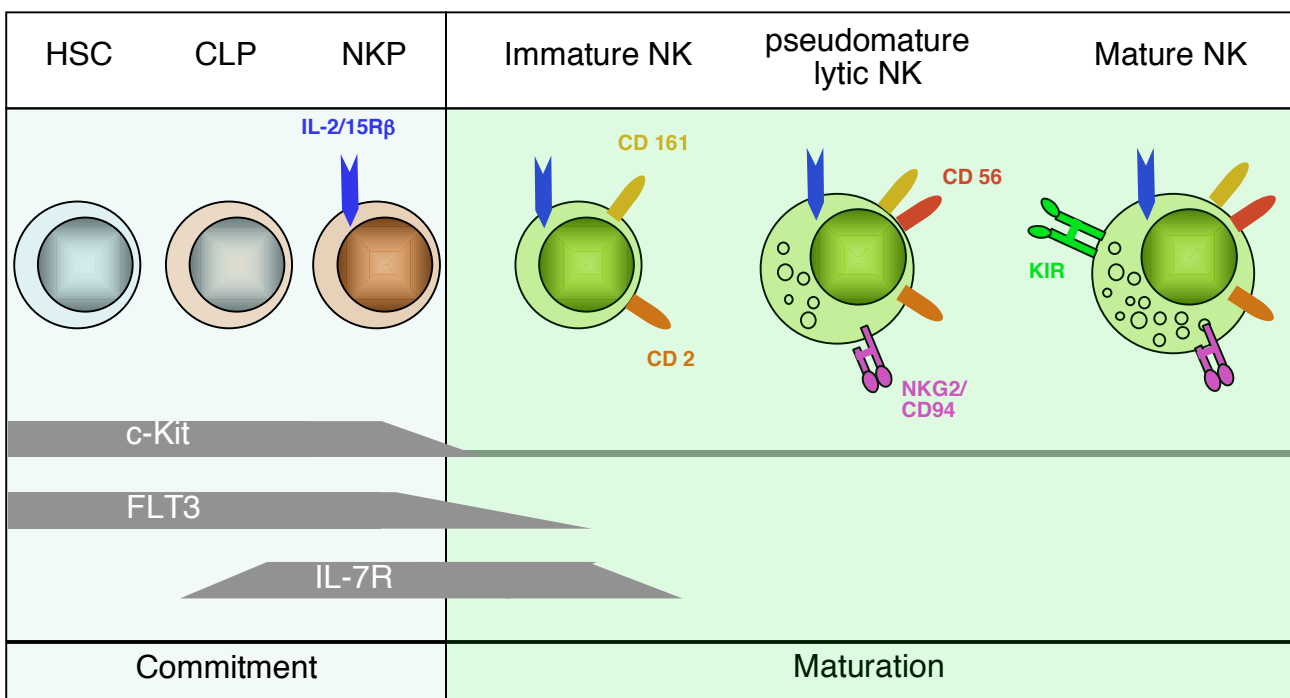


Figure 1-1: Model of NK cell development. NK cells originate in the bone marrow where hematopoietic stem cells (HSC) interact with cytokines and stromal cells to differentiate in an initial phase of cell commitment into common lymphoid precursors (CLP) and the NK cell precursors (NKP). NKPs are characterized by the expression of the IL-2/IL-15 receptor beta-chain subunit (IL-2/15R β), the progressive loss and acquisition of CD38, CD34 and of CD7, respectively (not shown). This early differentiation is accompanied by the regulated expression of the receptors for SCF (c-Kit), FL (FLT3) and IL-7 (IL-7R), as indicated by the horizontal grey bars. The phase of NK cell maturation is characterized by the expression of CD161, CD2 together with the loss of FL- and IL-7 receptors. The final differentiation into lytic and mature NK cells (characterized by the acquisition of cytolytic granules) is marked by the expression of CD56, the NKG2/CD94 complexes and the KIR repertoire together with the natural cytotoxicity receptors, NKG2D and CD16 (not shown). Mature NK cells enter the periphery where they can be divided into the functionally distinct subsets of cytolytic NK cells and the more immunoregulatory cytokine-producing killers (not shown). (adapted from F. Colucci/Colucci. 2003 #766)

In this linear model of development it is assumed that self-tolerance is achieved by the asynchronous expression of inhibitory and activating receptors together with a spatially and temporally regulated expression of their corresponding ligands. Moreover, upon the delivery of mature NK cells into the periphery, a differential expression of adhesion molecules, cytokine and chemokine receptors finally promote the generation of the functional distinct PB NK cell subsets (see above; chapter 1.2).

The *in vitro* generation of human NK cells can be initiated in the presence of SCF and FL, which induce the development of NKPs that respond to IL-2 and IL-15 to become mature NK cells. Precursors being CD34⁺CD38⁺CD7⁻ or even more differentiated CD34⁺CD7⁺ or CD34⁻CD7⁺ were isolated from different sources such as cord blood, adult bone marrow or murine fetal liver and fetal thymus [17] [18] [19] and were shown to be further differentiated into functional NK cells with IL-2 or IL-15 only. Several observations indicate that IL-2 and IL-15 could be redundant in their roles promoting the NK cell development, with IL-2 maintaining the NKP survival and IL-15 as the main differentiation inducing cytokine [16]. *In vitro* generated NK cells are consistently of the CD56^{bright} phenotype with a small subset of CD16⁺ cells, they lyse NK-sensitive targets and produce chemokines and cytokines upon stimulation. Even though such cells mostly resemble the phenotype of mature PB NK cells, they are reported to express low levels or even none of the inhibitory KIRs, thus often termed as “pseudomature” lytic NK cells. However, if “pseudomature” NK cells exist *in vivo* is unknown, as it is still controversial whether NK cells of the CD56^{dim} phenotype that predominates in the PB can be generated *in vitro*. In this context it also remains uncertain if during regular *in vivo* development the CD56^{dim} cells originate from the less differentiated CD56^{bright} phenotype or whether each subtype represent unique terminally differentiated NK cells with a distinct pathway of maturation [2].

1.4 Regulation of NK cell Function: The NK cell Receptors

The functions of NK cells are regulated by a balance of signals transmitted by activating and inhibitory receptors (see Table 1-1). In general, recognition of MHC class I molecules by inhibitory receptors dominates over activation signals and blocks the effector functions of NK cells, but the detailed mechanisms underlying this regulation of counteracting signalling pathways are not well understood. Activation signal is mediated by NK cell activating receptors that are non-covalently associated with transmembrane-anchored signalling adaptor proteins like CD3 ζ , Fc ϵ RI γ , DAP10 or DAP12. The engagement of activating receptors activates “first line” protein tyrosine kinases

(PTKs) of the Src-family, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM; consensus sequence: YxxL/I) in the cytoplasmic tail of the adaptor proteins (see Figure 1-2). Recruitment and activation of “second line” PTKs of the Syk-family like Syk and ZAP70 then results in the initiation of the downstream signalling cascade.

The inhibitory signal results from the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) in the cytoplasmic domain of inhibitory receptors (consensus sequence: I/VxYxxL/V). The phosphorylation of ITIMs upon HLA-ligand engagement of inhibitory receptors results in the recruitment and specifically binding of Src-Homology-2 (SH-2) domain containing protein phosphatases such as SHP-1, SHP-2 or SHIP. Activated phosphatases such as SHP-1 and -2 are able to dephosphorylate multiple targets in the activating pathway, thereby mediating its negative signalling. As a result, activating receptor signalling is directly inhibited by the de-phosphorylation of ITAM-recruited protein-tyrosine kinases like Syk, Zap70, SLP76 or LAT and their corresponding substrates (see Figure 1-2 and 1-3).

Inhibitory NK cell receptors		Activating NK cell receptors / co-receptors	
Receptor	Ligand specificity	Receptor	Ligand specificity
MHC class I-specific		MHC class I-specific	
a) KIR		a) KIR	
KIR2DL1 (CD158a)	HLA-C (w2, w4, w5, w6, and related alleles)	KIR2DS1	HLA-C (w2, w4, w5, w6, and related alleles)
KIR2DL2/3 (CD158b)	HLA-C (w1, w3, w7, w8, and related alleles)	KIR2DS2	HLA-C (w1, w3, w7, w8, and related alleles)
KIR2DL5	Unknown	KIR2DL4	HLA-G
KIR3DL1	HLA-Bw4	KIR2DS4	Unknown
KIR3DL2	HLA-A3, -A11	KIR2DS5	Unknown
KIR3DL7	Unknown	KIR3DS1	Unknown
b) C-type lectin receptors		b) C-type lectin receptors	
CD94/NKG2A/B*	HLA-E	CD94/NKG2C	HLA-E
CD161	Unknown	CD94/NKG2E/H*	Unknown
Immunoglobulinlike transcripts		Non-MHC class I-specific	
ILT-2 (LIR-1)	Unknown	(a) Natural cytotoxicity receptors (NCRs)	
Others		NKp46	
P75/AIRM	Unknown	NKp44	
IRp60	Unknown	NKp30	
LAIR-1	Ep-CAM	(b) C-type lectin receptor	
		NKG2D	
		MICA, MICB, ULBP-1, -2, -3	
		Others (coreceptors)	
		CD16 (FcRIII)	
		CD2	
		LFA-1	
		2B4	
		NKp80	
		CD69	
		CD40 ligand	
		CD40	

Table 1-1: Overview NK cell receptors

Inhibitory and activating NK cell receptors can be subdivided into two major groups, the MHC class I recognizing and the MHC class I non-specific receptors (see table 1-1). The class I specific receptors mainly consist of the superfamily of the immunoglobulin like receptors (KIRs) and the C-type lectin receptors that contain the inhibitory CD94-NKG2 receptor complexes and CD161, or the activating NKG2D. A huge panel of various MHC class I non-specific activating receptors and co-receptors exist with the natural cytotoxicity receptors (NCRs; immunoglobulin like superfamily) and NKG2D (C-type lectin receptor) representing the main NK cell function-triggering molecules. There are two major clusters of genes for these NK cell receptors on chromosome 19 and on chromosome 12. Unlike for T and B cells, the genes for NK cell receptors do not undergo rearrangements by somatic recombination.

1.4.1 Human killer cell immunoglobulin like receptors (KIRs)

The KIR family of NK cell receptors includes at least 13 members, of which 7 receptors are inhibitory and 6 are activating. KIRs are monomeric receptors and are characterized structurally by 2 or 3 extracellular immunoglobulin-like domains (KIR2D or KIR3D). Most KIRs specifically recognize groups of the „classical“ MHC class I alleles, including HLA-A, -B and -C. Each KIR2D or KIR3D receptor functions as inhibitory or activating receptor depending on the transmembrane and cytoplasmic domains they have.

Since the identical extracellular domain bind the same group of class I molecules, it is the long (L) ITIM containing cytoplasmic tail of KIR2DL or KIR3DL that induce an inhibitory response, whereas the short (S) cytoplasmic tail of KIR2DS and KIR3DS signal an activating response due to its association with ITAM bearing adaptor proteins. The only exception is represented by the “long-tailed” ITIM-containing KIR2DL4 receptor that recognizes the non-classical HLA-G and delivers an activation signal upon engagement (see Table1-1).

The most important KIR-mediated inhibitory interactions are the recognition of the HLA-Bw4 alleles by the KIR3DL1 receptor, of HLA-A3 /-A11 by KIR3DL2 and of the two different HLA-C epitopes by the KIR2DL receptors. HLA-C epitopes can be divided in two groups based on dimorphisms at the positions 77 and 80 in the alpha-1 helices of the HLA-C molecules:

Group 1 consists of HLA-C epitopes that carry each Ser 77 and Asn 80 (corresponding to the cw1-, cw3-, cw7- and cw8-serotypes) and are recognized by KIR2DL2 and KIR2DL3 receptors, whereas group 2 have Asn 77 and Lys 80 (cw2-, cw4-, cw5- and cw6-serotypes) and is recognized by KIR2DL1 only.

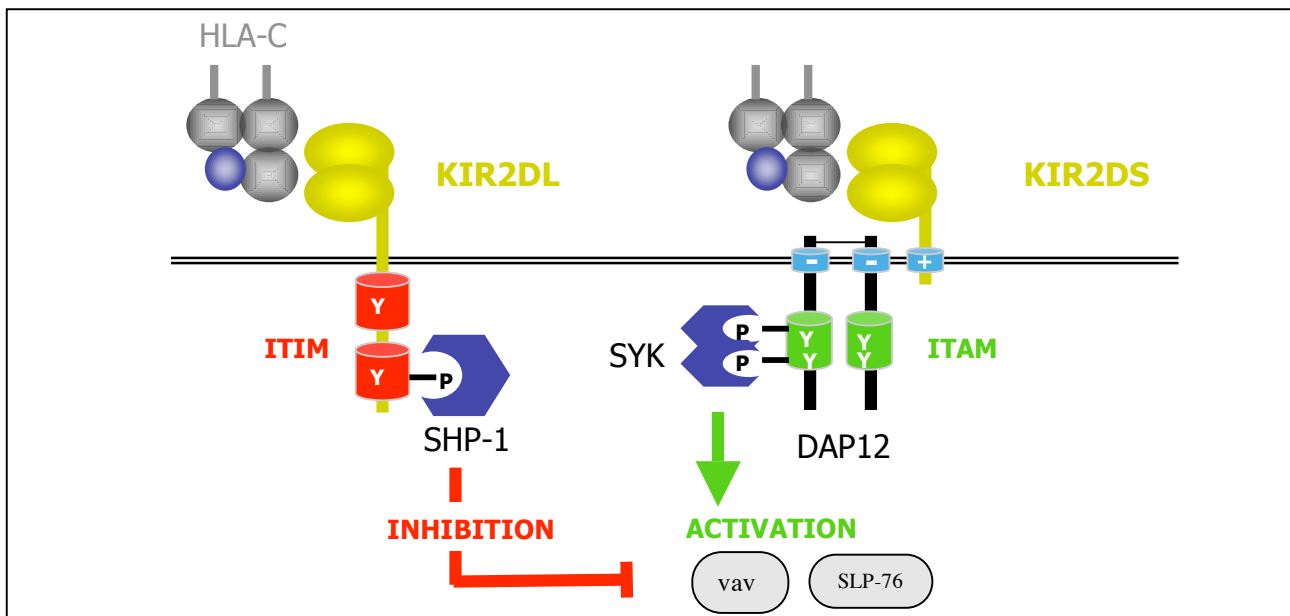


Figure 1-2: Signalling of inhibitory and activating KIRs in NK cells. KIR receptors either have 2 or 3 immunoglobulin domains (2D or 3D) and a long (L) or a short (S) cytoplasmic tail. KIR2/3DL contain ITIM motifs in the cytoplasmic tail, whereas KIR2/3DS receptors interact with the ITAM-containing adaptor molecule DAP12. Ligation of activating KIRs leads to the Src-family kinases-mediated phosphorylation of the ITAM-containing adaptor molecule DAP-12, which binds to and activates Syk-family tyrosine kinases that trigger the down stream activation cascade. When inhibitory KIRs bind their HLA ligand, Src-family kinases phosphorylate the ITIM, allowing to bind the tyrosine phosphatase SHP-1 (and possibly SHP-2). SHP-1/-2 mediates a negative signalling through the dephosphorylation of Vav and SLP-76. As inhibitory KIRs have higher affinities for the HLA class I ligands, co-ligation of both activating and inhibitory receptors result in an overall negative signal that blocks the cytotoxic activity or cytokine release of NK cells. (adapted from S.S. Farag[1])

Corresponding to this dimorphism of HLA molecules the specificity of each long- or short-tailed KIR2D is determined by a dimorphism at position 44 in the D₁ domain of the KIR receptors. Thus there are two activating short-tailed KIR2D receptors that carry the same dimorphism, resulting in the recognition of the HLA-C alleles of group 1 (KIR2DS1) and 2 (KIR2DS2), respectively.

The spectrum of HLA molecules covered by activating and inhibitory KIRs (and indirectly by CD94/NKG2 receptors; see below) is only partially overlapping, suggesting that both systems play a complementary role in monitoring the expression of HLA class I molecules. In both cases of KIRs (and CD94/NKG2 receptors) the affinity of the activating receptor is lower than that of the corresponding inhibitory receptor, ensuring a predominance of the inhibitory signal when both activating and inhibitory receptors recognizing HLA molecules are expressed on the same NK cell. However, only a minority of NK cell clones express both activating and inhibitory isoforms that recognize the same HLA allotype. More commonly, NK cell clones expressing an activating receptor for a certain HLA class I allele co-express at least one inhibitory receptor specific for a different one that predominates when engaged. Therefore, the MHC class I specific activating

receptors may only signal when target cells have lost the expression of an HLA allele recognized by the inhibitory receptor.

In this way, NK cell cytotoxicity is balanced and regulated by opposite signals delivered by inhibitory and activating receptors. This NK cell surveillance may be important for the removal of cells that have down-regulated or lost a single MHC class I allele while normal cells would be left unaffected.

1.4.2 C-type lectin-like receptors

In humans more than 15 type II transmembrane C-type lectin-like proteins are encoded on chromosome 19 where they are closely linked to the CD94 gene [20]. C-type lectin receptors are expressed as homodimers like the NKR-P1 receptor (CD161) or as heterodimers composed of monomers of the NKG2 family covalently bound to the common subunit CD94. CD94 is a product of a single nonpolymorphic gene and lacks a cytoplasmic domain for intrinsic signal transduction capacity [21]. Homodimers of CD94 exist but are of uncertain physiologic function. The extracellular and cytoplasmic domains of the NKG2 molecules are structurally diverse, consistent with differences in ligand recognition and signal transduction. Four closely related transcripts of the NKG2 family have been identified: NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F. NKG2D is a fifth distantly related member that displays only a low sequence similarity with the other NKG2 molecules and does not interact with CD94 (see below).

CD94/NKG2 heterodimers are constitutively expressed by all NK cells and cytotoxic T lymphocytes. Only CD94/NKG2A transmits inhibitory signals, whereas CD94/NKG2C and CD94/NKG2E are activating receptors. The inhibitory receptor dimer CD94/NKG2A and its activating counterpart CD94/NKG2C recognize the nonclassical class I molecule HLA-E, which binds nonamer peptides derived from the signal sequences of HLA-A, -B, -C, and -G [22]. Thus, CD94-NKG2A and -NKG2C dimers sense the overall expression of HLA class I molecules at the cell surface, a process that can be altered in virally infected or transformed cells. In addition, the ability of these receptors to discriminate among different peptide/HLA-E complexes might also influence the reactivity against allogeneic cells. As for the inhibitory and activating KIRs, the binding of the inhibitory receptor CD94/NKG2A to peptide/HLA-E complexes was shown to be stronger than binding of the activating receptor CD94/NKG2C to the corresponding complex [23].

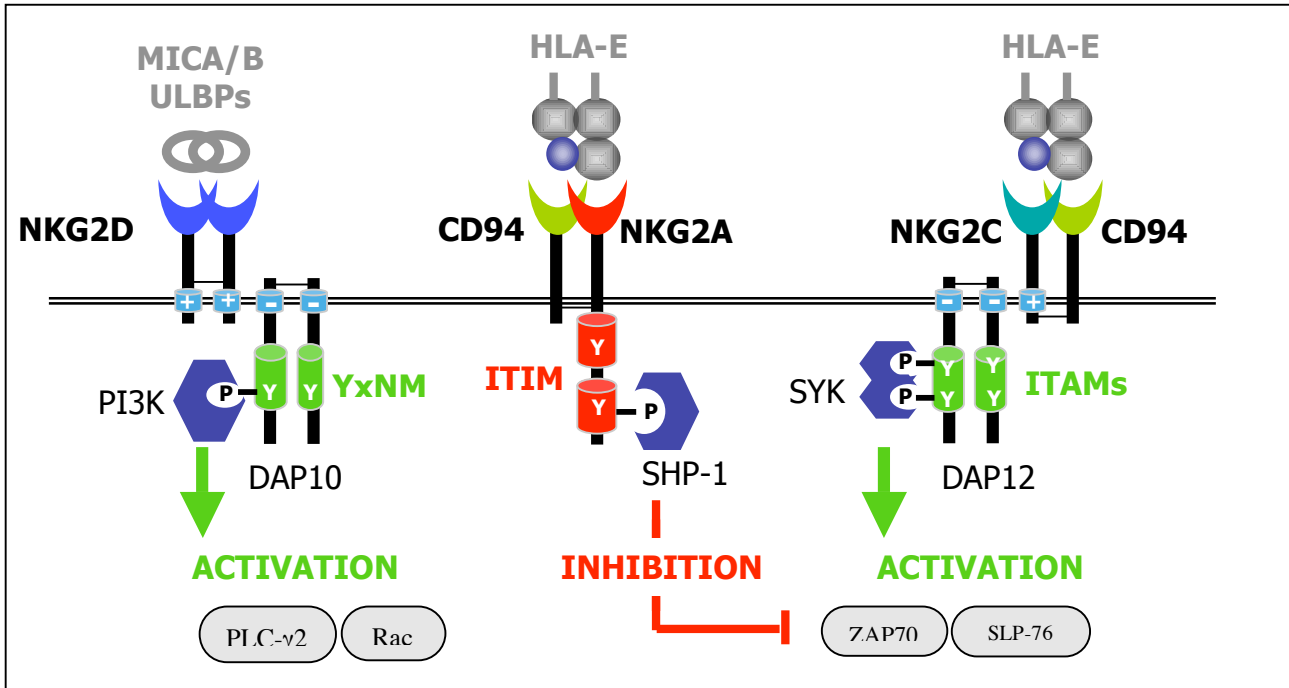


Figure 1-3: Signalling of inhibitory and activating C-type lectin-like receptors in NK cells. The C-type lectin-like receptors are disulfide-linked heterodimers of CD94 and members of the NKG2 family. The inhibitory NKG2A and the activating NKG2C both recognize the non-classical HLA class I molecule HLA-E. Similar to KIRs with long cytoplasmic tails, ITIM-containing NKG2A signals through SHP-1/2 that mediates inhibitory signals by the dephosphorylation of substrates such as Vav and SLP-76. NKG2C interacts with DAP-12 and transduce activating signals through Syk-family members. In contrast, homodimers of NKG2D associates with the adaptor molecule DAP-10 and binds to the MHC-like ligands MICA/B and to the ULBP family. DAP-10 contains an YxNM motif that bind PI-3 kinase (PI3K) upon phosphorylation thereby delivering an activation signal. As this alternative pathway through the PI3K cascade is not inhibited by SHP-1/2, NKG2D may be able to mediate activation signal that is dominant over the KIR- or NKG2A-mediated inhibition (adapted from S.S. Farag[1]).

1.4.3 The NKG2D receptor

NKG2D is the best-characterized non-MHC class I specific activating receptor described on human NK cells. NKG2D has little sequence homology to the NKG2 family and is not associated to CD94. The receptor is expressed as a disulfide-bonded homodimer and requires for the surface expression the association with the adaptor subunits DAP10 (DAP10 or DAP12 in mice) that mediate signalling, since the intracellular domain of NKG2D has no signalling motifs. NKG2D is constitutively expressed by all human and murine NK cells and CD8+ $\alpha\beta$ -T cells, by almost all human $\gamma\delta$ -T cells and by murine macrophages [24] [25].

In human NK cells triggering of NKG2D induces cytotoxicity but no cytokine release. Phosphorylation of the YxNM motif in DAP10 leads to binding of p85 subunit, the activation of PI3K and the subsequent activation of Rac, Rho-family GTPases and Phospholipase C- γ 2 (PLC- γ 2) resulting in the intracellular release of calcium and the induction of cytotoxicity [26]. Importantly, because NKG2D has a downstream signalling pathway that is distinct from the activating KIR and

C-type lectin receptors, triggering via NKG2D is less susceptible to blocking by KIR- or NKG2A-generated inhibitory signals (see Figure 1-3). As a consequence, signalling through human NKG2D was postulated to override inhibition signals generated by MHC class I engagement, and thus NKG2D functions as a primary cytotoxicity receptor rather than a co-receptor. This concept has been proven for the recognition of tumor cell lines [27] and by the transfection of NK cell resistant class I positive cell lines with ligands for NKG2D which rendered the targets susceptible to NK cell lysis [28, 29].

1.4.4 Ligands for NKG2D

The ligands for human NKG2D are the MHC class I chain related proteins A and B (MICA/B) and the UL16 binding proteins ULBP-1, -2, -3 and -4. In normal tissue low levels of MICA/B are found mainly on epithelial cells and fibroblasts where the expression is under the control of promoter elements related to those of heat shock genes [30]. MIC molecules are highly polymorphic since at least 50 different MICA and more than 15 MICB alleles are currently known [31]. MICA/B are glycoproteins that contain MHC-like α 1-, α 2- and α 3-domains but, in contrast to MHC class I molecules, do not require β 2-microglobulin or peptides for stable surface expression.

The family of the UL16-binding proteins (ULBPs) are NKG2D ligands that are glycoposphatidyl inositol (GPI)-linked surface molecules, which initially were identified by their ability to bind to the human CMV-derived membrane glycoprotein UL16.

Induction or up-regulation of NKG2D ligands may occur with pathogen related cellular stress, viral infection or tumor cell transformation. High MICA and MICB expression was found on epithelial tumors and on CMV infected epithelial tissues or fibroblasts [32] [33] [27]. It could be demonstrated that the CMV-derived UL16 binds to MICB, ULBP-1 and -2 and that this complex is selectively retained in the ER. As a consequence, CMV infected cells are no longer recognized through the surface-expression of these NKG2D ligands, indicating that the induction of UL16 expression upon infection represents an immune evasion mechanism of human and murine CMV [34, 35]. Indeed, the induced expression of NKG2D ligands were shown to markedly enhance the sensitivity of tumors to NK cells *in vitro* and *in vivo* in mouse models [36-38].

The human ULBP gene family is homologous to the murine retinoic acid early-induced transcript 1 (Rae1) gene family. However, only ULBP-1 and ULBP-2 but not ULBP-3 bind to UL16, and it is unknown if any polymorphisms within this gene family exists.

1.4.5 The Natural Cytotoxicity Receptors (NCRs)

The family of Natural Cytotoxicity Receptors (NCR) is characterized by the ability to trigger NK cell cytotoxicity in an MHC class I independent manner. NKp30, NKp44 and NKp46, all immunoglobulin-like type I transmembrane molecules, have been identified based on the screening of mAbs capable of inducing NK cell clone-mediated killing of Fc receptor bearing target cells. The NCRs share no sequence homology to each other [39] [40] [41]. The most important function of NCRs is the recognition and lysis of tumor cells by NK cells. This has become evident by the fact that monoclonal antibodies directed against the NCRs can block NK-mediated lysis of tumor cell lines („receptor-masking“). Cytotoxicity was shown to correlate strictly with the density of NCR surface expression on NK cells [27, 42]. Viral ligands specific for NKp44 and NKp46 are known (see below), but the cellular ligands for the NCRs have not yet been discovered. Accordingly, there is evidence that NCRs contribute to the defence against different pathogens, in particular in the case of viral infections [43-45]. NCRs signal through non-covalently associated adapter molecules that contain immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic tails. Src family-mediated phosphorylation (e.g. by p56lck) of the ITAM motifs in the adaptor molecules recruits protein-tyrosine kinases of the Syk-family (Syk-PTK) or zeta-chain associated protein of 70kDa (ZAP70) that activate the downstream signalling cascade to trigger NK cell cytotoxicity (see Figure 1-4).

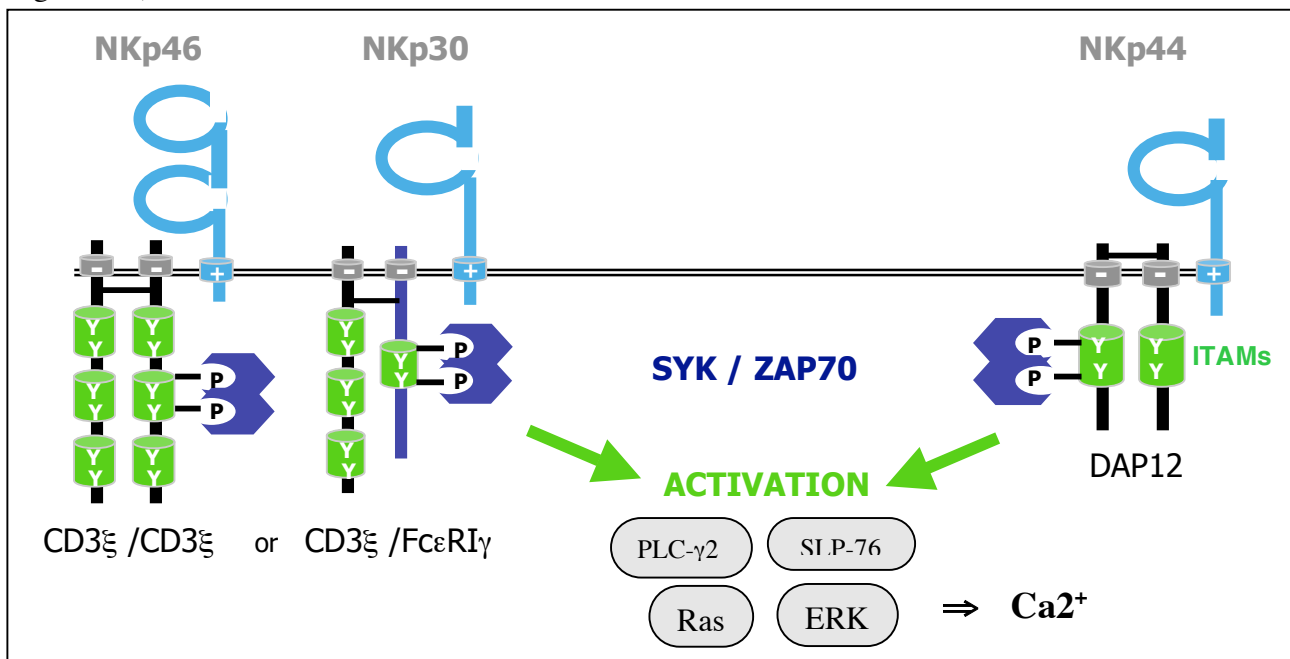


Figure 1-4: Signalling of Natural Cytotoxicity Receptors. NCRs either associate with homodimers of the CD3ξ chain and heterodimers of the FcεRIγ and CD3ξ chains (NKp46 and NKp30), or to the adapter protein DAP12 (NKp44). Ligand-engagement of the NCRs results in the phosphorylation of the ITAM-motifs (in green) and the recruitment and activation of Syk- and ZAP70 kinases with the subsequent initiation of the signalling cascade that induces NK cell cytotoxicity or cytokine release.

The subsequent downstream events include the phosphorylation and activation of different molecules like SLP76, p85-PI3K, LAT, the PLC- γ 1 and PLC- γ 2, the mobilization of Vav-1, Vav-2, the Rho-family GTPases, Grb2, and the intracellular release of calcium resulting in the induction of cytotoxicity or cytokine release.

The NKp30 receptor of 30 kDa associates with disulfide-bonded homodimers or heterodimers of the ITAM bearing adaptor molecule CD3 ζ and the Fc ϵ RI γ chain and is constitutively expressed by all activated and resting peripheral blood NK cells [39]. The receptor plays a role in the interaction of NK cells with dendritic cells (DC) in the initial phases of infection. In particular, activated NK cells were shown to lyse immature DCs through the NKp30 receptor [46].

NKp44 is almost absent in fresh isolated peripheral blood NK cells but can be detected on activated NK cells cultured in IL-2, and is also expressed by a subset of $\gamma\delta$ -T cells [47, 48]. NKp44 is a glycoprotein with a molecular size of approximately 44 kDa that associates with the ITAM bearing adaptor molecule DAP12. DAP12 exists solely as disulfide-bonded homodimers and mediates surface expression and signal transduction of NKp44. Although a recent report revealed the presence of an ITIM motif in the cytoplasmic domain of NKp44, this sequence was shown to lack inhibitory capacity and thus has no influence on the activating function of NKp44 [49].

NKp46 is thought to act as the main NCR and is constitutively expressed by all activated and resting peripheral blood NK cells. It associates with CD3 ζ and Fc ϵ RI γ , similar to NKp30 which was found to parallel expression levels of NKp46 [39, 40, 42]. The gene of the 46 kDa surface molecule is located in the leukocyte receptor complex of Ig-related genes (LRC) on chromosome 19 and has no significant homology to NKp30 and NKp44, which are encoded in the NKC on chromosome 12. NKp46 genes have also been identified in other mammals, including primates, rat and mouse. NKp46, and NKp44 (but not NKp30), has been reported to directly bind both influenza virus hemagglutinin (HA) and Sendai virus hemagglutinin-neuraminidase leading to enhanced killing of infected cells [50] [43]. To date no cellular ligands of NKp46 have been identified, although NKp46 has been shown to directly mediate the lysis of certain human tumor cells. A murine homolog of NKp46 exhibits a 58% identity to the human receptor, and it has been demonstrated that murine tumor cells are susceptible to killing via human NKp46 [51]. This cross-species reactivity may suggest a conserved ligand-binding site shared between the human and the mouse receptors [53]. In addition, results from CD3 ζ and Fc ϵ RI γ double knock out mice indicated a predominant role of NKp46 in tumor surveillance since these mice showed a profound loss of NK cell activity against most tumor targets [52].

2. Acute Myeloid Leukemia (AML)

Leukemia is a heterogeneous group of neoplasms affecting early stages of hematopoietic progenitors and includes the acute nonlymphoblastic or myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphoblastic leukemia (CLL). During the transformation process to leukemia the cells lose the ability to differentiate and mature into normal leukocytes. As a consequence, the malignant cells are no longer subjected to the normal cellular control mechanisms of cell growth, differentiation and maturation- or senescence-induced cell death with a final expansion of transformed cells that accumulate and suppress normal hematopoietic activity.

2.1 Characterization of AML

2.1.1 Classification

AML is characterized by a neoplastic proliferation of cells in the bone marrow that are arrested in their maturation, resulting in hematopoietic insufficiency (granulocytopenia, thrombocytopenia or anemia) with or without leukocytosis. AML is rapidly fatal without any treatment and most patients would die from infections and bleedings within a few months post diagnosis. The malignant cells mainly show a myeloid or monocytic differentiation but can also be of erythroid or megakaryocytic phenotype. AML is a rare disease, with an incidence of 2.4 per 100'000, but increases progressively with age to a peak of 12.6 per 100'000 in adults of 65 years or older. AML affects more men than women and despite effective treatment regimens the survival rate among patients who are less than 65 years of age is only around 40% (in the US; [54]). Although a precise aetiology of AML is unknown, several intrinsic and extrinsic factors have been implicated, including family history, Down's syndrome, Fanconi's anemia, myelodysplastic syndromes (MDS), exposure to high dose irradiation or low-frequency non-ionising radiation, chemicals (benzenes) and cytotoxic chemotherapy (alkylating agents, anthracyclines). The pathogenesis of AML is often associated with the formation of oncogenic fusion proteins generated as a consequence of specific chromosome translocations. AML can involve multiple gene rearrangements and chromosomal abnormalities are found in more than two-thirds of AML patients [55].

De novo AML is, by definition, a primary disorder and not associated with any other underlying disease. Secondary AML can arise after treatment of other malignancies and the transformation to AML is often seen in patients with MDS. Exposures to chemotherapy or radiation are particular risk

factors to develop a secondary leukemia [56]. The diagnosis and classification of acute myeloid leukemia is based on morphological and cytochemical analysis, as well as on the immunophenotypic, cytogenetic and molecular genetic analysis of the malignant cells in PB and BM. This classification was developed by the French-American-British (FAB) group [55, 57] and it divides AML into nine different subtypes. The distinction includes the reactivity of leukemic blasts with histochemical stains and the cytogenetic analysis of chromosomal aberrations. Four types namely M0, M1, M2 and M3 refer to granulocytic differentiation and differ in their extent of maturation.

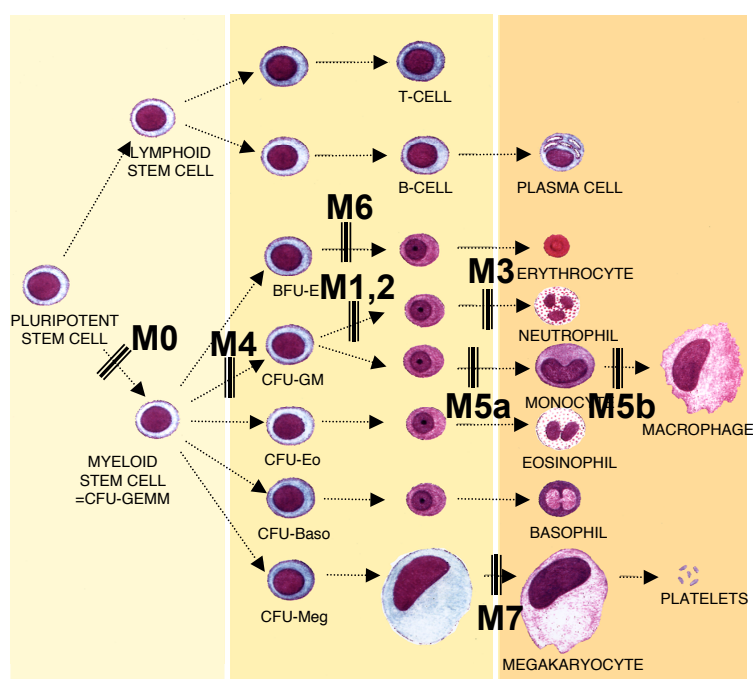


Figure 2-1: FAB-classification of AML

The system is based on *cytomorphological* and *cytochemical* observations, and subdivides AML in nine groups that differ with respect to the particular myeloid lineage involved and the degree of leukemic cell differentiation. The horizontal black bars represent the stage where an arrest developmental differentiation is present.

Table 2-1: WHO-classification of AML

AML is divided in 5 groups that are further distinguished in subgroups. The FAB-classification is used in the group of “not otherwise categorised” AML.

AML with recurrent genetic abnormalities	Translocations / inversions: e.g. t(8;21); t(15;17); inv(16); 11q23 abnormalities
AML with multilineage dysplasia	e.g. - secondary to MDS or MPD - without preexisting MDS
Therapy related secondary AML / MDS	related to prior therapy e.g. - alkylating agents - topoisomerase II inhibitors
AML not otherwise categorised	CORRESPONDS TO FAB-SYSTEM: M0-M7 in addition: acute myelofibrosis, myeloid Sarcoma, ...
AML with ambiguous lineage origin	e.g. - Acute undifferentiated leukemia - bilineal or biphenotypical acute leukemia

M4 is associated with both granulocytic and monocytic differentiation, whereas M5a/b has predominantly a monocytic character of blasts. M6 show an erythroid and M7 a megakaryocytic phenotype (see Figure 2-1).

A second classification is proposed by the World Health Organisation (WHO) and includes the FAB subtypes but broadens the classification to secondary leukemias and is more cytogenetically orientated. According to the WHO system, acute myeloid leukemias are categorised into five groups, which can be sub-divided into different minor groups (see Table 2-1).

2.1.2 Prognostic factors

Age and cytogenetic status are the two most important prognostic factors in assigning the appropriate therapy, in selection of the post-remission treatment and in predicting the outcome.

Children with AML have a better chance of cure than adults and are usually more tolerant of intensive therapy. Remission rates of 70 to 85% have been reported using conventional induction therapy. The most important prognostic factors in childhood AML are response to initial treatment and the karyotype [58]. As in adults, allogeneic HSCT from an HLA-identical or haploidentical sibling increases the long-term survival, and it is thought, that allogeneic HSCT is the treatment of choice for children and young adults in first relapse if a suitable donor exists.

Patients older than 60 years usually have a poorer outcome. There is a higher frequency of secondary AML and chromosomal abnormalities in elderly patients, and the death rates associated with remission induction tend to be higher. Good prognostic factors in this age group include a good physical condition, an age less than 80 years, primary rather than secondary AML, the absence of cytogenetic abnormalities and the absence of leukocytosis at diagnosis [54].

Of the karyotypes that have been identified, translocations between chromosomes 8 and 21, t(8;21), between 15 and 17, t(15;17) or the inversion of chromosome 16, inv(16) has been shown to indicate a favourable prognosis (roughly 20% of all AML). Conversely, loss of or deletions within chromosomes 5, 7 or both, and the translocations involving 11q23, typically define an unfavourable prognosis. These mutations are more common in older patients and in patients with secondary AML. A normal karyotype carries an intermediate prognosis [59]. Secondary AML is generally more resistant to treatment than de novo AML, and conventional induction therapy may produce

CR in only about 20% of patients [60]. High remission rates have been reported for an intensified induction therapy, but nearly all of the patients subsequently relapsed [61]. Moreover, the failure to achieve CR after two courses of chemotherapy has a very poor prognosis and is indicative of resistant disease [62].

2.2 Treatment of AML

2.2.1 Chemotherapeutic interventions

The primary goal in the treatment of AML is to induce disease remission (induction therapy) with the subsequent prevention of leukemia relapse (post remission consolidation therapy). Leukemia remission can be categorised based on defined response criterias. Complete remission (CR) is defined as the reduction of blasts to less than 5% of the nucleated bone marrow cells, regeneration of the blood lineages, an increase in peripheral blood neutrophils ($>1.5 \times 10^9/l$), platelets ($>100 \times 10^9/l$) and hemoglobin ($>110g/l$; no EPO) and the absence of detectable dysplasia or extramedullary leukemia. This definition of CR is based on morphology and has to be stable for at least 2 months [63]. Currently, more precise molecular techniques are used to define the remaining leukemic burden in patients with morphological remission, since it is estimated that one-half of the patients in remission may have clinically important residual disease [64].

Most standard induction regimens comprise an anthracyclin, such as daunorubicin or idarubicin, and cytosine arabinoside (ara-C). Between one-half and two-thirds of adults may achieve CR with one or two courses of standard induction chemotherapy, but CR is significantly influenced by age and cytogenetic risk factors (see above) [65].

2.2.2 Hematopoietic Stem Cell Transplantation (HSCT)

Since more than 30 years hematopoietic stem cell transplantation (HSCT) represents a curative treatment for hematologic disorders, including malignant and inherited genetic diseases. Despite the successful application in a large number of congenital and acquired disorders the use of allogeneic stem cell grafts is still limited by the availability of HLA-matched donors and due to treatment-related problems. In particular, allogeneic HSCT is associated with the danger of graft-versus-host-disease (GvHD) reflecting the response of donor-derived alloreactive cytotoxic T lymphocytes to the recipients HLA molecules in MHC-mismatched donor recipient pairs. Thus, the most severe form, the acute GvHD, can affect the skin, the liver and intestine and can be life threatening.

The source of stem cells can be either bone marrow (BM), placental blood obtained from the umbilical cord after birth (CB) or peripheral blood into which stem cells have been mobilized by the treatment with recombinant growth factors (mPB). The frequency of progenitors is lower in mobilized blood than in BM and cord blood, but the possibility to perform repeated lymphapheresis allows the collection and transplantation of higher numbers of stem cells. Peripheral blood stem cells are equally suitable for allogeneic transplantation as bone marrow derived cells, and in autologous transplantation mPB has largely replaced bone marrow as the source of hematopoietic progenitors [66]. Although HSCT with G-CSF-mobilized stem cells leads to faster engraftment than with BM cells [67], the higher content of T cells in mPB grafts may be associated with higher incidence of GvHD. This, on the other hand, is compensated by lower rate of leukemia relapse. Human umbilical CB is rich in progenitor cells, which were shown to be suitable for related and unrelated allogeneic transplantation [68]. The number of stem cells available from one CB donation is limited and often sufficient for pediatric patients only. Nevertheless, CB derived stem cells offer substantial advantages due to the high proliferative potential of stem cells and the immunological naïve status of accessory cells in the graft including a lower expression level of T cell-derived growth factors, that diminishes the risk of severe GvHD [69].

Development of acute GvHD upon allogeneic HSCT can be circumvented by the removal of T cells from the graft. However, this T cell depletion is associated with an increased risk of disease relapse, graft rejection and reactivation of endogenous cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infections.

Thus, in most cases acute GvHD is prevented by the careful selection of „HLA-identical“ or „HLA-matched“ donors. The preferred donor for any patient receiving a HSCT would be a syngeneic or at least HLA-identical sibling. Because the polymorphic HLA genes are closely linked and, for most practical purposes, constitute a single genetic locus, each pair of siblings has a 25% chance to be HLA-identical. This frequency allows approximately one third of patients to receive their transplants from an HLA-identical sibling. For patients for whom a suitable HLA-matched donor cannot be found, the use of autologous HSCs or partially HLA-matched or haploidentical grafts from the parents or siblings is considered.

The overall survival after allogeneic transplantations and the probability to live without acute GvHD of grades III-IV, positively correlate with the degree of HLA match [70]. But despite these detrimental effects of acute or chronic GvHD after allogeneic HSCT it was soon recognized, that patients could gain some benefit from a HLA-mismatch. In early reports it was demonstrated that

patients surviving acute GvHD benefited from a reduced tumor relapse rate [71]. The incidence of graft failure and of disease relapse is decreased compared to HSCT between HLA identical siblings or to allogeneic transplantation with intensive T cell depletion [72].

All these findings are strongly indicative of lymphocyte-mediated effects in the GvH direction, the so-called graft versus leukemia effect (GvL), which is thought to mediate the reduction in tumor relapse and increase in overall survival. Improvement of engraftment can be attributed to alloreactive T cells in the graft attacking host immune cells that otherwise would mount host-versus-graft (HvG) reactions. Direct evidence, that donor derived lymphocytes can prevent tumor relapse after transplantation was provided by donor lymphocyte infusions (DLI) in CML patients [73, 74] and has been subsequently confirmed by many reports [75-77].

2.3 The Role of NK cells in Leukemia

2.3.1 Evidence for an anti-leukemic activity of NK cells

Evidence for the important role of NK cells in the immune-surveillance of hematological malignancies in humans is provided by numerous reports on tumor-mediated suppression of NK cell activity or inherent defects in NK cell function in patients with leukemia.

In lymphoma for example, recent data have established the involvement of NK cells in the control of EBV-related lymphomas [78]. The X-linked lymphoproliferative disease (XLP) is a severe inherited immune deficiency characterized by abnormal immune responses to EBV. Most XLP patients succumb to fulminant infectious mononucleosis, whereas those who survive frequently develop lymphomas. During EBV infection infected B cells express at high levels CD48 the ligand for the NK cell co-receptor 2B4 (CD244). In XLP patients the CD48-2B4 interaction delivers a negative instead of an activating signal to NK cells due to the inability of the 2B4 molecule to associate to the SH2-domaine containing phosphatases, with the consequence that an efficient clearance of infected B cells is impeded.

In CML it was demonstrated that NK cells decrease in number and functional activity once CML progresses to the accelerated phase [79] [80-82]. In addition, an impaired proliferative capacity and lytic function was demonstrated for CML derived NK cells, which could be overcome by the *in vitro* activation with high doses of IL-2 [83]. Such CML-derived *in vitro* activated NK cells were shown to suppress autologous malignant but not normal hematopoiesis in long term culture assays.

Interestingly, activated NK cells from some patients that failed to lyse NK-sensitive targets also failed to suppress the malignant progenitors [84]. Indeed, according to a more recent finding, CML patients have alterations in the differentiation of NK cells from CD34+ progenitors, suggesting that an inherent defect of transformed progenitors is leading to the subsequent enrichment of functionally less effective NK cells [85]

In a study on 146 AML patients in complete remission the levels of NK cell activity against NK-sensitive targets was significantly lower than in normal blood donors and the loss of NK cell function was associated to a subsequent relapse. Thus, NK cell activity was suggested to serve as a possible risk-factor or indicator for leukemia relapse [86].

Similarly, blasts of AML-patients at diagnosis were found to be resistant to autologous killing [87], while the detection of cytolytic activity of NK cells obtained from PB of AML-patients in remission were shown to correlate to the duration of remission and survival.

A direct proof of an NK cell-mediated activity against autologous blasts *in vitro* and the association of this activity with the clinical outcome were first described in 1997 for three AML and ALL patients. The maintenance of complete remission after autologous HSCT was dependent on the sustained presence of anti-leukemic NK cells, since the loss of leukemia-reactive NK cells was followed by leukemia relapse, which in one patient could be reverted to complete remission by the IFN- α induced regain of anti-leukemic NK cells [89]. These results were confirmed on a larger group of 25 AML and ALL patients in complete remission either after chemotherapy or autologous HSCT, where the cytolytic activity of NK cells against the autologous blasts (isolated at diagnosis) were followed post remission. Low or absent activity was predictive of leukemia relapse with a high sensitivity and specificity [90]. More recently, in AML patients it was suggested that the observed defective expression of natural cytotoxicity receptors (NCRs) upon *in vitro* expansion might contribute to the lack of recognition of autologous leukemic blasts [88].

2.3.2 Mechanisms of tumors to escape recognition by cytotoxic effectors

Tumor cells are found to localize in immune privileged sites unreachable for effector cells, to down-regulate MHC class I molecules or to acquire an altered surface expression of ligands that are essential for NK cell recognition. However, down-regulation of MHC class I and class II molecules is a relatively infrequent event in acute leukemia, but alterations in antigenic peptide sequences may occur resulting in an impaired recognition by cytotoxic T lymphocytes [91]. Leukemic blasts can be deficient in co-stimulatory molecules such as CD80, CD83, CD86, CD40 and LFA-1 or ICAM-1

that leads to an insufficient priming or inhibition of CTL development. They were found to produce variable amounts of cytokines such as IL-10, which was shown to inhibit GvL reactions *in vitro* [92] or such as TGF-beta, a known potent inhibitor of activated lymphocytes. Some blasts are also known to abnormally secrete or to be resistant to TNF-alpha and IFN-gamma.

In AML patients it was shown that primary leukemic blasts could be resistant to NK cell mediated killing due to their reduced binding of perforin [93]. Culture supernatants derived from primary AML blasts and AML lines were demonstrated to have the potential to suppress T cell and NK cell proliferation, but without affecting their cytolytic function [94]. Further, it was shown that AML blasts are resistant to NK cell mediated killing due to the expression of Fas receptor as a non-functional surface molecule or in released form that “neutralizes” the Fas-ligand [95]. In a reciprocal way, expression of Fas-ligand by leukemic blasts was found to prevent cytotoxic activity of Fas receptor expressing T and NK cells [96]. In addition some lymphoid leukemias are known to be resistant due to their low surface expression of TRAIL molecules [97].

Shedding and the serum-accumulation of ligands for the activating receptor NKG2D was also shown to cause the down regulation of NKG2D in gastrointestinal tumors and leukemic blasts [34, 98, 99].

2.3.3 NK cells in HSCT: the role of KIR-MHC class I interactions

KIR-epitope mismatches are well known causes of NK cell alloreactivity [100-102] but their role in human transplantation has been evaluated only recently. NK cell alloreactivity was detected in transplantations where a mismatch in the killer cell immunoglobulin-like receptors (KIR) existed, which generated an NK cell response in the GvH direction. This alloreactivity was clearly correlated to higher engraftment rates without causing GvHD, it was mainly HLA-C directed and had a clear anti-tumor effect in AML but not in ALL [103].

The most prevalent and dominant pattern of NK cell alloreactivity is due to the recognition of the two HLA-C allotypes by KIRs (see section 1.4.1). Since KIR genes and HLA genes are located on different chromosomes, matching for HLA genotype does not necessarily result in matched KIR genes. Consequently, 75% of transplants from an HLA-identical sibling donor will be KIR mismatched, and for transplants from unrelated donors the frequency of KIR mismatch will be close to 100% [104]. In the haploidentical setting of transplantation the donor and recipient share at least one HLA-C allele but might differ in the second one. Thus, donors heterozygous in terms of the

HLA-C haplotype have subsets of NK cells that kill targets of homozygous recipients, whereas heterozygous recipients resist NK cell attack from either type of donors. This has the following implications for HSCT between HLA-identical or haploidentical siblings:

First, no alloreactivity at all, due to a full KIR-ligand (HLA-C) match. Second, alloreactivity occurs in the HvG direction. Here the correlation of certain HLA-C determinants to increased risk of graft failure was shown [105], but due to extensive conditioning regimens it is difficult to distinguish T cell- from NK cell-mediated HvG effects in rejection of allogeneic BM cells. In addition, since NK cells are sensitive to most conditioning reagents and the large amounts of HSC given in transplantation simply should „override“ potential residual NK cell reactivity.

The third possibility is represented by the alloreactivity in the GvH direction, which may be responsible for most of the beneficial effects on the outcome observed in haploidentical transplantation. Data from 92 haploidentical transplantations showed that mismatching in HLA-C allotypes correlated with a higher probability of an event-free five-year survival for patients with AML but not ALL [106, 107].

Why NK cell alloreactivity is observed in acute myeloid but not in lymphoblastic malignancies is not known, but may be due to the absence of the adhesion molecule leukocyte function-associated antigen 1 (LFA-1) on ALL blasts. Interestingly, LFA-1 is rarely expressed on non-hematopoietic cells, which may explain that NK cells do not kill these cells and, possibly, why NK cells do not cause GvHD. Another possibility is that in myeloid leukemia blast-derived dendritic cells directly present leukemia antigens to reactive T cells, which has been shown *in vitro* in AML or in CML where dendritic cells were found to be BcrAbl positive, thus of leukemic origin but still stimulatory for CTLs [108].

A controversy remains considering the consequences of KIR ligand incompatibility for the risk of severe GvHD, which was shown to be reduced [109] as well as increased [110]. In a case-control study on 556 bone marrow recipients from unrelated donors, mismatches for HLA-C correlated to a higher risk of graft rejection [111]. Even more striking, a recent study of 62 haploidentical transplanted leukemia patients revealed poorer engraftment rates and overall survival in KIR epitope mismatched than with matched transplants without any influence on the incidence of GvHD [112].

In general, depending on the protocol that is administered, allogeneic HSCT can produce an environment which favours a GvH directed immune response either dominated by T cells as in HLA-matched transplants and in mini-transplantation, or by NK cells in the case of haploidentical transplantations with T cell depletion, anti-thymocyte globulin treatment and KIR ligand

incompatibility. Taken together these observations suggest that alloreactive T and NK cells can contribute to the eradication of residual tumor cells and that patients receiving HSCT may benefit from DLI given at later time points.

3. Cellular Immunotherapy: The Adoptive Transfer of anti-Leukemic Effector Cells

The adoptive transfer of leukemia-directed effector cells to patients with hematologic malignancies includes different approaches. In principle, the infusion of cytotoxic effector cells is aimed at prevention of leukemia relapse and maintenance of remission after HLA-matched stem cell transplantation by the eradication of minimal residual disease. Moreover, the adoptive transfer of allogeneic or autologous effectors with GvL activity might be considered as a therapeutic tool in elderly patients who are mostly intolerant to intensive chemotherapy or as a potential therapy for recurrent chemotherapy-refractory leukemia.

Beside the well-established infusion of donor derived T cells there are attempts to use *in vitro* generated cytotoxic T lymphocytes that were selected for their tumor specificity. The alternative approach in the adoptive transfer of anti-leukemic effector cells is to infuse $\gamma\delta$ T cells, NK-T cells or CD3-CD56+ NK cells which are not MHC-restricted and therefore do not bear the risk to develop GvHD.

3.1 Donor-derived lymphocyte infusion (DLI) and leukemia-specific cytotoxic T lymphocytes (CTLs)

The most prominent approach is represented by the donor derived lymphocyte infusion (DLI) after T cell depleted allogeneic HSCT. Several studies demonstrated that donor derived CTL-precursors directed against leukemic blasts in transplanted patients emerged and persisted in the peripheral blood of recipients who maintained a state of remission. DLIs were initially applied to CML patients but are currently extended to a variety of hematologic malignancies [114]. In some CML patients a strong correlation was shown between the presence of anti-leukemic CTLs and the disappearance of the tumor cells. In contrast, in transplanted patients who experienced a relapse the frequency of CTL precursors rapidly declined [113].

Usually DLI consists of a crude mixture of lymphocytes collected by lymphapheresis and is applied in HLA-matched HSCT. T cell DLIs are not recommended in partially mismatched or haploidentical HSCT because of the high risk to develop GvHD. The adoptive transfer of donor T cells that specifically recognize viral antigens has been effective in preventing CMV and EBV disease after allogeneic HSCT without causing GvHD [115] and autologous tumor-reactive T cells have been transferred to successfully treat patients with melanoma [116].

3.2 The adoptive transfer of $\gamma\delta$ -T cells and cytokine-induced NK-T cells

The TCR positive $\gamma\delta$ -T cells were demonstrated to be important mediators of alloengraftment [117] without causing GvHD [118]. The anti-tumor activity of $\gamma\delta$ T cells was shown for many tumors of different origin, where the reactivity was not MHC restricted but depended on interaction of LFA-1 with its ligand ICAM-1. In addition, $\gamma\delta$ -T cell clones (V δ 1 cells) could be raised against acute lymphoblastic leukemias (ALL) but not against myeloid blasts indicating a possible specificity of $\gamma\delta$ T cells to lymphoid cells [119]. No reports so far demonstrated the immunotherapeutic use of expanded and activated $\gamma\delta$ -T cells.

NK-T cells are TCR $\alpha\beta$ + CD3+ CD56+ cytotoxic T cells that are abundant in the liver, where they make up to 40% of total lymphocytes and localize to the sinusoidal walls. They are also found in the BM, in smaller numbers in the spleen, lymph nodes and the thymus [120]. NK-T cells may represent an intermediate stage in phylogenetic development between NK cells and T cells. Their function is not yet clearly defined, but they may play an important role in the surveillance of malignant cells and cells infected with intracellular pathogens as well as in the control of autoreactive lymphocytes [121]. Early reports on *ex vivo* expanded and activated T cells, at that time not defined as the NK-T cell subset, demonstrated a MHC-unrestricted and unspecific cytotoxicity against a variety of malignant cells including autologous leukemic blasts [122].

Cytokine induced killer (CIK) cells are NK-T cells expanded from PBMNC by the timed addition of IFN- γ , IL-2 and the anti-CD3 monoclonal antibody OKT3. The CIK cells are CD16- and are derived from T cells and not from NK cells, which retain their phenotype under these conditions [123]. A typical bulk CIK culture contains approximately one third of CD3+CD56+ cells, two thirds of CD3+CD56-cells and below 10% of CD3-CD56+ NK cells [124].

Such bulk cultures exert a marked non-MHC restricted cytotoxicity against a variety of tumor cells including B-lymphoma lines [125], fresh tumors and autologous or allogeneic CML progenitors, but have only minor effects on normal hematopoietic progenitor cells [126]. The adoptive transfer

of CIK cells to SCID mice challenged with human lymphoma cell lines protected against the development of tumors and prolonged the survival [123]. In a HLA class I and class II mismatched mouse transplantation model it was clearly demonstrated that CIK cells do not mediate GvHD but are able to exert a strong GvL effect against a mouse lymphoma cell line [127].

In CML patients it could be demonstrated that the large-scale *ex vivo* generation and expansion of BcrAbl negative non-malignant CIK cells is possible, and that these cells have potent *in vitro* and *in vivo* effects against autologous tumor cells. In tumor-inoculated immunodeficient SCID mice a single CIK infusion could significantly delay the progression of leukemia [126]. CIK cells could also be generated during relapse phase of patients treated with chemotherapy for a variety of malignancies including lymphoma, acute leukemia, myeloma and breast carcinoma. CIK cells could be obtained from peripheral blood samples with a minimal pool of residual normal lymphocytes and were demonstrated to be cytotoxic against autologous and allogeneic leukemic blasts from AML [128], ALL and B-CLL patients [129].

3.3 The adoptive transfer of NK cells

3.3.1 Results from mouse transplantation models

Expanded and activated CD3-CD56+ NK cells are known to mediate numerous anti-tumor effects *in vitro* and *in vivo*, particularly against metastatic tumors and autologous or allogeneic leukemic blasts [130-132]. The adoptive transfer of activated NK cells was shown to promote the engraftment in mice receiving syngeneic as well as allogeneic grafts [133, 134].

In an MHC mismatched transplantation model NK cells were shown to play a pivotal role in tumor suppression and the augmentation of allogeneic BM engraftment in mice [106]. In this model (donor F1: H-2d/b transplanted to parental: H-2b/b) donor T cells were tolerant of the recipients MHC, but donor NK cells included alloreactive cells (recognizing H-2d allotype, hence not inhibited by the H-2b allotype). A single dose of alloreactive but not of syngeneic NK cells could eradicate advanced human CML in NOD/SCID mice. These alloreactive NK cells did not cause GvHD, even when infused in large amounts into lethally irradiated and transplanted recipients (all mice with 100% survival), whereas the co-transplantation of alloreactive T cells caused GvHD and the rapid death in all mice. However, the alloreactive NK cells could reduce recipient type T cells and granulocytes in bone marrow and spleen to levels only observed after lethal irradiation, demonstrating a strong immune- and myeloablating effect. These results implicated the use of

alloreactive NK cells as a preconditioning infusion to obviate high-intensity regimens while still inducing efficient myeloablation. Indeed, the co-transplantation of as few as 2×10^5 alloreactive NK cells to non-lethally irradiated recipients resulted in high levels of mixed donor chimerisms (up to 80 %), which could be converted to a full donor chimerism by an additional post-engraftment infusion of alloreactive NK cells. This MHC-mismatched F1 to parent transplantation model could clearly demonstrate that the conditioning by alloreactive NK cells could prevent GvHD by the elimination of recipients' antigen presenting cells (APC).

Similar results were obtained in an allogeneic transplantation model using tumor-bearing mice as bone marrow recipients [131]. It could be demonstrated that donor derived NK cells inhibited GvHD and substantially promoted graft-versus-tumor (GvT) reactions. Mice receiving NK cells had significantly improved survival, and this could be further augmented with the co-administration of donor T cells. In contrast, donor T cells alone gave a certain protection from the tumor, but the mice ultimately died due to GvHD. If the administration of NK cells was delayed after initiation of GvHD by transferred donor T cells, the incidence and severity of GvHD was increased. Importantly, this allogeneic transplantation model used an adenocarcinoma, suggesting that the adoptive transfer of alloreactive NK cells may be effective in HSCT for the treatment of hematological disorders as well as metastatic solid tumors.

Taken together, these results in mice implicate that the adoptive transfer of NK cells can have a protective effect against GvHD through the inactivation of recipients APCs such as dendritic cells and is suggested as a mechanism of suppression of GvHD by NK cells in allogeneic transplantations while inducing strong graft versus tumor activity [135].

3.3.2 Transfer of ex vivo expanded NK cells in humans: NK cell DLI

One possible immunotherapeutic application of donor derived NK cells in humans which can be directly concluded from mouse transplantation models would be the preconditioning in HSC transplantation with donor NK cell infusions. Such NK cell DLIs would aim at the consolidation of engraftment with a less severe conditioning or even without high-intensity regimen and without T cell depletion while still inducing an efficient myeloablation.

Moreover, the adoptive transfer of NK cells after allogeneic HSCT offers the possibility to induce a strong GvL activity against residual disease without the development of GvHD. Thus, NK cell infusions can be given as a „pre-emptive NK-DLI“ at predetermined time points after transplantation or as „therapeutic NK-DLI“ upon evidence of minimal residual disease or a mixed

chimerism. First prospective ongoing studies and clinical trials are concerning the safety and efficacy of donor NK cell collections and the tolerability of NK cell infusions to cancer patients or recipients of allogeneic or autologous HSCT. Recent reports demonstrated the feasibility and safety of the adoptive transfer of donor lymphocyte preparations, which were *ex vivo* IL-2 activated and enriched for CD56+ effector cells. The adoptive transfer of isolated, enriched and *ex vivo* IL-2 activated haploidentical CD56+CD3- NK cells to AML patients was shown to be safe without any severe adverse events. Here the persistence without any further *in vivo* expansion of donor cells up to several months was demonstrated [136]. In addition, these studies are designed to determine the practicability of large-scaled graft engineering to prepare highly purified NK cells for the use in NK-DLIs [137, 138].

Furthermore, NK cell infusions could be applied to AML patients at high risk, particularly in elderly patients or patients in a bad physical condition who are usually not eligible to allogeneic transplantations since they are not able to tolerate the severity of a myeloablative regimen. Such an adoptive transfer of alloreactive, *ex vivo* expanded and activated allogeneic or autologous NK cells may serve as an alternative approach in the treatment of disease. The first reports on the adoptive transfer of *ex vivo* IL-2 activated and expanded NK cells in humans, of so called Lymphokine Activated Killer (LAK) cells in 1993, demonstrated the feasibility of NK cell infusions as an alternative approach to high-dose *in vivo* IL-2 administration even though no direct benefit could be assigned to the transferred cells [139]. More recently, the *ex vivo* expansion of AML patient-derived NK cells with a cytolytic activity against autologous leukemic blasts was shown [140]. Similarly, the expansion of NK cells and NK-T cells from patients with chronic B cell lymphocytic leukemia (B-CLL) was reported. NK cells were expanded from PBMC cultures with no difference in expansion rates of NK cells derived from patients either in indolent or progressive state of disease [141].

Importantly, since CIK cells and NK cells can be generated at the time point of diagnosis it is worth to note, that patient derived effector cells are derived from normal resting NK cells or NK-precursor cells, thus are non-leukemic in nature which would justify their clinical application [129]. This is most feasible in the autologous transplantation setting, in which peripheral blood lymphocytes harvested by leukapheresis could be expanded for re-infusion post transplantation after hematopoietic recovery at a time of minimal residual disease. In general, with the demonstration of potent cytotoxicity against autologous leukemic blasts in AML, it is conceivable that IL-2 activated NK-like T cells (CIK) or NK cells will find a place in clinical use.

4. Gene Therapy and Genetic Modification with Lentiviral vectors

4.1 The concept of gene therapy

Gene therapy protocols have been designed to correct inherited or acquired metabolic, infectious, or malignant diseases. The primary aim of gene therapy, namely the insertion and expression of a therapeutic copy of a mutant or deleted gene has been extended to additional clinical applications of gene transfer. Such therapeutic interventions may concern the genetic modification of tumor cells or terminally differentiated effector cells of the immune system. New strategies include for example the replacement or inactivation of mutant tumor suppressor genes and oncogenes in tumor cells, the introduction of genes encoding prodrug-metabolizing enzymes as new forms of drug delivery [142] as well as the transfer of genes encoding co-stimulatory molecules [143] to modify tumor cells for the use as cancer vaccines.

The genetic modification of immune cells such as dendritic cells (DC), T cells or NK cells could provide new immunotherapeutic approaches for an effective anti-cancer therapy. For instance, genetically modified DCs expressing defined tumor-associated antigens may serve as cellular vaccines in eliciting a tumor specific CTL response [144]. Further, the transfer of T cell receptor (TCR) genes to T cells which are additionally genetically modified to express the HSV thymidine kinase (HSV-tk) “suicide-gene” were successfully applied in immunotherapeutic DLIs after allogeneic HSCT [146] and offer the potential to direct T cells to any antigen of interest [145].

4.2 Viral vectors for gene transfer

4.2.1 Viral gene delivery systems

Successful gene therapy primarily depends on the safe and efficient transfer of the selected gene and the precise targeting into the cells of interest. If long-term expression is needed the transferred DNA has to integrate into the genome of the target cell, ideally in a specific site, and the vectors should allow the integration of large exogenous DNA inserts without the induction of gene silencing. Moreover, in some therapeutic applications the transgene should be able to respond to cellular mechanisms of regulation of gene expression or should allow to be regulated by the exogenous administration of drugs, so that the gene product will be produced in correct quantities and at appropriate times. Among the wide variety of gene delivery systems viral vectors have

proven most useful since they fulfil the majority of the requirements. However, the most important constraints of all vector systems including viral vectors are their inability to be targeted to a specific cell type or tissue together with their random insertion to the host genome. This integration represents a mutagenic event that has the potential to disrupt or to transcriptional activate genes. Each viral vector system available today has certain advantages that make it valuable for a particular application in gene therapy (see Table 5-1), but only retroviral derived vectors have the capability to mediate the stable integration of the transgene into the host genome.

vector	characteristics	disadvantage
Adenoviruses	high vector titers, with large DNA inserts (up to 15kb); high level of expression; infection of non-dividing cells; high target cell range	no genomic integration, thus toxic and inflammatory <i>in vivo</i> reactions possible, with neutralisation of vector particles by the immune system
Adeno-associated viruses	High vector titers; Infection of non-dividing cells with genomic integration; Non-toxic	Small vector genome allows only small DNA inserts; Requirement for helper adenovirus, with low gene transfer efficiencies
Herpes simplex virus	large DNA inserts possible (up to 30kb)	no genomic integration; toxic and inflammatory <i>in vivo</i> reactions possible
Retroviruses	Genomic Integration of viral genome; high vector titers possible with large DNA inserts (up to 9kb); high target cell range	genomic integration in dividing cells only; random insertion with low gene transfer efficiencies
HIV-derived lentiviruses	High vector titers; Infection of non-dividing cells with genomic integration; large DNA inserts (more than 10kb)	random insertion

Table 5-1: Characteristics of viral gene delivery systems

4.2.2 The lentivirus-based vector system

Retroviruses are lipid-enveloped particles comprising a homodimer of two single stranded RNA genomes of 7 to 11 kb and virus replication enzymes within a protein core. Following infection, the viral genome is reverse-transcribed into DNA that is transported to the nucleus where it randomly integrates into the chromatin. The family of retroviruses includes several varieties being exploited for gene therapy, the mammalian and avian C-type retroviruses (oncoretroviruses), spumaviruses and the lentiviruses. All retroviral genomes have two long terminal repeats at the 5' and 3' ends (5'-/3'LTR) with closely located cis-acting sequences that are active during viral gene expression, reverse transcription, packaging and integration. The LTR frames the three groups of structural genes *gag*, *pol* and *env* that encode the capsid proteins, the reverse transcriptase, polymerases, the integrases and the surface glycoproteins, respectively (see Figure 5-1). The 5'LTR (R-5U) contains the promoter that drives the transcription of the viral genes, whereas the 3'LTR (U3'-R) contains

the transcription termination site and the polyA signals. Lentiviruses have a more complex genome encoding two additional groups of regulatory and accessory genes that are essential during the viral life cycle and pathogenesis. The regulatory gene product *tat* is responsible for the promoter activity and initiates transcription from the 5'LTR, whereas the *rev* protein binds to the *rev* responsible element (RRE) within the viral RNA which is necessary for efficient *gag* and *pol* expression and allows the transport of unspliced RNA out of the nucleus. The accessory genes *vpr*, *vif*, *vpu* and *nef* have function in nuclear transport, particle assembly and release as well as in the growth arrest of infected cells, thus representing virulence factors for the infectivity of the virus. Upon infection of eukaryotic cells the retroviral RNA is reverse transcribed within the cytoplasm and the viral genome enters the nucleus to integrate into the host DNA. Unlike the common oncoretroviruses such as the murine leukemia virus (MLV) that cannot cross the nuclear membrane, lentiviruses use a mechanism of active transport of the viral genome through the pores of the intact nucleus. The reverse transcribed viral DNA together with the *gag*-encoded matrix protein, the enzyme integrase and the product of the *vpr* gene form the pre-integration complex that is recognized by the nuclear import machinery and translocated to the nucleus (see Figure 5-2).

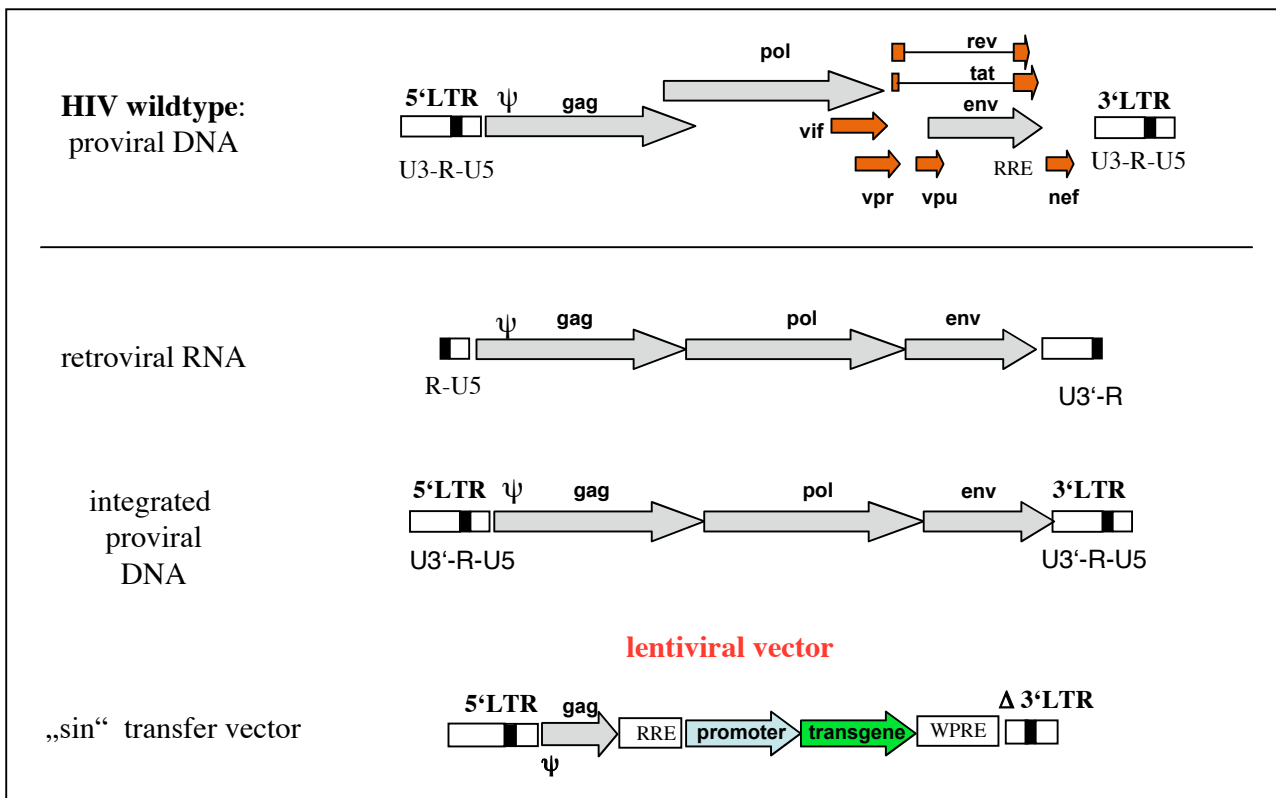


Figure 5-1: Schematic organisation of the retroviral genome. In addition to the *gag*, *pol* and *env* genes found in all retroviruses accessory (*vpr*, *vif*, *vpu* and *nef*) and regulatory genes (*tat*, *rev*; all in red) are present in the wild-type HIV. Retroviral RNA that is packed into a virus particle contains shortened LTRs with the U5-and the U3' regions duplicated in proviral DNA after reverse transcription. A prototypical self-inactivating (*sin*) lentiviral vector is shown below. The only regions left from the HIV are the LTRs are a small part of the *gag* gene, the packaging signal sequence ψ (both necessary for RNA encapsidation), and the *rev*-responsible element (*RRE*). A 400bp deletion in the 3'LTR (Δ 3'LTR) results in the abolishment of the promoter activity of the (duplicated) U3'-R-U5 LTR after integration.

Lentiviruses are therefore able to infect and integrate into non-dividing cells such as terminally differentiated somatic cells or hematopoietic stem cells, while oncoretroviruses need mitotically active cells (with the disassembly of nuclear membrane during mitosis) to complete their replication cycle. During the process of reverse transcription the U5-region of the 5'LTR (R-U5) and the U3'-region of the 3'LTR (U3'-R) are both duplicated, so that a proviral DNA integrates that contains two identical LTRs, each consisting of the regions U3 (viral promoter/enhancer), R (transcriptional start sequence) and U5. This is especially important for the generation of so-called “self-inactivating” (sin) viral vectors where a deletion in the U3'-region results in the loss of promoter activity after reverse transcription and integration (see Figure 5-1). Such engineered vectors are able to transduce a foreign gene after infection of target cells, but are replicative defective and thus unable to multiply and spread to other cells[149].

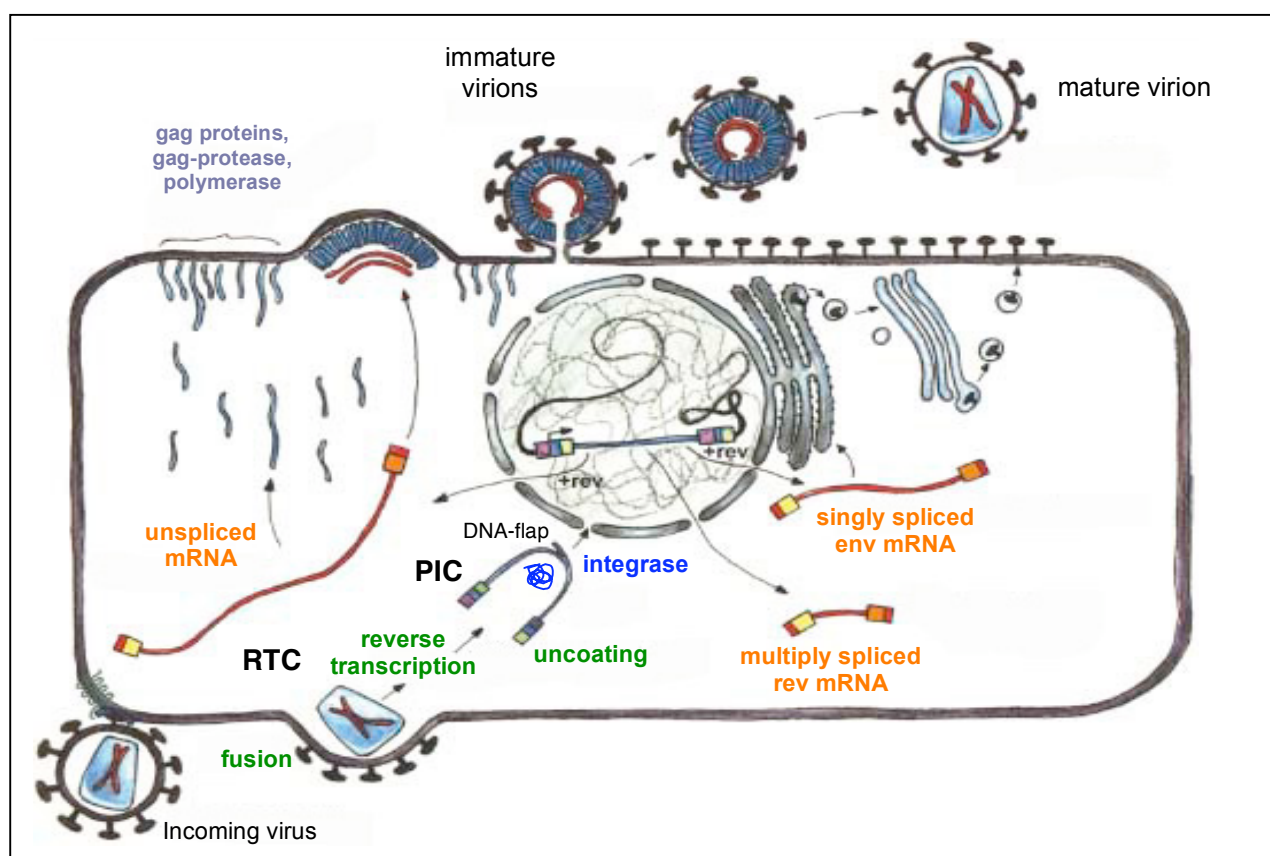


Figure 5-2: Lentivirus replication cycle. Infection begins with the recognition of specific cell surface receptors by the viral envelope glycoproteins, followed by the fusion with the target cell membrane. Viral RNA is reverse transcribed to DNA in the reverse transcription complex (RTC). Proviral DNA is uncoated and forms together with DNA binding viral proteins (e.g. integrase; associate with the central DNA flap) the pre-integration complex (PIC) that is translocated to the nucleus where the provirus integrates randomly. Integration to the host genome enables the provirus to be stably maintained and passed to the progeny cells by host DNA replication during mitosis. Viral RNA is transcribed into unspliced and spliced mRNA variants. The unspliced RNA is translated into structural core proteins (matrix, capsid and nucleocapsid) and the viral enzymes (protease and integrase) and two copies are packed as genomic RNA into new virions. The spliced mRNA variants code either for the packed reverse transcriptase or the envelope proteins. Mature virions are released from the cell (virus budding) after the acquisition of their envelopes that consists of cellular membrane components and viral *env* proteins.

The currently used lentiviral vectors are derived from the immunodeficiency viruses of human (HIV-1 or HIV-2), simian (SIV), feline (FIV) or bovine (BIV) origin. The rationale to develop lentiviral vectors from the non-human viruses is that they would be more acceptable for clinical application since the parental viruses are not infectious to humans.

Lentiviral vector particles are generated by the co-transfection of a producer cell line with three different vector plasmids [147, 148] (see Figure 5-3; section 2.4.1). The first plasmid represents the transfer vector that carries the gene of interest under the control of a strong internal promoter. Most viral genes are deleted, leaving a backbone of the LTRs with the packaging signal sequence ψ (encapsidation-site; 3' of the 5'LTR), a small part of the gag coding region and a variety of regulatory sequences. Such *cis*-acting elements like the rev responsible element (RRE) regulate and improve the nuclear translocation of reverse transcribed DNA as well as the nuclear export and stabilization of vector mRNA and their propagation into viral particles. Additional important improvements in such vectors are the insertion of the central polypurine tract and central termination site element (cPPT/CTS) that facilitates nuclear translocation of the pre-integration complex and the insertion of the post-transcriptional regulatory element (PRE) of the woodchuck hepatitis B virus (WPRE) [150] that enhances the transgene expression through the stabilization of vector mRNA. The second plasmid, the packaging construct, provides the viral *gag*, *pol* and *rev* genes in *trans*, whereas the third plasmid encodes the envelope protein that is mostly either the amphotropic MLV envelope glycoprotein or the vesicular stomatitis virus G-protein (VSV-G) that broaden the types of cells that can be infected.

This split of the genetic information on three different transcriptional units improves the biosafety since it minimizes the probability of the reconstitution of a replication competent retrovirus (RCR). For additional safety aspects all accessory genes (*vpr*, *vif*, *vpu* and *nef*) are deleted in the so-called 2nd generation packaging and vector plasmids without affecting the vector yield or the transduction efficacy of most cell types.

4.3 Genetic modification of hematopoietic stem cells

The hematopoietic stem cell is one of the preferred targets for gene therapy, since this cell has the capacity to self renew and to differentiate into all mature cell lineages of the blood and the immune system. Many of the genetic diseases that affect these systems could be treated by the stable introduction of genes into HSCs with the potential to achieve a long-term therapeutic effect. HSCs

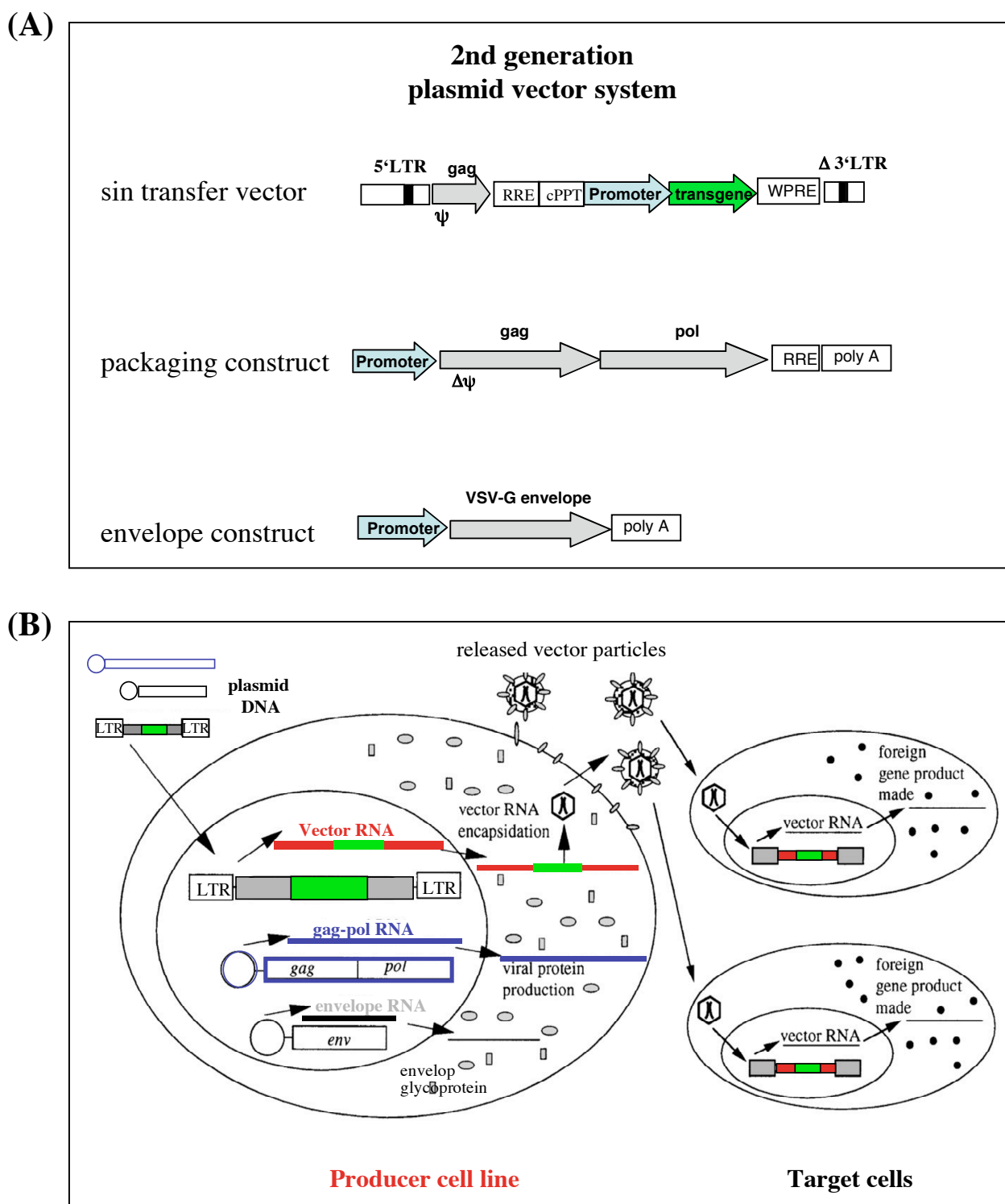


Figure 5-3: Lentiviral vector production in producer cell lines. Schematic drawing of the “2nd generation” three-plasmid lentiviral vector system is shown in (A). All information for the production of viral particles is split into three separate DNA plasmids. The transfer vector carries the gene of interest flanked by the HIV-derived LTRs, the regulatory RRE sequence, a shortened form of the *gag* gene with the encapsidation site (ψ) and the accessory sequences cPPT to achieve genomic integration in the target cells. The packaging construct provides the *gag* and *pol* genes in *trans* but is deleted for the ψ -signal to avoid the encapsidation of mRNA. The third plasmid codes for the amphotropic vesicular stomatitis virus G-protein (VSV-G), that allows the infection of a broad range of target cells. In (B) the process of virus production is illustrated. The plasmids are inserted by Ca-phosphate transfection to a producer cell line that releases high amounts of infective viral particles. Cell culture supernatant is collected and concentrated virus (ultracentrifugation step) is used to transduce target cells with the desired vector construct.

are easy accessible for *ex vivo* manipulation, and in theory a small number of modified cells would be sufficient to repopulate the whole hematopoietic system of a recipient for his entire life.

However, human HSC therapy is still hampered by many obstacles concerning the gene delivery systems used. A stable long-term transgene expression *in vivo* is only achieved when the gene transfer is mediated into the relatively rare population of primitive stem cells. Only these cells would be able to long-term reconstitute the hematopoiesis after transplantation leading to the sustained expression of the transgene in all blood cell lineages. Common viral and non-viral gene delivery systems are inefficient in the transduction of quiescent stem cells, since these types of vectors do require the breakdown of the nuclear membrane at mitotic cell division to enter the nucleus. Genomic integration of DNA from such vectors needs the *ex vivo* growth factor stimulation to induce cell division prior to transduction, that leads to a lineage commitment and differentiation of stem cells and finally results in a weak long-term engraftment of transduced cells in the recipient. Transduction with lentivirus-derived vectors could circumvent these limitations since these vectors integrate into the genome of non-dividing cells. Even though many modifications improved safety and efficacy of gene transfer and elevated the acceptance of lentiviral vectors, only a few clinical trials with lentiviral vectors have been initiated so far [151, 152]. However, recent clinical experiences with gene therapy trials on X-linked SCID have recalled into question the ultimate biosafety of retrovirus-mediated genetic modifications of human cells. Two cases of acute T cell leukemia were reported in patients who had undergone retrovirus-mediated gene therapy of CD34+ selected hematopoietic stem cells to cure inherited X-linked SCID [153, 154]. In these patients' cancerous T cells it was found that the vector had inserted itself into a gene called *LMO-2*, mutations in which are known to be involved in childhood cancers. Such processes of insertional mutagenesis in oncogenic regions are thus the most worried side effects of retroviral mediated gene transfer. On the other hand, no serious adverse effects have been associated to date with the adoptive transfer of gene-modified mature cells [155]. In contrast to HSC, mature cells have a limited life span, they do not pass through multiple rounds of proliferation and differentiation and may thus have a lower risk of malignant transformation following retroviral integration.

III. RESEARCH OBJECTIVES

Natural killer (NK) cells are important effectors of innate immunity mediating the spontaneous killing of virus-infected and malignant cells [156]. The cytotoxic activity of NK cells is regulated by the complex balance between activating and inhibitory receptors. The normal expression of HLA class I molecules, which function as the ligands for inhibitory receptors, protects healthy cells from NK cell lysis by blocking the signalling pathways of activating receptors [1]. In the absence of HLA class I expression, as a consequence of viral infection or tumor transformation, the effects of activating receptors are no longer counterbalanced and target cell lysis is initiated (“missing self recognition”).

Most tumors have evolved strategies to escape the immune surveillance by NK cells. In hematological malignancies such as acute myeloid leukemia (AML), several mechanisms can be responsible for the failure of recognition of malignant cells. First, unlike most solid tumors, leukemic blasts maintain the expression of HLA class I molecules that mediate NK cell inhibition [91, 157]. Second, the malignant cells may display a low or absent expression of the ligands for NK cell triggering receptors [34, 37]. Alternatively, NK cells may fail to eliminate AML blasts due to the defective expression or the down-regulation of activating receptors involved in the recognition of tumor antigens [88].

In this context we hypothesize that the anti-tumor activity of AML-derived NK (AML-NK) cells could be enhanced by shifting the receptor balance towards a status that “overrides” the inhibitory signalling and favours NK cell activation. Hence, the major objective of this work was to characterize the function of AML-NK cells and to manipulate the expression of activating receptors on NK cells in order to confirm the model of a receptor-balanced NK cell regulation.

The goal of the first part of this thesis was to achieve an enhanced cell surface expression of the Natural Cytotoxicity Receptor NKp46 by the genetic modification of NK cells. For this purpose, we used the HIV-derived lentiviral vector system for the gene transfer to NK cell lines, primary peripheral blood NK cells and NK cells generated *in vitro* from hematopoietic progenitor cells. With regard to the potential application of genetically modified NK cells in cellular immunotherapy protocols, we investigated the conditions for the expansion of NK cell populations with the concomitant maintenance of transgene expression.

In the second part of the thesis we focused on the phenotypic and functional characterization of AML- NK cells. The main goal was to define whether abnormalities in NK cell effector functions are responsible for the insufficient recognition and lysis of autologous leukemic blasts. Next we investigated the possibility to expand AML-NK cells *in vitro* and examined the influence of cytokines on the pattern of expression of NK cell receptors. The activity of cytokine activated AML-NK cells was determined *in vitro* by the IFN- γ release and the cytotoxicity against NK cell sensitive target cell lines and autologous leukemic blasts. The anti-leukemic activity of AML-NK cells *in vivo* was determined upon the adoptive transfer to NOD/SCID mice transplanted with human leukemic blasts.

In a third part of this thesis we investigated the conditions that lead to the development of human NK cell precursors and mature NK cells in the murine bone marrow microenvironment.

Suitable models for the *in vivo* analysis of human NK cell effector functions and their development have been lacking. The NOD/SCID transplantation system has not been useful since the lymphoid differentiation in mice repopulated with human progenitors cells is restricted to the B cell lineage whereas T and NK cells are produced at a minimum level or not at all. We administered human cytokines that are known to act during NK cell development *in vitro* to NOD/SCID mice engrafted with human cord blood derived CD34+ progenitors and characterized the NK cell lineage derived from the human graft *in vivo*.

Results of these studies on the characterization of NK cells in AML patients and their genetic modification as well as the establishment of an NOD/SCID *in vivo* system of human NK cell development may contribute to the improvement of immunotherapeutic strategies for the treatment of acute leukemia.

IV. MATERIALS AND METHODS

1. Molecular Biology

1.1 RNA Isolation and cDNA Synthesis

1.1.1 Total RNA isolation and DNaseI treatment

Approximately 5×10^6 - 1×10^7 cells were used for the extraction of total cellular RNA. Cells were washed in PBS and centrifuged at maximum speed (12'000rpm) for 1-2 min. The pellet was resuspended in 1ml of TRIzol Reagent (Gibco, Life Technologies, Inc.), vortexed vigorously for 1-2 min and incubated for 15min at RT. 200 μ l of chloroform was added, the tubes were vortexed again vigorously for 2-3 min and incubated for additional 10-15 min at RT. The cell lysate was centrifuged at maximum speed for 15 min at 4°C. The upper RNA containing (colourless) hydrophilic phase was carefully transferred into a new tube and 500 μ l (1:1 ratio) of isopropanol was added and the tubes shortly vortexed. After an incubation time of 10 min at RT the tubes were centrifuged at maximum speed for 15 min at 4°C. The supernatant was discarded and the RNA pellet washed in 1ml of 70% ethanol and again centrifuged at maximum speed for 15 min at 4°C. The ethanol was completely removed and the pellet air-dried for 5-10 min in the flow of a sterile hood. RNA was dissolved in 50 μ l DEPC-H₂O and stored at -70°C for further use in RT-PCR.

DNaseI treatment was done with 5-8 μ g of total cellular RNA in a total volume of 20 μ l. 2 μ l of 10x buffer and 5-8 units of DNaseI (1U/ μ g RNA) were added to the RNA and the volume adjusted with DEPC-H₂O to final 20 μ l. RNA was incubated for 15min at RT, reaction was stopped by the addition of 25mM EDTA and the enzyme heat-inactivated at 65°C for 10min.

1.1.2 Small scale isolation of polyadenylated mRNA

When only small amounts of cells were available for RNA isolation (e.g. from cord blood differentiation cultures initiated with 2×10^5 transduced CD34+ progenitors) the QuickPrep® Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc.) was used. The mRNA was isolated according to the manufacturers protocol; briefly, microcentrifuge tubes were prepared with 1ml of oligo- (dT)-cellulose suspension and cells were homogenized in 0,4ml of "extraction buffer" (buffered aqueous solution containing guanidinium thiocyanate). 0,8ml of "elution buffer" (10mM

Tris-HCl, 1mM EDTA) was added, the cleared homogenate (centrifuged at maximum speed for 1min) was mixed with the oligo- (dT)-cellulose and gently mixed for 3min. Tubes were centrifuged at maximum speed for 1min, the supernatant discarded and the cellulose-pellet washed 5 times with 1ml of “high-salt” buffer (10mM Tris-HCl, 1mM EDTA, 0,5M NaCl) and 2 times with 1ml of “low-salt buffer” (10mM Tris-HCl, 1mM EDTA, 0,1M NaCl). The pellet was transferred to a MicroSpin column and washed again 3 times with “low-salt buffer”. The mRNA was finally eluted with preheated (65°C) “elution buffer” (10mM Tris-HCl, 1mM EDTA).

1.1.3 RT-PCR

Reverse transcription (RT) was performed in a total volume of 20 μ l. 1-2 μ g RNA was used in a maximum volume of 8 μ l, 2 μ l 10x buffer (; Perkin Elmer), 0,75 μ l RNase inhibitor (40U/ μ l; Promega), 1 μ l of Random Hexamers (...; 50 μ M), 0,25 μ l RTase (SuperScript™II RT, 200U/ μ l; GibcoBRL) and 4x2 μ l of dNTPs (10mM each; Promega) were added. The RT reaction was incubated at 21°C for 12min, followed by 45min at 42°C and stopped at 95°C for 5min. 2 μ l of this RT-mix was used in the subsequent PCR which was performed in a total volume of 20 μ l. 2 μ l of 10x buffer (; Perkin Elmer), 2 μ l of a dNTP mix (2mM each), 0,5 μ l Taq polymerase (5U/ μ l; Promega) and 1 μ l of each primer (2-10 μ M) were added and the volume adjusted with DEPC-H₂O to final 20 μ l. Standard PCR was performed with 5min preheating at 94°C and 30 cycles of 94°C/1min, 60°C/1min and 72°C/1min. The reaction was finished at 72°C for 7min.

1.2 Isolation of genomic DNA

Genomic DNA was isolated using the DNazol® Reagent (GibcoBRL, Life Technologies) according to the manufacturers protocol. Briefly, up to 1x10⁷ cells were washed in PBS and the pellet was resuspended in 1ml of DNazol. Cells were lysed by gently vortexing and inverting the tube. The homogenate was centrifuged at maximum speed for 10min and the viscous supernatant was transferred to a fresh tube for ethanol precipitation. 0,5ml of ethanol (100%) was added leading to a cloudy DNA precipitate that was wrapped around a pipette tip and attached to the tube wall. Ethanol was discarded, the remaining cell lysate was aspirated and the genomic DNA was washed 2 times with 95% ethanol. Alcohol was completely removed and the DNA air-dried for 15min. DNA was dissolved in 200 μ l DEPC-H₂O.

Small-scale isolation of genomic DNA was done by a cell-lysis procedure using a two detergent “Whole cell lysis buffer”. For 100ml of buffer 5ml of KCL (1M), 1ml of TrisHCl (pH 8,3; 1M), 150 μ l of MgCl₂, 10mg gelatine and 450 μ l Nonidet p40 and 450 μ l Tween 20 were added to 93ml H₂O. The cells (< 10000) were resuspended in 20 μ l of the lysis buffer and 3 μ l of proteinase K (conc.:) was added. The lysates were incubated for 1hour at 56°C and the enzyme was inactivated at 100°C for 10min. In standard PCR reactions 1 μ l of the cell lysate was used.

1.3 Western Blotting

1.3.1 Preparation of total cell lysates

Cell lysates were prepared using a “triple-detergent” extraction buffer (EB; for total 10ml: 7.5ml milli-Q water; 0.5ml Tris [1M, pH8.0; final:50 mM]; 0.3ml NaCl [5 M; final 150 mM]; 0.1ml EDTA [0.5 M, final 5 mM]; 1.0 ml DOX [5 %, final 0.5 %]; 100 μ l SDS [10 %, final 0.1 %]; 400 μ l NP-40 [25 %, final 1.0 %]; 20 μ l Aprotinin [1mg/ml, final 2ng/ml]; 50 μ l Leupeptin [1mg/ml, final 5ng/ml]). Cells were washed in ice cold PBS, centrifuged and all liquid was carefully removed. Per 1-2,5 x10⁶ cells in suspension 100 μ l EB was added, vortexed and incubated for 10 min on ice. The lysate was centrifuged at 10'000 rpm and the supernatant was immediately put on dry ice or stored at -70°C (in 25 μ l aliquots).

1.3.2 SDS-PAGE separation and membrane blotting

The gels were prepared according to Laemmli (Laemmli 1970) with some modifications. The resolving gel contained 12% Acrylamide/Bis, 375 mM Tris, pH 8.8, and 0.1 % SDS, and was polymerised with 50-75 μ l 10 % APS and 5 μ l TEMED per 10 ml gel. The stacking gel contained 4% Acrylamide/Bis, 125 mM Tris, pH 6.8, and 0.1 % SDS, and was polymerised with 50 μ l 10 % APS and 10 μ l TEMED per 10 ml gel. The 4x sample buffer contained 10% glycerol, 2 % SDS, 0.5 % bromophenol blue, 60 mM Tris, pH 6.8, and 5% β -mercaptoethanol (added just before use). The samples were denatured for 4min at 95°C. The separation buffer contained 25 mM Tris-base, 14.4 % glycine and 1 % SDS. The gels were run for 15 min at 100V followed by 1hr at 160-200V. Before the protein transfer, the gels were washed once in running buffer and once in transfer buffer (25 mM Tris-base, 14.4 % glycine). The proteins were blotted to Immun-Blot PVDF Membrane (Biorad) using the Mini Trans-Blot Electrophoretic Transfer Cell (Biorad) with 100V for 1hr. The blots were blocked over night in blocking solution, BS (1g BSA and 5g dry milk in 100ml PBS,

0.05% Tween20). The membrane was washed 3 times in PBS-Tween and incubated with the primary antibody for 1-3 hrs at RT (purified mAbs: 2,5 μ l/ml; polyclonal Abs: 1 in 2000-5000 dilution) in BS. The primary antibody was removed by washing 3 times in PBS-Tween before the secondary antibody was incubated for 1 h at RT in PBS-0,1 % BSA (HRP-conjugates: 1 in 4000 dilution; SAV-HRP- conjugates: 1 in 5000 dilution). After washing three times with PBS-Tween, the protein bands were revealed with the Super Signal Reagent® (Amersham Pharmacia) and exposed to Hyperfilm ECL (Amersham Pharmacia).

2. Cell Biology

2.1 Flow Cytometry

Three-colour FACScalibur® analysis (Becton Dickinson) was used to phenotypically characterize freshly isolated NK cells, activated and expanded polyclonal NK cell lines or patient-derived PB samples. Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)- and peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies (mAbs) against human CD3, CD16, CD33, CD34, CD45, CD56, CD69, CD94 and CD161 or isotype control antibodies (all from BD PharMingen, San Jose, CA). Staining with the unlabeled mAbs anti-HLA-A,B,C (clone G46-2.6; BD PharMingen), anti-MHC class II (clone L243; hybridoma supernatant) anti-CD158a, -CD158b, -NKG-1 (clones EB6, GL183, DX9; BD PharMingen), anti-NKp46 (clone 9E2; hybridoma supernatant provided by Marco Collona, Basel Institute for Immunology) and anti-NKG2D (clone M585, IgG1; 10 μ g/ml) or anti-ULBP-1 (clone M295, IgG1; 10 μ g/ml), -ULBP-2 (clone M311, IgG1; 20 μ g/ml) and -ULBP-3 (clone M250, IgG1; 10 μ g/ml) (all provided by David Cosman, Amgen Washington Inc., Seattle) was revealed with secondary PE- or FITC-conjugated goat anti-mouse (gt α ms) antibodies (Southern Biotechnology Associates, Birmingham, AL). Incubation with normal mouse serum (1:10 diluted; Jackson ImmunoResearch, West Grove, PA) allowed the subsequent staining with directly labeled mAbs. MICA/B expression was analysed using an anti-MICA/B hybridoma supernatant (rat anti-human mAb; provided by Marco Collona, Basel Institute for Immunology) combined with a FITC-conjugated gt α rat secondary IgG (1:100 diluted; Jackson ImmunoResearch, West Grove, PA). The putative ligands for the natural cytotoxicity receptors (NCR) were measured using the solubilized(s) dimeric complexes of the recombinant BirA1.4-tagged receptor molecules sNKp30, sNKp44 and

sNKp46 as staining reagent (anti-BirA mAb [clone BirA1.4] crosslinked; 5-10 μ g/ml). Binding was revealed with secondary FITC-conjugated α gt α ms antibodies (Southern Biotechnology Associates, Birmingham, AL). All stainings were incubated on ice for 20 minutes in FACS buffer containing PBS, 2% fetal calf serum (FCS, Invitrogen, Carlsbad, CA) and 0.02% NaN₃ (Fluka, Buchs, Switzerland). Propidium Iodide (Sigma) was used to exclude dead cells from analysis. Staining of patient samples was performed in 100 μ l aliquots of fresh heparinized PB followed by red blood cell lysis (FACS lysis buffer; BD PharMingen, San Jose, CA). FACS data were analysed using the CellQuestPro[®] software (Becton Dickinson).

2.2 Preparation of cord blood CD34⁺ cells

Cord blood (CB) was kindly provided by the Department of Obstetrics and Gynecology, University Hospital Basel and the Department of Obstetrics and Gynecology, Kantonsspital Bruderholz, with informed consent of the mothers. The Ethical Committee of the University Hospital Basel approved all investigations.

CB was harvested aseptically by umbilical vein puncture right after delivery and between 10 to 50 ml was collected into heparin-containing bags. The delay between collection and sample processing did not exceed 24 hours. CB mononuclear cells were separated by Histopaque (Sigma, St Louis, MO) density-gradient centrifugation and the subsequent red blood cell lysis (lysis buffer; KBS, Kantonsspital Basel). The cells were cryopreserved in liquid nitrogen until use.

Frozen samples were pooled after thawing and left overnight in IMDM (20% FCS) containing 100U DNase (Sigma) per 1x10⁷ cells/ml at 4°C. Cells were washed 2 times in PBS and resuspended in MACS buffer (PBS, containing 2mM EDTA and 0,5% BSA). CD34⁺ cells were isolated with MACS (magnetic cell sorting) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the CD34⁺ cell population was determined by FACS analysis and ranged from 85 to 95%.

2.3 NK cell *in vitro* differentiation from CB derived CD34⁺ Cells

Cord blood derived CD34⁺ progenitor cells were seeded at 1 to 2 x10⁶ cells /mL in 24-well plates in Iscove modified Dulbecco medium (IMDM) containing 5% FCS. The NK cell differentiation

medium was supplemented with 5% human AB⁺ serum (Blutspendezentrum Basel), 380 ug/mL iron-saturated human transferrin, 1% bovine serum albumin (BSA) and with IL-15, FL, and SCF (each at 100 ng/mL). After 1 week, cells were transferred to 6-well plates, and half the medium was replaced once a week for another 2 to 5 weeks. The development of CD56⁺ NK cells was determined weekly by FACS analysis. NK cell lines were expanded by restimulation as described below (see 2.5.2).

2.4 Lentiviral Transduction

2.4.1 Preparation of concentrated lentivirus

Lentiviral particles pseudotyped with the VSV-G envelope protein were produced by the transient co-transfection of the human embryonic kidney cell line 293T with three different plasmids. The packaging plasmid pCMVΔR8.91, the VSV-G encoding plasmid pMD.G (or pMD2.G) and a transfer vector containing the gene of interest were used to transfect 293T cells by Ca-phosphate precipitation. All plasmids were kindly provided by D. Trono and P. Salmon, University of Geneva, Switzerland. The transfer vector constructs are derivatives either from the single-gene vector pLox/EW-GFP or the bicistronic constructs pWP-IRES-GFP or pWP-IRES-mCD8 and were generated as described above (see 1.2). The 293T cells were split four and one day prior to the transfection to obtain mono-layers of actively proliferating cells which were optimally covering approximately 2/3 of the surface area of 10cm culture Petri-dishes. 5μg of pMD.G, 15μg of pCMVΔR8.91 and 2μg of the transfer vector plasmid were mixed and the volume was adjusted to 250μl with ultra pure H₂O and 250μl of Ca-phosphate buffer was added. The mixture was added slowly and drop-wise to 500μl of 2x concentrated HBSS buffer in a 15ml Falcon polystyrene tube while permanently vortexing the tube. The mixture was incubated for 20 to 25 minutes at RT to allow the formation of Ca-phosphate-DNA crystals. The precipitated DNA was spread on the 293T cell layers and the plates were incubated at 37°C. After eight hours the transfection medium (DMEM, 10% FCS) was completely replaced by fresh medium and the cells were incubated for additional 16 to 20 hours. At that time, if GFP encoding transfer vector constructs were used, the transfection efficiency of the 293T cells was checked by fluorescence microscopy. The viral supernatant was collected and replaced by fresh medium for an additional incubation period of 16 to 20 hours. The supernatant was filtered through 0,45μm syringe-filters (Nalgene) pooled to 40ml “Ultra-clear open-top” tubes (Beckman-Coulter, Nyon) and centrifuged at 100000 rpm for 90

minutes at 4°C in an ultra-centrifuge. The supernatant was discarded and the (non-visible) virus pellet was vigorously vortexed in the remaining volume of 400 to 500µl. Tubes were covered with parafilm and stored at 4°C overnight to let the viral particles readily disaggregate and dissolve. 400-600µl of the virus preparation were transferred to one spinex filter-tube and filtered by the centrifugation at 8000 rpm for 2-3 minutes. The filter cartridge was removed and the tubes were additionally centrifuged for 60 minutes with 13000 rpm at 4°C. The viral particles usually formed at that time a visible pellet and all of the supernatant was carefully removed. The pellet was resuspended in approx. 100µl medium and aliquots of 50µl were stored at -70°C until use in transduction experiments.

2.4.2 Titration of concentrated lentivirus

The titers of the concentrated LV preparations of GFP-encoding virus were determined on HeLa cells. In 6 well plates HeLa cells were seeded with 1×10^5 cells per well (day -1) and transduced with serial dilutions of the concentrated virus (day 0). Concentrated virus in serial 1/3 dilutions starting from 1/300 to 1/10'000 was used in a total infection volume of 500µl per well with 5µg/ml protamine sulfate (Sigma). After the HeLa cells were incubated for 4 hours the culture volume was completed to 3-4 ml. Transduction efficiency was determined by FACS analysis on day 3 post infection. For the FACS analysis cells were trypsinized, washed two times in PBS and fixed in 2% paraformaldehyde (Sigma) for ten minutes at RT. The titers were given as transducing units per ml (TU/ml) and were determined for each virus-dilution according to the following formula:

$[\% \text{ (GFP}^+ \text{ cells)} \times (\text{virus dilution factor}) \times 2(\text{infection was done in } 0,5 \text{ ml})] \text{ TU / ml.}$

The final viral titers were calculated as the average of all titers determined for each dilution. On average the lentiviral titers usually obtained were 5×10^7 TU/ml (range: $0,5-40 \times 10^7$ TU/ml).

2.4.3 Transduction of CB-derived CD34⁺ progenitors

Before the lentiviral transduction of purified CD34+ cord blood derived hematopoietic progenitors (see 2.2) the cells were prestimulated for 48 hours to induce cell cycling. The 2x concentrated prestimulation-medium consisted of IMDM 20% FCS, supplemented with 380 µg/ml iron saturated human transferrin, 1% bovine serum albumin (BSA) and the cytokines in the following concentrations: IL-3 20ng/ml; IL-6 20ng/ml; SCF 100ng/ml; FL 100ng/ml. As an alternative, if the

differentiation into NK cells was the goal, the progenitors were prestimulated for 48 hours in NK cell differentiation medium (see 2.3).

$2-3 \times 10^5$ CD34⁺ progenitors were resuspended in 30-50 μ l of the corresponding medium and transferred to 96 well round bottom plates. Concentrated lentivirus preparation was added to an MOI of 20-30 in a total volume of 50-80 μ l depending on the viral titer. Protamine sulfate (PS) was added as a 10x concentrated stock-solution to final 5 μ g/ml. The progenitors were incubated for 4 hours, resuspended in fresh medium and transferred to 96 well flat bottom plates in a total volume of 200 μ l. On day 2 to 4 post infection the transduction efficiency was determined by FACS analysis.

2.4.4 Transduction of NK cell lines and primary NK cells

As for hematopoietic progenitors the transduction of NK cell lines and primary NK cells was done in 96 well round bottom plates in a total volume of 50-80 μ l in the presence of PS (final 5 μ g/ml). Per transduction $2-3 \times 10^5$ cells were infected with lentivirus at an MOI of 20-30. Cells were incubated for 4 hours, resuspended in fresh medium, transferred to 96 well flat bottom plates and routinely expanded and split in regular time intervals (after 3-5 days of culture). FACS analysis of the transduction efficiency was done on day 4 post infection.

Primary NK cells were separated from peripheral blood mononuclear cells (PBMNC) by an immunomagnetic negative selection procedure (see 2.5.1) and infected upon the stimulation with 100 U/ml IL-2 for 2 days. After the 4-hour infection period NK cells were either immediately restimulated with PHA, IL-2 and irradiated feeders as described below (see 2.6.2) or they were cultured for additional 3 days in IL-2 containing medium prior to restimulation.

2.5 NK cell Isolation from Peripheral Blood Samples

2.5.1 MACS separation

Human mononuclear cells (MNC) obtained from patients after informed consent were isolated from total blood samples by Ficoll-Histopaque (Sigma, St. Louis, MO) density-gradient centrifugation and cryopreserved in liquid nitrogen until use. NK cells were isolated from 14 patients with AML, 13 newly diagnosed patients and 1 in relapse. The diagnosis and division into the AML subtypes of M1-M7 was based on morphologic, cytogenetic and immuno-phenotypic criteria.

All patient-NK cells (AML-NKs) were purified starting from thawed MNCs by immunomagnetic negative selection using the MACS system („NK cell isolation kit“, Milteny Biotech, Bergisch Gladbach, Germany) according to the manufacture’s instructions. CD3, CD14, CD19, CD36 and IgE positive MNCs were retained in a LD depletion column whereas untouched CD56⁺CD3⁻ NK cells were collected in the flow-through. The purity of NK cell preparations from healthy donors was at least 95% and less than 0.5% were positive for CD3. NK cell separation from AML patient samples resulted in variable levels of purity (2-10%) due to the high proportions of leukemic blasts (characterized by the CD45^{dim} phenotype). The combination of negative selection and a subsequent positive selection for the NK cell marker CD16 or CD56 resulted in an enrichment of NK cells to a purity of 42-70%. These mixtures of MACS-enriched NK cells and residual AML blasts were expanded in restimulation cultures as described below.

2.5.2 In vitro cultivation: PHA restimulation

Purified NK cells were cultured on irradiated (30Gy) allogeneic peripheral blood MNCs in „NK cell medium“ (IMDM, 5% human AB⁺ serum (Blutspendezentrum Basel) containing 100U/ml IL-2 (Novartis, Basel, Switzerland) and phytohemagglutinin (2ug/ml; H16, Murex Biotech, Datford, England). The medium was supplemented with nonessential aminoacids, sodium pyruvate, L-glutamine and penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). Preparations containing 2x10⁴ to 3x10⁵ CD56⁺CD3⁻ NK cells were seeded onto 2x10⁶ irradiated feeders in a total volume of 2ml in 24 well plates. After 8 to 10 days cells were expanded to 6 well plates and between day 14 and 28 activated NK cell lines were phenotypically and functionally analysed. Repeated stimulations were done after 3 to 4 weeks of culture.

NK cell expansion was either performed in the general restimulation-mix alone (irradiated feeders, PHA and IL-2) or together with different combinations of the cytokines IL-12 (Roche, Nutley, NJ), IL-15 and IL-21 (both from Immunex, Seattle, WA). The final cytokine concentrations were 1ng/ml for IL-12, 10ng/ml for IL-15 and 100ng/ml for IL-21, respectively.

2.6 Measurement of Intracellular Ca²⁺-Mobilization

Cells were washed 2 times in PBS and adjusted to 5-10x10⁶ cells/ml in “flux-medium”(RPMI without phenol red; 5% FCS). Indo-1AM (Indo-1-penta-acetoxy-methylester; 10mg/ml in DMSO) was added to a final concentration of 2ug/ml and incubated for 30 minutes at 37°C (during loading

cellular esterases cleave the AM moiety of the membrane-permeant Indo-1AM resulting in the trapping of the highly charged Indo-1 in the cells). Cells were washed, gently pelleted (centrifuged below 1100 rpm) and resuspended in flux-medium to a concentration of 1×10^7 cells/ml. Indo-1 loaded cells were stored at RT in the dark until analysis in a FACS Vantage SE (Becton Dickinson). Each measurement of intracellular calcium release was performed on 5×10^5 cells in total $500 \mu\text{l}$ ($450 \mu\text{l}$ to 37°C pre-warmed flux-medium + $50 \mu\text{l}$ Indo-1 loaded cell suspension). Purified azid-free primary mAb at a concentration of $0,5 \mu\text{g/ml}$ (anti CD3; clone TR66) or $25 \mu\text{l}$ of undiluted hybridoma supernatant (anti NKp46; clone 9E2) was added. To obtain the baseline of intracellular calcium, cells were acquired in the FACS Vantage SE for approximately 2 minutes. Then the secondary cross-linking mAb was added (goat anti mouse; S418) and acquisition was continued for additional 10 minutes. For a positive control the calcium ionophor ionomycin (1mg/ml in DMSO; Sigma) was added to one sample in a final concentration of $2 \mu\text{g/ml}$. The Indo-1 ratio of $395 \text{nm}/500 \text{nm}$ fluorescence emission (FL5/FL4) was calculated and plotted versus time.

2.7 Measurement of IFN- γ production in activated NK cells

2.7.1 NK cell activation by target co-cultures

Co-cultures of PHA restimulated NK cells and target cells were performed in 96 well flat bottom plates at an effector to target cell ratio of 2:1 in a total volume of $200 \mu\text{l}$. NK cells were used at day 15 to 25 post restimulation and seeded at 1×10^5 cells per well in a volume of $100 \mu\text{l}$. Target cells were washed in PBS, resuspended in complete NK cell medium (see 2.6.2) and added to the NK cells at 5×10^4 cells per well in a volume of $100 \mu\text{l}$. As a positive control for IFN- γ release NK cells were stimulated with final 20ng/ml PMA (Sigma) and final $1 \mu\text{M}$ Ca^{2+} -ionophor (Calbiochem). To determine the background IFN- γ release, NK cells were cultured without any targets. All co-cultures and controls were performed in triplicates.

To analyse IFN- γ release upon the antibody-mediated “re-directed” NK cell-target interaction (with murine P815 targets), NK cells were either pre-incubated for 30 min with anti-NKp46 before the target cells were added or anti-NKp46 was directly added to the co-culture (clone 9E2, mAb-SN; $10 \mu\text{l}/\text{well}$). Culture supernatants for the measurement of IFN- γ release were taken after 36 hrs of co-culture and stored at -20°C until use in ELISA. If NK cells were analysed for intracellular IFN- γ

(see 2.7.4) Brefeldin A ($5\mu\text{g/ml}$) was added to the co-culture for the last 4 hrs of the 36 hrs incubation time.

2.7.2 NK cell activation by antibody mediated receptor cross-link

Receptor cross-link was performed in 96 well flat bottom plates coated either with receptor-specific mAbs or with mAbs in solution. For the coating purified mAbs were used at a concentration of final $20\mu\text{g/ml}$ in PBS in a volume of $20\mu\text{l}$ per well whereas hybridoma supernatants were used undiluted at $50\mu\text{l}$ per well. Plates were incubated over night at 4°C . Coated plates were washed once with PBS and cells were added at a concentration of 5×10^4 cells per well in total $200\mu\text{l}$. If the mAbs were used in solution, cells were pre-incubated with the corresponding mAbs for 30 minutes at RT, washed and plated. Secondary cross-linking mAbs were added at a final concentration of $20\mu\text{g/ml}$. Monoclonal antibodies used as cross-linking reagents were anti-CD3 (TR66) anti-CD28, anti-NKp46 hybridoma supernatant (clone 9E2; Marco Collona, Basel Institute of Immunology, Basel) and goat anti-mouse IgG1 PE labelled mAb (Southern Biotechnology), all as NaN_3 -free preparations. Culture supernatants for the measurement of IFN- γ release were taken 36 hrs after receptor cross-link and stored at -20°C until use in ELISA (see 2.7.3). For the FACS analysis of CD69 surface expression or intracellular IFN- γ the cells were harvested after 36 hrs. Brefeldin A was added for the last 4 hrs if intracellular IFN- γ was measured (see 2.7.4).

2.7.3 ELISA detection of released IFN- γ

Culture supernatants were stored at 4°C until use in the IFN- γ ELISA. 96-well ELISA plates were coated at 4°C over night with anti huINF γ (clone 43-11, mIgG1; kindly provided by Ch. Heusser, Novartis, Basel) at a concentration of $5\mu\text{g/ml}$ in a volume of $100\mu\text{l}$ per well. Plates were washed and blocked with RIA-buffer for one hour at room temperature. Each washing step was done 4 times with PBS and all incubation were at RT. Plates were washed and culture supernatants or the IFN- γ standard were added. Serial 1/3 dilutions of the IFN- γ standard (recombinant huINF- γ BD PharMingen San Jose, CA; cat. no. 554616) were made starting with a final concentration of 30ng/ml in RIA-buffer and added in a volume of $100\mu\text{l}$ per well. After an incubation time of 3 hrs at RT plates were washed and the biotinylated anti huINF- γ revealing mAb was added (clone 45-15, mIgG1-biotin; Ch. Heusser, Novartis, Basel). The biotinylated mAb was 1/10000 diluted and added

in a volume of 100 μ l per well. Plates were incubated for 2 hrs, washed and 100 μ l per well streptavidine-alkaline phosphatase (Sigma) was added at a 1/10000 dilution in RIA-buffer and incubated for 1 hour. After an additional washing step the substrate, p-nitrophenyl phosphate (Sigma) was added in a volume of 150 μ l per well and incubated for 30 minutes. The enzymatic reaction was stopped by the addition of 1N NaOH (50 μ l per well) and the plates were analysed for the absorbance at 405 nm in an ELISA reader.

2.7.4 Intracellular FACS staining of IFN- γ production

NK cells were used on day 15 to 25 post PHA-expansion and 1-1.5 $\times 10^6$ cells/ml were incubated for 36 hrs in 96 well plates either with 10U/ml IL-12 and 100ng/ml IL-18 (PeproTech, Rocky Hill, NY) or with 100U/ml IL-2 only. Brefeldin A (Sigma) was added at 5 μ g/ml for the last 4 hours of culture. Cells were fixed in 2% paraformaldehyde (PFA) for 15 minutes at RT, washed 3 times in FACS-buffer and 2 times in FACS buffer containing 0.1% saponin to permeabilize the cells. Anti-IFN- γ FITC-conjugated and isotype control mAbs (BD PharMingen, diluted in permeabilization buffer) were added for 30 minutes at RT. Cells were washed twice in permeabilization buffer and 3 times in FACS buffer and analysed with FACScalibur. Staining for cell surface markers were done before PFA fixation and intracellular IFN- γ staining.

2.8 Up-regulation of NKG2D ligands on primary AML blasts and the HL60 cell line

Primary AML blasts were seeded at 6 x 10⁵ per mL in 24-well plates containing 2 mL Dulbecco's modified eagle medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/mL streptomycin, 10% fetal calf serum (FCS; all from Invitrogen, Carlsbad, CA). Cells were cultured with growth factors (flt3 ligand, (FL) at 100 ng/mL, stem cell factor (SCF) at 100 ng/mL and granulocyte-macrophage colony stimulating factor (GM-CSF) at 20 ng/mL; all kind gifts of Amgen Inc, Thousand Oaks, CA), and IFN- γ at 100 U/mL (PeproTech, City, UK).

The AML derived cell line HL60 was cultured with Bryostatin-1 (LKT laboratories, St. Paul, USA), at a final concentration of 10⁻⁸ M for 2 days. NKG2D ligand up-regulation was determined by FACS analysis after 1, 2 and 4 days of culture.

2.9 Cytotoxicity Assays

2.9.1 ⁵¹Chromium release killing assay

Cytotoxicity of activated AML-NK and control-NK cells was determined at day 14 to 28 of expansion culture against the NK sensitive target cell line K562 and the primary autologous AML-blasts in a 4-hour ⁵¹chromium-release assay. Cryopreserved patient-derived MNC (Ficoll density-gradient purified) were thawed and maintained 1 to 2 days in IMDM supplemented with 5% FCS, nonessential aminoacids (1:100), 1mM sodium pyruvate, 2mM L-glutamine and 100µg/ml each penicillin/streptomycin (all Gibco Life Technologies, Gaithersburg, MD, USA) until blasts were prepared for the ⁵¹chromium release assays. The MNCs were directly used for the ⁵¹Cr-loading when the percentage of leukemic blasts (CD45^{dim} phenotype) was above 85% of total. Alternatively, MNCs were enriched for blasts by the selection of CD34⁺ or CD33⁺ cells using MACS microbeads (Milteny Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For the target cell loading 2x10⁶ cells were incubated at 37°C for 2 hours with 250µCurie of Na ⁵¹CrO₄ (Amersham, Little Chalfont, UK) in a total volume of 200-300µl. The effector to target ratio ranged from 20:1 to 0.6:1 for the AML blasts and from 10:1 to 0.3 for the K562 erythroleukemia target cell line. All experimental wells were set up in triplicates and contained 3x10³ target cells in a final volume of 200µl. Plates were incubated at 37°C for 4 hours. To determine maximum ⁵¹Cr-loading, a triplicate of target cells ("target maximal release") was lysed in final 0.1% Triton X-100 (20µl/well of a 10x solution; Sigma). 30µl of culture supernatant of each well was transferred to Luma® scintillation-plates (Perkin Elmer) and analysed in a gamma counter. Results are expressed as percentages of specific ⁵¹Cr-release and calculated as follows: [(cpm experimental release – cpm spontaneous release) / (cpm target maximal release – cpm spontaneous release)] x 100.

To block the inhibitory KIR-HLA interaction and to induce killing of the autologous AML target cells anti-HLA class I monoclonal antibodies were used (W6-32; IgG2a, ATCC). Blocking was achieved by the preincubation of target cells for 15 minutes prior to the addition of the effectors (10µg/ml during the 4-hrs incubation). Anti-HLA class II mAb was used as a negative control (10µg/ml; clone L423, IgG1, ATCC).

2.9.2 Redirected killing of murine P815 cell line

The murine Fc γ RII/III⁺ mastocytoma cell line P815 was used in reverse cytotoxicity assays (redirected killing). Reverse cytotoxicity is mediated upon stimulation of CD16 or other activatory/cytotoxicity receptors on NK cells with NK cell receptor-specific mAbs. The Fc part of these mAbs binds to Fc γ RII (mCD32) on the mastocytoma cells and thereby elicits the lysis of the otherwise NK cell-resistant targets. P815 target cells were labelled with 250 μ Ci of Na ⁵¹Chromat at 37°C for 1 hour, washed and resuspended in complete “killing medium” (as described in 2.9.1). Purified and NaN₃-free NK cell receptor-specific mAbs were added to the targets at a 2x final concentration, cells were incubated for 15 minutes at RT and added in a volume of 100 μ l to the prepared effectors to a final volume of 200 μ l per well. The final concentration of the mAbs anti CD16, anti CD56 (both BD PharMingen), anti NKG2D (clone M585) was 1 μ g/ml. In the case of the anti NKp46 hybridoma supernatant (clone 9E2) targets were pre-incubated with 10 μ l SN per 3x10³ cells.

The redirected killing was further performed as described for the standard ⁵¹chromium-release assay (see 2.9.1).

2.10 Transplantation of human leukemia to NOD/SCID mice and NK cell transfer

NOD/LtSz-scid/scid (NOD/SCID) mice (The Jackson Laboratory, Bar Harbor, ME) were bred and maintained under specific pathogen-free conditions in the animal facility of the Research Department, University Hospital Basel. Mice were kept on acidified drinking water supplemented with Bactrim (32/160 mg/L; Roche Pharma AG, Reinach, Switzerland) for the duration of the experiments.

K562 erythroleukemia cells were washed and resuspended in PBS and 1x10⁷ cells in a total volume of 100 μ l were injected subcutaneously into the dorsal lateral thorax of NOD/SCID mice. 3-5x10⁶ *in vitro* activated and expanded AML- and control-NK cells on day 14 to 21 of restimulation culture were resuspended in 150 μ l PBS and injected intravenously on day 1 post tumor inoculation. Tumor growth was monitored weekly by determining the tumor surface area as follows: [(short diameter/2 x long diameter/2) x π]. Unpaired student t test was used to confirm statistical significance between the different transplantation groups of mice.

PBMNCs from AML patients containing 1×10^7 blasts (only patient samples with a content of more than 75% blasts were used) were resuspended in PBS and were injected intravenously in a total volume of 100 μ l into the tail veins of 7-10 week old NOD/SCID mice sub-lethally irradiated with 375 cGy (^{60}Co source; 2cGy/min). Engraftment of the human leukemia was monitored on week 4, 8, 10 and 12 post transplantation by the FACS analysis of peripheral blood samples as described in section 2.1. In mice repopulated with AML blasts as indicated by $> 0,5\%$ CD45+ cells in the PB, bone marrow samples were aspirated from one femur by puncture through the knee joint [158] under the intraperitoneal anaesthesia using Ketalar (150mg/kg; Parke-Davies) and Xylasol (30mg/kg; Gräub AG, Bern). BM cells were collected in FACS buffer and phenotypically analysed by flow cytometry. If engraftment of AML-blasts was confirmed in BM aspirates, *in vitro* activated and expanded autologous AML-NK cells on day 14 to 21 of restimulation culture were resuspended in 200 μ l PBS and injected intravenously on week 8–12 post transplantation. Transplanted human NK cells were supported by the i.p. administration of human rhIL-2 (Novartis, Basel, Switzerland) and rhIL-15 (Immunex, Seattle, WA). 10 μ g of each cytokine in 100 μ l PBS was given in three doses, along with the injection of NK cells and 24 and 48 hours later. Mice were sacrificed on day 7 post NK cell transfer and the content of AML blasts in the BM, spleen and PB was determined by FACS analysis.

V. RESULTS

(A) GENETIC MODIFICATION WITH LENTIVIRAL VECTORS

1. Cloning of Transfer Vector Constructs

The lentiviral transfer vector constructs used in this study are based on the vector plasmids pLox/EW-GFP Δ Sall (single gene vectors) and pWP-IRES-GFP (bicistronic vectors) that were kindly provided by Didier Trono, Department of Genetics and Microbiology, CMU, Geneva. Both transfer vectors belong to the self-inactivating (SIN) lentiviral vectors, which lose the transcriptional capacity of the viral long terminal repeat (LTR) once integrated in the genome of the target cells. This SIN activity is due to a 400bp deletion in the U3 region of the 3'LTR (Δ LTR; Gene Bank accession no.: AF 237862). Transgene expression is either regulated by the elongation factor 1-alpha (EF1-alpha) promoter (in pLox-GFP vectors) or its shorter version, the intron-less EF1-alpha ("EF1-alpha short"; in pWP-IRES-GFP vectors). All vectors contain the post-transcriptional regulatory element (WPRE) of woodchuck hepatitis virus that allows an enhanced transgene expression (Zufferey R, J. of Vir., 1999). The bicistronic vectors additionally contain the central polypurine tract (cPPT), a cis-acting element that improves the efficiency of gene transfer to the target cell nucleus.

1.1 The "single-gene" transfer vectors

The single gene transfer vector for the cDNA of the natural cytotoxicity receptor NKp46 was constructed based on the green fluorescence protein (GFP) encoding lentiviral vector plasmid pLox/EW-GFP Δ Sall. The FLAG-tagged NKp46^{FLAG} cDNA was PCR-amplified from a pFLAG-CMV1 expression vector containing the cDNA of NKp46 in frame to the sequences of an N-terminal FLAG tag and the preprotrypsin leader peptide (a gift of Marco Colonna; Basel Institute of Immunology, Basel, Switzerland). A 935 bp fragment flanked by a 5' BamHI and a 3' ClaI restriction site was amplified using primers carrying overhangs to generate the corresponding sites (primer: 5'BamFLAG sense; 3'p46Cla antisense; see "Materials and methods"). The amplified fragment was BamHI-ClaI sub-cloned in the pBluescript[®] II KS expression vector (Figure 1-1 a). In a second step the GFP encoding cDNA in the pLox/EW-GFP Δ Sall vector was exchanged by the 940 bp BamHI-SalI NKp46^{FLAG} fragment excised from the pBluescript plasmid (Figure 1-1 b). The correct sequence of the NKp46 cDNA insert in the pBluescript plasmid as well as in the final transfer vector construct was confirmed by automated sequencing.

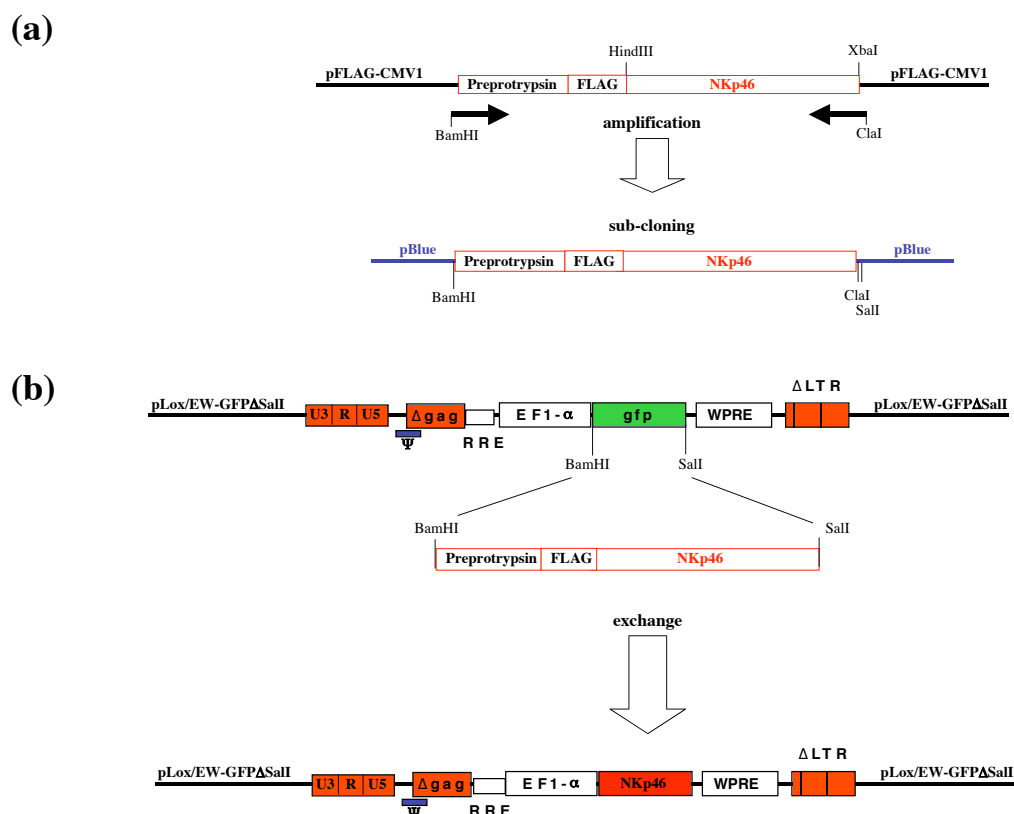


Figure 1-1: Cloning of the single gene transfer vector pLox-NKp46. A 935bp fragment containing the NKp46 cDNA fused to an N-terminal FLAG-tag and the preprotrypsin leader sequence was PCR amplified with primers generating 3' BamHI and 5' ClaI restriction sites. The insert was sub-cloned and sequenced in the pBluescript expression vector (a). The pLox NKp46 transfer vector was generated by the BamHI-SalI replacement of GFP in the pLox/EW-GFPΔSalI plasmid with the NKp46 insert (b).

1.2 Bicistronic transfer vectors

The bicistronic lentiviral transfer vector construct is based on the pWP-IRES-GFP vector plasmid (Fig. 1-2). The pWP-constructs contain additionally the central polypurine tract (cPPT) sequence and the short form of the EF1 alpha promoter regulates expression of the transgene-IRES-GFP cassette. This vector construct contains the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (ECMV) and allows the simultaneous expression of the gene of interest together with the GFP marker gene. Transcription leads to the formation one bicistronic mRNA with the IRES sequence functioning as the initiation site for translation of the GFP gene. To generate an NKp46 encoding bicistronic vector, the 940 bp BamHI-SalI NKp46^{FLAG} fragment excised from the pBluescript plasmid was inserted to the pWP-IRES-GFP backbone upstream to the

IRES sequence. Correct sequence and integration of the inserted fragment was confirmed by automated sequencing of the EF1 α -IRES region in the pWP-NKp46-IRES-GFP vector.

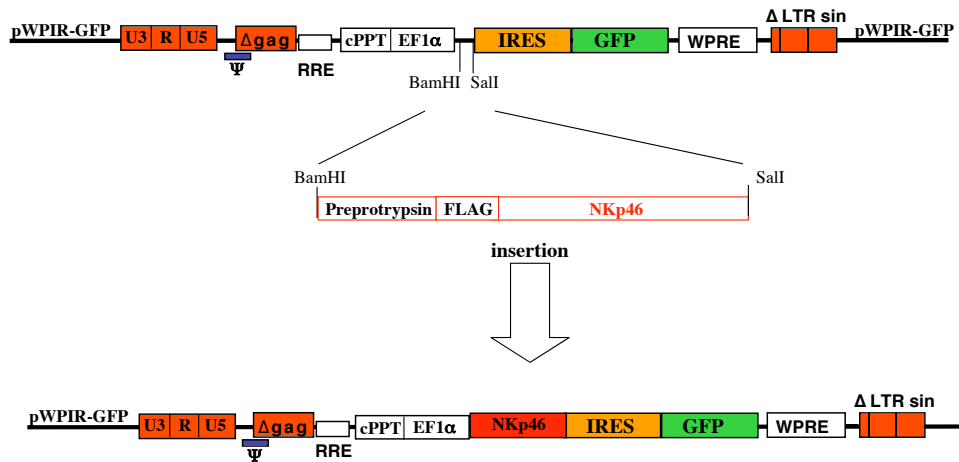


Figure 1-2: Generation of the bicistronic transfer vector pWP-NKp46-IRES-GFP. The BamHI-Sall NKp46 cDNA fragment was inserted upstream to the IRES sequence into the pWP-IRES-GFP plasmid.

2. Genetic Modification of Cell Lines

To investigate the potential of the generated lentiviral vectors to transduce NK cells with the cDNA for the natural cytotoxicity receptor NKp46, we first chose the T-lymphocytic Jurkat cell line and the NK cell lines NK92 and NKL as a model for gene transfer. These cell lines can be easily cultured *in vitro* and, in contrast to primary lymphocytes, they actively proliferate and therefore should be highly susceptible to lentiviral transduction. Like all primary T cells, the Jurkat T cell line does not express NKp46, but the CD3- ζ subunit that associates as an adaptor protein with NKp46. Thus, the Jurkat cell line provides a useful model system to explore the lentiviral gene transfer of the activating NK cell receptor NKp46 into lymphocytes.

The NK cell lines NKL and NK92 express endogenous NKp46 at very low levels, and therefore should allow the study of functional consequences of the expression of a transgenic receptor.

2.1 Lentiviral transduction of Jurkat cells

2.1.1 Transduction of Jurkat cells with single-gene transfer vectors

Lentiviral transduction of Jurkat cells was performed either with viral supernatants or with preparations of concentrated virus. Concentrated virus of the pLox-GFP was titrated using HeLa cells. Viral titers of concentrated virus of the pLox-NKp46 vectors were determined based on the RT assay.

Transduction efficiencies with the pLox-GFP vector were on average higher than that of the pLox-NKp46 vector (see Table 2-1). Transductions done with viral supernatant of the pLox-GFP vector on average lead to $51,3 \pm 27,2\%$ (range: 14-83,6%) of GFP positive cells, while a poor transduction efficiency was obtained with viral supernatant of the pLox-NKp46 vector yielding $1,2 \pm 0,9\%$ (range: 0,2-1,8%) of NKp46⁺ cells. When concentrated virus preparations at an MOI of 20 were used, the frequency of GFP positive Jurkat cells was $97,7 \pm 3,5\%$ (range: 91-98%) whereas transduction with concentrated pLox-NKp46 virus preparations resulted in an output of transgene expressing Jurkat cells that was $34,8 \pm 9,7\%$ (range: 24-83,8%).

	pLox-GFP GFP+	pLox-NKp46 NKp46+
LV supernatant	$51,3 \pm 27,2\%$	$1,2 \pm 0,9 \%$
concentrated LV MOI 10-20	$97,7 \pm 3,5\%$	$34,8 \pm 9,7\%$

Table 2-1: Transduction efficiencies of Jurkat cells. Cells were transduced with the pLox/EW-GFPΔSalI based transfer vector constructs and percentage of transgenic cells was determined by FACS analysis. Mean percentages and SD are shown (pLox-GFP; SN; n=7) (pLox-GFP, conc. virus; pLoxNKp46, SN or conc. virus n=3).

To obtain pure populations of NKp46-expressing Jurkat cells, we repeatedly sorted for transgene expressing cells that were transduced with concentrated pLox-NKp46 virus. FACS sort of transduced Jurkat cells in combination with single cell cloning by limiting dilution technique generated clones that showed a stable and homogenous surface expression of the NKp46 receptor (Figure 2-1).

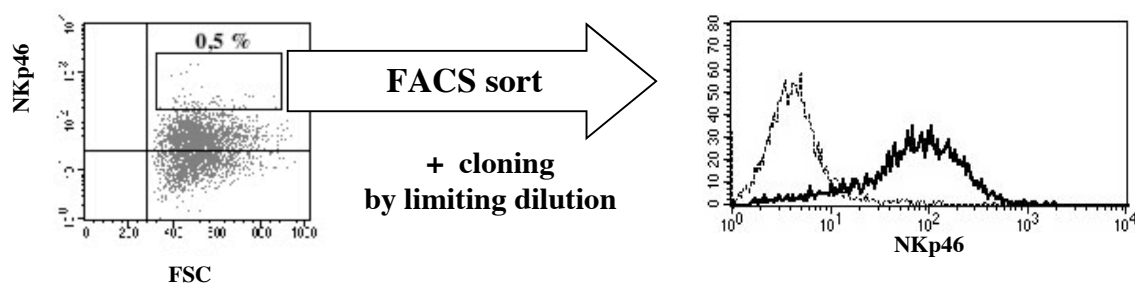


Figure 2-1: Generation of transduced Jurkat clones. Cells were transduced with concentrated pLox-NKp46 lentivirus and the subjected to FACS sorting and single cell cloning by limiting dilution to generate a homogenously NKp46 expressing clone. Jurkat-NKp46 clone #3 (0,3 cell/well) is shown.

Detection of the NKp46 transgene in the Jurkat-p46.3 clone by western blot analysis is shown in Figure 2-2.

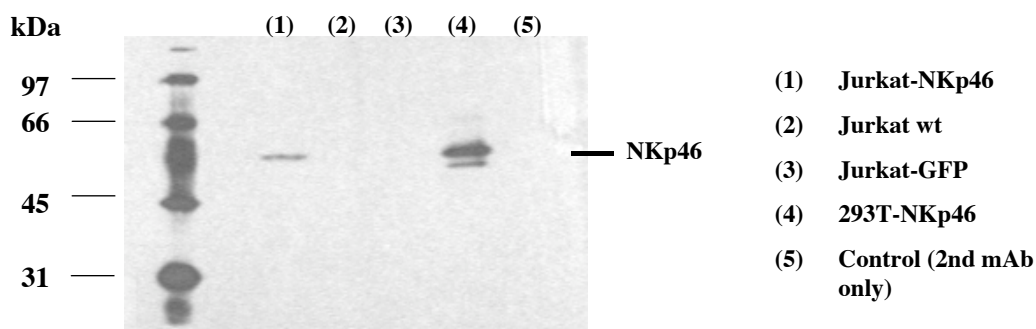


Figure 2-2: Western Blot detection of transgenic NKp46 receptor. pLox-NKp46 transduced Jurkat cells (clone Jurkat-p46.3) were lysed and electrophoretically separated by SDS-PAGE (approximately $2,5 \times 10^5$ cells per lane). The proteins were blotted to a nitrocellulose membrane and NKp46 was detected with anti-NKp46 hybridoma supernatant (mIgG1; clone 9E2) combined with a horse-radish peroxidase (HRP)-conjugated secondary antibody.

The genomic integration of lentivirus derived DNA was confirmed by PCR on genomic DNA of the Jurkat-p46.3 clone and on pLox-GFP transduced Jurkat cells. Figure 2-3 shows the agarose gel analysis of the PCR products generated with primer pairs specific for the integrated EGFP transgene or the exogenous, FLAG-tagged NKp46 receptor cDNA. PCR was also performed on genomic DNA of wt Jurkat cells that served as a negative control.

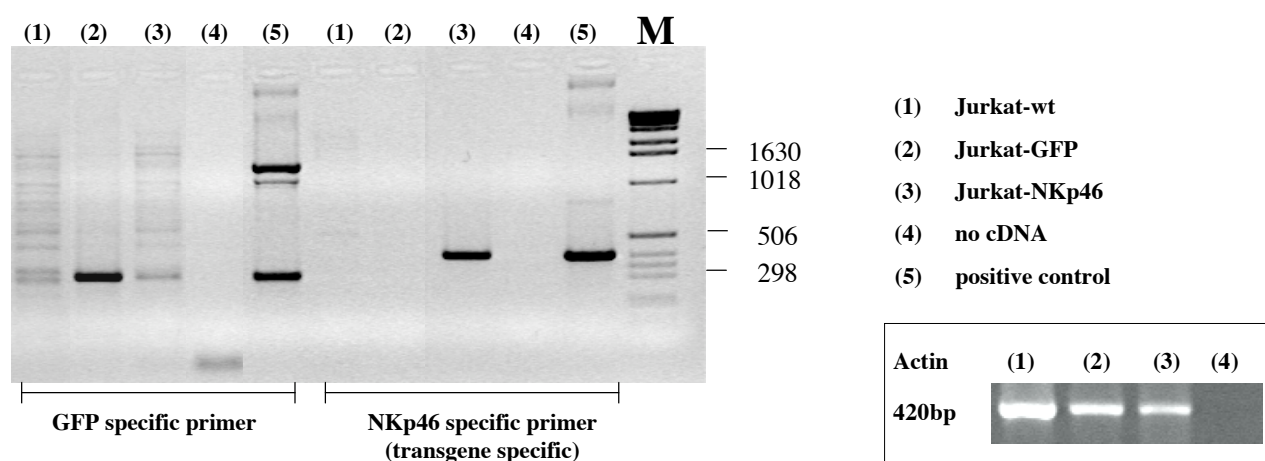


Figure 2-3: PCR detection of genomic integrated lentiviral provirus cDNA. Integration was confirmed by the use of transgene specific primer pairs. The GFP encoding vector cDNA was detected with the 3'EGFPsense-EGFP5'antisense primer combination, whereas the integration of NKp46-cDNA was confirmed by the BamFLAG 3'sense-ClaI5'antisense primer pair specific for the vector-encoded exogenous NKp46. As a positive control the plasmid DNA of the corresponding transfer vector construct was used. Comparable amounts of genomic DNA were used in the PCR reaction as shown by the amplification of the β -Actin gene (gel insert below).

2.1.2 Transduction of Jurkat cells with bicistronic transfer vectors

The transduction of Jurkat cells with lentivirus preparations of the bicistronic pWP-IRES-GFP transfer vector resulted in almost pure transgenic populations. The transduction with LV supernatant yielded between 88% and 98% GFP+ cells (not shown). Figure 2-4 shows a typical transduction result with concentrated virus of the bicistronic pWP-NKp46-IRES-GFP transfer vector at a MOI of 2-5. FACS analysis of GFP expression on day 5 after infection revealed a transduction efficiency of 97%. As expected, the mean fluorescence of the GFP expression observed with the bicistronic vectors was approximately one log lower compared to the high GFP expression levels achieved with the single-gene pLox-GFP vector (MFI $\sim 10^3$). A lower expression of the GFP marker gene was also seen in transductions using concentrated virus of the bicistronic control vector pWP-IRES-GFP (data not shown). This reduced expression may reflect the fact that the initiation of translation from the IRES site on the bicistronic mRNA is less efficient compared to the translation starting at the 5'end of mRNA. Importantly, expression of both genes was stable over time as measured for up to 7 weeks after transduction.

We observed that a certain fraction of transduced cells did not express the upstream NKp46 receptor gene (Figure 2-4). Despite nearly 100% of the cells express the GFP gene, less than 90% of the GFP+ cells showed the surface expression of the NKp46 receptor (85% NKp46+ vs. 97% GFP+). Since bicistronic vectors were used and the opposite was to be expected, this “incomplete” surface expression of the NKp46 receptor suggests that in a subpopulation of Jurkat cells the transport to the cell surface is impaired, possibly due to a limited availability of adaptor molecules. This is supported by the results obtained in transduction experiments using concentrated virus of the pLox single-gene vectors. The GFP-encoding vectors readily transduced Jurkat cells even at low multiplicities of infection, whereas homogenous expression of the NKp46 receptor was only achieved upon the cloning of transduced cells after FACS sorting.

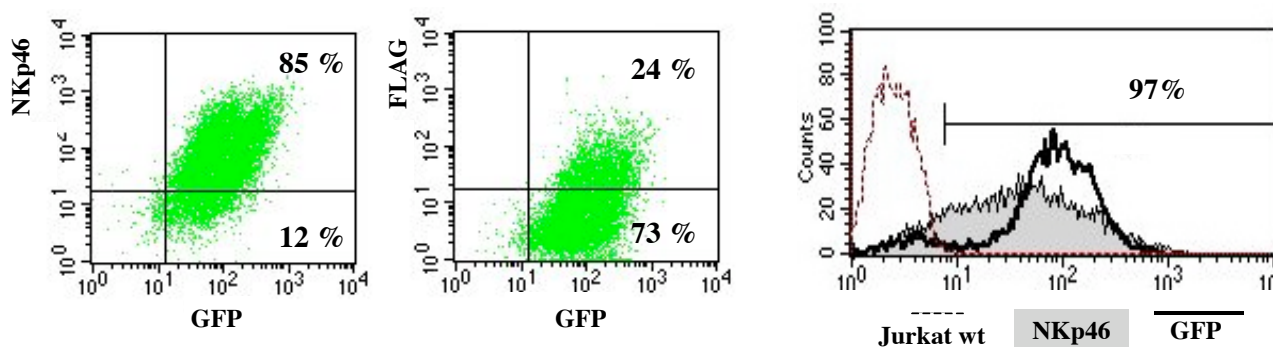


Figure 2-4: Transduction of Jurkat cells with concentrated virus of the bicistronic construct pWP-NKp46-IRES-GFP. FACS analysis of transgene expression was measured on day 5 post infection (MOI 2-5). Staining was done with unlabelled anti-NKp46 and anti-FLAG (M2; Sigma) mAbs using the same PE-conjugated goat anti-mouse IgG1 secondary reagent. Detection of transgenic NKp46 receptor with the anti-FLAG mAb as the primary reagent is less efficient compared to the anti-NKp46 mAb.

2.2 Lentiviral transduction of the NK cell lines NK92 and NKL

2.2.1 Transduction efficiency with “single-gene” and “bicistronic” transfer vectors

In contrast to the results obtained with Jurkat cells, only poor transduction efficiencies were achieved in NK cell lines. Transductions with concentrated lentivirus of the single-gene pLox-GFP vector at an MOI of 20 resulted in 0,8-1,0% of GFP+ cells on day 3 post infection. Results for

concentrated virus of the bicistronic LV transfer vectors were up to 45% measured on day 3 post infection (Figure 2-5), but rapidly dropped on day 8 to below 1% GFP+ cells for both NKL and NK92 (Table 2-2). This phenomenon of “pseudo-transduction” was observed upon the infection with concentrated virus preparations of the pWP-NKp46-IRES-GFP vector as well as with virus of the “empty” pWP IRES-GFP control vector (data not shown). The high initial transgene expression may be indicative of a highly efficient transfer of vector DNA into the nucleus of target cells without the integration into the hosts’ genome or with high rates of gene-silencing. No pseudo-transduction was observed when concentrated virus of the single gene vectors was used. Indeed, unlike the bicistronic constructs, these vectors do not carry the central polypurine tract (cPPT) sequence that allows an increased transfer of the vector preintegration-complex through the nuclear pores.

Thus, the NK cell lines NK92 and NKL are highly susceptible to the nuclear translocation of vector DNA derived from the bicistronic lentivirus constructs. This indicates that the NK cell lines may possess a mechanism to minimize vector DNA integration, although “silencing” of integrated vector DNA cannot be ruled out since the integration of vector DNA into the genome of cells with a high initial “pseudo-transduction” was not evaluated.

	pLox-GFP GFP+	pWP-NKp46-IRES-GFP GFP+
NKL	< 1,0%	< 1% d8
NK92	< 1,0%	< 1% d8

Table 2-2 : Transduction efficiencies of NK 92 and NKL cell lines. Cells were transduced with concentrated lentivirus of the single-gene or the bicistronic vector constructs at an MOI of 20. The percentages of GFP+ cells at day 3 (pLox-GFP) or day 8 post transduction (pWP-constructs) are shown.

2.2.2 Generation of stably transduced NK92 and NKL

Stably transduced NK92 and NKL cells were generated by the repeated FACS sort of GFP+ cells resulting in almost pure transgenic NK cell lines with 94% (NK92) to 98,5% (NKL) of cells positive for GFP expression. Figure 2-5 illustrates the results obtained for NK92. Transduction of about 2×10^5 cells resulted in a very high initial transduction rate that rapidly declined within the following 8 days to below 1% (Figure 2-5a). The subsequent FACS sort of about $1,3 \times 10^4$ GFP positive cells resulted in the enrichment of transgenic cells to a level of 4,1% after an expansion period of 16 days. In contrast to day 8 post infection the transgenic cells were now visible as a

distinct and highly GFP positive population that allowed the subsequent re-sort to a purity of 94% GFP positive NK92 cells (Figure 2-5b).

Similar results were obtained for the NKL cell line transduced with the same vector. A high purity of transduced cells (> 98%) was achieved even after a single FACS sort (data not shown).

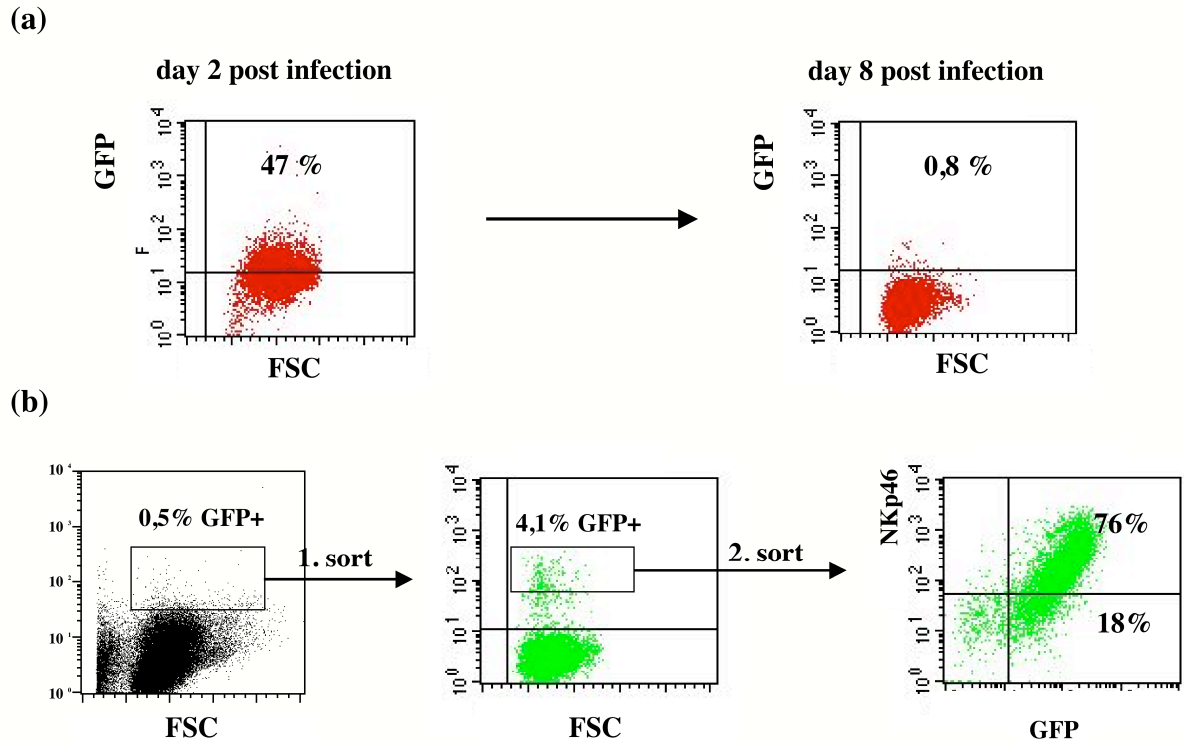


Figure 2-5: Generation of a stably transduced NK cell line NK92. NK92 cells were transduced with concentrated virus of the vector construct pWP-NKp46-IRES-GFP at an MOI of 20. FACS analysis of the transduction efficiency at the indicated days is shown in (a), the repeated FACS sort of transduced NK92 on day 8 and day 24 post infection in (b).

As observed in Jurkat cells, FACS analysis revealed a lower expression of the upstream NKp46 gene, when compared to expression of the downstream GFP marker. This diminished surface expression of transgenic NKp46 in a proportion of transduced cells suggests either a limited availability of adaptor molecules necessary for the surface expression or simply reflects an inefficient transport of receptor molecules to the cell surface in NKL and NK92.

2.3 Analysis of the functional activity of transgenic NKp46 in cell lines

By the combination of repeated FACS sorting and cloning by limiting dilution stably transduced clones of the Jurkat cell line were generated. These Jurkat-p46 clones showed a high and homogenous surface expression of the transgenic NKp46 receptor whereas the wild type cells were negative (Figure 2-1). Accordingly, a high expression of NKp46 together with the GFP reporter gene was achieved after FACS sorting of GFP⁺ cells of the NK cell lines NK92 and NKL transduced with the bicistronic pWP transfer vector constructs. NKp46 expressed by the modified NK cell lines represents mainly exogenous receptor since wild type NKL and NK92 express low levels of endogenous NKp46.

To determine the functionality of the transgenic receptor, we measured the induction of the lymphocyte activation marker CD69, the production of IFN- γ or the mobilization of intracellular calcium-ions upon monoclonal antibody-mediated receptor crosslinking or in target cell co-cultures. Further we analysed the potential of the transgenic NKp46 receptor to induce the monoclonal antibody mediated re-directed lysis of murine target cells.

2.3.1 CD69 expression and intracellular Ca^{2+} -release in the Jurkat cell line

Resting primary lymphocytes as well as the cultured Jurkat T cell line do not express the activation marker CD69. Strong surface expression of CD69 was induced by the unspecific stimulation with PMA and PHA, which served as a positive control treatment. Jurkat-NKp46 cells were either incubated with mAbs in solution or with immobilized mAbs in antibody-coated plates. To allow optimal receptor crosslink on cells pre-stained with mAbs a secondary goat anti-mouse polyclonal Ab (GaM) was added. Crosslinking with anti-CD3 only or in combination to a crosslinking secondary mAb served as a positive control for antibody mediated CD69 expression. The anti CD3 mediated induction of CD69 expression was as high as in response to PMA-PHA when the cells were incubated on antibody-coated plates (Figure 2-6b), but was lower when anti CD3 was used in solution (Figure 2-6a), reflecting a stronger cross-linking effect through plastic adherent mAbs. In contrast, the mAb mediated crosslink of the transgenic NKp46 receptor in the Jurkat-p46.3 clone did not result in the induction of CD69. Neither the anti-NKp46 mediated receptor cross-link alone (Figure 2-6c), nor the simultaneous stimulation of the co-receptor CD28 (Figure 2-6d) induced any CD69 expression on the transgenic Jurkat clones.

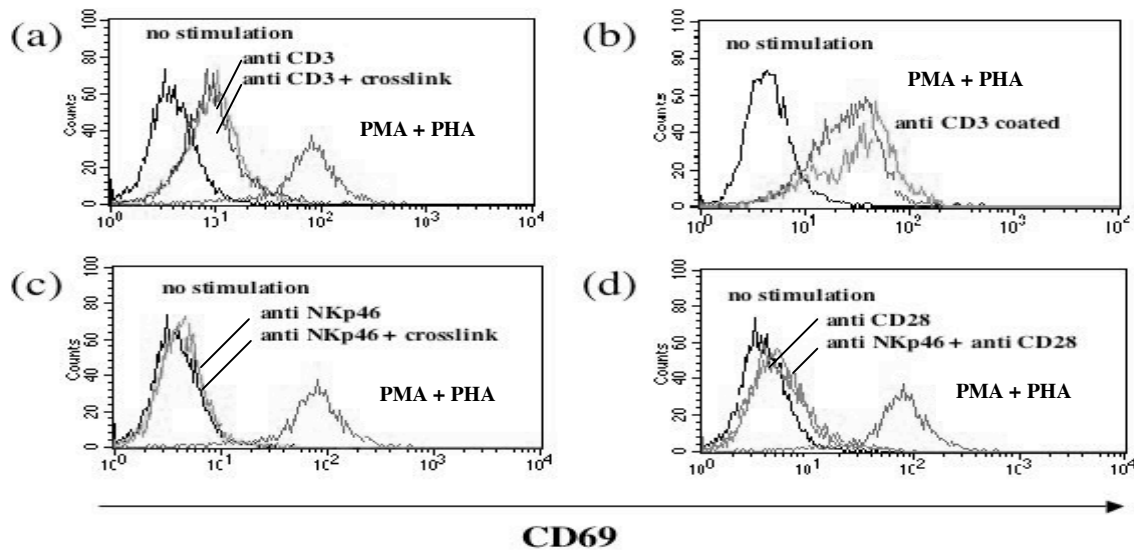


Figure 2-6: FACS analysis of the expression of the activation marker CD69 on Jurkat cells transgenic for NKp46 (clone Jurkat-p46.3). Crosslink of NKp46 (c), NKp46±CD28 (d) and CD3 (a,b) was achieved either through plastic adherent (“coated”) anti-NKp46 (mAb-SN; 60 μ l/well) or anti-CD3 and anti CD28 (purified mAb; 20 μ g/ml), or through the addition of a secondary goat anti mouse (GAM, final 20 μ g/ml) mAb to Jurkat cells pre-stained with the corresponding mAbs (“crosslink” in a, b and c). As a positive control CD69 expression was induced by the unspecific activation with PMA (20ng/ml) and PHA (1 μ g/ml). FACS analysis of CD69 expression was done on day 36 hours post stimulation.

We next analysed whether the crosslink of the transgenic NKp46 receptor could induce the mobilization of intracellular Ca^{2+} ions. As shown in Figure 2-7b a sharp increase in free intracellular Ca^{2+} was detected upon the addition of the calcium ionophor ionomycin. The pre-staining with anti CD3 and the subsequent cross-link with a secondary GaM mAb resulted in an immediate but lower Ca^{2+} mobilization (Figure 2-7c) as compared to the ionomycin treatment. Treatment with anti-NKp46 or the secondary GaM mAb only did not result in any detectable change in free intracellular Ca^{2+} (Figures 2-7a, d). In contrast, the addition of cross-linking GaM to NKp46 pre-stained cells induced a weak but significant Ca^{2+} mobilization (Figure 2-7e). This result clearly suggests that the transgenic NKp46 receptor is functional in the Jurkat cell line in terms of mediating an immediate activation of the signal transduction cascade upon receptor triggering.

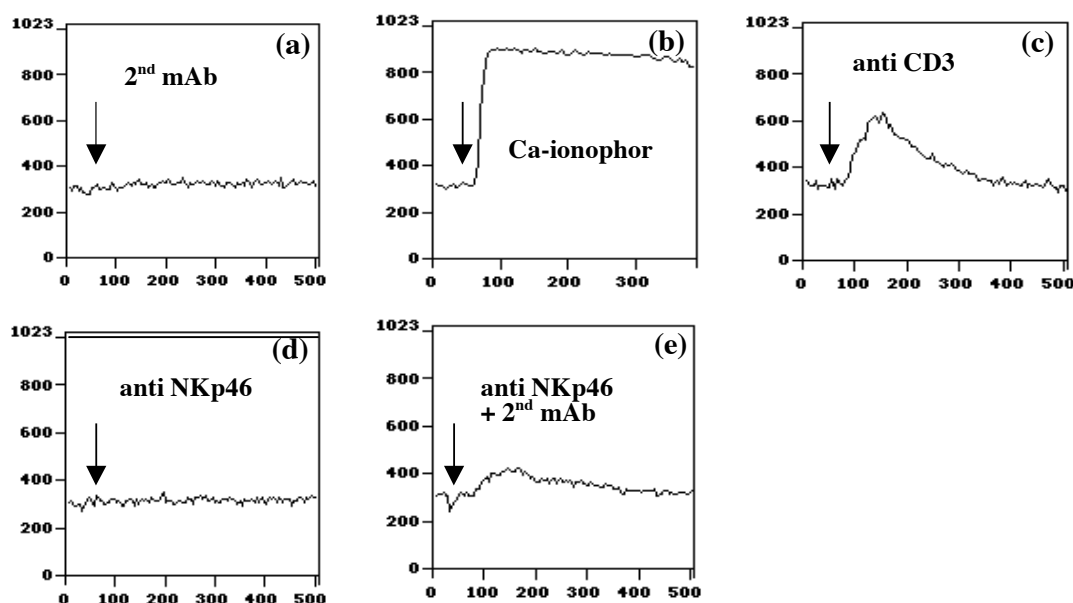


Figure 2-7: Measurement of intracellular Ca^{2+} ion mobilization in the Jurkat-p46.3 clone. Ca^{2+} -release was measured upon the treatment with secondary mAb only (a), calcium-ionophor (b), anti CD3 crosslink (c), anti NKp46 only (d) or in the presence of anti NKp46 together with secondary mAb crosslink (e). Arrows indicate the time point of the addition of the secondary cross-linking mAb.

However, the relatively weak response to the used monoclonal antibody might indicate that this particular mAb is not an agonistic type of antibody or that the transgenic NKp46 receptor is ineffective or even defective in its capacity to trigger a strong intracellular signalling.

2.3.2 Crosslinking of transgenic NKp46 and $\text{IFN-}\gamma$ release by NKL

In contrast to the transgenic Jurkat clone Jurkat-p46.3 (see 2.3.1) intracellular Ca^{2+} -ion mobilization upon the anti-NKp46 mediated receptor crosslink could not be detected with the transduced NK cell line NKL-p46-IRES-GFP. All obtained results were inconclusive and even the calcium ionophor stimulation did not elicit any reproducible Ca^{2+} -fluxes (data not shown).

To assess whether the stimulation of the exogenous receptor induces $\text{IFN-}\gamma$ secretion of transgenic NKL we mediated the receptor crosslinking. Figure 2-8 shows the secretion of $\text{IFN-}\gamma$ by primary PB NK cells and by NKL wild type (wt) and transduced NKL-p46-IRES-GFP cells in response to anti-NKp46 mediated crosslinking, that was either mediated through plastic adherent primary antibodies (anti-NKp46; anti-CD38; anti CD16) or by the stimulation of pre-stained NKL cells with secondary goat anti-mouse antibodies (Figures 2-8a,c). Alternatively, anti-NKp46 mediated crosslinking was induced by the “re-directed” use of the antibody in co-cultures with the murine target P851 pre-

incubated with anti-NKp46 (Figure 2-8b). Here the K562 cell line served as a negative control for the induction of IFN- γ release.

IFN- γ secretion was determined by ELISA of culture supernatants collected 36 hours after stimulation. For a positive control, cells were stimulated with PMA and ionomycin, which resulted in a high IFN- γ release that was up to 28'000 pg/ml for NKL and 59'000 pg/ml for PB NK cells. In contrast, unstimulated PB NK cells as well as wt and transduced NKL cells displayed a very low background-release of IFN- γ (300-500 pg/ml) and crosslink of NKp46 did not lead to any further induction of the cytokine. Neither plastic adherent or soluble anti-NKp46 nor the “re-directed” anti-NKp46 mAb (bound to the murine P851 target cells via the Fc γ RII-receptors) triggered the cells to release IFN- γ .

Similarly, the crosslinking of CD38 by anti-CD38 mAb did not result in the induction of cytokine release in NKL. In NKL it was shown that signalling via CD38 is strictly dependent on the expression of CD16 that provides the signalling components (Deaglio, S. Blood 2002). Since the NKL cell line we used is negative for CD16, and the crosslink of CD38 did not reveal any cytokine release, a possible effect of the “over-expressed” transgenic NKp46 receptor in the delivery of signalling components to CD38 in NKL can be ruled out.

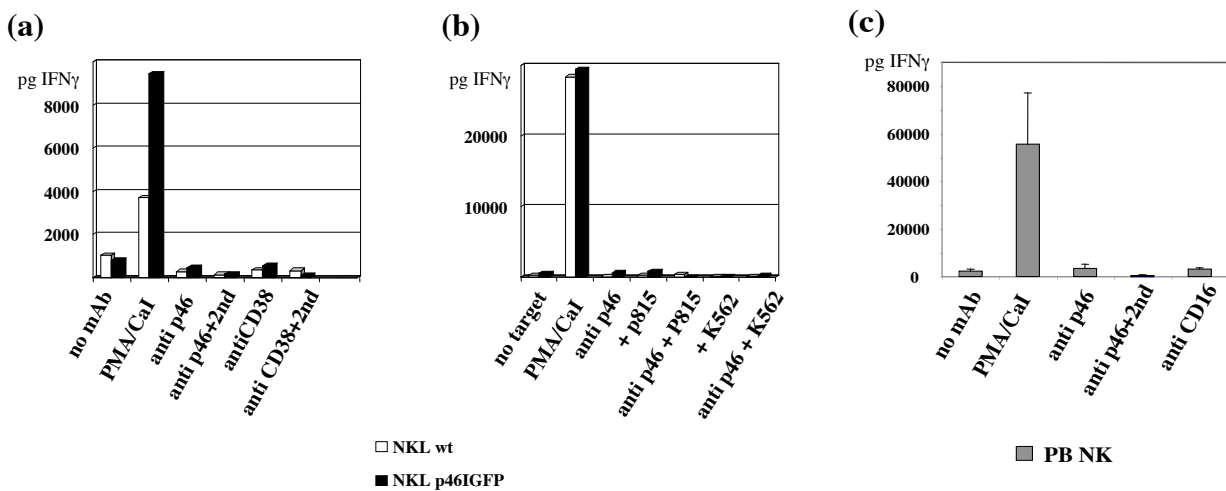


Figure 2-8: ELISA measurement of IFN- γ -release upon mAb mediated receptor crosslink and upon co-culture with target cells. For receptor crosslink in (a) and (c) $0.5 \cdot 10^5$ cells per well were seeded in 96 well plates coated with anti-NKp46 (mAb-SN; $60 \mu\text{l/well}$) or anti CD38 and anti CD16 (purified mAb; $20 \mu\text{g/ml}$). Alternatively, cells were pre-stained with anti-NKp46 or anti-CD38 and the secondary reagent (goat anti-mouse IgG1) was added to a final concentration of $20 \mu\text{g/ml}$. Co-cultures were done at an E:T ratio of 2:1 with $1 \cdot 10^5$ effector cells (b). Target cells were pre-incubated for 30 min with anti-NKp46 before NK cells were added (mAb-SN; $10 \mu\text{l/well}$). Culture supernatants were taken after 36 hrs. Maximal IFN- γ -release was induced by the unspecific stimulation with PMA (20ng/ml) and calcium ionophor (Ionomycin; $1 \mu\text{M}$).

As already hypothesized from the Ca^{2+} -flux measurements in the Jurkat-p46.3 clone, the transgenic NKp46 receptor might be functionally defective in terms of signal transduction. At least, the antibody-mediated crosslinking of the exogenous receptor might be insufficient to transmit a full activation signal that could lead to the release of cytokines. One possible explanation for the dysfunction of the transgenic receptor molecule might be the fact that the exogenous receptor contains a FLAG tag at the N-terminus. The six additional amino acid residues of the tag do not interfere with the specific binding of the antibody to the receptor, but may affect the process of signal transduction.

However, in control experiments with primary PB NK cells that express the endogenous NKp46 receptor and CD16 at normal high levels no $\text{IFN}\gamma$ release was detectable upon the anti-NKp46 or anti-CD16 mediated receptor crosslink (Figure 2-9c). Thus, it cannot be excluded that the crosslink with anti-NKp46 mAb is insufficient to induce any cytokine release in NK cells or that the mAb we used (anti NKp46, clone 9E2) have no agonistic capacity.

2.3.3 Redirected killing of murine targets by NKp46 transduced NK92

In order to determine whether the monoclonal antibody specific for NKp46 is capable to function as an agonistic reagent, we performed “re-directed” cytotoxicity assays with normal primary NK cells. These IL-2 activated and *in vitro* expanded peripheral blood NK cells displayed normal surface expression levels of CD16 and NKp46. In NK cells a “re-directed” cytotoxicity against the NK-insensitive murine target cell P815 can be induced by the addition of mouse anti-human monoclonal antibodies specific for activating NK cell receptors. In Figure 2-9 the “re-directed” killing of P815 by activated and expanded primary PB NK cells (a) and by wt NK92 and transgenic NK92 (b) is shown. For the induction of the cytotoxic activity of the NK cells, purified anti-CD16 mAb was used as a positive control reagent. The addition of anti-CD56 served as a negative control. Since CD56 is not a cell-activating molecule, the tight contact between NK cells and the targets mediated by anti-CD56 should not lead to cytolysis.

As expected, the spontaneous killing was around 1% or almost undetectable, but a high specific lysis was induced by anti-CD16 that was up to 30% at an E:T ratio of 20:1 (Figure 2-9a). In contrast, no lysis was measured when anti-CD56 was added. The addition of anti-NKp46 mAb induced a cytolytic activity against P815 at different E:T ratios with a maximal specific lysis of 8% at the E:T ratio of 20:1, corresponding on average to 40% of the anti-CD16 induced response.

These results strongly indicate that anti-NKp46 indeed represents an agonistic antibody suitable to trigger cytotoxic activity in NK cells. However, the level of mAb-induced activation is clearly below that mediated by anti-CD16. This may be due to the fact that the mAb was used as an unpurified hybridoma supernatant of unknown protein concentration.

We next analysed the re-directed cytotoxicity of the NK92 wt cell line compared to the lentivirus-transduced counterpart NK92-NKp46-IRES-GFP. Since the NK92 cell line is negative for CD16, no antibody was available to serve as a positive control for a maximal inducible specific lysis. The transduced cell line displayed a high surface expression of NKp46 (MFI ratio 15,0) compared to the wt line expressing low levels of endogenous NKp46 (MFI ratio 2,8; not shown). Background activity against P851 (without the addition of antibody or in the presence of anti-CD56) was low or almost absent and equal for the wt and the transgenic cells (Figure 2.9a).

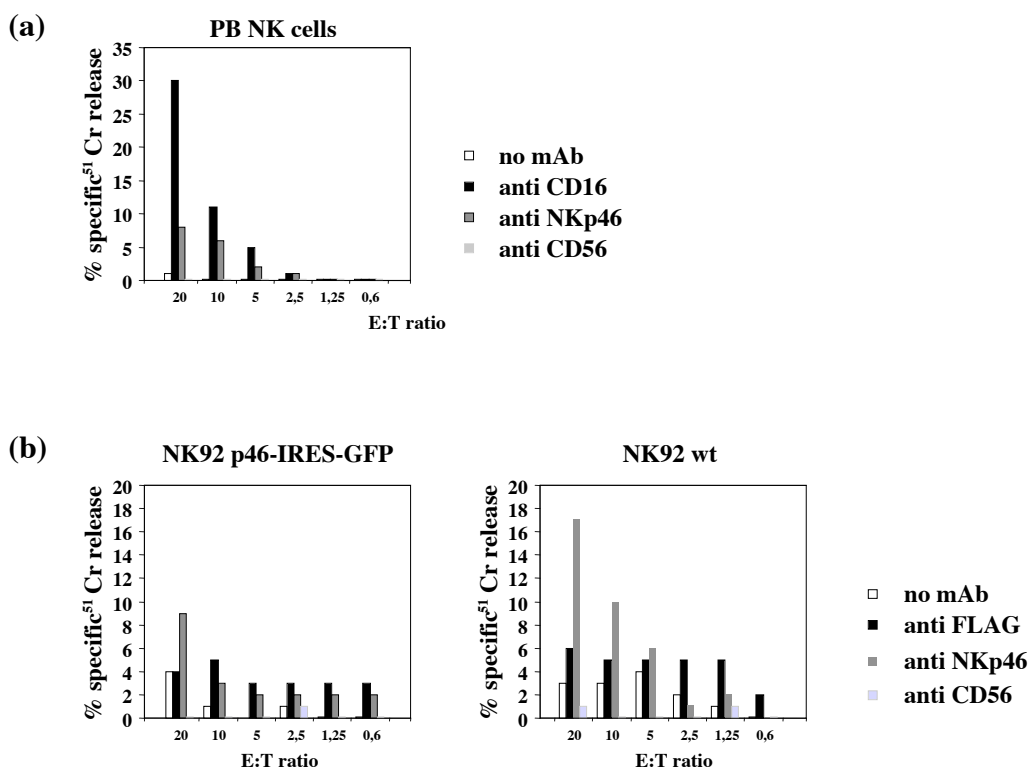


Figure 2.9: “Re-directed killing” of the murine target cell line P851. The “re-directed” killing by activated and expanded primary PB NK cells (a) and by wt NK92 and transgenic NK92-NKp46-IRES-GFP (b) is shown. Percentage of specific lysis was measured in standard 4 hrs Cr⁵¹ release assays. The P851 target cells were pre-incubated with the indicated mAbs for 30 min before they were added to the effector cells (purified anti-CD16, anti-FLAG and anti-CD56 at final 1 μg/ml; anti-NKp46 SN at 10 μl/100 μl, corresponding to final 0,5-1 μg/ml). FACS analysis of the surface expression of the activating receptors NKp46 and CD16 is shown in (b).

No difference between wt NK92 and transgenic cells was observed with the anti-FLAG mAb that led to a E:T ratio-independent unspecific triggering. The addition of anti-NKp46 induced a cytolytic activity in both cell lines, and surprisingly the specific lysis of wt NK92 was stronger compared to the lysis observed with the transduced line. At the highest E:T ratio of 20:1 for instance, specific lysis of wt NK92 was 17%, whereas that of NK92-NKp46-IRES-GFP was only 9%.

This unexpectedly low killing despite higher NKp46 surface expression might be explained by the hypothesis that the transgenic NKp46 receptor is indeed functionally inactive. If one assumes that the availability of anti-NKp46 mAb is not limited, all Fc γ RII-receptors on the P851 targets should have bound anti-NKp46 mAb. Thus, the high surface expression of inactive receptor molecules on NK92-NKp46-IRES-GFP cells may compete with the endogenous, functionally active NKp46 for the binding to the triggering antibody and thereby may lead to the observed reduction in “re-directed” cytotoxicity.

3. Genetic Modification of Primary Peripheral Blood NK Cells

3.1 Phenotypic characterization of activated and expanded PB NK cells

3.1.1 Phenotypic characterization of PHA and IL-2 expanded polyclonal NK cell cultures

To determine the ability of lentiviral vectors to transduce primary NK cells we isolated NK cells from fresh or cryopreserved ficolled peripheral blood samples of healthy donors. NK cells purified by MACS technology were stimulated and expanded with phytohemagglutinin (PHA) and IL-2 in the presence of irradiated allogeneic PBMCs. Expansion of NK cells was measured on day 14, 21 and 28 post stimulation. In a typical polyclonal stimulation culture NK cells started to form (PHA-) blasts on day 4 to 6 and entered the phase of exponential growth on day 8 to 10 of culture period. Figure 3-1 shows the average expansion of normal NK cells upon stimulation. NK cells usually reach a plateau-phase of expansion between day 21 and 28, thereafter they stop to proliferate, decrease in size and enter a resting phase, in which they can be repeatedly stimulated.

Results

Regular restimulations with the same polyclonal NK cell line were done up to for times resulting in comparable proliferative responses (data not shown). NK cells were phenotypically characterized on day 14, 21 and 28 during culture period by FACS analysis for the cell surface expression of different NK cell and lymphocyte markers. Cells were stained for the NK cell specific CD56, the natural cytotoxicity receptor NKp46, the activatory receptors NKG2D and CD16 (FcγRIII) and the lymphocyte activation marker CD69.

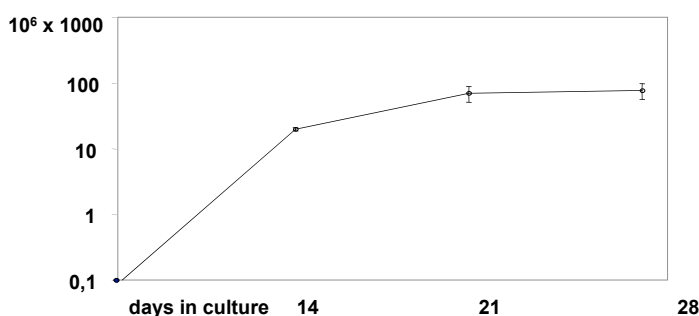


Figure 3-1: Average expansion rates of normal peripheral blood NK cells. $1-3 \times 10^5$ NK cells isolated from healthy donors ($n=4$), were stimulated with phytohemagglutinin (PHA; $1 \mu\text{g/ml}$) and 2×10^6 irradiated allogeneic PBMCs in the presence of rhIL-2 (100U/ml). Cell counts were done on the indicated days of expansion culture and standardized for the expansion of $0,1 \times 10^6$ cells.

NK cells were also stained for the inhibitory receptors CD158a (KIR2DL1), CD158b (KIR2DL2) and NKB1 (KIR3DL1) of the killer cell immunoglobulin-like receptor (KIR) family, for the lectin-like inhibitory receptor CD161 (NKR-P1) and for CD94 that forms heterodimers either with the inhibitory NKG2A or with the activatory NKG2C receptors (Figure 3-2a). The surface expression levels (measured as percentage positive cells or by the mean fluorescence intensity ratio; MFIR) of the inhibitory KIRs, of CD161, and of CD94 were unchanged during *in vitro* expansion, while the activation marker CD69 was strongly upregulated when compared to freshly isolated resting NK cells (data not shown).

Freshly isolated peripheral blood NK cells belong to different subsets according to the expression pattern of CD56 and CD16. However, in all stimulation cultures analysed, the NK cell subsets characterized by the $\text{CD56}^{\text{bright}}/\text{CD16}^{\text{dim/negative}}$ and the $\text{CD56}^{\text{dim}}/\text{CD16}^{\text{bright}}$ phenotype were no more distinguishable since almost all cells acquired a uniform $\text{CD56}^{\text{bright}}/\text{CD16}^{\text{bright}}$ phenotype (Figure 3-2b). The surface expression of the natural cytotoxicity receptor NKp46 showed a transient down-regulation during the process of restimulation but reached in almost all expansion cultures the initial or even higher levels between day 20 and day 28 (Figure 3-2c). The average expression level of NKp46 during the 28 days of *in vitro* culture was $8,1 \pm 2,3$ (MFIR \pm SEM) and thus comparable to

freshly isolated PB NK cells with an average MFIR of $10,0 \pm 0,9$. In contrast, surface expression level of the activatory receptor NKG2D was strongly upregulated in all cultures analysed. The average MFIR increased from $6,8 \pm 0,7$ at day 0 to a MFIR of $25,2 \pm 3,4$ without any down-modulation during the 28 days of *in vitro* expansion (Figure 3-2d). FACS analysis of freshly isolated PB NK cells revealed three different patterns of NKp46 surface expression (Figure 3-3a). NK cells either showed a homogenous receptor expression that was of a “dim” or “bright” phenotype or consisted of two distinct subpopulations, representing a “bimodal” phenotype of NKp46 surface expression.

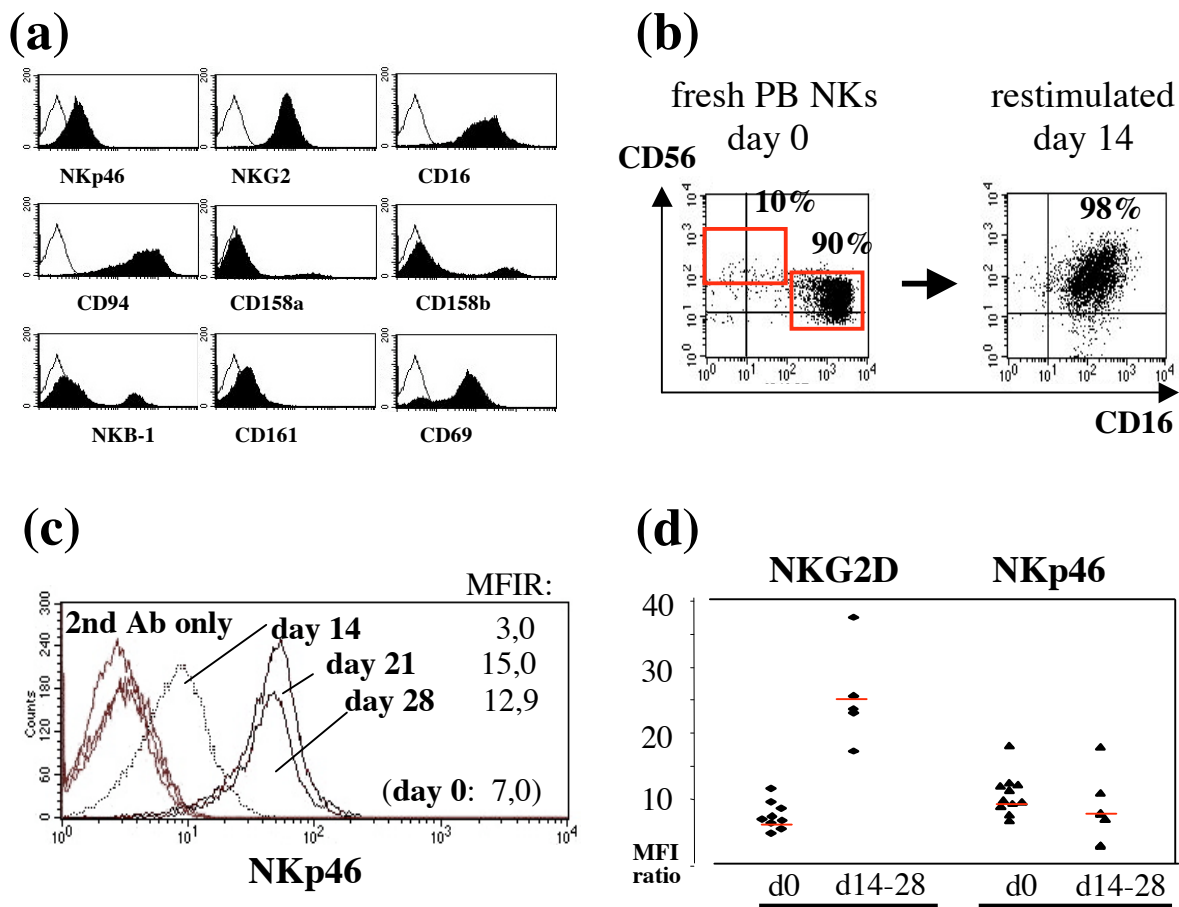


Figure 3-2: Typical surface expression pattern of different NK cell markers. PHA-stimulated normal peripheral blood NK cells were analysed by FACS. Expression of the markers is shown as histograms on day 14 of restimulation culture (a); open curves represent the staining with isotype mAb (if directly labelled mAbs were used) or with secondary reagent only (in case of unlabeled primary mAbs were used; e.g. for the detection of NKp46). Compared to the different subsets of PB NK cells found in freshly isolated NK cells, activated and expanded cells showed a uniform CD56 and CD16 bright phenotype (b). NKp46 surface expression during the process of restimulation was transiently down regulated but reached initial or higher levels between day21 and day28 of culture; a typical kinetic of surface expression is shown in (c). The surface expression of the activatory receptors NKG2D and NKp46 on freshly isolated peripheral blood NK cells compared to the average expression levels upon *in vitro* expansion between day14 and day28 is shown in (d); red bars represent the mean values.

3.1.2 Modulation of the NKp46 surface expression by repeated PHA stimulations

To further expand and propagate NK cell cultures *in vitro*, cells were regularly restimulated between day 21 and day 28 of expansion culture. As described in 3.1.1, the average expression levels of NKp46 after the first PHA/IL-2 stimulation reached the initial “*ex vivo*” values, but in some cases (2/8 healthy donors) the overall surface expression level decreased with repeated stimulations.

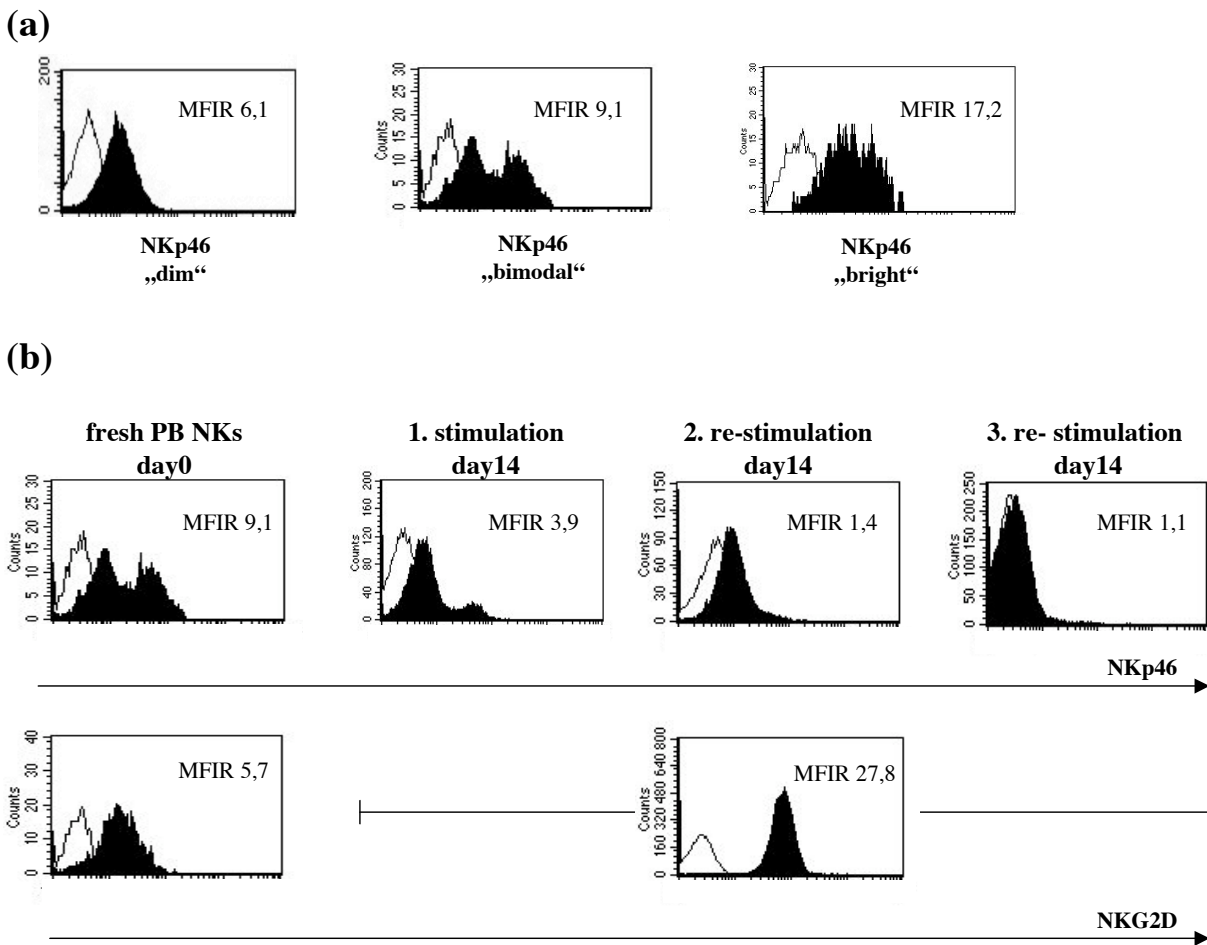


Figure 3-3: Flow cytometry analysis of NKp46 surface expression on freshly isolated expanded PB NK cells. Filled histograms show the NKp46 surface expression, with the indicated mean fluorescence ratio (MFIR), whereas the open histograms represent background staining with the secondary reagent only (FITC-labelled goat anti mouse IgG1). In freshly isolated NK cells the expression of NKp46 was either of the “dim”, the “bimodal” or was of the “bright” phenotype (a). Repeated PHA/IL-2 stimulations lead to the complete loss of surface expression of the receptor as shown for one normal donor with an initial “bimodal” type of NKp46 expression (b). In all PHA/IL-2 expansion cultures the initial NKG2D dim expression was upregulated to a “bright” phenotype that stayed at high levels even upon repeated stimulations.

Figure 3-3b shows the repeated expansion of a polyclonal NK cell line with a bimodal type of NKp46 expression. After first expansion the bimodal distribution of NKp46 expression was maintained, but the overall MFIR decreased from 9,1 to an MFIR of 3,9. NKp46 surface expression was progressively decreasing with further restimulations to and was virtually lost after the third stimulation. Similar results were obtained upon the repeated PHA/IL-2 expansion of a polyclonal NK cell population with a bright NKp46 surface expression.

In this case the initial bright expression after the first expansion changed to a bimodal distribution and finally was lost after the third restimulation (data not shown). In contrast, in all PHA/IL-2 expansion cultures NKG2D surface expression was upregulated after the first expansion and remained at high levels even upon the repeated stimulation (Figure 3-3b).

3.1.3 Modulation of the NKG2D surface expression upon PHA-stimulation in combination with different cytokines

The finding that the repeated *in vitro* expansion of PB NK cells with PHA and IL-2 resulted in a strong up-regulation of NKG2D and in a transient down modulation or even a complete loss of NKp46 prompted us to test the effect of different cytokines in combination with the PHA/IL-2 stimulation. Among the cytokines that are known to regulate the function and homeostasis of NK cells, we used IL-12, IL-15 and IL-21. We analysed the influence of these cytokines on the cell expansion and focused on the expression levels of the activatory receptors NKG2D and NKp46.

Figure 3-4a shows the expansion of normal PB NK cells upon PHA stimulation either in the presence of IL-2 alone or with IL-2 in various combinations with IL-12, IL-15 and IL-21. In all tested combinations of the different cytokines similar NK cell expansion rates were obtained. FACS analysis of the receptor expression revealed no changes in the surface expression of NKp46, but a strong upregulation of NKG2D in cultures containing IL-2 only, together with IL-15 or IL-21 or IL-2 in combination with both cytokines (Figure 3-4 b). In contrast, in all cultures that contained IL-12 the up-regulation of NKG2D surface expression was suppressed

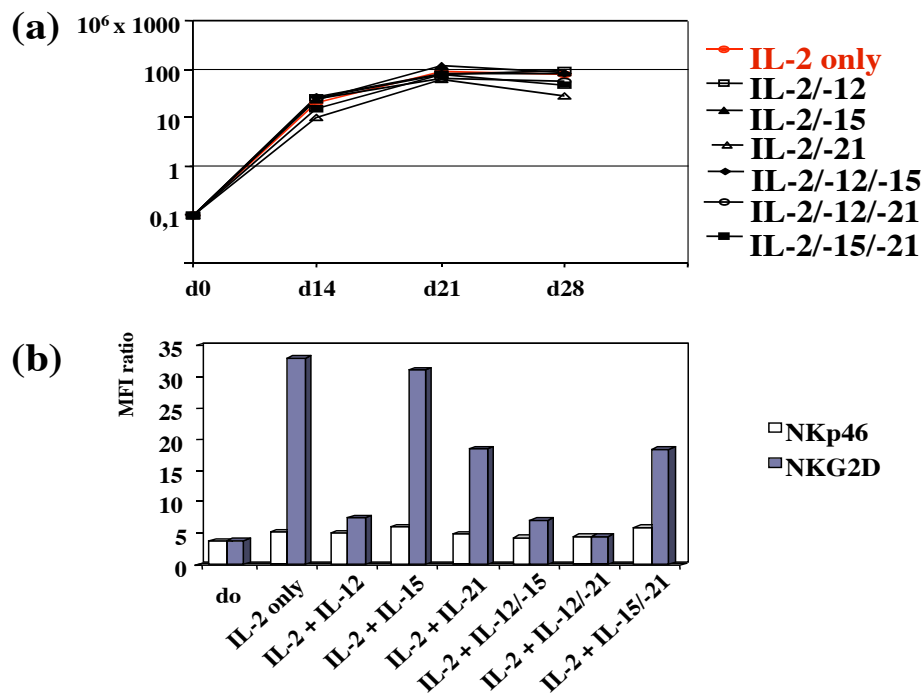


Figure 3-4: Expansion rates and receptor expression in response to different cytokines. NK cells were stimulated with phytohemagglutinin (PHA; 1 μ g/ml) IL-2 (100U/ml) and irradiated allogeneic PBMCs in the presence of the indicated cytokines. The final cytokine concentrations were 1ng/ml for IL-12, 10ng/ml for IL-15 and 100ng/ml for IL-21, respectively. Expression levels of NKp46 and NKG2D were determined by FACS analysis as mean fluorescence ratio (MFIR) on day 14 of expansion cultures (b).

3.1.4 Functional consequences of NKG2D surface modulation

To assess whether the observed cytokine mediated upregulation or suppression of the NKG2D receptor has any influence on the cytotoxicity, we analysed the cytolytic activity of receptor-modulated NK cells against the NK-susceptible AML cell line HL60. HL60 cells express the NKG2D ligands ULBP-1, -2 and -3 as well as MIC/A and MIC/B at low levels. The ligands can be upregulated upon treatment with growth factors promoting the myeloid differentiation (such as SCF, Flt3-ligand and GM-CSF) or with the monocyte-activating cytokine IFN γ . In addition, the transcription enhancing compounds like the 5-aza-2-deoxycytidine (AZA; inhibitor of DNA methyltransferase), Trichostatin (TSA; histone deacetylase inhibitor, HDACi), Bryostatin-1 (phosphokinase-C activator) or all-trans retinoic acid (ATRA) and 1-alpha-2,5-dihydroxyvitamin-D3 (vitamin D), which are all known to induce the differentiation of myeloid progenitors or

malignant myeloblasts, were also found to induce the up-regulation of NKG2D ligands on HL60 cells (dissertation A.Rohner, 2004).

The HL60 cell line untreated and treated with Bryostatin-1 for 3 days was used in cytotoxicity assays to determine functional differences upon the modulation of NKG2D surface expression. Figure 3-5 shows the cytokine-modulated “low” and “high” NKG2D expression phenotypes on expanded NK cells isolated from the same donor (Figure 3-5a) and the upregulation of the NKG2D ligands ULBP-1, -2 and-3 on Bryostatin-1 treated HL60 cells (Figure 3-5b). In contrast to the treatment with vitamin D or IFN γ , the surface expression of MHC class I molecules was not affected and remained at initial levels (data not shown). Figures 3-6a and 3-6b show the cytolysis of untreated HL60 cells by the two different types of NK cells. At all E:T ratios the specific lysis of the “NKG2D low” NK cells was significantly below that of the “NKG2D high” NK cells, indicating that the extent of cytolysis may be NKG2D-dependent.

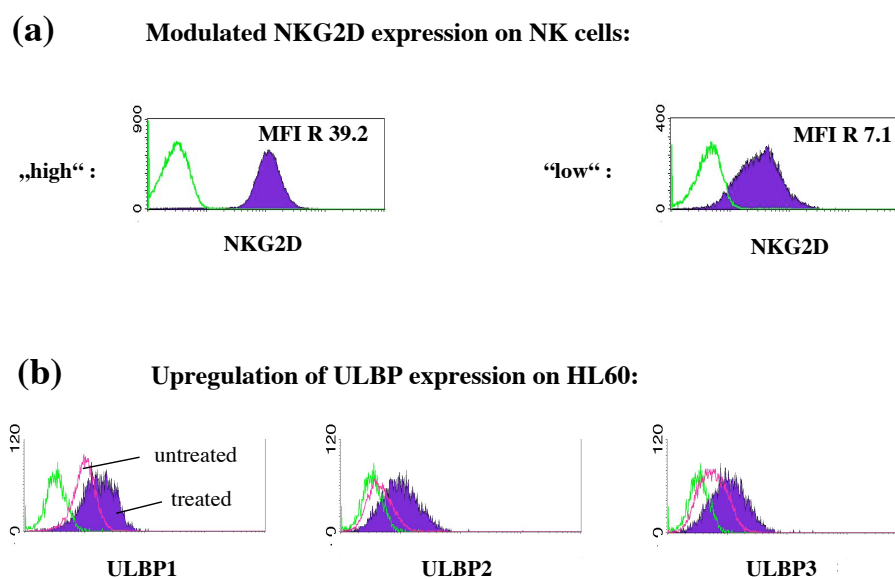


Figure 3.5: FACS analysis of the surface expression of NKG2D on cytokine-modulated NK cells. Expression levels of NKG2D (a) and the corresponding NKG2D ligands on Bryostatin-1 treated HL60 cells (b) were measured on day14 of expansion culture. The “high” NKG2D phenotype was induced by PHA-stimulation and expansion with IL-2, whereas the “low” phenotype was achieved by PHA-stimulation and expansion in the presence of the cytokines IL-2, IL-12 and IL-21 (filled histograms). Upregulation of the surface expression levels of the NKG2D ligands ULBP-1, -2, and -3 on HL60 cells was induced by the treatment with Bryostatin-1 for 3 days. The thin line represents the background level of the ligands (“untreated”) whereas the filled areas show the ligand expression upon treatment (“treated”).

To test this hypothesis, we blocked the NKG2D-ligand interaction by the addition of anti-ULBP and anti-NKG2D mAbs. A slight decrease of the cytotoxicity by the “NKG2D low” but not by “NKG2D high” NK cells was observed (Figure 3-6b). This implies that the “NKG2D high” phenotype characterizes an activation status with an almost maximal NK cell activity that might be mediated by the concerted activation of several different receptor-ligand interactions. In contrast, the “NKG2D low” phenotype may characterize an activation status with an overall low cytotoxic activity that might be enhanced by the upregulation of receptor ligands. Indeed, Bryostatatin-1 treatment of HL60 target cells that causes the upregulation of NKG2D ligands resulted in a clear increase in cytotoxicity by the “NKG2D low” NK cells at most E:T ratios, while the cytotoxic potential of the NKG2D “high” effectors was only marginally enhanced.

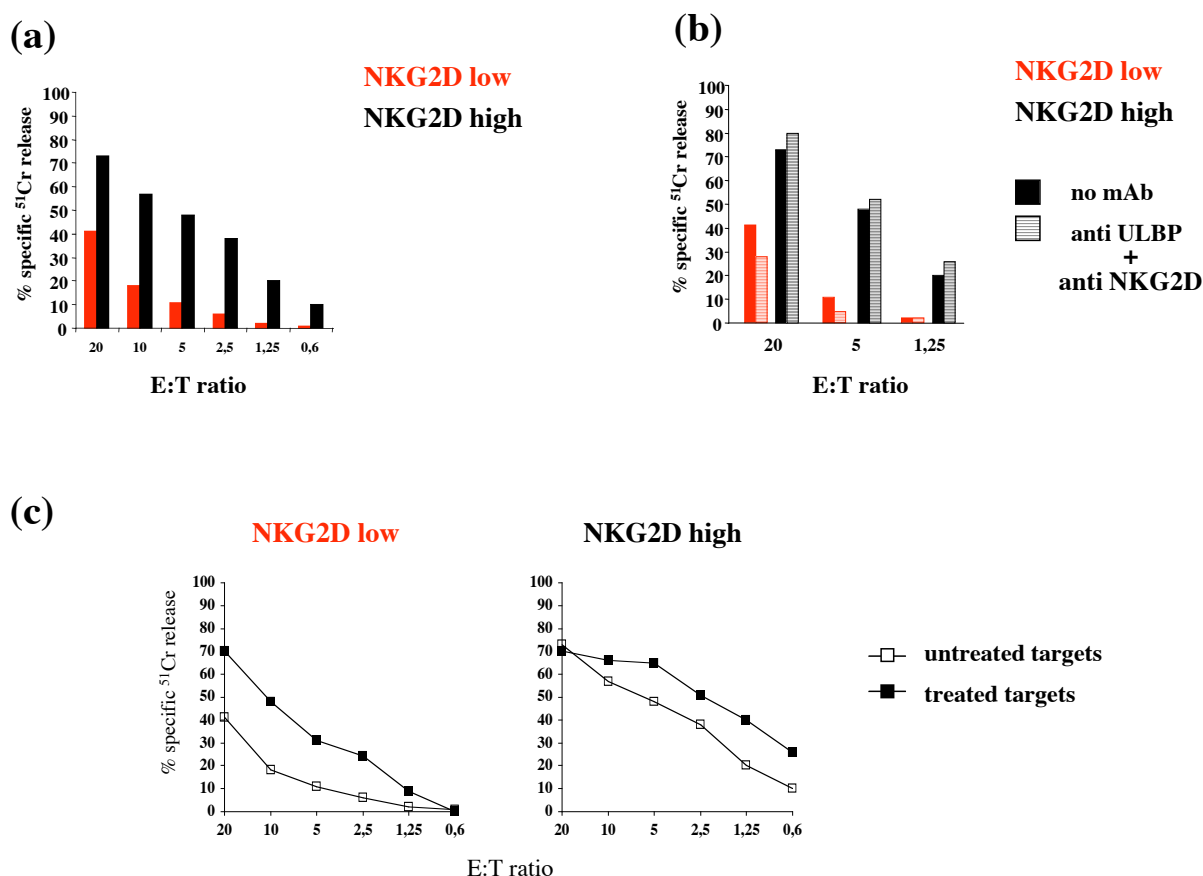


Figure 3-6: Cytotoxic activity against the NK sensitive target cell line HL60. In (a) the specific lysis of untreated HL60 targets by NK cells with “low” and “high” NKG2D surface expression is shown. NK cells were used on day14 of expansion cultures for the analysis in ⁵¹chromium release assays. Effect upon the addition of NKG2D blocking mAbs (anti-ULBP-1,-2 and anti-NKG2D; each 10µg/ml) at the indicated E:T ratios is shown in (b). The graphs in (c) illustrate the cytotoxic activity of the two types of NK cells against Bryostatatin-1 treated (filled symbols) compared to untreated HL60 targets (open symbols).

Taken together, the 60 to 70% specific lysis of HL60 by “NKG2D high” NK cells may represent the maximal inducible lysis and, more importantly, NK cells having a low NKG2D surface expression can induce an almost maximal killing only if a sufficient amount of ligands is present on the target cells. These ligands can be upregulated resulting in an enhancement of the susceptibility to NK cell lysis. Thus, the weak cytolytic activity of cytokine-modulated NK cells is at least in parts due to the low expression of NKG2D.

3.2 Lentiviral transduction of peripheral blood NK cells

3.2.1 Transduction with single-gene and bicistronic transfer vectors

As described for NK92 and NKL (see 2.2), single-gene or bicistronic lentiviral vectors were used to transduce primary peripheral blood NK cells. The transduction efficiency of stimulated and *in vitro* expanded PB NK cells with the single-gene vector pLox-GFP on day 3 to 7 post infection was as low as observed with NK92 or NKL ($2,2 \pm 1,0\%$; Table 3-1). Transduction with the bicistronic transfer vectors showed high initial rates of gene transfer which were most likely due to “pseudotransduction” since transgene expression rapidly declined within the first 7 days post infection to levels achieved with the single-gene vectors (from $35,3 \pm 16,7\%$ to $3,2 \pm 1,5\%$). As expected, the mean expression level of GFP was approximately one log higher after transduction with the single gene vectors than with bicistronic vectors containing GFP as a downstream gene with the IRES sequence as the starting site of translation.

Figure 3-7 shows the typical FACS analysis results for the transduction of *in vitro* expanded PB NK cells. 2×10^5 NK cells were transduced at an MOI of 20-30 on day 14 to 21 of expansion culture. Since NK cells stop to proliferate between day 14 to 21 in expansion cultures the cells were restimulated immediately after the 4 hours of infection or on day 6 post infection. For both types of transfer vectors FACS analysis on day 14 post restimulation revealed that the percentages of transduced cells were equally ($5,2 \pm 1,4\%$ vs. $4,6 \pm 2,6\%$) and maintained through the process of expansion at the levels measured before (Table 3-1).

Results

	GFP+		
	day 3 post infection	day 7 post infection	day 14 post restimulation
pLox-GFP (n=4)	2,2 ±1,0%	n.d.	5,2 ±1,4%
pWP-IRES-GFP (all constructs; n= 7)	35,3 ±16,7%	3,2 ±1,5%	4,6 ±2,6%

Table 3-1: Transduction efficiencies of expanded polyclonal primary NK cells. GFP expression was determined by FACS analysis at the indicated time points. PHA and IL-2 expanded PB NK cells were transduced on day14 to 21 of expansion culture at an MOI of 20-30. Transduced NK cells were repeatedly stimulated with PHA/IL-2 either on day 7 post infection or immediately after the 4 hours of infection. Mean expression levels of GFP are shown ± SD.

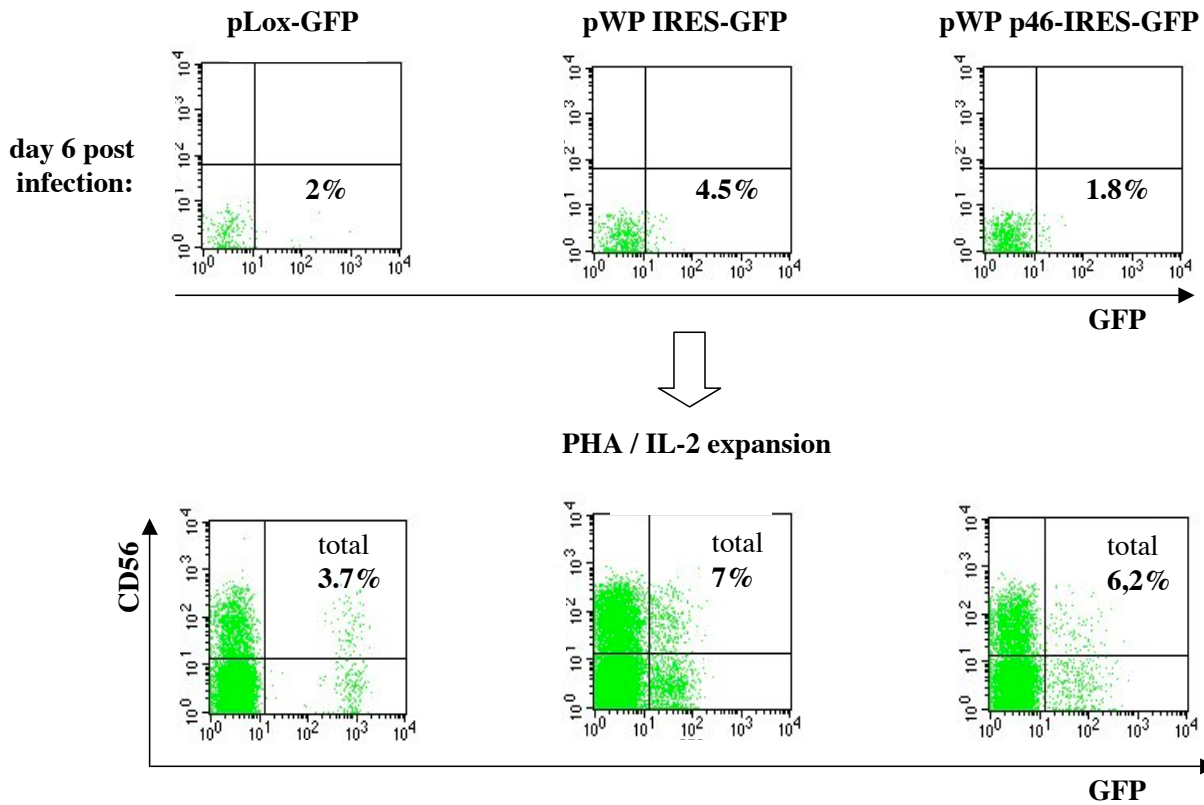


Figure 3-7: Transduction and propagation of *in vitro* expanded primary peripheral blood NK cells. PHA/IL-2 expanded polyclonal NK cell lines were transduced at day14 to 21 of expansion culture. 2x10⁵ NK cells were transduced at an MOI of 20-30 and restimulated with PHA and IL-2 on day 6 post infection. FACS analysis was performed on day 14 post restimulation.

In contrast, the transduction of freshly isolated peripheral blood NK cells resulted in higher efficiencies compared to those obtained with PHA/IL-2 expanded cells. PB NK cells were isolated from cryopreserved (ficolled) blood samples, stimulated for 2 days in IL-2 containing medium and transduced with concentrated virus. Figure 3-8 illustrates the transduction of freshly isolated NK cells with the bicistronic vector pWP-NKp46-IRES-GFP at an MOI of 20. Transduction efficiency was measured on day 4 post infection and NK cells were PHA/IL-2 stimulated 3 days later. FACS analysis on day 14 post stimulation showed an overall efficiency of about 25,2% GFP+ cells with 30,1% of NK cells transduced. Since in NK cell stimulation cultures residual CD3+ T cells and NK-T cells are expanded as well, it is possible to compare the transduction efficiencies of primary CD3-CD56+ NK cells with that of primary CD3+ cells. Interestingly, the high transduction efficiency achieved with the bicistronic vector in the CD3-CD56+ NK cell population (30,1%) was significantly higher compared to the efficiency observed in the CD3+ T cell and NK-T cell subsets which was only around 7,5%.

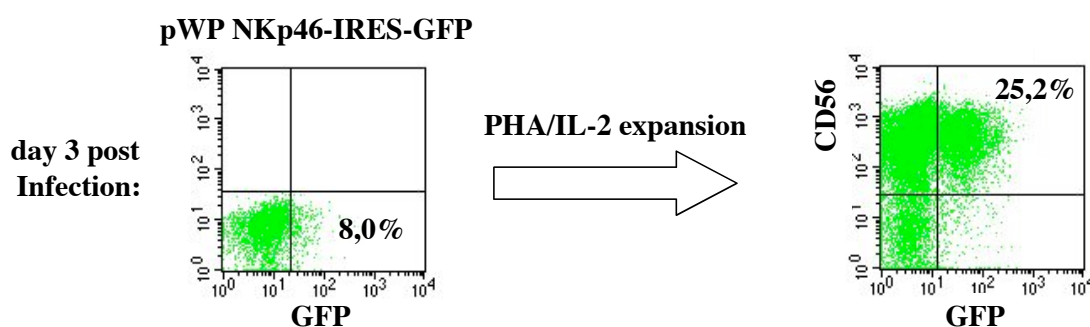


Figure 3-8: Transduction and propagation of freshly isolated primary PB NK cells. NK cells were separated from cryopreserved PB samples and stimulated for 2 days in IL-2. $2-3 \times 10^5$ NK cells were transduced with concentrated pWP-NKp46-IRES-GFP virus at an MOI of 20. NK cells were PHA/IL-2 stimulated on day3 post infection and FACS analysis of expanded NK cells was done on day14 of stimulation culture. The indicated percentages correspond to the overall content of GFP+ cells.

3.2.2 Selection of stably transduced NK cells by FACS sorting

In order to maintain and propagate the transduced CD56+CD3- NK cell population in a polyclonal NK cell expansion culture, the transgenic cells were FACS sorter purified before repeated stimulation. If the transgenic cells were not selected or enriched, the percentage of transduced cells gradually declined during the process of PHA/IL-2 expansion.

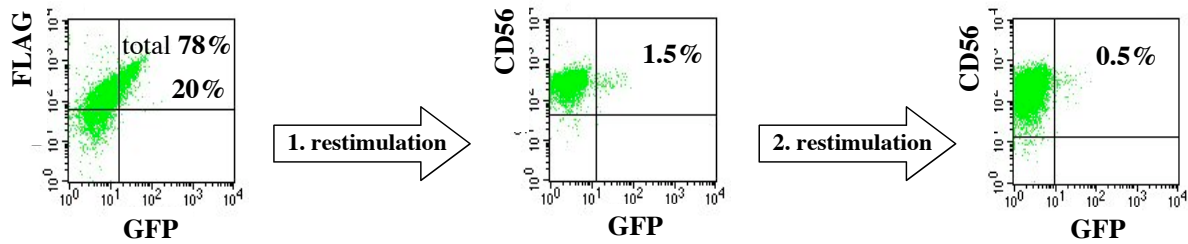
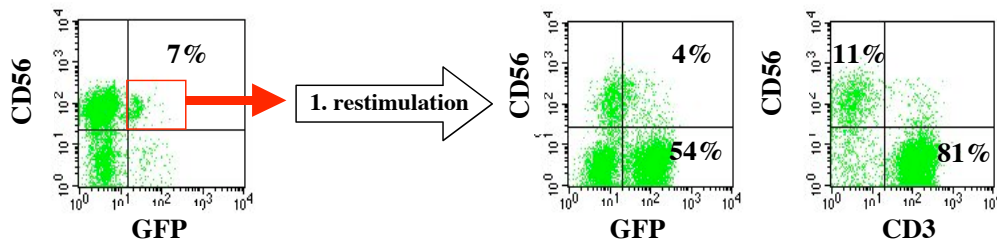
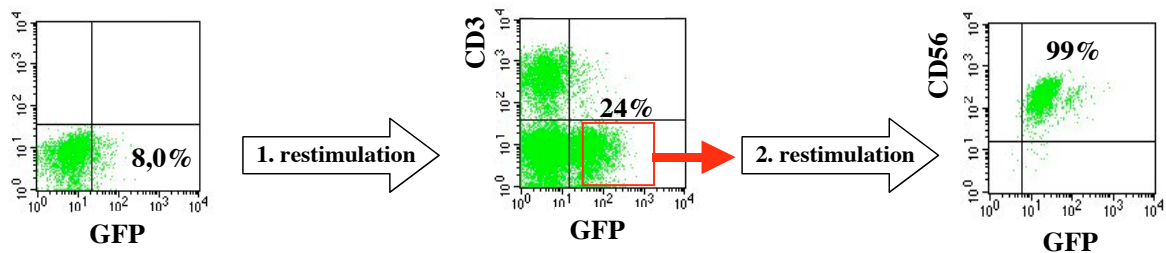
(a) Loss of transgenic cells by repeated stimulations:**(b)** No CD3 depletion: preferential expansion of CD3+ NK-T cells:**(c)** Generation of a stably transduced CD3-CD56+ NK cell population:

Figure 3-9: Transduction and maintenance of transgenic primary PB NK cells. NK cells were infected with the bicistronic pWP-NKp46-IRES-GFP vector at an MOI of 20. In (a) the transduction of *in vitro* expanded PB NK cells and the repeated PHA/IL-2 restimulation is shown. After the first expansion on day 6 p.i., the bulk population contained about 1,5% transgene expressing NK cells, which were almost lost upon the second restimulation. FACS sorter enrichment of GFP+CD56+ cells without the depletion of CD3+ cells resulted in the preferential expansion of CD3+ (NK)-T cells (b). In (c) the expansion and maintenance of transduced CD3-CD56+ PB NK cells upon FACS sort and restimulation of CD3-GFP+ cells is shown.

Figure 3-9a shows the progressive loss of a transgenic NK cell population in a repeatedly stimulated expansion culture transduced with the bicistronic lentiviral vector pWP-NKp46-IRES-GFP. Typically for this vector, very high initial transduction efficiency on day 3 post infection (78% FLAG+; 20% GFP+) declined to about 1,5% on day 14 after *in vitro* expansion. This small population of GFP+ NK cells was almost lost upon the second restimulation. For the maintenance of transduced NK cell cultures it is important to ensure that the *in vitro* cultures are depleted of CD3+ NK-T and NK cells before the repeated stimulation.

The depletion is necessary since CD3⁺ cells preferentially expand under these conditions and overgrow the CD3-CD56⁺ NK cells. The FACS sort of CD56⁺ cells including the CD3⁺ NK-T and NK cells with the subsequent restimulation is shown in Figure 3-9b. This particular sort for transgenic cells on day 14 post restimulation yielded about 60% GFP⁺ cells, which mainly comprised T cells (>80%) and contained only 11% CD3-CD56⁺ NK cells.

An example for the enrichment of a transgenic CD3-CD56⁺ NK cell population by FACS-sorting is shown in Figure 3-9c. In this experiment freshly isolated and IL-2 activated PB NK cells were transduced with the bicistronic pWP-NKp46-IRES-GFP vector and restimulated on day 6 post infection. The content of GFP⁺ NK cells on day 14 of expansion culture was around 24%, which allowed an efficient separation of transduced cells. The FACS sort of CD3-CD56⁺GFP⁺ NK cells with the subsequent restimulation resulted in a stably transduced population of NK cells with a proportion of GFP⁺ cells that was almost 100%. Figure 3-10 shows the analysis of the surface expression levels of NKp46 on these transgenic NK cells on day 14 after the second (upon the FACS sort) and third restimulation, respectively.

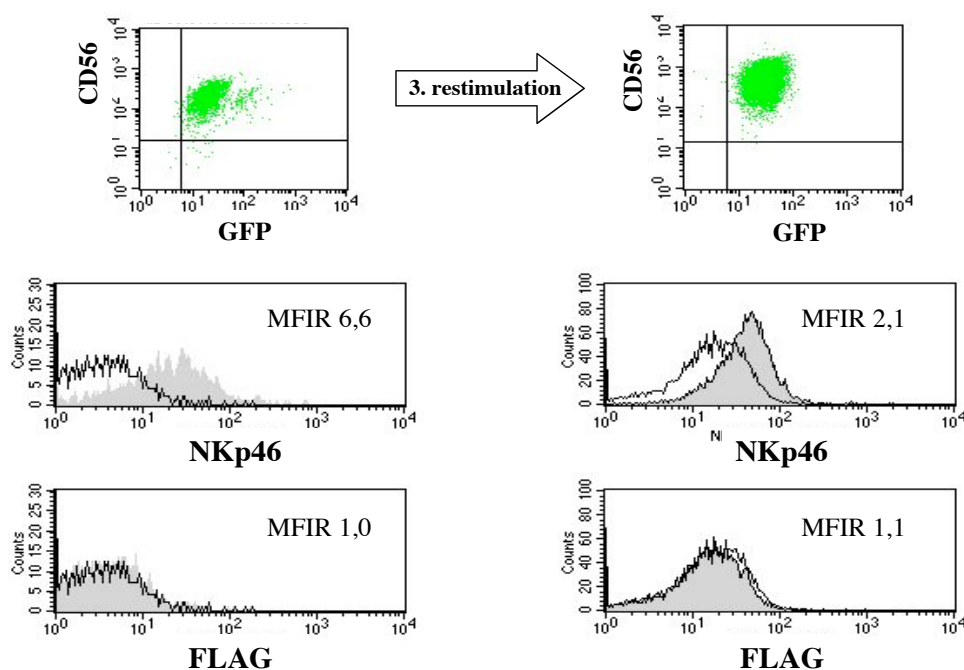


Figure 3-10: NKp46 surface expression on transgenic NK cells upon repeated in vitro expansion. NK cells were transduced with concentrated pWP-NKp46-IRES-GFP virus at an MOI of 20, PHA/IL-2 stimulated on day 6 post infection, FACS sorted for GFP⁺ NK cells and repeatedly stimulated for a second and third time. NKp46 expression was determined by FACS analysis on day 14 of each stimulation procedure and is indicated as the mean fluorescence intensity ratio (MFIR). The open histograms represent the staining with the secondary reagent only (GaM IgG1-PE).

At both time points the NK cells homogenously expressed the GFP marker and even after the third restimulation RT-PCR on total RNA clearly revealed the presence of lentivirus derived mRNA for exogenous NKp46 (data not shown). However, the transgenic cells not only were negative for the surface expression of exogenous NKp46 (as indicated by the absence of FLAG staining) but also showed a decrease in endogenous NKp46 to low levels (MFIR of 2,1).

Thus, it remains to be elucidated whether the observed down regulation of endogenous NKp46 in turn influenced the expression of the transgenic receptor through post-transcriptional mechanisms or whether the lentiviral vector derived mRNA may interfere with the endogenous NKp46 messenger in a way that only low levels of endogenous NKp46 are present on the cell surface.

3.2.3 Transient transductions: restoration of down-modulated NKp46 surface expression

In order to obtain a sufficient amount of NK cells transgenic for NKp46 to perform functional assays, we took advantage of the high rates of pseudo-transduction observed with the bicistronic lentiviral vectors. As mentioned in section 3.2.1, infections with concentrated virus of the bicistronic pWP-constructs resulted in high transduction efficiencies on day 2 post infection. This efficient transgene expression was only transient, since the percentage of transgenic cells rapidly declined within 8 days after infection (see Table 3-1). High pseudo-transduction was achieved even upon the infection of 3×10^6 NK cells at an MOI of 1-2 for 8 hours.

To assess any potential functional differences due to the expression of the transgenic NKp46 receptor, we transduced repeatedly *in vitro* expanded primary PB NK cells, which lost their NKp46 surface expression (see 3.1.2). Figure 3-11 illustrates the transient transduction of primary PB NK cells that displayed a practically complete down-modulation of NKp46 surface expression upon three consecutive PHA/IL-2 stimulations. FACS analysis two days post infection revealed a high expression of exogenous NKp46 with about 78% of the cells positive for the FLAG-tag and more than 85% of the cells positive for the receptor, corresponding to an MFI ratio of 7,2 (versus 1,4 for the not transduced control cells; Figure 3-11a,b). Like in all transductions performed with the pWP-vectors, this high transgene expression was not stable and NKp46 expression declined to background levels on day 7 to 8 post infection as shown in Figure 3-11c.

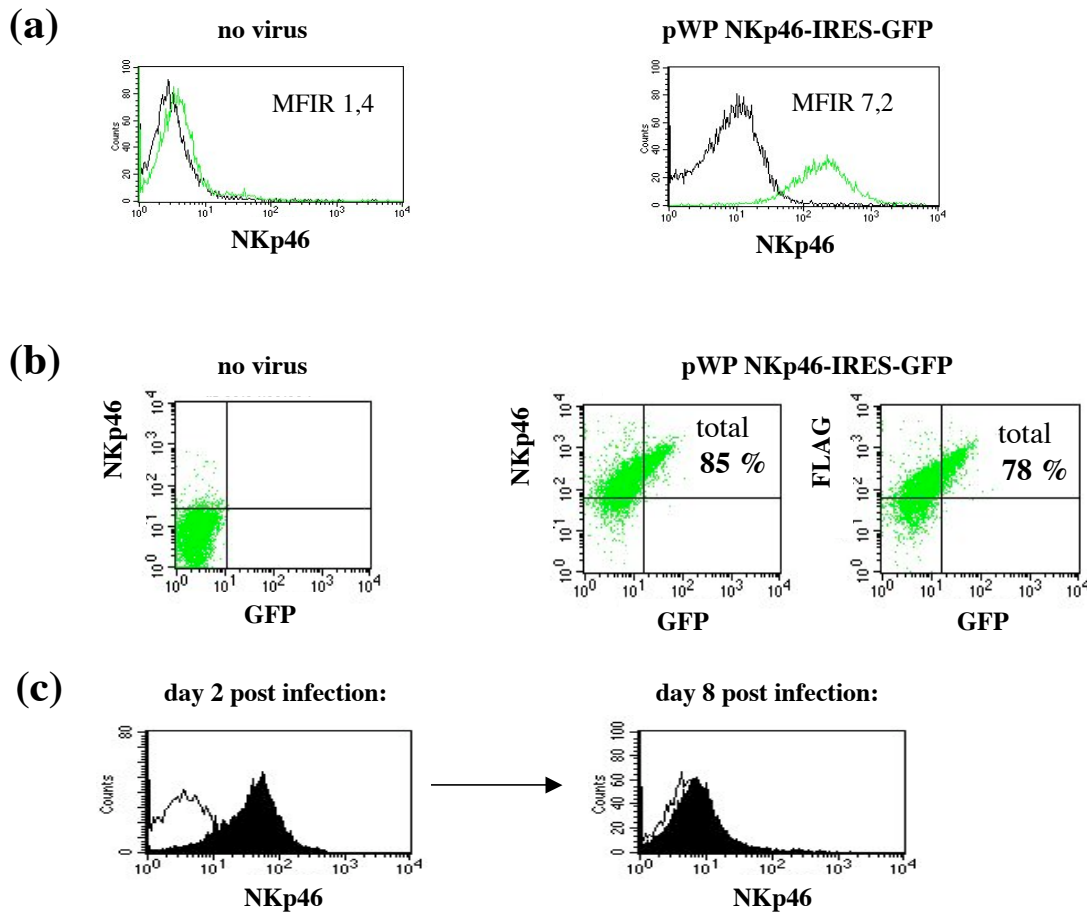


Figure 3-11: Transient expression of NKp46 in PHA/IL-2 expanded PB NK cells. High “pseudo-transduction” was achieved by the transduction of 3×10^6 NK cells with concentrated virus of the lentiviral vector pWP-NKp46-IRES-GFP at an MOI of 1-2 in a total volume of $500 \mu\text{l}$ (a). FACS analysis of NKp46 surface expression on day 2 post infection of untransduced (no virus) and virus transduced NK cells as mean fluorescence intensity ratio (MFIR; a) or as percentages of positive cells (b). Surface expression of transgenic NKp46 declined within 8 day to background levels (c). Black histograms represent the NKp46 staining, whereas open histograms show the secondary reagent (GaM IgG1) only.

Thus, transductions with the bicistronic pWP-vectors result in high short-term transgene expression levels and represent an efficient approach to generate transgenic NK cells suitable for functional assays.

3.2.4 Functional consequences upon restoration of NKp46 surface expression

We first analysed if the lentivirus-mediated restoration of NKp46 surface expression has any influence on the cytotoxic activity in general, using the NK cell sensitive target cell line K562 in ^{51}Cr -release cytotoxicity assays. Therefore, PHA/IL-2 expanded primary PB NK cells with a down-modulated NKp46 surface expression were “short-term” transduced with the lentiviral transfer vector pWP-NKp46-IRES-GFP. Figure 3-12a shows the surface expression levels of NKp46 on the NK cells before and after the lentiviral infection. In this particular experiment the relatively low surface expression of NKp46 (MFI ratio 2,3) could be increased to an intermediate level with an MFI ratio of 9,7. As shown in Figure 3-12b the high NKp46 expression did not result in any detectable differences in the cytolytic activity against K562 targets. Both NK cell populations displayed a high activity that was in a normal range with a specific lysis of up to 80% at the upper effector to target ratios (E:T ratio). This result shows that the virus infection per se did not interfere with the cytolytic capacity of NK cells.

The high and NKp46-independent cytotoxicity was confirmed by the finding that the K562 target cell line we used did not express the ligands either for NKp46, nor for the other NCRs NKp44 and NKp30 (as determined by the use of recombinant soluble NCR receptors-dimers; sol p30/44/46) or the activating receptor NKG2D (data not shown).

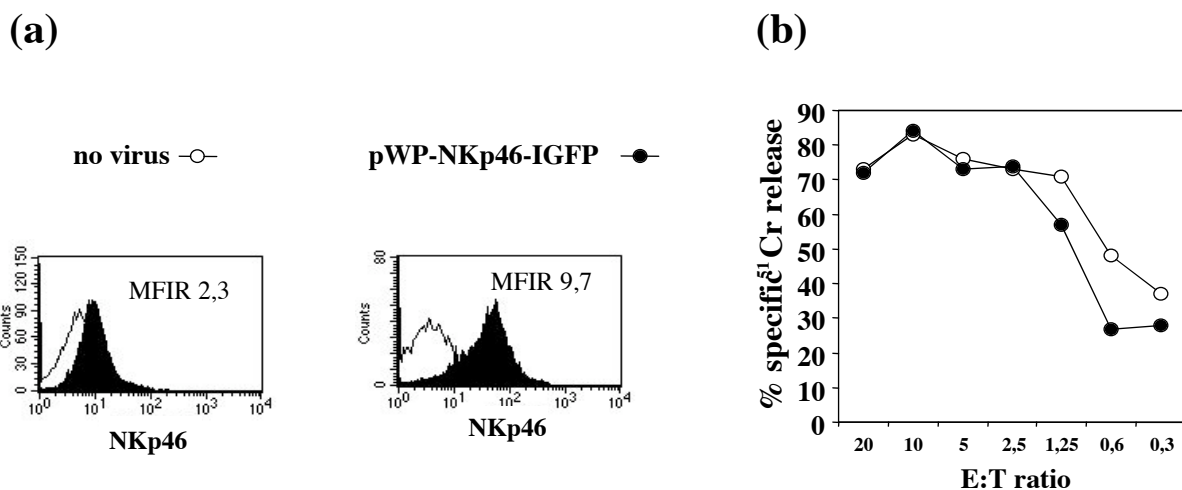


Figure 3-12: Cytolytic activity against the NK sensitive target cell line K562. PHA/IL-2 expanded primary PB NK cells that had a down-modulated NKp46 expression were transduced with concentrated pWP NKp46-IRES-GFP virus at an MOI of 1-2 on day 14 of expansion culture. FACS analysis of NKp46 expression was done on day 2 post infection (a). The specific lysis of K562 target cells was measured in standard 4 hrs ^{51}Cr release assays (b).

Next, we performed ^{51}Cr -release cytotoxicity assays with the THP-1 target cell line, which expresses ULBP-1 and -3, the ligands for NKG2D at substantial levels and is highly positive for the ligands for NKp30, NKp46 and NKp44 (Figure 3-13a). The cytolytic activity of untransduced and transduced NK cells against THP-1 cell is shown in Figure 3-13b. For both NK cell populations the specific lysis was below that against the K562 targets and was comparable to the lysis measured with freshly isolated and IL-2 activated control PB NK cells. No major difference in the cytolytic potential was detectable for the transduced NK cells in comparison to the untransduced cells. Since THP-1 target cells are positive for the NKp46 ligand(s) this strongly indicates the possibility that the transgenic NKp46 receptor is not capable to recognize the corresponding ligand(s) or to trigger an activating signal that could result in an enhanced cytolysis.

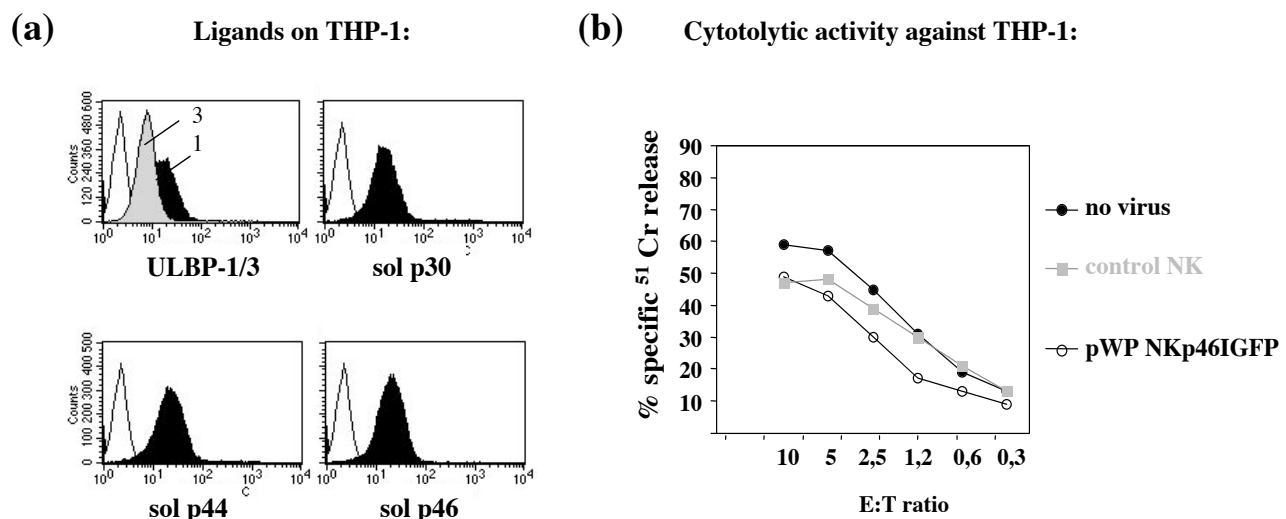


Figure 3-13: Cytolytic activity against the NK sensitive target cell line THP-1. THP-1 expresses ULBP-1 and -3, the ligands for NKG2D as well as the ligands for NKp30, NKp46 and NKp44 (a) as measured by the use of recombinant receptor proteins (soluble receptors-dimers; sol p30/44/46). In (b) the cytolytic activity of virus transduced vs. untransduced PB NK cells (see Figure 3.11) compared to the lytic activity of freshly isolated IL-2 stimulated (for 3 days) PB NK cells is shown.

As demonstrated in section 2.3.3, hybridoma supernatant of the anti-NKp46 mAb (clone 9E2) showed an “agonistic” capacity and could induce the cytolysis of the NK cell resistant murine target cell line P815 by normal PB NK cells or by wild type NK92 when used against the endogenous NKp46 receptor in a “redirected” way. From such “redirected” cytotoxicity assays with the transgenic NK92 cell line we already concluded that the exogenous NKp46 receptor might be inactive probably due to a deficiency in signal transduction.

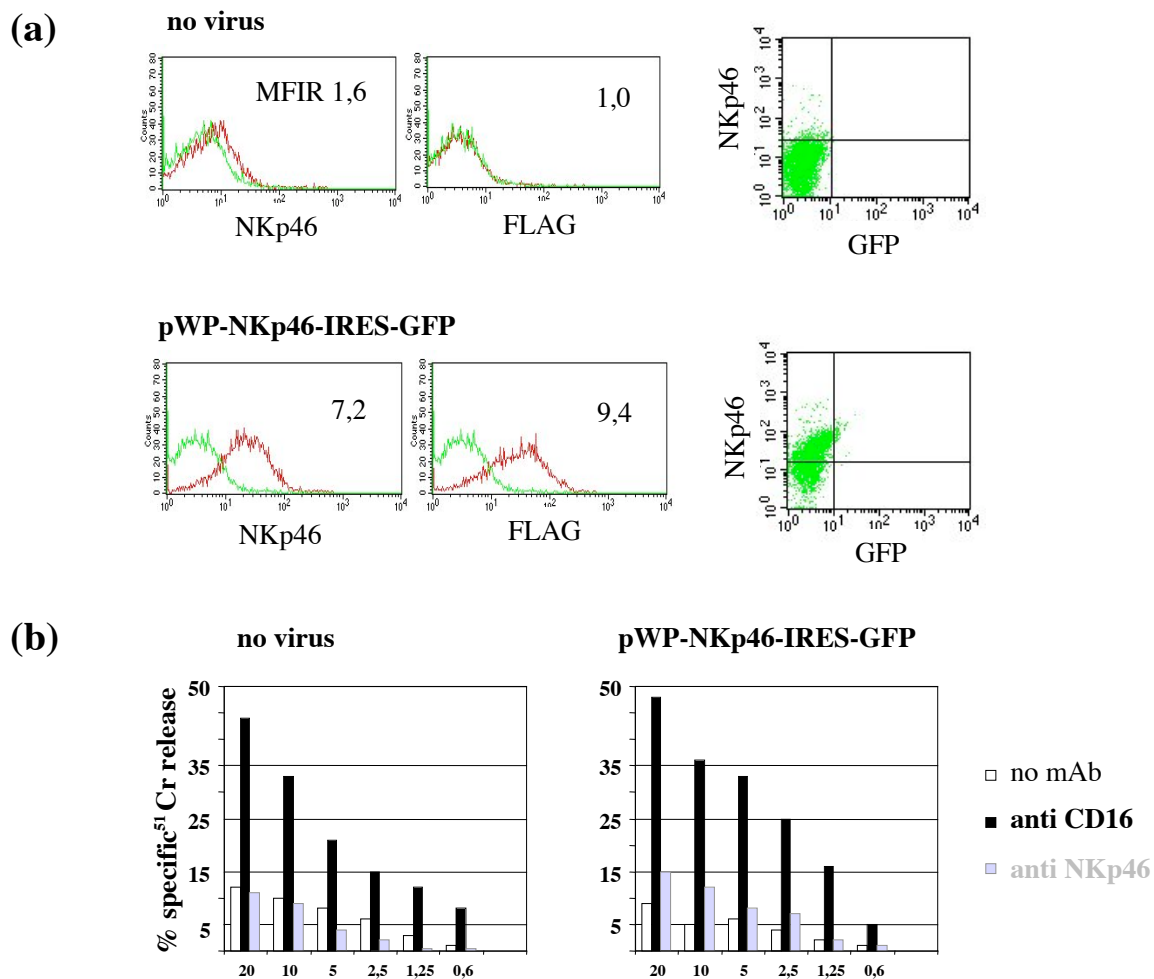


Figure 3-14: “Re-directed killing” of the murine target cell line P851. FACS analysis of the surface expression of NKp46 was done on day 2 post infection (a). The “re-directed” killing by PHA/IL-2 expanded primary PB NK cells is shown in (b). Percentage of specific lysis was measured in standard 4 hrs Cr⁵¹ release assays. The P851 target cells were pre-incubated with the indicated mAbs for 30 min before they were added to the effector cells (purified anti-CD16, anti-CD56 at final 1μg/ml; anti-NKp46 SN at 10μl/well).

Figure 3-14 illustrates the lentiviral modification of primary NK cells and the evaluation of the “redirected” cytotoxicity against the P815 target cells upon the expression of exogenous NKp46 receptor. FACS analysis on day 2 post infection (Figure 3-14a) revealed an efficient lentiviral gene transfer to the primary PB NK cells.

Untransduced NK cells were almost negative for endogenous NKp46 (MFIR 1,6) whereas the transduced cells displayed an at least 5-fold increase in the surface density of exogenous NKp46 as indicated by the MFIR of 9,4 obtained with the anti-FLAG staining.

However, this significant difference in NKp46 surface expression did not result in any substantial change in the “redirected” killing of P815 cells (Figure 3-14b). For both NK cell populations the anti-CD16 induced cytolysis as well as the background lysis without the addition of any mAbs was in the same range for most E:T ratios. As expected from the experiments performed with transgenic NK92 (section 2.3.3; Figure 2-10), the addition of anti-NKp46 mAb could not induce any cytolysis by the transduced cells that was significantly higher compared to untransduced cells or compared to background lysis. In contrast to the transgenic NK92, the primary NK cells used in this experiment were practically negative for endogenous NKp46 and thus allowed the assessment of the functionality of the transgenic NKp46 without any interference due to the expression of endogenous receptor. Therefore, we could confirm the results obtained with the transgenic NK cell line, but the results suggest that the transgenic NKp46 receptor, at least as a FLAG-tagged version, is functionally inactive.

Taken together, the high cytolytic activity against K562 and THP-1 observed with lentivirus modified NK cell populations indicates that the vector-mediated surface expression of NKp46 does not interfere in any way with the cytolytic activity of NK cells. However, since the over-expression of NKp46 did not correlate to an enhanced cytotoxicity against target cells that are positive for the NKp46 ligand(s) and, more strikingly, since the “redirected” triggering of exogenous NKp46 did not result in any significant enhancement in the cytolysis of P815 targets, we conclude that the transgenic receptor is indeed functionally inactive.

4. Genetic Modification of Cord Blood Derived Hematopoietic Precursor Cells

In the previous chapter we described the limited effectiveness in transduction of mature PB NK cells, confirming that NK cells are refractory to genetic modification. In the following a highly efficient approach of transfer to and the stable expression of the green fluorescent protein (GFP) marker gene in NK cells is described. Since functional NK cells can be differentiated from hematopoietic progenitor cells (HPCs) *in vitro* [18], this goal was achieved by the lentiviral transfer of the GFP gene to cord blood derived progenitors with the subsequent differentiation towards the NK cell lineage (see Figure 1-1). The selection of transduced GFP-expressing precursors by FACS sorting resulted in almost pure transgenic populations of NK cells after the *in vitro* differentiation. This approach was extended to the gene transfer of the natural cytotoxicity receptor NKp46 and to explore its influence on the effectiveness to recognize and lyse target cells by *in vitro* generated NK cells. For this purpose the single gene and bicistronic lentiviral vectors described in the previous chapters were used.

4.1 Transduction with single-gene transfer vectors and *in vitro* differentiation

Our first goal was to determine the effectiveness in generation of transgenic NK cells by the lentiviral transduction of CD34+ HPCs and the subsequent *in vitro* differentiation towards the NK cell lineage. We used concentrated lentivirus preparations of the single gene transfer vectors pLox-GFP and pLox-NKp46. In all transduction experiments CD34+ cord blood derived HPCs were prestimulated with “NK cell differentiation medium” (containing SCF, FL and IL-15; see Materials and Methods) for 2 to 3 days prior to exposure to the lentiviral vectors. $2-3 \times 10^5$ cells were transduced at an MOI of 20-30. Immediately after the infection cells were expanded in the “NK cell differentiation medium” for 5 to 6 weeks.

Figure 4-1 shows a typical FACS analysis of differentiation cultures of pLox-GFP transduced and untransduced CD34+ HPCs. The content of developing CD56+ NK cells and the GFP expression were determined weekly over a time period of 4 to 5 weeks. In all cultures analysed differentiation did not lead to the development of CD3+ cells (Figure 4-2, top). Gene transfer efficiency in pLox-GFP transduced cultures was on average $16,8 \pm 9,9\%$ (n= 5) as measured on day 3 post infection.

Importantly, the percentage of GFP expressing cells stayed throughout the differentiation culture at levels comparable to the initial efficiency of transduction. In the depicted differentiation culture for instance (Figure 4-1, bottom), the percentage of GFP positive cells is maintained in all cells as well as in the developing NK cell population at a level of about 20%. This indicates that the transgene expression in the transduced progenitors is stable and not affected by the process of cell differentiation.

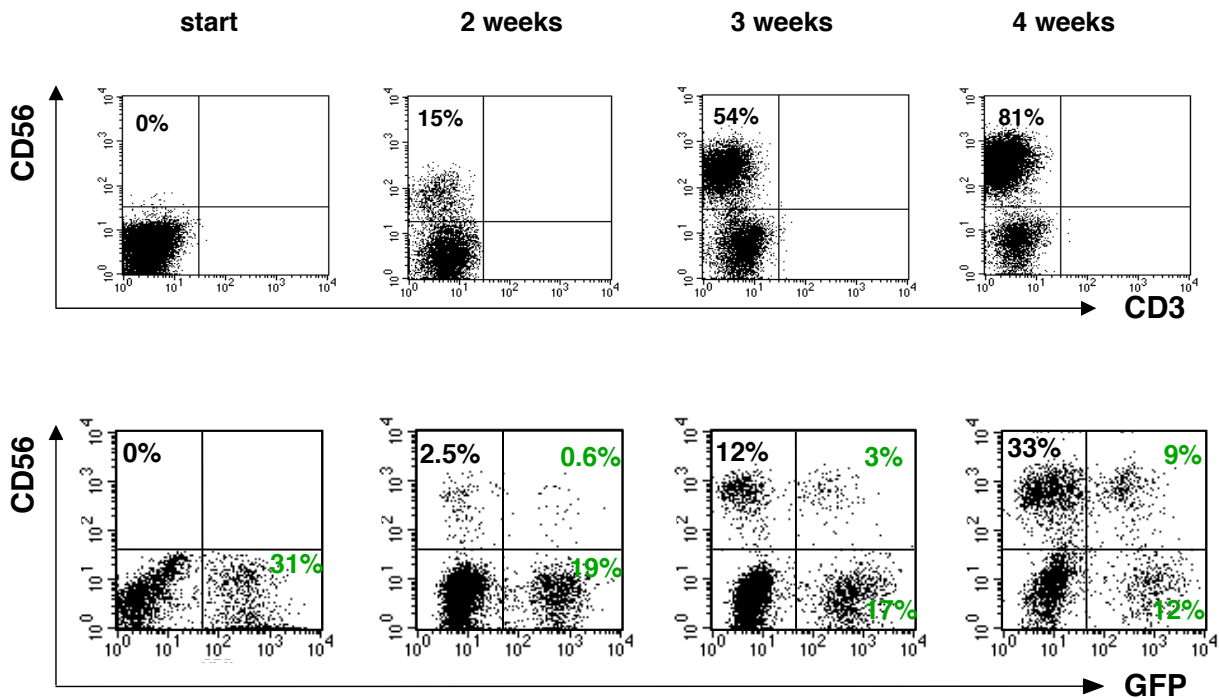


Figure 4-1: FACS analysis of two independent differentiation cultures of CB-derived hematopoietic progenitors. The upper panel shows the FACS analysis a control differentiation culture without transduction. In the lower panel a differentiation culture starting with pLox-GFP transduced progenitors is shown. Cells were prestimulated 3 days with IL-15, SCF and FL prior to infection and initial transduction efficiency was measured on day 2 post transduction. Expression of CD56, CD3 and GFP was measured before and during *in vitro* culture at week 2, 3 and 4. The percentages of cells positive for each marker are indicated in the corresponding quadrants

Transduction efficiencies achieved with the pLox-NKp46 vectors could not be determined since we were not able to detect the exogenous NKp46 receptor in the differentiation cultures by FACS analysis. This may be due to different reasons. First, at the time the transduction experiments were performed the titers of the different batches of concentrated pLox-NKp46 lentivirus could not be determined as done for the GFP-encoding constructs. Therefore pLox-NKp46 virus preparations were used in amounts (volumes) similar to those of pLox-GFP vectors, but possibly below the intended MOI of 20 to 30. This may have resulted in a content of transgenic cells that was below the detection level in FACS analysis which may be confirmed by the fact that transductions of the Jurkat cell line performed with concentrated virus preparations (see 2.2) resulted in efficiencies that were clearly below that of the GFP encoding vector.

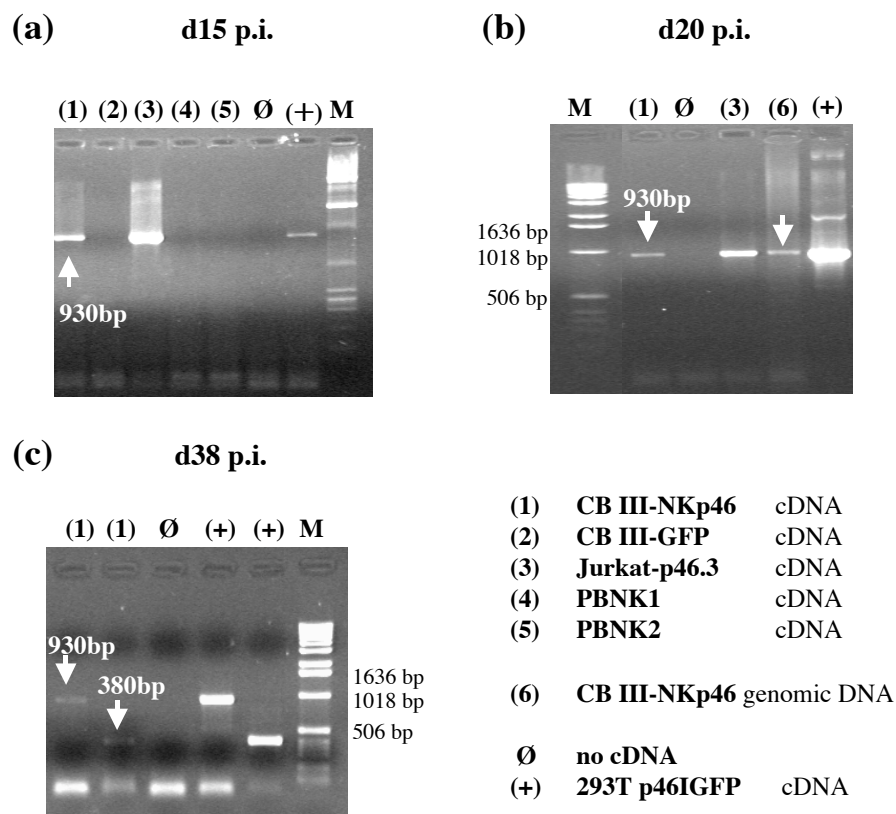


Figure 4-2: Detection of integrated vector cDNA and of exogenous NKp46 transcripts. Total cell lysates of differentiation cultures transduced with the pLox-NKp46 transfer vector were analysed (pLox-GFP transduced cultures, CBIII-GFP, served as a negative control). On the indicated days post infection (p.i.) mRNA was isolated (lanes 1 and 2) and analysed by RT-PCR. Genomic DNA was isolated on day 38 p.i. and analysed by PCR (lane 6). Arrows indicate PCR products that confirm transduction of CBIII-NKp46 culture. The control cDNA (lanes 3, 4, 5) was generated by isolation of total RNA of the indicated cells and analysed by RT-PCR. The two primer combinations used, are both specific for vector sequences (5'BamFLAG sense/Cla3'anti-sense, 930bp; 5'pLNKp46 sense/WPRE3'anti-sense, 380bp). As a positive control (+) for the primer combinations cDNA isolated from 293T cells transfected with pWP NKp46-IRES-GFP plasmid DNA was used.

Second, the expression of exogenous NKp46 receptor in the CD34+ HPCs may suppress or interfere with the development of early NK cell progenitors and thus may lead to a loss of transduced progenitors that would give rise to transgenic NK cells. Similar conclusions can be drawn from results obtained in transductions with bicistronic transfer vectors (see 4.1.4). Despite the failure to detect pLox-NKp46 transduced cells by FACS analysis in differentiation cultures, the amplification of PCR products indicates that vector cDNA has integrated in the progenitors. In independent differentiation cultures transcripts of exogenous NKp46 could be confirmed by RT-PCR on mRNA and the integration of the transgene was confirmed by PCR on genomic DNA. Figure 4-2 shows the PCR detection of exogenous NKp46-transcripts (Figure 4-3a,c) and of vector cDNA integrated to genomic DNA (Figure 4-3b). Moreover, RT-PCR on mRNA isolated on day 38 post infection indicates the persistent transcription of the exogenous NKp46 transgene throughout the process of differentiation as it was observed in pLox-GFP transduced cultures.

4.2 Restimulation of *in vitro* generated transduced NK cells

The *in vitro* differentiation towards the NK cell lineage is accompanied by an intensive proliferation and expansion to high cell numbers. Table 4-1 summarizes the average content of CD56+ NK cells and the average overall cell expansion of *in vitro* differentiation cultures. Within 4 weeks the content of CD56+ NK cells reaches an average of $41,2 \pm 18,7\%$ of total cells, with a range of 12-81%.

	day0	week 1	week 2	week 3	week 4	week 5
% CD56+ (n=10) (range)	--	1,5±1,2 (0,5-5)	6,9±3,8 (0,7-16,7)	16,9±13,7 (4-64)	41,2±18,7 (12-81)	52,6±23,3 (21-94,5)
fold expansion (n=4)	--	65±24	164±76	208±57	347±96	n.d.
calculated abs. cell numbers	2×10^5	13×10^6	$32,8 \times 10^6$	$4,16 \times 10^7$	$6,94 \times 10^7$	n.d.
estimated abs. numbers CD56+	--	$1,9 \times 10^5$	$2,3 \times 10^6$	$7,1 \times 10^6$	$28,5 \times 10^6$	

Table 4-1: Average content of CD56+ NK cells and the average total cell expansion rates. Two measured in independent CB CD34+ NK cell differentiation cultures were initiated with $2-3 \times 10^5$ progenitors. The average content of developing NK cells and expansion rates were determined based on the analysis of vector transduced and non-transduced differentiation cultures.

Together with an average expansion rate of 347 ± 96 fold and a starting population of about 2×10^5 progenitor cells one can calculate the total cell number in differentiation cultures after 4 weeks to roughly 7×10^7 cells. Thus, the estimated output of NK cells within such a differentiation culture can be estimated to at least 3×10^6 cells after 4 weeks of expansion.

In order to propagate and to further expand the in vitro generated NK cells, the differentiation cultures can be restimulated as described for primary PB NK cells (see 3.1.1). Restimulations were performed as early as at week 2 of in vitro culture. Despite the NK cell content at this time point is usually below 20% (Table 4-1) restimulations resulted in the expansion of almost pure populations of CD56+CD3- NK cells on day 14 to 21 (see Figure 4-4).

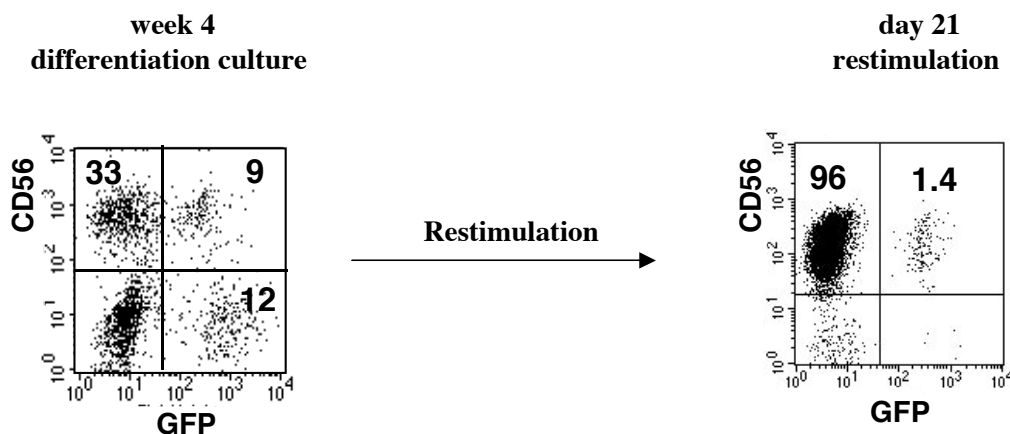


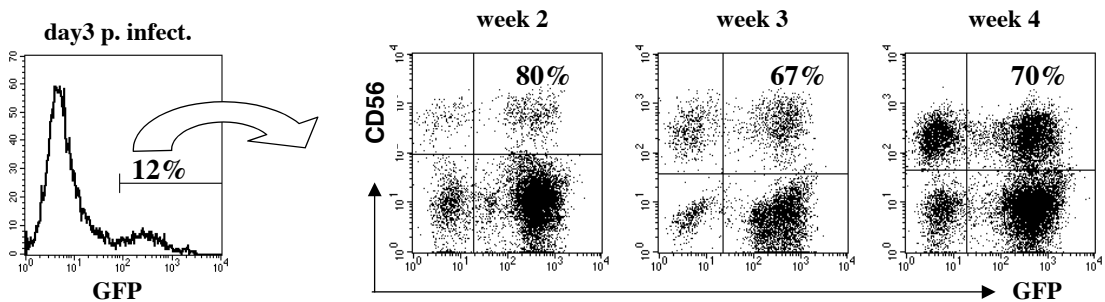
Figure 4-4: Loss of transgenic NK cells in differentiation cultures after restimulation. At week 4 of differentiation culture $1.5\text{-}2 \times 10^6$ cells were PHA/IL-2 stimulated as a “bulk” unseparated population. The percentages of cells positive for each marker are indicated in the corresponding quadrants. The content of CD3+ cells was usually below 3% (not shown). The content of transgene expressing cells dropped from initially 21% to 1.4% after stimulation.

However, in lentivirus transduced differentiation cultures the expansion of CD56+CD3- NK cells was as efficient as for untransduced cultures, but PHA/IL-2 stimulation lead to the loss of transgene expressing cells. Figure 4-4 illustrates a differentiation culture transduced with the pLox-GFP vector and restimulated at week 4 post infection. The content of NK cells in this particular differentiation culture was 42% with about 20% of cells positive for the GFP marker. Restimulation resulted in the expansion of the CD56+CD3- NK cells to a purity of more than 97%, but the transgene expressing NK cells were almost all lost (1.4%), indicating that the process of restimulation preferentially expanded the non-transduced NK cells.

Thus, as already concluded for transduced primary PB NK cells (see 3.2.2), the restimulation of transgenic NK cell populations needs the separation or at least the enrichment of transgenic cells prior to restimulation to avoid the loss of transduced cells during expansion. To reach this goal, we purified transgenic NK cells by FACS based cell sorting.

In a first step we purified the transduced progenitor cells by FACS 2 days after the lentiviral transduction. In Figure 4-5 the *in vitro* differentiation of FACS sorted pLox-GFP transduced CD34+ progenitor cells is shown. The sort of a 12% population of GFP+ progenitors on day 3 after lentiviral infection resulted in the enrichment of transduced cells to 70-80% at week 4 (Figure 4-5a).

(a) FACS sort and *in vitro* differentiation



(b) FACS sort and restimulation

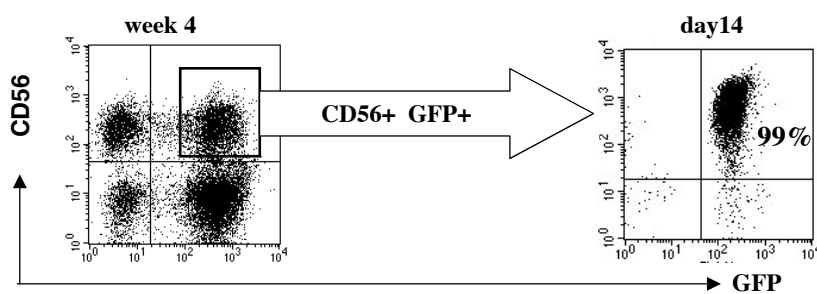


Figure 4-5: FACS based enrichment of transgenic precursor cells after lentiviral infection. Developing NK cells were separated and expanded during *in vitro* differentiation. Cord blood derived CD34+ HPCs were transduced with the pLox-GFP vector at an MOI of 20. On day 3 post infection GFP+ progenitors were FACS sorted and subjected to *in vitro* differentiation (a). The indicated percentages represent the content of GFP+ cells within the developing NK cell population. The content of CD56+ NK cells was 7, 24 and 40% at week 2, 3 and 4, respectively. Figure (b) illustrates the FACS sort of transgenic NK cells at week 4 and the subsequent expansion. FACS analysis on day 14 upon expansion is shown.

This content of transgenic cells was maintained in all subpopulations during *in vitro* differentiation and thus 70% of the developing NK cells were transgenic for the GFP marker. In a second step, the generated NK cells were sorted at week 4 to obtain a pure population of transgene expressing cells. At that time point the overall content of NK cells was about 40% that allowed an efficient FACS sort of transgenic NK cells. The subsequent restimulation of the sorted cells lead to the expansion of a nearly 100% GFP+ NK cell population (Figure 4-5b).

In conclusion, the FACS based enrichment of transduced progenitor cells combined with a second purification step of transduced NK cells during development represents a highly efficient approach in the generation of genetically modified NK cells. The early sort of transduced progenitor cells significantly enhances the output of transgenic NK cells in terms of absolute numbers, which in turn enhances the effectiveness of the subsequent cell purification for PHA/IL-2 expansion. Thus, this approach does not only allow to use a relatively low number of progenitor cells with a maximal output, but also helps to reduce the consumption of lentivirus, the preparation of which to high titers is representing one of the limiting factors in retroviral gene transfer technology.

4.3 Transduction with bicistronic transfer vectors

Figure 4-6 illustrates the transduction of CB derived CD34+ cells with the bicistronic vectors pWP-IRES-GFP and pWP-NKp46-IRES-GFP. As observed for the infection of primary PB NK cells (see 3.2.1), the transduction efficiencies measured on day 3 post infection reached very high levels in CD34+ cells but dropped during the *in vitro* differentiation to below 10% GFP+ cells after 2 to 3 weeks (see Figure 4-4). This effect was observed for both vectors and it remains to be elucidated whether this decrease is due the failure of vector integration or whether the loss of transgene expression is due to an ineffective or even suppressed transcriptional activity. Although the overall differentiation towards the NK cell lineage in cultures transduced with the bicistronic constructs seemed to be unaffected, there is evidence that the transgene is transcriptional inactive in the developing NK cells. Figure 4-7 shows the FACS based analysis of the NK cell content and GFP expression in the NK cell populations of differentiation cultures transduced with the pWP-IRES-GFP and pWP-NKp46-IRES-GFP vectors.

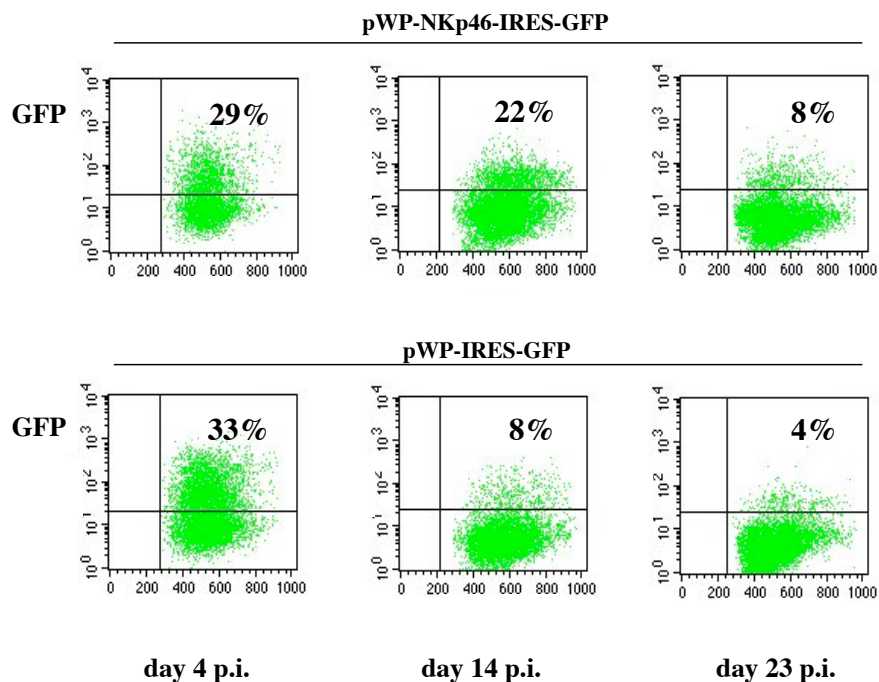


Figure 4-6: Progressive loss of transgene expression. Differentiation cultures of cord blood derived CD34+ HPCs transduced with bicistronic pWP-NKp46-IRES-GFP transfer vector (top panel) and the corresponding control vector pWP-IRES-GFP (lower panel). FACS analysis of GFP expression was done at the indicated time points post infection (p.i.).

The content of *in vitro* generated NK cells is comparable for both bicistronic constructs reaching 70 to 94% at week 4 to 5 (Figure 4-7a) similar to cultures transduced with the single gene vectors (see Table 4.1). But in contrast to differentiation cultures initiated from pLox-GFP transduced progenitors, the percentage of transgenic NK cells was dramatically reduced in cultures transduced with bicistronic vectors. As shown for the pWP-IRES-GFP vector, a roughly 10-fold reduction in the content of transgene expressing NK cells was observed when compared to the overall content of GFP+ cells or to that of the residual (non-NK) cells (Figure 4-7b). In pLox-GFP transduced cultures the percentage of transgene expressing NK cells did not differ significantly from the overall content of GFP+ cells.

The pWP-constructs contain the (intron-less) short form of the EF-1 alpha promoter (see section 1.1), which may explain different expression levels of the transgene in different cell populations. Since stable transduction of mature PB NK cells with the bicistronic pWP-NKp46-IRES-GFP vector was only successful for freshly isolated IL-2 activated but not for *in vitro* expanded cells (see 3.2.2), it is possible that the “EF-1alpha short” promoter is transcriptional inactive in not terminally differentiated, developing NK cells.

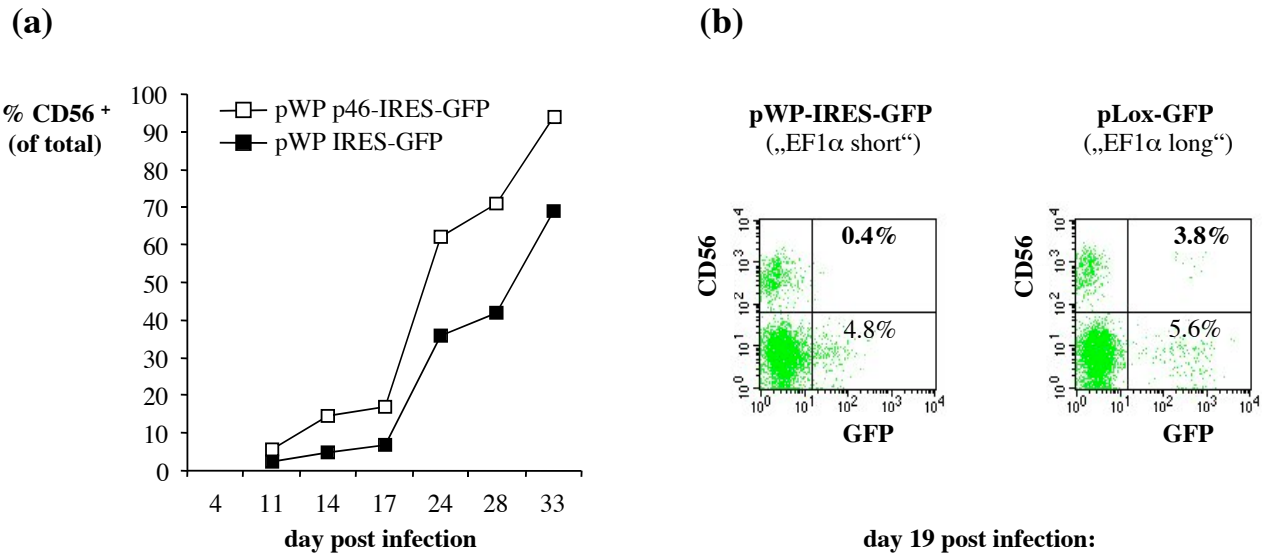


Figure 4-7: NK cell content and GFP expression in the NK cell populations of differentiation cultures. CB derived CD34+ HSCs were transduced with the vectors pWP-IRES-GFP, pWP-NKp46-IRES-GFP and pLox-GFP, respectively. Content of CD56+ NK cells during in vitro differentiation culture is shown in (a). In (b) the percentages of GFP+ NK cells in pWP-IRES-GFP transduced cultures is compared to the content of transgenic NK cells in pLox-GFP transduced cultures.

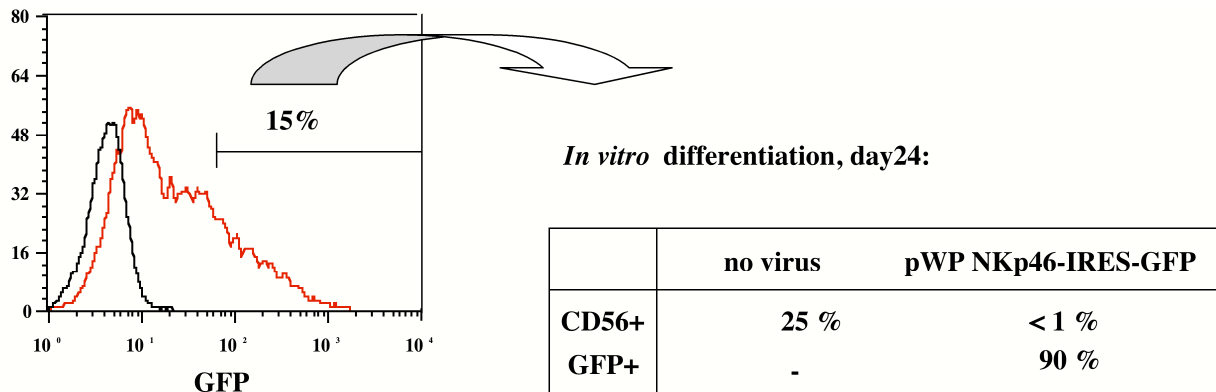


Figure 4-8: FACS sorting of transgenic precursor cells and the subsequent *in vitro* differentiation. The upper 15% of GFP+ progenitors transduced with the pWP NKp46-IRES-GFP vector were sorted on day 3 post infection. At day 24 of in vitro differentiation the content of NK cells was 25% in the control culture that was not transduced, whereas in the FACS sorted and GFP+ differentiation culture the content of CD56+ NK cells was below 1%.

This hypothesis cannot fully explain the fact that the decrease in transgene expression is preferentially observed in the developing NK cell population. If transcription is not suppressed in mature NK cells and assuming that *in vitro* generated NK cells acquire a full state of maturation, one would expect the detection of transgenic cells at later time points during the process of differentiation. Thus, it cannot be ruled out, that the exogenous NKp46 receptor interferes with the development of early NK cell progenitors leading to the loss of transgenic cells.

Indeed, differentiation cultures that were FACS sorted for transgenic precursors early after lentiviral infection contained more than 90% transgenic GFP+ cells after a culture period of 3 weeks. In contrast to the untransduced control culture starting from the same CD34+ progenitors that comprised of 24% NK cells, the content of NK cells in the transduced differentiation culture was below 1% (Figure 4-8). This strongly indicates that the expression of exogenous NKp46 receptor may have a suppressive or even a toxic effect on early NK cell precursors. In the case of a pure transgenic progenitor population the differentiation towards NK cells may be totally suppressed resulting in the complete failure of NK cell development.

V. RESULTS

(B) THE CYTOTOXIC POTENTIAL OF NK CELLS FROM AML PATIENTS

5. Isolation and Expansion of NK cells from AML patients

5.1 Patient cohort, NK cell isolation and *in vitro* expansion

Peripheral blood NK cells were isolated from patients with newly diagnosed or relapsed acute myeloid leukemia (AML). 14 patients were included into the study, 12 newly diagnosed and 2 patients with recurrent leukemia from which 1 relapsed after allogeneic stem cell transplantation. All PB samples were taken before chemotherapeutic intervention. Table 5-1 summarizes the main characteristics of the selected patients. Diagnosis and the division into AML FAB-subtypes of M1 to M7 were based on morphologic, cytogenetic and immuno-phenotypic criteria (determined by the Hämatologie Labor, University Hospital Basel). The main immuno-phenotypic lineage markers in AML are CD33, CD117, CD15 and CD65 for the myeloid, CD61, CD41 (CD42b) for the megakaryocytic and CD14, CD11b, CD64 for monocytic lineage.

A highly reliable way to gate on leukemic blasts in flowcytometry is the use of the pan-leukocyte marker CD45, which is expressed at low levels (CD45dim) in all leukemic blasts and almost absent on some ALL and megakaryocytic AML. Myeloblasts in PB, BM and other body liquids are phenotypically identical [63]. The content of myeloblasts in PB samples was on average 37,4% per total MNCs. The CD45bright population was defined as the compartment of residual healthy cells (see Figure 5-1). The content of CD56+CD3- NK cells was below 1% of mononuclear cells (%NK per MNC) and of total leukocytes (%NK per total). These values significantly differed from the NK cell contents measured per MNCs in PB of healthy donors (2,6 vs. 7,0%), corresponding to a reduction that was at least 2,5-fold. For all 14 patients depicted in Table 5-1 NK cells were separated using the magnetic immuno-bead (“MACS-beads”) technology in a procedure that combined the depletion of blasts and residual healthy non-NK cells with the positive selection of CD56+ NK cells. The separations were all done from thawed cryopreserved samples starting with 2-20 x10⁷ MNCs.

In a first step a cocktail of lineage marker specific immuno-beads was used to mainly deplete for B- and T lymphocytes and cells of the myeloid lineages (see “Materials and Methods”). By this initial depletion we were able to enrich the CD3-CD56+ NK cell subset in the PB samples to 2-10% of total. Depletion of CD3+ NK-T and T cells was almost complete in all separations (95-99% CD3-).

patient characteristics								healthy donors		
patient	age	sex	FAB-type	cytogenetic abnormalities	% blasts per MNCs	%NK per MNCs	%NK per total		%NK per MNCs	%NK per total
BeT	68	m	2 nd OMP	?	78.2	0.7	0.1	BS	5.5	1.6
BT#	57	m	M5b; relapsed	+8; t(2;11), t(3;9)	92.9	0.7	nd	CA	8.6	2.8
CC	43	f	M5	?	93.5	nd	nd	FC	7.6	3.9
CL	67	f	M2	?	73.5	1.4	1.2	FA	1.2	0.3
DC	52	f	M4	?	37.7	2.1	1.7	KC	7.1	2.8
SH	73	f	M1	-	84.3	0.3	0.3	CV	5.2	1.8
SC	69	f	M2	+4; del9q; del11q	88.3	0.3	0.3	NP	3.2	1.4
RM	86	f	2 nd MDS	?	55.4	1.0	0.6	RA	20.3	6.7
SR	74	m	M1; relapsed	?	58.9	(10.9	5.2)	SG	3.4	1.7
EA	68	f	M4	+8	76.5	0.4	0.4	SU	7.3	2.5
FE	37	f	M4	?	74.8	0.3	nd	SS	7.8	2.9
MU	46	f	M5b	?	37.2	nd	nd			
SF	54	m	M5a	-	73.1	0.8	nd			
SD	62	f	2 nd M4 (CML)	-	93.4	0.2	nd			
median	64,5			mean	37,4	0,7	0,6		7.0	2.6
				StDev	19,4	0,6	0,5		5,0	1.7
				range	37,2-93,5	0,2-2,1	0,1-1,7		1.2-20.3	0.3-6.7
				n	14	11	7		11	11

Table 5-1: AML Patient characteristics and NK cell content in peripheral blood samples of patients compared to healthy donors. FACS analysis was done on thawed samples of cryopreserved (ficolled) MNCs or on whole blood of freshly isolated PB. Patient BT# relapsed after allogeneic transplantation; thus NK cells are of donor origin. Values of NK cell content for patient SR were excluded since the blasts of this patient expressed the CD56 NK cell marker.

This was absolute crucial since CD3+ cells preferentially expand during the subsequent *in vitro* expansion of separated NK cells. In rare cases the initial step allowed an efficient depletion of blast cells and resulted in highly enriched NK cell preparations (> 80%).

We further tried to deplete for the myeloid blasts by the use of immuno-beads specific for CD33 or CD34 depending on the phenotype of the AML blasts. However, for not explained reasons in the majority of the performed separations these additional depletions were completely inefficient and resulted in a reduction of blast content that was only marginal. This may be simply due to the enormous amount of myeloblasts that might skew the ratio between immuno-beads and the “target cells” to levels that are below the proportions recommended for separations of normal cell subsets.

In the next step we further enriched for the NK cells by positive selection using CD56 or alternatively CD16 specific MACS beads. This two-step NK cell enrichment resulted in a purity of NK cells that ranged from 10 to 70%. However, the overall efficiency of AML-NK cell purification was further reduced due to the fact that each MACS separation step is accompanied with a considerable loss of cells. Thus, since the actual output of separated NK cells in terms of the absolute numbers was 2×10^4 - 3×10^5 , and starting from 1×10^8 cells in the beginning with an considered theoretically yield that should be about 10×10^5 , this corresponds to an overall efficiency of 2 to 30%.

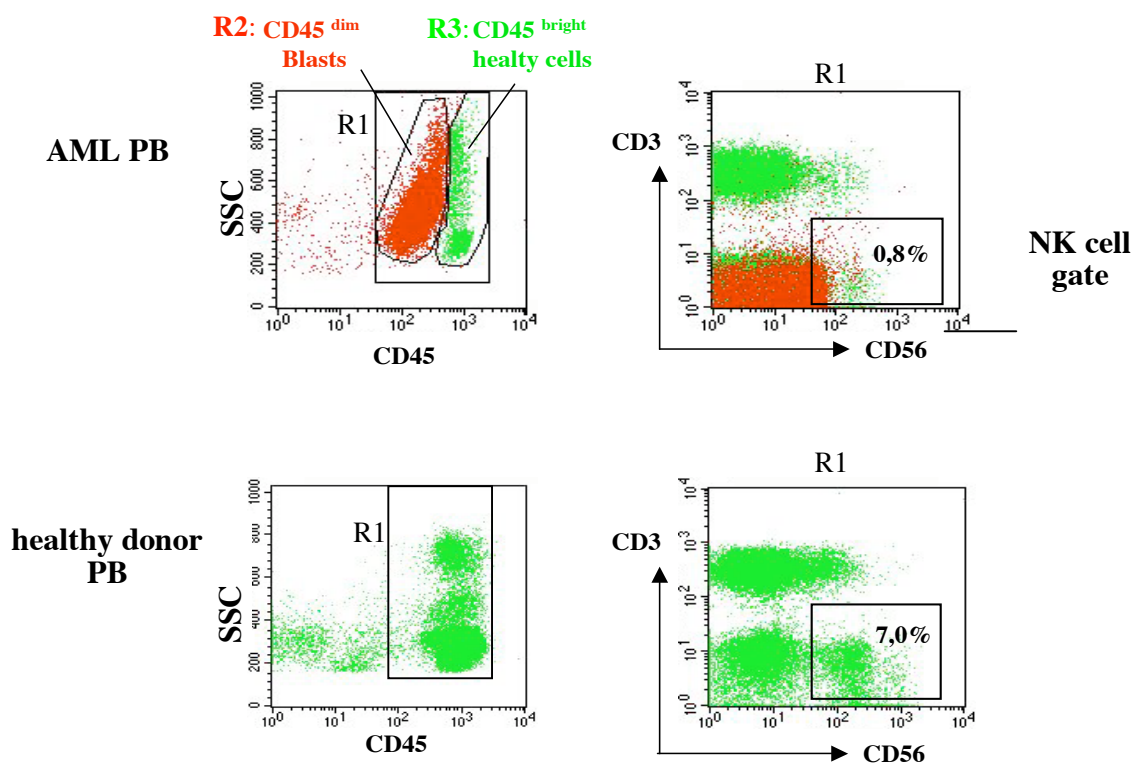


Figure 5-1: FACS analysis of the NK cell content as measured on whole blood samples. Representative examples of AML patient derived PB (top) and of PB from a normal healthy donor (bottom) are shown. R1 represents the gate on total mononuclear cells (based on FSC/SSC-plot; granulocytes excluded). the dark coloured dots represent the blasts gate R1. The percentages indicate the content of CD3-CD56+ NK cells in the “NK cell gate”.

As a consequence of the poor yield and contamination with a substantial proportion of blasts, we were not able to perform any functional assays with *ex vivo* isolated AML-NK cells. Instead, the separated cells were immediately subjected to *in vitro* expansion as described for the expansion of normal PB NK cells (see section 3.1.1). Except for two patients, all stimulations of enriched NK cell preparations resulted in the successful expansion of CD3-CD56+ NK cells.

The fact that in some cultures the proportion of CD3+CD56- T cells and CD3+CD56+ NK-T cells reached 20-30 % of total cells emphasizes the importance of CD3 depletion prior to stimulation. In contrast, the depletion of “contaminating” blasts seems to be not necessary. Even though some AML-NK cell preparations consisted of mixtures with up to 90% of blasts, these cells disappeared like the irradiated feeder cells within 14 days of culture. Figure 5-2 shows the expansion curves of AML-NK cells compared to the expansion of NK cells from normal healthy donors and illustrates the disappearance of residual blasts during *in vitro* expansion. No significant difference in the expansion potential of AML-NK cells and control NK cells was observed. In both “types” of polyclonal NK cell cultures the cells start to proliferate between day 6 to 8 and expand within the next 10 to 14 days of culture. The plateau of cell expansion is reached between day 14 and 21. Cell counts on day 14, 21 and 28 revealed an expansion rate that was on average 2000 ± 790 fold-increase in cell numbers for AML-NK cells and about 750 ± 195 fold-increase for control NK cells.

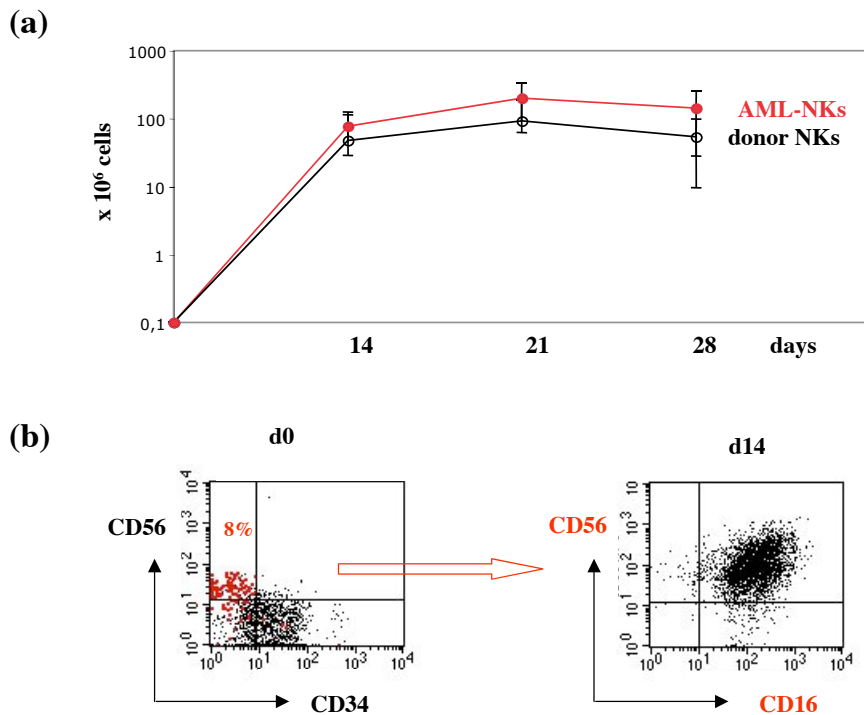


Figure 5-2: *In vitro* expansion of separated AML-NK cells. MACS separated PB AML-NK cells (2×10^4 - 3×10^5) were stimulated with PHA/IL-2 in the presence of 2×10^6 irradiated allogeneic PBMNCs. In (a) the cell expansion of AML-NK cells is compared to the expansion of normal healthy donor derived PB NK. Cell counts were done on the indicated time points. The cell expansion is “normalized” for 1×10^5 cells. The expansion of AML-NK cells as a mixture of NK cells and residual leukemic blasts is exemplified in (b). Blast content of more than 90% did not influence the expansion of as few as 8% NK cells (red highlighted) to pure NK cell populations within 14 days of culture.

5.2 Phenotypic characterization of expanded polyclonal NK cell cultures

PHA stimulated NK cells were phenotypically characterized during expansion on day 14, 21 and 28 by FACS analysis for the cell surface expression of different NK cell and lymphocyte markers. Cells were stained for the NK cell specific marker CD56 and CD16, for CD3, the activation marker CD69 and for the activating receptors NKp46 and NKG2D. The NK cells were also stained for the inhibitory receptors CD158a, CD158b, NKB1, CD161 and for CD94. Figure 5-3 summarizes the FACS results for the analysed surface markers. No significant difference between AML-NK cells and control NK cells was found. Moreover, as observed for the expansion of PB NK cells from normal healthy donors (see section 3.1.1) surface expression levels of the inhibitory KIRs, of CD161, and of CD94 were unchanged during *in vitro* expansion of AML-NK cells, while the activation marker CD69 was upregulated when compared to freshly isolated AML-NK cells (data not shown). Accordingly, all expanded AML-NK cells acquired a CD56^{bright}/CD16^{bright} phenotype upon expansion.

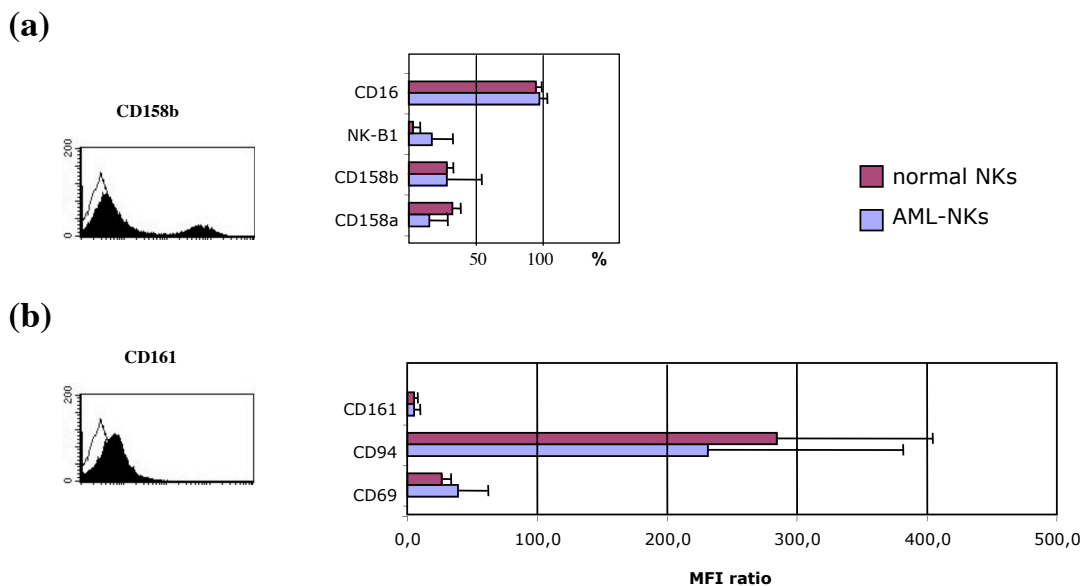


Figure 5-3: FACS analysis of the surface expression of activating and inhibitory NK cell receptors on stimulated NK cells. Freshly isolated cells were stimulated and analysed on day 14 to 21 of expansion culture. In (a) the expression levels of the activating receptor CD16 and the inhibitory receptors CD158a/b and NK-B1 is shown. As indicated by the histogram for CD158a, the polyclonal NK cell populations consisted of negative and positive cells and thus the percentages for the positive cells are indicated. In (b) the inhibitory receptor CD161, the NK cell receptor associated CD94 and the lymphocyte activation marker CD69 is shown. Here the MFI ratios are indicated since for these markers the populations stained homogeneously. Open histograms represent the isotype staining.

The two NK cell subsets that can be distinguished based on the differential expression of CD56 and CD16 were found in freshly isolated AML-NK cells (data not shown) but disappeared upon *in vitro* expansion (Figure 5-2). FACS analysis results of the surface expression of the activating receptors NKp46 and NKG2D in AML-NK cells and healthy control NK cells are summarized in Table 5-3 and Figure 5-4. Expression levels were measured at the day of NK cell separation (“freshly isolated”) and between day 14 to 28 during *in vitro* expansion (“PHA expanded”/ “stimulated”). The MFI ratios of NKp46 surface expression stayed at the initial “*ex vivo*” levels during expansion of AML-NK cells (6,9 “stimulated” vs. 7,0 “*ex vivo*”) and of the control NK cells (8,7 vs. 6,8). Importantly, the absolute mean values of expression were at an equal level for both (Figure 5-3, left). In contrast, the surface expression of NKG2D was strongly upregulated in response to the restimulation process in AML-NK cells as well as in NK cells from healthy controls (Figure 5-3, right). In accordance to NKp46, the absolute expression levels of NKG2D as measured “*ex vivo*” or during the expansion were almost identical for both “types” of NK cells (9,8 vs. 27,0 for AML-NK cells; and 10,0 vs. 27,1 for control NK cells).

freshly isolated NK cells					
Patient	NKp46	NKG2D	healthy donors	NKp46	NKG2D
AG	6.0	17.5	BS	10.9	11.7
BH	6.4	nd	CA	6.1	10.6
BeT	6.1	18.0	FC	nd	6.9
BT#	7.5	9.6	FA	7.9	17.2
CA	5.2	nd	KC	4.2	8.6
CL	6.5	nd	KR	nd	nd
DR	14.0	9.9	NP	8.8	11.2
DC	2.9	2.9	RA	6.7	8.8
EA	8.7	8.6	SG	4.9	6.1
FE	6.3	5.3	SU	5.7	9.1
HI	6.3	10.7	SS	nd	8.2
JJ	11.5	11.0	CV	6.2	11.4
KA	7.9	nd			
LE	nd	6.9			
OE	nd	7.2			
RA	4.1	8.5			
RM	3.0	2.7			
SH	7.3	7.3			
SC	nd	6.2			
SR	5.2	2.7			
SJ	11.1	10.6			
WG	6.8	11.2			
ZC	3.4	nd			
ZP	7.2	18.2			
Mean	7.0	9.8		6.8	10.0
StDev	3.09	4.59		2.1	3.0
Range	2.9-14.0	2.7-18.2		4.2-10.9	6.1-17.2
n	18	16		9	11

PHA expanded NK cells					
patient	NKp46	NKG2D	healthy donor	NKp46	NKG2D
BeT	3,5	24,3	I	7,4	23,2
BT#	10,8	37,0	III	10,5	25,5
CC	2,3	32,3	IV	2,7	22,9
CL	2,2	13,3	VI	2,6	19,6
DC	10,3	23,8	VII	5,2	33,0
SH	5,1	26,4	VIII	23,6	38,5
SC	13,5	34,3			
RM	15,2	27,3			
SR	2,2	19,7			
EA	8,7	25,0			
FE	8,0	27,9			
MU	nd	nd			
SF	5,8	30,2			
SD	4,6	23,5			
Mean	6,9	27,0		8,7	27,1
StDev	4,3	6,2		7,2	15,4
Range	2,2-15,2	13,3-37,0		2,6-23,6	19,6-38,5
n	13	13		6	6

Table 5-2: FACS analysis results of the surface expression of NKp46 and NKG2D on AML-NK cells and on NK cells from healthy donors. Expression levels are shown as the MFI ratio at the day of NK cell separation (left side) or as the average of MFI ratios between day14 and day28 upon *in vitro* expansion (right side). The highlighted AML patients on the left side correspond to the patients on the right side from which NK cells were isolated and *in vitro* expanded. *In vivo*- and expansion-values for healthy control NK cells were determined on different individuals.

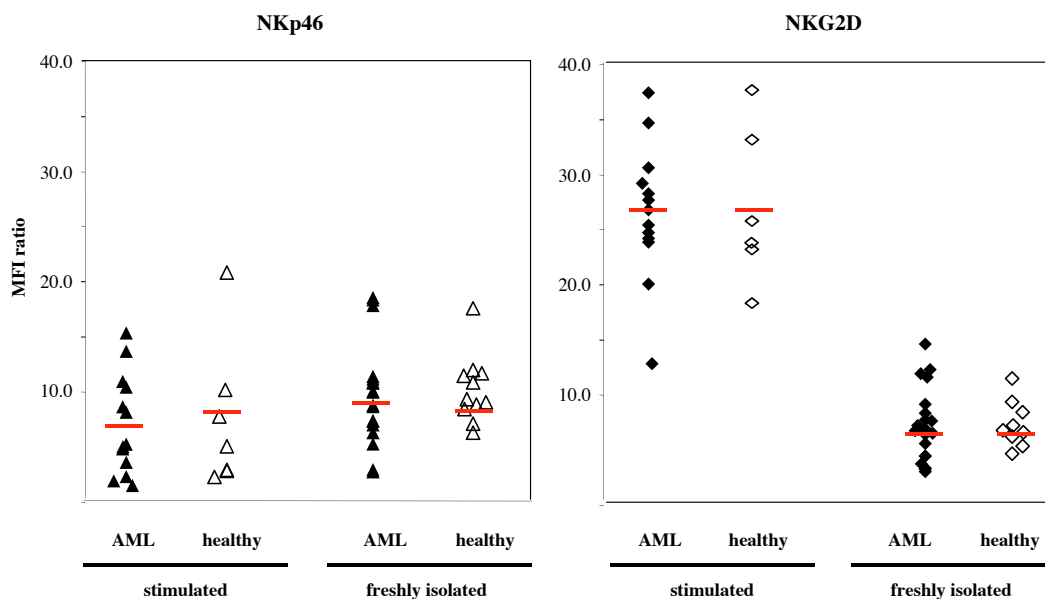


Figure 5-3: FACS analysis of the of activating receptors NKp46 and NKG2D. Surface expression on AML-NK cells (filled symbols) compared to NK cells from healthy donors (open symbols). Expression levels are shown as the MFI ratio at the day of NK cell separation (“freshly isolated”) or as the average of MFI ratios between day14 and day28 upon *in vitro* expansion (“stimulated”).

Taken together, AML-NK cells display a proliferative capacity that is analogous to or even higher than that of healthy donor derived NK cells. AML-NK cells also do not differ from healthy donor derived control NK cells, in terms of surface marker expression including the expression of activating and inhibitory receptors.

6. Functional characterization of AML-NK cells

In addition to the phenotypic characterization of *in vitro* expanded AML-NK cells we determined their functional properties compared to expanded healthy donor derived PB NK cells.

Functional activity of NK cells can be defined by their ability to lyse virus infected or transformed cells and by the capacity to produce a variety of immunoregulatory cytokines. Thus, in the following the potential of expanded AML-NK cells for the production of IFN- γ and the *in vitro* cytotoxicity against different target cells is described.

6.1 IFN- γ production upon IL-12/-18 stimulation

To assess the potential of *in vitro* expanded AML-NK cells to produce IFN- γ , we stimulated the cells for 36 hours with the cytokines IL-12 and IL-18 which are known to induce the release of IFN- γ by NK cells [2]. The cells were stimulated between day 14 and 21 during *in vitro* expansion and the proportion of IFN- γ producing cells was measured by intracellular FACS analysis. Figure 6-1 shows the results obtained for AML-NK cells and healthy donor derived NK cells. As a control NK cells were incubated with IL-2 alone or in the absence of any cytokines. The proportion of IFN- γ producing cells among AML-NK and donor NK cells without cytokine stimulation or with IL-2 was below 1%. In contrast, stimulation with IL-12 and IL-18 resulted in the IFN- γ production by $26,1\pm 13,2\%$ of AML-NK cells and $22,7\pm 8,6\%$ of the healthy donor derived NK cells.

From this we conclude that AML-NK cells are functional in terms of IFN- γ production and do not differ from NK cells isolated from healthy donors since they respond equally well to the stimulation with the cytokines IL-12 and IL-18.

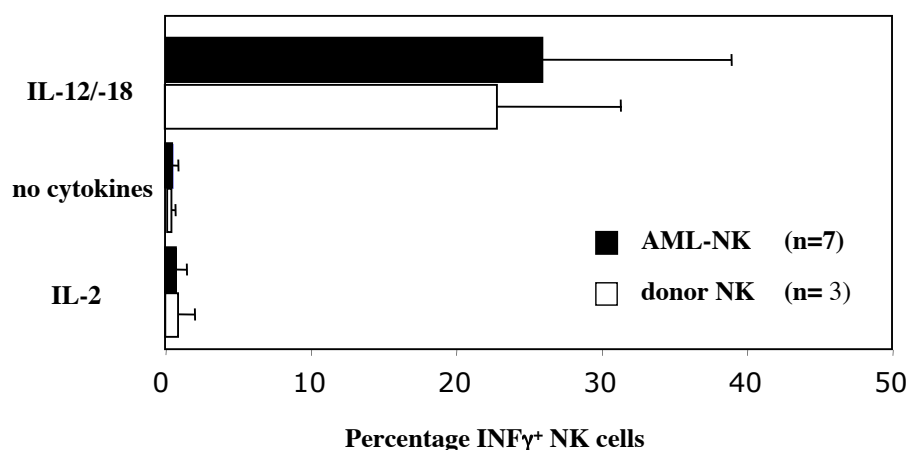


Figure 6-1: IFN- γ production of AML-NK cells and of NK cells derived from healthy donors. The percentage of IFN- γ producing cells measured by intracellular FACS analysis is indicated. NK cells were either stimulated with IL-12 and IL-18 for 36 hours or were incubated without any cytokines. Incubation with IL-2 only served as a control of background level for IFN- γ producing NK cells. BrefeldinA was added for the last 4 hours of culture period to induce the intracellular retention of IFN- γ .

6.2 *In vitro* cytotoxicity of AML-NK cells

6.2.1 *In vitro* cytotoxicity against the target cell line K562

We next analysed the cytolytic capacity of AML-NK cells against the target cell line K562. Due to a deficiency in MHC class I expression K562 erythroleukemia cells are highly sensitive to NK cell mediated lysis. Killing of K562 target cells thus can serve as a control for the integrity of the cytolytic activity of a given NK cell population. Figure 6-2 shows the cytolytic activity of AML-NK cells and of NK cells from healthy donors at different effector to target ratios. The cytotoxicity was measured by ^{51}Cr release assays on day 14 to 21 during *in vitro* expansion. AML-NK cells displayed a high cytolytic activity against K562 with an average specific lysis that ranged from $45 \pm 12,9\%$ to $74 \pm 9,5\%$ at the E:T ratios of 0,6 to 10 (Figure 6-2a).

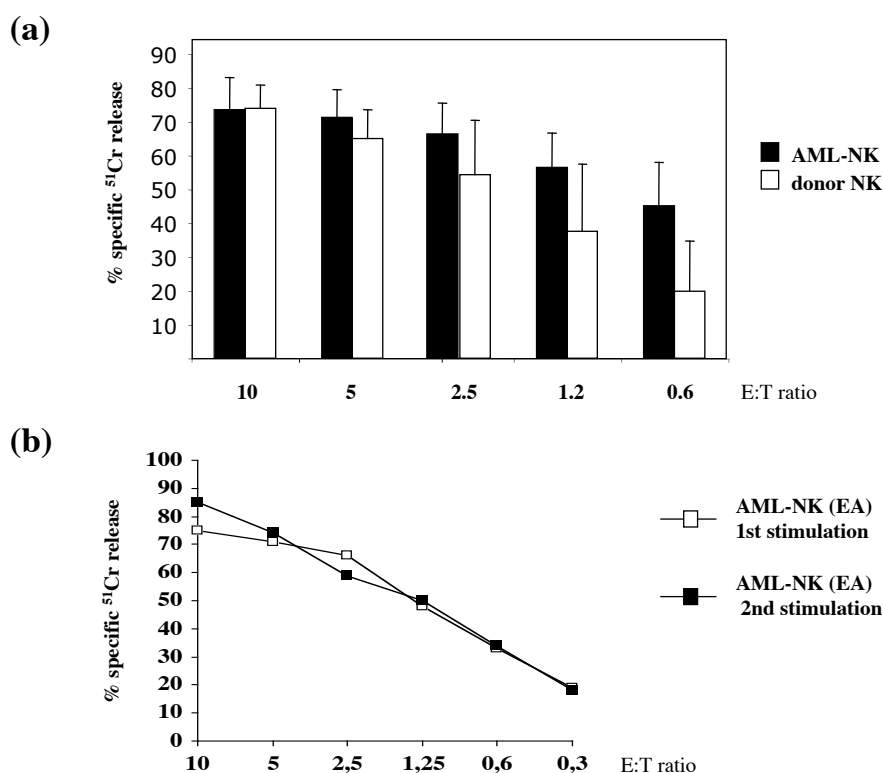


Figure 6-2: *In vitro* cytotoxicity of expanded AML-NK cells against K562. In (a) the cytotoxic activity of AML-NK cells (black bars) and of NK cells derived from healthy donors (white bars) is shown. Percentage of specific lysis at the indicated effector to target ratios was measured in standard 4 hrs ^{51}Cr -release assays. NK cells were assayed for their cytolytic activity on day 14 to 21 during restimulation culture. In (b) the percentages of specific ^{51}Cr -release for a particular *in vitro* expanded AML-NK cell line in first (open symbols) and in second restimulation (filled symbols) is shown.

This cytolytic activity was even higher than the activity measured for control NK cells ($18,5\pm 14,7\%$ to $73,9\pm 6,9\%$). Importantly, this high cytolytic capacity is retained through the process of repeated *in vitro* expansion (Figure 6-2b).

In conclusion, AML-NK cells are highly cytotoxic against NK cell sensitive targets, indicating that the NK cells do not bear any functional deficiencies in their cytolytic potential.

6.2.2 Cytolytic *in vitro* activity against autologous primary AML blasts

We next investigated the cytolytic *in vitro* activity of AML-NK cells against the autologous leukemic blasts. The main goal was to define whether AML-NK cells are characterized by an inherent defect in target cell recognition that may contribute to the escape of malignant cells from the immune surveillance.

To address this issue, we performed ^{51}Cr release cytotoxicity assays with expanded AML-NK cells for 9 out of the 14 patients that were included in the study (see Table 5-1). Due to methodological limitations of the ^{51}Cr release technique (^{51}Cr uptake by the AML blasts was too low; spontaneous ^{51}Cr -release exceeded 25% of maximal release) we could reliably analyse the cytolytic activity for 5 out of these 9 patients. Since we expected a dominant effect of KIR-MHC class I interaction that inhibits the cytotoxicity upon NK cell target interaction, we included an anti-class I mAb in the cytotoxicity assays to block the induction of any inhibitory signalling.

Figure 6-3 illustrates the results in cytotoxicity assays obtained for the AML-NK cells generated from patient “EA”. The cytolytic activity of AML-NK cells on day 14 after initial stimulation is shown as the percentages of specific ^{51}Cr -release at different E:T ratios. As expected from the high levels of MHC class I expression displayed by the AML blasts (see section 6.3; Table 6-4), the spontaneous lysis by the particular AML-NK cells was very low (from about 16% to below 2%).

In contrast, cytolytic activity measured in the presence of MHC class I mAb was highly elevated and ranged from 77% to 12%. The observed induction of cytotoxicity upon the addition of monoclonal antibodies was not due to an Ab-mediated “re-directed” triggering of NK cell activity, since control experiments with anti-MHC class II mAbs that did not result in any significant induction of cytotoxicity (data not shown). In addition, as observed for the cytotoxicity of K562 target cells the repeated stimulation and expansion did not impede the cytolytic activity of AML-NK cells against the autologous blasts (Figure 6-3b).

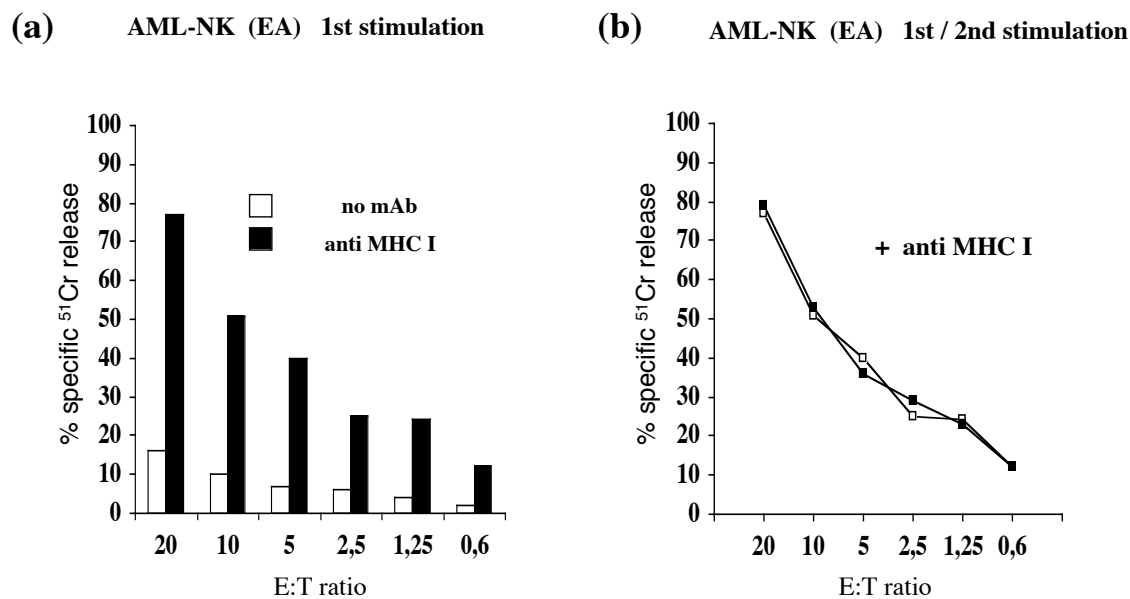


Figure 6-3: *In vitro* cytotoxicity of expanded AML-NK cells against autologous leukemic blasts. In (a) the cytolytic activity of AML-NK cells of patient “EA” after initial restimulation is shown as the percentages of specific ^{51}Cr -release (4 hrs assays) at the indicated E:T ratios. Lysis in the presence of MHC class I blocking mAb (clone W6/32; $10\mu\text{g/ml}$) is represented by the black bars, without the addition of mAbs by the white bars. In (b) the cytolytic activity of AML-NK cells of patient “EA” generated by two subsequent restimulations are compared to each other. Open symbols show the activity of AML-NK cells of the initial first stimulation whereas the filled symbols represent lysis of NK cells upon the second stimulation.

In Figure 6-4 the results of cytotoxicity assays obtained for all 5 AML-NK cells and their corresponding blasts are summarized. In addition to the specific lysis by the autologous NK cells (AML-NK cells; left side), the specific lysis of the indicated blasts by allogeneic healthy donor derived NK cells at the E:T ratio of 10 is shown (donor NK cells; right side).

For all patients (except “CC”) the spontaneous autologous killing of AML-NK cells was below 10%, but a strong cytolytic activity could be induced by the addition of MHC class I specific mAbs. A homogenous and high specific lysis was observed that reached levels between 43 and 59%. The high spontaneous killing of blasts from patient “CC” may be due to the fact that these leukemic cells displayed relative high levels of surface expression of ligands for the activating receptors NKp30, NKp46 and NKG2D (see section 6.3.4; Table 6-4), whereas only low levels of ligand expression were measured for the blasts of the remaining patients.

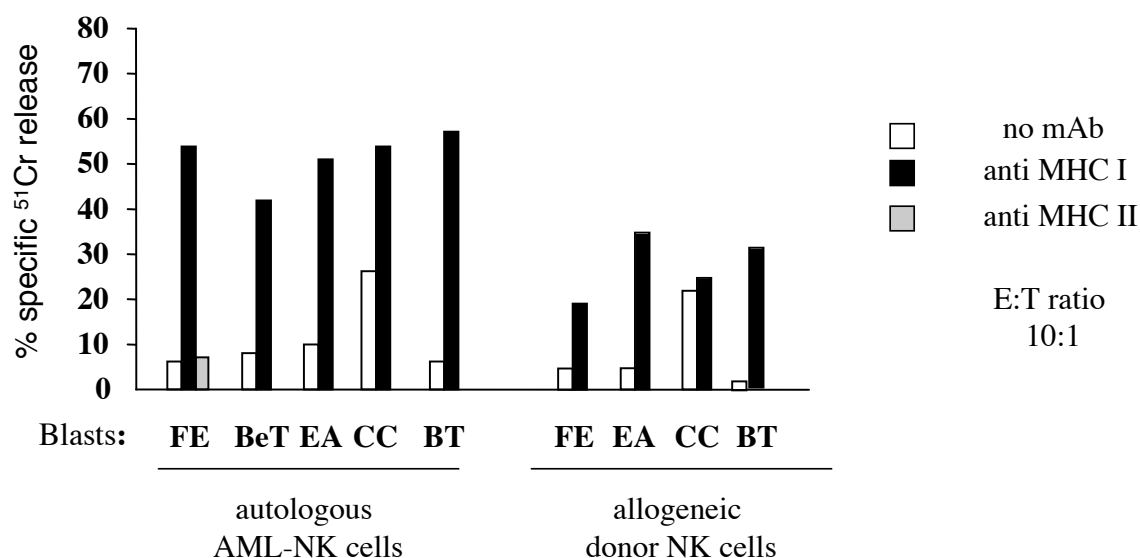


Figure 6-4: Lysis of AML blasts by expanded autologous AML-NK cells. The cytolytic activity of autologous AML-NKs (left) and allogeneic healthy donor-derived NK cells (right) is shown. White bars represent the spontaneous specific lysis whereas the black bars indicate the lysis in the presence of KIR-MHC class I interaction blocking mAb (clone W6/32; 10 μ g/ml) at the E:T ratio of 10. As a control the lysis measured in the presence of anti-MHC class II mAb is shown for autologous killing by AML-NK cells of patient “FE”. Below the bars the blasts used in the corresponding cytotoxicity assay are indicated.

Surprisingly, the spontaneous lysis measured for allogeneic donor NK cells was as low as for autologous AML-NK cells. This was not necessarily expected since the high probability for KIR-mismatches between unrelated individuals - as the case in these experiments - should favour an increased killing of allogeneic targets.

However, no exact KIR-typing was done so that we cannot exclude the possibility of matches in KIR-MHC class I recognition that would explain the observed MHC class I dependent inhibition of cytotoxicity.

In addition, the specific lysis by allogeneic donor NK cells that was induced upon MHC class I blocking (Figure 6-4, right) was below the high levels of cytotoxicity that were observed with autologous AML-NK cells. This phenomenon may be explained by a higher overall activity of the AML-NK cells compared to healthy donor NK cells as it was already observed for the killing of K562 target cells. Thus, we determined the cytolytic activity of AML-NK cell against allogeneic AML blasts.

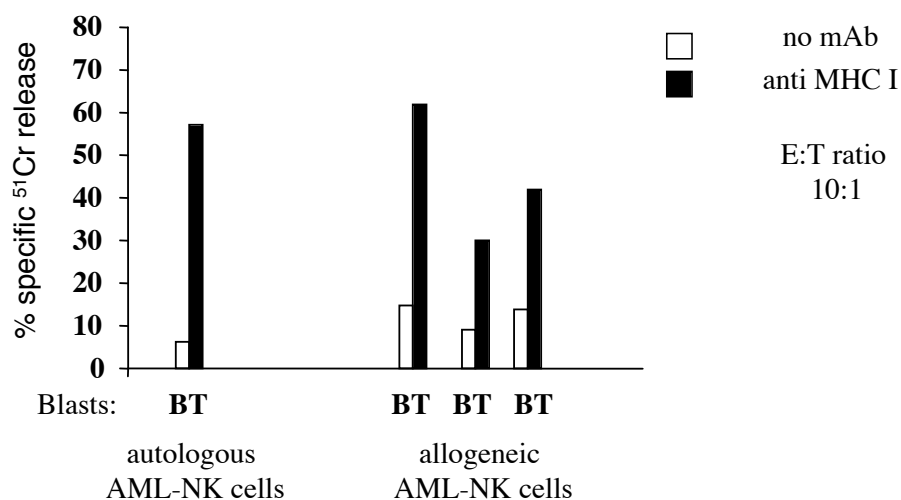


Figure 6-5: Lysis of AML blasts from patient “BT” by autologous and allogeneic AML-NK cells. Spontaneous specific lysis (white bars) and specific lysis in the presence of MHC class I blocking mAb (clone W6/32; 10 μ g/ml) (black bars) at the E:T ratio of 10 is shown. “FE”, “BeT”, and “SR” indicates AML-NK cells from the corresponding patients.

Figure 6-5 shows the cytotoxic *in vitro* activity of allogeneic AML-NK cells against the blasts of patient “BT”. The anti-MHC class I induced killing of the allogeneic blasts was as high as observed for the autologous setting, but compared to the activity of healthy donor derived NK cells the spontaneous as well as the mAb-induced killing of AML-NK cells was indeed higher.

In conclusion, these results strongly indicate that AML-NK cells are capable to recognize and efficiently lyse autologous leukemic blasts. Since the blockage of the KIR-MHC class I interaction can induce a strong autologous killing it appears that AML-NK cells are readily triggered by their targets through the interaction of NK cell activating receptors and their corresponding ligands on the target cells. However, the fact that only the block of inhibitory signalling renders the leukemic blasts susceptible to NK cell lysis underlines the predominance of KIR-mediated inhibition.

It remains to be elucidated if the shift in receptor balance by the over-expression of activating receptors or the upregulation of the corresponding ligands on leukemic blasts can contribute to an enhanced activation signal that might override the otherwise dominant inhibition of NK cell cytotoxicity.

6.3 In vivo activity of AML-NK cells in the NOD/SCID transplantation model

To investigate the cytotoxic effect of AML-NK cells against leukemic target cells *in vivo*, we used the NOD/SCID transplantation model. We first determined the anti-tumor activity of NK cells in mice inoculated with the erythroleukemia cell line K562. Further we transplanted leukemic blasts of the AML patients described in section 5.1 into NOD/SCID mice to assess the *in vivo* activity of the AML-NK cells against the autologous leukemic blasts.

6.3.1 In vivo suppression of K562 tumor formation in NOD/SCID mice

As described in section 6.2.1, AML-NK cells displayed a high cytotoxic activity against the MHC class 1-deficient human erythroleukemia cell line K562 *in vitro*. Thus, to confirm the observed anti-leukemic activity *in vivo* and to establish an animal model for the transplantation of human leukemias we transplanted NOD/SCID mice with the K562 cell line. Here we took advantage of the fact that the K562 cells upon the subcutaneous (s.c.) injection to NOD/SCID mice form solid tumors whose growth can be easily followed over time (Weichold, FF Blood). Six- to eight-week-old NOD/SCID mice received 1×10^7 K562 cells s.c. and the growth of K562 tumors was followed over a time period of 3 to 4 weeks post transplantation. To investigate the *in vivo* activity of adoptively transferred NK cells we injected $2-5 \times 10^6$ NK cells 24 hours after the K562 inoculation. Figure 6-6 shows the results for tumor transplanted NOD/SCID mice that either remained without any further treatment or received a single dose of AML-NK cells or of NK cells derived from healthy donors. Tumor growth was apparent 3 to 4 weeks after K562 injection. In untreated mice tumor load reached an average size of 300mm^2 in diameter. At that stage of tumor expansion the observation was stopped and mice were sacrificed. In contrast, in mice treated with adoptively transferred AML-NK cells or donor NK cells the tumor growth was significantly reduced to about 60-70% of the tumor size in the control group of untreated mice. No human NK cells could be detected in tumors recovered from treated mice using FACS analysis of tumor preparations and immuno-histochemical analysis of tumor sections (data not shown). Therefore reduction in tumor size is likely to reflect an NK cell-mediated reduction of the initial tumor growth early after NK cell infusion.

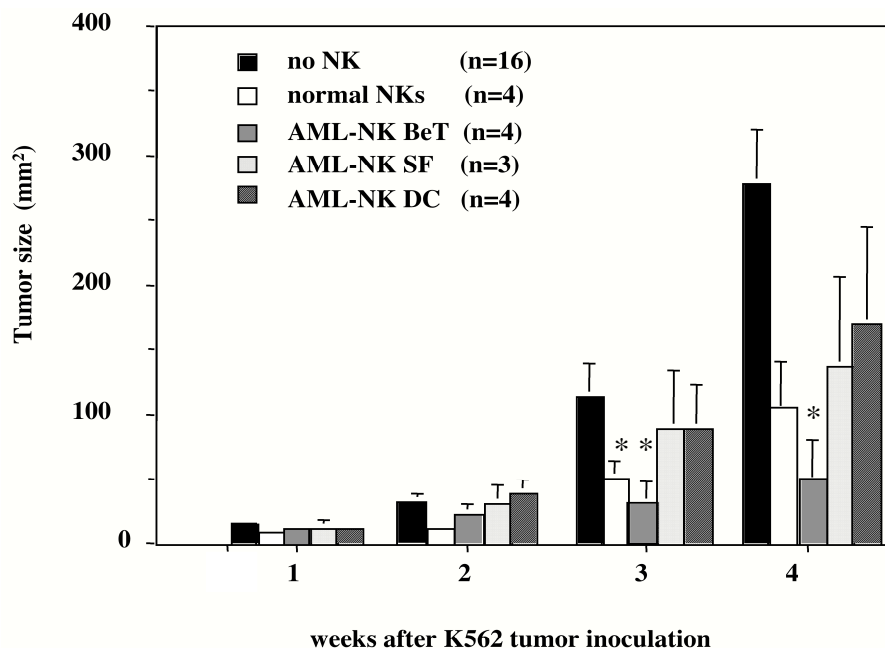


Figure 6-6: Cytotoxic activity of AML-NK cells against the K562 erythroleukemia cell line *in vivo* in the NOD/SCID transplantation model. NOD/SCID mice were inoculated subcutaneously with 1×10^7 K562 cells. Tumor formation was measured (as mm^2) at the indicated time points post inoculation. AML-NK cells or healthy donor derived NK cells were injected i.v. on day 1 post tumor inoculation. The NK cells were used at day 14 to 21 of *in vitro* expansion cultures. Numbers in parenthesis (n) indicates the number of mice per group. Tumor size is expressed as a mean \pm St.Dev.. Significant differences between the NK cell infused groups and the control group are marked.

6.3.2 Engraftment of NOD/SCID mice with primary human AML blasts

We next transplanted primary human acute leukemic blasts to NOD/SCID mice and determined the levels of engraftment eight to ten weeks after the injection. Six- to eight-week-old NOD/SCID mice were irradiated 2-6 hours before the intravenous injection of 1×10^7 AML blasts. We transplanted mice with blast preparations of six of the 14 patients listed in Table 5-1. For all transplantations we used thawed samples of cryopreserved ficolled patient PBMCs. If the blast content was below 90% the absolute numbers of injected cells were adjusted to achieve the transplantation of 1×10^7 leukemic cells.

In Figure 6-7 the detection of human AML blasts in bone marrow preparations of NOD/SCID mice 8 weeks post transplantation is shown. Leukemic blasts can be easily distinguished from the murine cells by the FACS analysis of cell suspensions of the whole BM (Figure 6-7a). The level of engraftment was determined as the percentage of human CD45+CD33+ cells per total cells. Human leukemic blasts are also visible in BM cytopsin preparations of transplanted mice (Figure 6-7b).

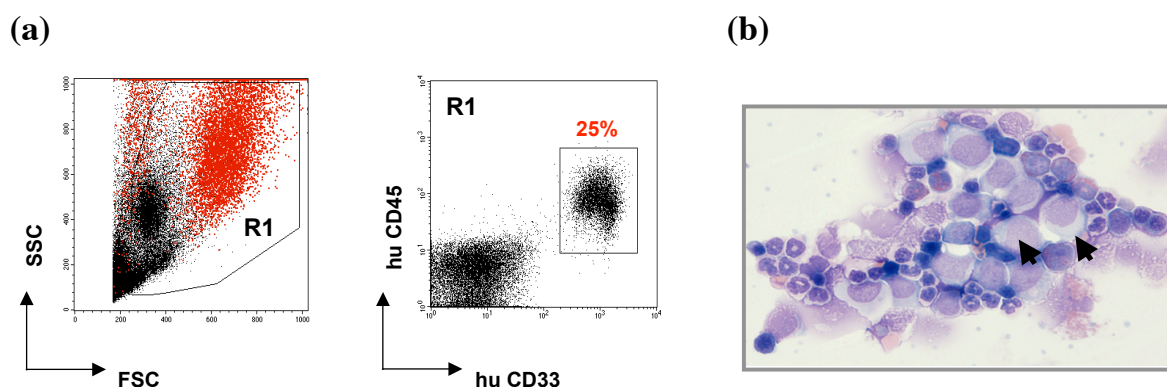


Figure 6-7: Engraftment of human AML blasts in the bone marrow of NOD/SCID mice. Detection of human cells by FACS analysis of bone marrow aspirates (a) and microscopally by histochemical staining of a BM cytopsin preparation (b). In (a) murine BM was analysed on week 8 post transplantation and human cells were identified in total bone marrow by the use of anti-huCD45 and anti-huCD33 mAbs. The percentage indicates the content of blasts per total mouse BM. Black arrow-heads in (b) indicates the human leukemic blasts.

The histochemical analysis revealed that the blasts retained their morphological properties after the repopulation of the murine BM. The percentage of human blast-engraftment in each transplanted NOD/SCID mouse on week 6 to 8 after injection is summarized in Figure 6-8. On average 5 mice (range 3 to 8) were transplanted per patient sample. Engraftment was achieved in almost all mice transplanted with blasts derived from patients of different AML-subtypes. The proportions of human cells in murine BM were highly variable, even within each single patient group resulting in a median blast content of 26,6% with a range from 20,8% to 37,8%. However, for two patient samples no engraftment was detected at all (“CL”, “RM”).

6.3.3 Adoptive transfer of expanded AML-NK cells to leukemia-repopulated NOD/SCID mice

To evaluate the activity of AML-NK cells against the autologous AML blast *in vivo* we transplanted NOD/SCID mice as described in the previous section and transferred AML-NK cells to mice that were engrafted with human leukemic cells. Figure 6-9a illustrates the experimental outline for the adoptive transfer of AML-NK cells to leukemia-repopulated NOD/SCID mice.

Figure 6-9b summarizes the results obtained with AML-NK cell transfer to mice repopulated with blasts of the patients “BT”, “EA” “SD” and “CC”. 10 mice received expanded NK cells and 8 mice were analysed as controls without the infusion of NK cells. All mice infused with AML-NK cells showed a significant overall reduction of tumor load. For seven out of the 10 mice that were treated with transferred NK cells we determined the blast content at “day-7” before infusion to an average of about 30,6% (Figure 6-9b; right).

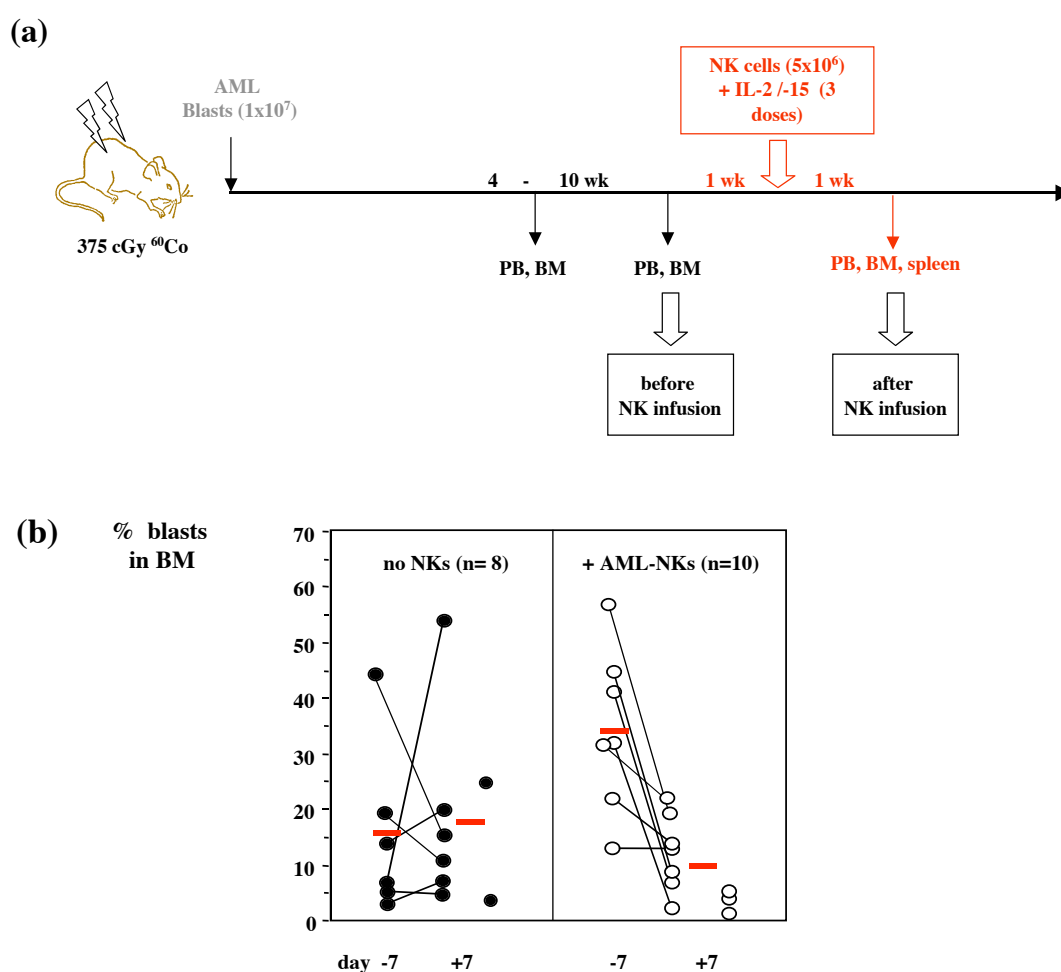


Figure 6-9: Adoptive transfer of AML-NK cells to leukemia-repopulated NOD/SCID mice. In (a) the time schedule of NK cell infusion is illustrated. Open arrows “before” and after NK infusions represent bone marrow aspirations one week before or end-point analysis one week after the NK cell infusion. In (b) the levels of bone marrow engraftment are shown. The filled circles on the left side represent the control mice that received no NK cell infusions, whereas the open symbols represent the mice treated with NK cell infusions. The connection between the symbols correspond to the measurements of one individual mouse performed before NK cell infusion (at the day of bone marrow aspiration) and one week after NK cell infusion at the end-point analysis. Horizontal bars represent the average of BM engraftment.

Mice with high blast content were selected for the NK cell infusion-group in order to increase the chance to determine any significant effect due to the treatment. On “day +7” the average content of human leukemic blasts in the BM of all (n=10) treated mice was significantly reduced to about 10,0%, corresponding to an average reduction of tumor-load of 68%. All mice treated with NK cell infusions contained below 1% of blasts in PB and spleen. Importantly, the infused AML-NK cells were no more detectable at the day of analysis neither in the BM, nor in spleen or peripheral blood. In the control-group the content of blasts was on average 15,8% at “day -7” as measured for 6 mice by BM aspiration. The content of human cells measured in PB was below 1%, except for the two mice with the highest BM-content (20 and 38%) where the percentage in PB was about 2,1 and 3,0%, respectively. Two weeks later at “day +7” the average content of human cells in the BM of all 8 control-mice was marginally increased to about 18,3% (Figure 6-9; left). Despite this low overall increase, there is a tendency to elevated levels of blast contents. In 4 out of the 6 mice where the “pre-values” were determined the content of blasts in the BM remained at the initial level or was clearly increased, whereas in two mice a strong reduction within the 2 week period was measured (the two control-mice with the highest initial tumor load).

In conclusion, AML-NK cells display a strong anti-leukemic effect against autologous blasts *in vivo* in leukemia-repopulated NOD/SCID mice. This *in vivo*-activity was unexpected, since the cytolytic activity measured *in vitro* was mostly dependent on the block of the inhibitory signalling, indicating that the interaction of HLA class I displayed by the leukemic blasts and the KIRs on the NK cells exert a dominant inhibitory effect (see section 6.2.2). One possible explanation for the observed *in vivo* activity could be that apoptosis inducing NK cell-target interactions might contribute to the reduction of tumor load. Such mechanisms act in an HLA class I independent way and would explain the observed discrepancy of the determined *in vitro* activity and the anti-leukemic *in vivo* effect. Another possibility is that the murine BM microenvironment influenced the transplanted leukemic blasts in a way that rendered them more susceptible to NK cell lysis. This would imply that AML blasts may undergo a change in HLA class I expression or in the expression of the different ligands for activating NK cell receptors during the repopulation of the murine BM.

6.3.4 Ligands for NKG2D and the NCRs on primary AML blasts and blasts recovered from transplanted NOD/SCID mice

In order to evaluate the possibility that the NOD/SCID BM environment induces a change in ligand expression or a down-modulation of HLA class I molecules on the transplanted leukemic blasts we first analysed the freshly isolated AML blasts for the expression of HLA class I molecules and for the ligands for NKG2D and for the NCRs (Table 6-1). The yet unknown ligands for the NCRs NKp30, NKp44 and NKp46 were detected by the use of dimers of soluble recombinant NCRs (“solp30/p44/p46”). Analysis of HLA class I expression on the leukemic blast revealed MFI ratios

Patient	FAB-type	MHC class I		NKG2D ligands			NCR ligands		
		blasts	CD45 ^{bright}	ULBP-1	ULBP-2	ULBP-3	sol p30	sol p44	sol p46
AG	M2	163	166	1.2	nd	2.1	1,3	1,3	1,2
BeT	2 nd	92	70	3.6	nd	4.0	4,4	3,3	3,9
BT	M5b	113	181	10.1	3.7	1.0	nd	nd	nd
CA	M2	380	490	2.0	1.4	1.7	nd	nd	nd
CC	M5	82	n.d	4.6	2.5	3.5	4,8	nd	5,2
CL	M2	175	193	1.1	1.4	1.5	nd	nd	nd
DC	M4	40	48	1.9	nd	1.6	2,0	1,7	1,7
DR	M5	n.d	n.d	1.7	nd	3.5	1,6	1,6	1,6
EA	M4	36	27	1.2	2,8	2.4	1,2	nd	2,4
HI	M1	116	143	1.7	nd	1.9	nd	nd	1,6
JJ	M1	612	415	2.7	nd	3.0	nd	1,7	1,9
KA	?	103	85	1.1	1.0	1.6	nd	nd	nd
LE	M2	n.d	n.d	1.2	nd	1.2	nd	nd	nd
MM	2 nd	236	382	1.4	1.2	2.1	nd	nd	nd
MU	M5b	n.d	n.d	1.3	1.1	1.4	nd	nd	nd
RA	?	58	68	1.6	nd	1.9	1,4	nd	nd
RM	2 nd	18	32	1.1	nd	1.2	nd	nd	1,1
SJ	M4	n.d	n.d	1.3	nd	1.4	1,4	1,4	1,3
SR	M2	29	306	1.3	nd	1.4	1,5	nd	nd
SD	M2 (2 nd)	81	n.d	1,3	1,0	1,1	1,3	nd	1,3
SH	M1	60	108	1,6	nd	2,3	1,7	1,6	1,4
SC	M2	n.d	n.d	nd	nd	nd	nd	nd	nd
TL	M7	n.d	n.d	(10,9	3	11,4)	nd	(32,8	21,5
VP	M2	n.d	n.d	1.1	1.2	1.1	nd	nd	nd
WG	MD	135	373	1.4	nd	1.3	1,9	1,3	1,5
ZC	M4	n.d	n.d	1.1	1.2	1.6	nd	nd	nd
ZP	MD	158	194	nd	nd	nd	nd	nd	nd
Mean		145	193	2.4	1.7	2.3	1.9	1.7	2.0
StDev		142	143	2,6	0,9	2.1	0,3	0,6	1,2
Range		18-612	27-490	1.1-10,9	1.0-3,7	1.0-11.4	1,3-4,4	1,3-3,3	1,1-5,2
n		19	17	25	12	25	10	8	13

Table 6-1: Expression levels of HLA class I molecules and the ligands for NKG2D and the NCRs.

Expression of the indicated molecules were determined by FACS analysis and are shown as MFI ratio. CD45 bright indicates the residual healthy cell compartment in AML patients. The highlighted patients correspond to the patient cohort for which the AML-NK cells were isolated (see also Table 5-1).

that were comparable or even below to that found on the residual healthy cell compartment (indicated by the CD45^{bright} phenotype) but nevertheless, high enough to induce a dominant inhibitory signalling (145±32 vs. 193±32; see Table 6-1). This is in accordance to the obtained functional *in vitro* data, which suggest that primary AML blasts are protected from autologous NK cell lysis through the HLA class I mediated inhibition of NK cell triggering. In addition, the low or even absent surface expression of ligands for the activating receptors NKG2D and the NCRs support the hypothesis that acute leukemic blasts are poorly recognized by NK cells and thus might escape NK cell lysis in the patients.

We next analysed the expression levels of these molecules on blasts recovered from the BM of leukemia-repopulated NOD/SCID mice. Table 6-2 summarizes the FACS determined expression levels of HLA class I molecules and the ligands for NKG2D determined for the transplanted blasts derived from the patients “EA”, “BT”, “CC” and “SD” (see also Figure 6-8). HLA class I expression by two of the transplanted blasts was upregulated during the 3 month process of BM repopulation. Even though this would suggest an increased resistance to NK cell mediated lysis, a

Blasts	MHC class I	NKG2D ligands				NCR ligands		
		MICA/B	ULBP-1	ULBP-2	ULBP-3	sol p30	sol p44	sol p46
EA								
d0	36	1,1	1,2	n.d.	1,1	1,2	n.d.	2,4
ex NOD/SCID	426	n.d.	7,0	1,7	2,3	n.d.	n.d.	n.d.
BT								
d0	113	n.d.	10,1	3,7	1,0	n.d.	n.d.	n.d.
ex NOD/SCID	243	1,5	8,9	3,5	3,4	8,0	3,5	4,6
CC								
d0	82	n.d.	4,6	2,5	3,5	4,8	n.d.	5,2
ex NOD/SCID	115	27	12,1	5,4	5,6	12,1	n.d.	12,4
SD								
d0	81	1,1	1,3	1,0	1,1	1,3	n.d.	1,3
ex NOD/SCID								

Table 6-2: Expression levels of HLA class I molecules and the ligands for NKG2D and the NCRs on AML blasts recovered from transplanted NOD/SCID mice. FACS analysis of total BM isolated from the leukemia-repopulated mice 10 to 12 weeks post transplantation. Expression of the indicated molecules is shown as MFI ratio. Values “d0” indicate the expression levels determined for the freshly isolated patient samples.

high *in vivo* susceptibility to the adoptively transferred autologous AML-NK cells was also observed for these particular AML blasts (see Figure 6-9). This could be a further indication that mainly HLA class I independent processes may contribute to the observed anti-leukemic effect.

However, when the expression levels of the ligands for NKG2D and the NCRs were evaluated, it turned out that for all analysed blasts the ligands for at least one of these receptors were upregulated. Blasts of patient “EA” showed a high upregulation of ULBP-1 (MFI ratio 7,0 vs. 1,2). Similarly, blasts of patient “BT” were already high for ULBP-1 and –2, but upregulated the expression of ULBP-3 upon the transplantation to NOD/SCID mice (3,4 vs 1,0). Although for these particular blasts the initial expression levels of all three NCR ligands were not determined it is likely that the ligands were upregulated upon transplantation, since the high levels measured after BM repopulation were clearly above the average values determined for a panel of AML patients (8,0 vs.1,9 solp30; 3,5 vs.1,7 solp44; 4,6 vs.2,0 solp46; see Table 6-1). For the blasts of patient “CC” almost all NKG2D ligands as well as the ligands for the NCRs were upregulated upon transplantation to the mice. In accordance to the high initial expression levels of the ligands, these particular blasts showed a relative high susceptibility to the killing by allogeneic and autologous NK cells indicated by a high spontaneous *in vitro* lysis (see Figures 6-4 and 6-5).

In conclusion, these data suggest that the observed *in vivo* activity of adoptively transferred AML-NK cells against the autologous blasts might be explained by an enhanced susceptibility due to the increase of ligand levels for activating receptors taking place in the murine BM microenvironment.

V. RESULTS (C) HUMAN NK CELL DEVELOPMENT IN THE NOD/SCID MOUSE TRANSPLANTATION MODELL

Human NK cell development in NOD/SCID mice receiving grafts of cord blood CD34⁺ cells

Christian P. Kalberer, Uwe Siegler, and Aleksandra Wodnar-Filipowicz

Definition of the cytokine environment, which regulates the maturation of human natural killer (NK) cells, has been largely based on *in vitro* assays because of the lack of suitable animal models. Here we describe conditions leading to the development of human NK cells in NOD/SCID mice receiving grafts of hematopoietic CD34⁺ precursor cells from cord blood. After 1-week-long *in vivo* treatment with various combinations of interleukin (IL)-15, β 13 ligand, stem cell factor, IL-2, IL-12, and megakaryocyte growth and differentiation factor, CD56⁺CD3⁻ cells were detected in bone marrow (BM), spleen, and

peripheral blood (PB), comprising 5% to 15% of human CD45⁺ cells. Human NK cells of NOD/SCID mouse origin closely resembled NK cells from human PB with respect to phenotypic characteristics, interferon (IFN)- γ production, and cytotoxicity against HLA class 1-deficient K562 targets *in vitro* and antitumor activity against K562 erythroleukemia *in vivo*. In the absence of growth factor treatment, CD56⁺ cells were present only at background levels, but CD34⁺CD7⁺ and CD34⁻CD7⁺ lymphoid precursors with NK cell differentiation potential were detected in BM and spleen of chimeric NOD/

SCID mice for up to 5 months after transplantation. Our results demonstrate that limitations in human NK cell development in the murine microenvironment can be overcome by treatment with NK cell growth-promoting human cytokines, resulting in the maturation of IFN- γ -producing cytotoxic NK cells. These studies establish conditions to explore human NK cell development and function *in vivo* in the NOD/SCID mouse model. (Blood. 2003;102:127-135)

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Introduction

Human natural killer (NK) cells comprise approximately 10% of peripheral blood (PB) lymphocytes and are characterized phenotypically by the expression of CD56 and the lack of CD3 cell surface antigens.¹ They are important effectors of the innate immune system and contribute to the first line of defense against infections and malignancy.² In contrast to T lymphocytes, NK cells are able to kill cancer and virus-infected target cells without the need for prior antigen stimulation. NK cell precursors have been identified within the CD34⁺ hematopoietic cell population in adult bone marrow (BM) and umbilical cord blood (CB). These precursors can efficiently generate mature NK cells *in vitro* in the presence of interleukin-15 (IL-15) and early-acting cytokines such as β 13 ligand (FL) or stem cell factor (SCF), which increase the frequency of NK cell precursors responding to IL-15.^{3,5} IL-15 is a key cytokine in NK cell development.⁶ Targeted disruption of the IL-15 or IL-15 receptor genes in mice^{7,8} and spontaneously occurring mutations in the signaling components of the receptor in humans⁹ cause blocks in early NK cell development. FL and SCF play important roles in the early differentiation steps of NK cells and in their subsequent expansion and functional maturation as revealed in mice rendered deficient in FL or carrying mutations in the *c-kit* receptor.^{10,11} Other important regulatory cytokines are IL-12 and IL-18, which enhance cytotoxicity and trigger cytokine release by mature NK cells.^{12,13}

Biologic responses of NK cells are controlled by a balance of signals from inhibitory and activatory receptors. The inhibitory

receptors recognize epitopes shared by different alleles of HLA class I molecules and deliver negative signals, thereby suppressing NK cell function and blocking lysis of normal cells.¹⁴ In the absence of inhibitory signals, the ability of NK cells to lyse cells altered by virus infection and tumor transformation is mediated by the activatory receptors, including Fc γ RIII (CD16) and natural cytotoxicity receptors (NCRs), which were recently identified as unique NK-specific cell surface molecules.¹⁵ In human leukemia, the expression of NCRs is down-regulated and correlates with low NK cell cytotoxicity,¹⁶ whereas, conversely, antileukemic responses are enhanced by NK cell alloreactivity in mismatched stem cell transplants.^{17,18} Therefore, NK cells appear important for tumor surveillance and might be exploited in the immunotherapy of diseases.

However, understanding the development and function of human NK cells is largely based on *in vitro* analyses, and models to study human NK cells *in vivo* are lacking. Results of murine NK cell studies are not always applicable to the human NK cell system because of the lack of a murine homologue of CD56 and because of differences in the inhibitory and activatory receptors.^{14,19} Furthermore, it has not been possible to use nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice repopulated with human hematopoietic progenitors for studies on human NK cells because the lymphoid differentiation in these mice is restricted to the B-cell lineage, whereas T and NK cells are produced at a minimum level or not at all.²⁰⁻²² In this study, we show that

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NOD/SCID mice repopulated with CB CD34⁺ cells contain human NK cell precursors and that the administration of recombinant human IL-15, together with other NK cell growth-promoting cytokines, leads to the *in vivo* development of NK cells in BM, spleen, and blood circulation. The combination of IL-15 and FL is sufficient to generate NK cells at levels comparable to the NK cell content in human PB. NK cells generated in NOD/SCID mice are CD56⁺CD3⁻, express CD16 and NKp46, and are functional with respect to interferon- γ (IFN- γ) production in response to IL-12 and IL-18. Furthermore, NK cells of NOD/SCID origin show cytotoxic activity against K562 cells *in vitro* and reduce the growth of K562 erythroleukemia *in vivo*. Establishment of human NK cell development in NOD/SCID mice provides an *in vivo* system to investigate NK cells in immunotherapeutic strategies against infectious diseases and cancer.

Materials and methods

Cord blood cell preparation

CB was kindly provided by the Department of Obstetrics and Gynecology, University Hospital Basel and the Department of Obstetrics and Gynecology, Kantonsspital Bruderholz, Switzerland with informed consent of the mothers. Investigations were approved by the Ethical Committee of the University Hospital Basel. CB mononuclear cells were separated by Histopaque (less than 1.077 g/cm³; Sigma, St Louis, MO) density-gradient centrifugation and subsequent red blood cell lysis. Frozen samples were pooled after thawing, and CD34⁺ cells were isolated with superparamagnetic MACS (magnetic cell sorting) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the CD34⁺ cell population ranged from 80 to 95%.

Transplantation of CD34⁺ cells into NOD/SCID mice

NOD.LtSz-scid/scid (NOD/SCID) mice²³ (The Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal facility of the Research Department, University Hospital Basel. CB CD34⁺ cells ($1-2 \times 10^6$) together with 1×10^6 irradiated (1500 cGy) human PB mononuclear carrier cells were injected intravenously into the tail veins of 8-week-old NOD/SCID mice previously given 375 cGy from a ⁶⁰Co source (2 cGy/min). Mice were kept on acidified drinking water supplemented with Bactrim (32/160 mg/L; Roche Pharma AG, Reinach, Switzerland) for the duration of the experiment.

In vivo treatment with human growth factors

Eight to 10 weeks after transplantation, NOD/SCID mice were injected intraperitoneally with phosphate-buffered saline (PBS) or the following recombinant human growth factors: IL-15, FL (both from Immux, Seattle, WA), SCF (Amgen, Thousand Oaks, CA), PEGylated megakaryocyte growth and development factor (MGDF; Amgen and Kirin Brewery, Tokyo, Japan), IL-2 (Novartis, Basel, Switzerland), and IL-12 (Roche, Nutley, NJ). IL-15, FL, SCF, MGDF, and IL-2 were administered for 7 consecutive days, each at 10 μ g daily, and IL-12 was administered at 1 μ g per day for the last 2 days of the treatment. Twenty-four hours after the last injection, spleen and BM cells were harvested, and single-cell suspensions of spleen and BM were prepared. To measure circulating human NK cells, PB was drawn weekly up to 3 weeks after growth factor injections. Statistical analyses for comparison of treatment groups were performed with the Mann-Whitney *U* test.

Flow cytometry and cell sorting

Three-color fluorescence-activated cell sorter (FACS) analysis was used to characterize human engraftment of NOD/SCID mice that underwent

transplantation. Single-cell suspensions from BM and spleen were resuspended in FACS buffer containing PBS, 2% fetal calf serum (FCS; both from Invitrogen, Carlsbad, CA), and 0.02% NaN₃ (Fluka, Buchs, Switzerland) and were stained on ice for 20 minutes with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) against human CD2, CD3, CD7, CD16, CD19, CD33, CD34, CD38, CD45, CD56, CD62L, CD69, CD138a, CD138b, NK1, and HLA-DR or isotype control antibodies (all from BD Pharmingen, San Jose, CA). Staining with unlabeled mAb anti-CD94 (clone 39B10), anti-NKp46 (clone 9E2; each a generous gift from M. Colonna and A. Bouchon, Basel Institute for Immunology, Switzerland) and anti-IL-2R γ chain (clone CP.88; kindly provided by D. Baker, Biogen, Cambridge, MA) was revealed with secondary PE- or FITC-conjugated goat antimouse antibodies (Southern Biotechnology Associates, Birmingham, AL). Normal mouse serum was used to saturate free binding sites of secondary antibodies before cells were subsequently incubated with directly labeled mAbs. Propidium iodide (PI; Sigma) staining was used to exclude dead cells from the analysis. FACS analysis of circulating NK cells was performed by incubating whole blood with labeled antibodies at room temperature for 20 minutes, followed by erythrocyte lysis with FACS Lysing Solution (BD Pharmingen) and subsequent washing with FACS buffer. FACS analysis was performed on a FACScalibur (Becton Dickinson), and data were analyzed using CellQuest Pro software (Becton Dickinson). For cell-sorting experiments, BM cells from NOD/SCID that underwent transplantation were resuspended in FACS buffer without NaN₃ and were stained with the appropriate antibodies. Cells were washed, incubated with PI, and sorted on a FACS Vantage SE (Becton Dickinson).

NK cell differentiation *in vitro*

Cell suspensions from NOD/SCID mouse BM or spleen containing 20% to 90% human CD45⁺ cells were seeded at 1 to 2×10^6 /mL cells in 24-well plates in Isocove modified Dulbecco medium (IMDM) containing 5% FCS, 5% human AB⁺ serum (Blutspendezentrum Basel), 380 μ g/mL iron-saturated human transferrin, and 1% bovine serum albumin and were supplemented with IL-15, FL, and SCF (each at 100 ng/mL). After 1 week, cells were transferred to 6-well plates, and half the medium was replaced once a week for another 2 to 5 weeks, as specified in "Results." The development of CD56⁺ NK cells was determined at the indicated time points by FACS.

NK cell lines were cultured by restimulation with irradiated mononuclear cells from human PB and phytohemagglutinin HA 16 (2 μ g/mL; Murex Biotech, Dartford, England) in the presence of 100 U IL-2 every 3 to 4 weeks.

NK cell cytotoxicity and IFN- γ production

After 4 to 5 weeks of differentiation in culture, CD56⁺ cells were purified by positive selection with MACS CD56-microbeads (Miltenyi Biotec). Cells were washed and resuspended in IMDM containing 2% FCS, and cytotoxicity against the NK-sensitive target K562 was determined in a 4-hour lactate dehydrogenase (LDH) release assay (Cytotoxic 96; Promega, Madison, WI) according to the manufacturer's instructions. The effector-target ratio ranged from 5:1 to 0.6:1.

IFN- γ production was measured by intracellular flow cytometry. MACS-purified NK cells from differentiation cultures (purity, 80%-95%) or FACS sorter-purified NK cells from BM of growth-factor-treated NOD/SCID mice were washed, and 1×10^6 cells/mL were plated in 96-well plates for 36 hours in IMDM containing 5% FCS, 5% AB⁺ human serum, 10 U/mL IL-12, and 100 ng/mL IL-18 (PeproTech, Rocky Hill, NJ). Brefeldin A (Sigma) was added at 5 μ g/mL for the final 8 hours of culture. Cells were washed with FACS buffer, stained with anti-CD56-PE mAb for 20 minutes on ice, and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Cells were washed twice and permeabilized in saponin-containing FACS buffer. Anti-IFN- γ -FITC and isotype control mAb (BD Pharmingen) were added for 30 minutes at room temperature. Cells were

washed twice in permeabilization buffer and once in FACS buffer and were analyzed with FACScalibur.

K562 tumor formation

K562 erythroleukemia cells were resuspended in 100 μ L PBS and injected subcutaneously into the dorsal lateral thorax of NOD/SCID mice. NK cells, resuspended in 200 μ L PBS, were injected intravenously 1 day after tumor cell inoculation. In mice that received grafts of CB CD34⁺ cells, K562 cells were inoculated 1 day after 7-day-long treatment with IL-15 and FL. The effect of NK cells on tumor growth was determined in groups of 2 to 4 animals. Tumor growth was monitored weekly, and tumor surface area was calculated using the formula ($a/2 \times b/2 \times \pi$, where *a* and *b* are the long and short diameters (in millimeters)). Statistical analysis for comparison of treatment groups were performed using the unpaired Student *t* test.

Results

Growth factors induce maturation of human NK cells in NOD/SCID mice

To investigate the requirements for human NK cell development *in vivo*, the NOD/SCID xenotransplantation system was chosen to test the effect of human growth factors known to be involved in human NK cell development *in vitro*. Groups of NOD/SCID mice that underwent transplantation 2 months earlier with highly enriched CB CD34⁺ cells were injected for 7 consecutive days with one of the following 4 combinations of cytokines: IL-15/FL, IL-15/FL/SCF, IL-15/FL/IL-12, and IL-15/FL/SCF/MGDF/IL-2. On day 8, BM and spleens were examined for the presence of human NK cells (Table 1). Among the CD45⁺ human lymphocytes, only background levels of 0.3% \pm 0.1% human CD56⁺ NK cells were detected in untreated control animals (Figure 1A; Table 1). With the administration of growth factors, the frequency of CD56⁺ cells increased approximately 15-fold and was similar among mice tested with the 4 growth factor combinations (range, 3.9% \pm 0.9% to 4.9% \pm 2.0%). The content of NK cells generated in the BM of NOD/SCID mice with human growth factor treatment was comparable to that in human BM and CB (1%-10% and 4%-12% CD56⁺ cells, respectively, as determined using 6-8 samples from both blood sources; Figure 1C and results not shown). Human NK cells were also found in spleens of NOD/SCID mice, in which they constituted 3.2% \pm 1.4% to 5.0% \pm 1.4% of human CD45⁺ cells, depending on the growth factors used. IL-15/FL/IL-12 led to NK cell development mainly in the BM but only marginally in the spleen (Table 1). Additionally, in the blood circulation of NOD/SCID mice, 12.2% \pm 0.8% (*n* = 8) of CD45⁺ human cells were CD56⁺ NK cells, which corresponds to the number of NK cells in

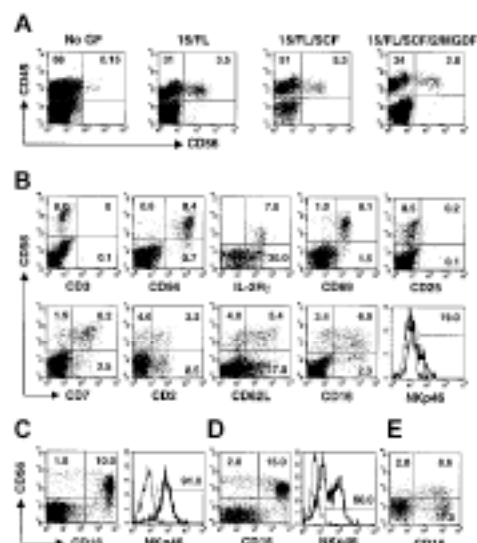


Figure 1. Phenotype of human NK cells in BM of growth-factor-treated NOD/SCID mice. Two months after transplantation of CB CD34⁺ cells, NOD/SCID mice were treated with combinations of growth factors (A) for 7 consecutive days, and BM, spleen, and PB cells were analyzed 1 day later by flow cytometry. (A) Human NK cells in untreated control (no growth factor [GF]) and growth-factor-treated mice were detected by staining with the human leukocyte marker CD45 and the NK-cell-specific marker CD56. Analysis was performed with total mononuclear cells; numbers indicate the percentages of total BM cells. (B) Human NK cells were characterized by staining with antibodies against CD56 and the indicated NK surface marker. Numbers indicate the percentages of human CD45⁺ cells. For comparison, human NK cells from (C) CB and (D) PB were stained with antibodies against CD56 and CD15 (gated on lymphocytes) and NKp46 (gated on CD56⁺ cells). The thin line indicates staining with isotype-matched control antibody. (E) NK cells in PB of NOD/SCID mice treated with IL-15 and FL. Numbers indicate the percentages of human CD45⁺ cells.

human PB (7%-15%; Figure 1D-E), and they were detectable for at least 3 weeks after growth factor treatment.

Human NK cells that developed in NOD/SCID mice were examined for the expression of a panel of cell surface markers. Staining patterns of NK cells residing in BM and spleen were indistinguishable, and there were no marked differences in NK cell phenotype with respect to the growth factor combination used to generate them. NK cells from the BM of IL-15/FL-treated mice are shown in Figure 1B. CD56⁺ cells were always CD3⁻, indicating that these cytokine combinations promoted only the development of bona fide NK cells and not the CD56⁺CD3⁺ NK T-cell population. CD94 and the IL-2R γ chain were expressed on all CD56⁺ cells from NOD/SCID mice. The early activation marker CD69 was expressed on virtually all NK

Table 1. Effect of growth factor administration on human hematopoietic lineages in BM and spleen of NOD/SCID mice with transplanted CD34 cord blood cells

Treatment (no. mice)	Bone marrow			Spleen		
	CD56 ⁺	CD19 ⁺	CD33 ⁺	CD56 ⁺	CD19 ⁺	CD33 ⁺
No treatment (20)	0.3 \pm 0.1	87.0 \pm 1.5	11.5 \pm 1.5	0.3 \pm 0.1	93.0 \pm 1.2	3.0 \pm 0.7
IL-15/FL (7)	3.9 \pm 0.9*	62.0 \pm 4.0*	27.0 \pm 5.0*	3.2 \pm 1.4†	80.0 \pm 5.0*	6.6 \pm 1.3†
IL-15/FL/SCF (5)	4.1 \pm 1.7*	64.0 \pm 4.5*	28.0 \pm 3.0*	4.4 \pm 1.6*	81.0 \pm 4.0†	9.4 \pm 2.5†
IL-15/FL/IL-12 (5)	4.9 \pm 2.0*	86.0 \pm 4.0	6.3 \pm 2.6	1.1 \pm 0.2†	92.0 \pm 1.0	2.6 \pm 0.5
IL-15/FL/SCF/MGDF/IL-2 (3)	4.1 \pm 1.3†	70.0 \pm 3.5†	25.0 \pm 3.5†	5.0 \pm 1.4†	80.0 \pm 1.5†	10.0 \pm 2.0†

Values are mean percentages of human CD45⁺ cells \pm SEMs.
**P* < .005.
†*P* < .05.

cells, likely as a response to IL-15,²⁴ whereas CD25, the low-affinity IL-2R α chain, was not present. Of the other tested markers, CD2, CD7, and CD62L selectin were expressed by 30% to 45%, 65% to 80%, and up to 60% of NK cells, respectively (Figure 1B). Intracellular perforin was expressed by all NK cells. HLA-DR was expressed only at low levels or not at all, and c-kit was absent (not shown). Interestingly, staining of CD16 and CD56 surface markers revealed that the prevalence of the CD56^{dim}CD16^{dim} population, which is characteristic of human CB and PB NK cells (Figure 1C-D), was not apparent in NK cells generated in NOD/SCID mice. Instead, CD16 was expressed at various cell surface densities on 50% to 70% of all CD56⁺ NK cells (Figure 1B, E). NKp46, the major NCR selectively expressed by NK cells, showed a heterogeneous expression pattern of 5% to 19% (Figure 1B), lower than that found in human NK cells from PB and CB (Figure 1C-D). This lower NKp46 expression was not attributed to a limiting availability of the CD3 ζ chain, the coreceptor for NKp46, because FACS staining revealed CD3 ζ expression in all human NK cells generated in mice that underwent transplantation (data not shown). Based on these results we conclude that human recombinant growth factors, including IL-15 and FL, specifically promoted the maturation of human NK cells in the BM and spleen microenvironments of NOD/SCID mice. The phenotype of these cells closely resembled, but was not identical to, NK cells from human PB.

Effect of growth factor treatment on human cell engraftment and lineage composition

The effect of growth factor treatment on the cellularity of BM and spleen and on engraftment with human cells of NOD/SCID mice that underwent transplantation was also examined (Figure 2). The BM of 4 long bones of untreated control NOD/SCID mice contained $22 \pm 4 \times 10^6$ cells, of which $64\% \pm 5\%$ were of human origin. Growth factor injections reduced the cellularity of BM but not of spleens. In particular, quick and dramatic 10-fold cell loss was observed with the administration of IL-15/FL/IL-12 involving 2 days of treatment with IL-12 (Figure 2A). This is in accord with the reported hypoplasia in BM but not spleen with IL-12 administration in humans and mice.²⁵⁻²⁷ The overall level of human CD45⁺ cells was reduced by 30% to 50% in BM and spleens in all groups of growth-factor-treated mice except the IL-15/FL/IL-12 group (Figure 2B). This reduction was associated mainly with a decrease in CD19⁺ B lymphocytes, which constituted most human cells in untreated and treated mice and which were reduced by 15% to 30% after growth factor injections (Table 1). Concomitantly, the proportion of myeloid cells expressing CD33 was increased up to 3-fold. The lower engraftment and changes in the proportions between B cells and myeloid lineages on growth factor treatment are reminiscent of the effects of FL alone and with IL-7 and SCF, reported to have an even greater effect when injected over several weeks.²⁸

Lymphoid precursors with NK cell differentiation potential are present in BM and spleens of NOD/SCID mice that underwent transplantation

The finding that human NK cells developed within 7 days of growth factor administration prompted us to characterize the NK precursor cells in NOD/SCID mice that received grafts of human hematopoietic cells. In the first series of experiments, we investigated whether CD56⁺ NK cells could also be generated in vitro

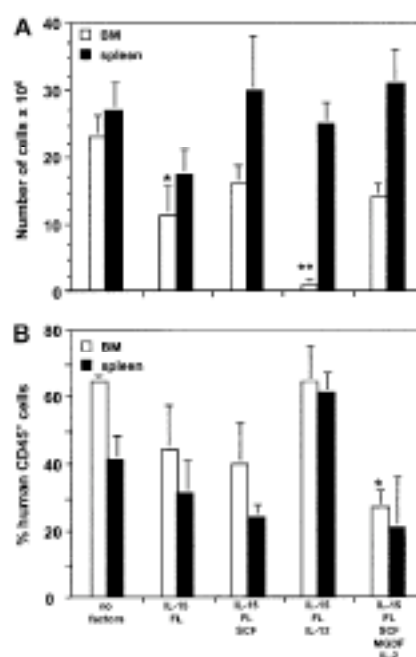
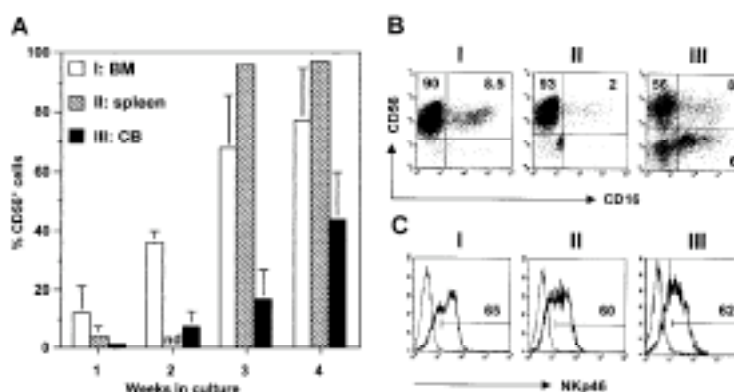


Figure 2. Cellularity and level of human engraftment in BM and spleen of NOD/SCID mice after growth factor treatment. Two months after transplantation of CB CD34⁺ cells, NOD/SCID mice were treated with the indicated human growth factor combinations. (A) Total cell number was determined from the BM (4 long bones, □) and from spleen (■). (B) Human engraftment was defined by staining BM (□) and spleen (■) cells with human anti-CD45 antibody. Mean \pm SEM percentages are shown. Significant differences between untreated and treated groups are marked (* $P < .05$; ** $P < .005$).

from BM and spleen cells obtained from NOD/SCID mice not treated with growth factors (Figure 3). In cultures containing IL-15, FL, and SCF, human CD56⁺ cells developed within the first week and reached 68% to 96% by 3 weeks (Figure 3A). This was faster and more efficient than the development of NK cells from CB CD34⁺ progenitors cultured under the same conditions ($17\% \pm 10\%$ CD56⁺ cells at week 3), particularly considering the fact that the purity of CB CD34⁺ cells at the start of culture was at least 80%, whereas CD34⁺ human cells constituted only $19.0\% \pm 1.1\%$ and $6.4\% \pm 0.9\%$ of all cells in NOD/SCID BM and spleen, respectively (Table 2). The phenotypes of NK cells of NOD/SCID mice origin and those generated from CB CD34⁺ cells were similar (Figure 3B). CD16 was expressed only at low levels in 2% to 15% of all NK cells. Remarkably, NKp46 was expressed by up to 60% of NK cells generated in vitro (Figure 3C), indicating that NKp46 is present on CD16⁻ and CD16⁺ NK cells. The surface markers CD7, CD62L, CD69, and CD161 were expressed at various levels on 5% to 30% of NK cells generated in vitro from NOD/SCID BM. The killer immunoglobulin-like receptors CD158a, CD158b, and NK1 were not detected (data not shown), in agreement with reports that NK cells differentiated in vitro from CD34⁺ cells are mainly negative for these receptors.³ CD56⁺CD3⁺ NK T cells or CD3⁺ T cells were never found in these cultures (data not shown).

To define the precursor cell populations able to give rise to NK cells in vivo and in vitro, we performed FACS analysis of human cells from BM of growth-factor-untreated NOD/SCID mice (Figure 4A). CD34⁺CD38⁻ and CD34⁺CD38⁺ hematopoietic

Figure 3. In vitro differentiation of human NK cells from NOD/SCID BM and spleen and from human CB. (A) Single-cell suspensions of BM (I: n = 13, □ and spleen (II: n = 5, ▨) of NOD/SCID that underwent transplantation and purified CB CD34⁺ cells (III: n = 7, ■) were cultured for 4 weeks in NK differentiation medium containing recombinant human IL-15, FL, and SCF. The appearance of CD56⁺ NK cells was determined by FACS analysis. Shown are the mean ± SEM percentages of total cells in the cultures. nd indicates not determined. (B) Expression of CD16 and CD56 and of (C) NKp46 on CD56⁺ NK cells generated in cultures I, II, and III at 4 weeks. This line indicates staining with isotype-matched control antibody. Numbers indicate the percentages of NKp46⁺ NK cells.



progenitor cells and CD34⁺CD7⁺ and CD34⁺CD7⁻ cells, all reported to contain NK precursor cells,²⁹⁻³¹ were cultured in the presence of IL-15, FL, and SCF, and NK cell development was examined. The fastest differentiation toward CD56⁺ cells, apparent after 3 days, was obtained with CD34⁺CD7⁺ cells, suggesting the highest frequency of NK precursors in this cell fraction (Figure 4B). CD34⁺CD7⁺ cells also gave rapid rise to 75% of CD56⁺ cells after 14 days. In contrast, in cultures initiated with primitive hematopoietic CD34⁺CD38⁻ progenitors, only 20% of all cells expressed CD56 by day 14. These results indicate that even in the absence of growth factor treatment, human NK cell precursors had progressed in the NOD/SCID microenvironment to an advanced differentiation stage, providing an explanation for the faster response of unseparated NOD/SCID-derived cells compared with CB CD34⁺ cells cultured under the same conditions (Figure 3A). Despite different kinetics, NK cells generated in vitro from sorted cell populations (R1-R4) of NOD/SCID mouse BM acquired the same phenotypic characteristics as those from bulk cultures described in Figure 3 (results not shown).

Treatment of NOD/SCID that underwent transplantation with IL-15/FL, IL-15/FL/SCF, and IL-15/FL/IL-12 combinations in vivo specifically increased the population of human CD34⁺CD7⁺ NK cell precursors up to 5-fold in BM and spleens (Table 2). This increase is in accord with the increase in CD56⁺ cells observed after growth factor injections (Table 1), some of which were CD56⁺CD7⁺ (data not shown). The proportion of total CD34⁺ cells was significantly decreased in BM, primarily because of a reduction in CD34⁺CD19⁺ pre-B-cell levels. Neither the CD34⁺CD19⁻ nor the CD34⁺CD7⁺ cell population was affected by the cytokine treatment in either organ. We conclude that the tested growth factors acted on a CD7⁺ NK precursor at the transition from the CD34⁺ to the CD56⁺ stage.

IFN- γ production by human NK cells generated in NOD/SCID mice

A hallmark of NK cell function is the ability to produce IFN- γ . To assess this feature, CD56⁺ NK cells from the BM of growth-factor-treated NOD/SCID mice were isolated by cell sorting and were stimulated in vitro for 36 hours with IL-12 and IL-18. Additionally, NK cells were isolated after 4 weeks of culture of BM from NOD/SCID mice, both growth factor treated and untreated. The proportion of IFN- γ -producing NK cells was measured by intracellular FACS analysis. NK cells differentiated in vivo and in vitro responded equally well to the cytokine stimulus (Figure 5). Of the 3 types of tested NK cells, production of IFN- γ was found in 14% \pm 6% and 15% \pm 5% of cells purified directly or after culture of growth-factor-treated mouse BM and in 6.4% \pm 1.7% of NK cells generated in vitro from the BM of untreated NOD/SCID mice. For comparison, purified NK cells from human PB were included in the assay and responded more readily to IL-12 and IL-18 (33% \pm 10%). The differences in IFN- γ production may be related to the maturation stages of the NK cell populations generated under these different experimental conditions.

Cytotoxic activity of NOD/SCID-mouse-derived human NK cells

Another characteristic of NK cells is the capacity to kill target cells without prior sensitization. To study the cytotoxic activity of NOD/SCID-mouse-derived human NK cells, we used HLA class 1-deficient human K562 erythroleukemia cells, which represent a sensitive NK cell target in vitro and, on subcutaneous inoculation in vivo, form a solid tumor whose growth can be easily followed over time.³²

For the analysis of K562 lysis in vitro, NK cells generated in growth-factor-treated mice were not directly examined because of

Table 2. Effect of growth factor administration on hematopoietic progenitor and lymphoid precursor cells in BM and spleen of NOD/SCID mice with transplanted CD34⁺ cord blood cells

Treatment (no. mice)	Bone marrow*					Spleen*			
	CD34 ⁺	CD34 ⁺ CD19 ⁺	CD34 ⁺ CD19 ⁻	CD34 ⁺ CD7 ⁺	CD34 ⁺ CD7 ⁻	CD34 ⁺	CD34 ⁺ CD19 ⁺	CD34 ⁺ CD7 ⁺	CD34 ⁺ CD7 ⁻
No treatment (20)	19.0 \pm 1.1	13.2 \pm 1.2	4.9 \pm 0.4	0.9 \pm 0.1	0.8 \pm 0.1	6.4 \pm 0.9	2.7 \pm 0.6	< 0.1	1.1 \pm 0.2
IL-15/FL (7)	7.7 \pm 1.8 [†]	2.1 \pm 0.4 [†]	5.5 \pm 1.8	1.3 \pm 0.5	4.0 \pm 1.1 [†]	7.3 \pm 0.7	2.4 \pm 0.4	< 0.1	5.3 \pm 2.0 [†]
IL-15/FL/SCF (5)	9.7 \pm 1.0 [†]	2.7 \pm 0.7 [†]	7.1 \pm 1.4	1.1 \pm 0.4	4.5 \pm 1.3 [†]	5.5 \pm 0.3	3.0 \pm 0.5	< 0.1	4.5 \pm 0.9 [†]
IL-15/FL/IL-12 (5)	8.3 \pm 0.9 [†]	4.2 \pm 1.1 [†]	4.2 \pm 0.5	0.7 \pm 0.15	3.9 \pm 1.3 [†]	7.3 \pm 2.4	1.5 \pm 0.3	0.15 \pm 0.02	2.4 \pm 1.1

Values are mean percentages of human CD45⁺ cells \pm SEMs.

[†]P < .005.

[‡]P < .05.

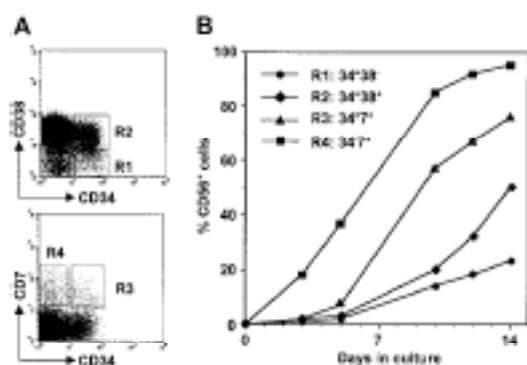


Figure 4. In vitro differentiation of human NK cells derived from FACS sorted-purified progenitor cells of NOD/SCID mouse BM. (A) Hematopoietic progenitor cells and NK precursor cells were identified in the BM of untreated NOD/SCID mice by staining with CD34/CD38 (top panel) and CD34/CD7 (bottom panel). Cells falling into the gates R1 (CD34⁺CD38⁺; 1.5% of total BM cells), R2 (CD34⁺CD38⁺; 12%), R3 (CD34⁺CD7⁺; 0.5%), and R4 (CD34⁺CD7⁺; 1.5%) were sorted and cultured for 14 days in NK differentiation medium (see "Materials and methods"). (B) The development of CD56⁺ NK cells in vitro was followed by FACS analysis at the indicated time points.

the limited number of cells that could be isolated by FACS sorting. Instead, we tested the cytotoxicity of human NK cells obtained in higher numbers in differentiation cultures of BM and spleen cells of growth-factor-untreated NOD/SCID mice (Figure 6A). These NK cells were highly cytotoxic; in particular, the killing potential of BM-derived cells was even higher than that of human PB NK

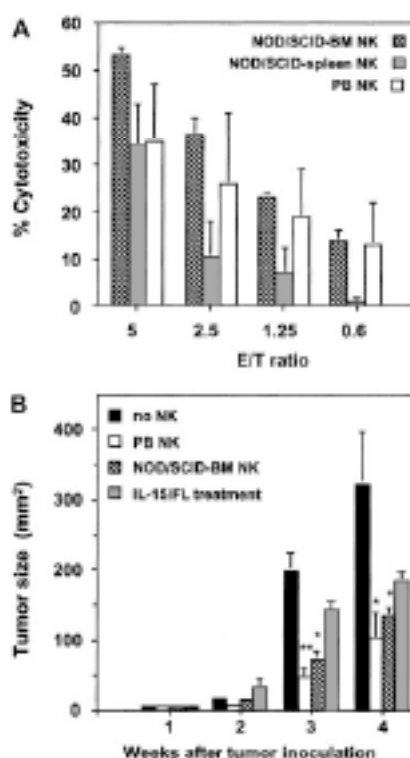


Figure 6. Cytotoxicity of NK cells from NOD/SCID mice. (A) NK cells were harvested after 20-day culture of NOD/SCID BM ($n = 2$, \blacksquare) or spleen ($n = 3$, \square) cells in the presence of IL-15, FL, and SCF. For comparison, NK cells were purified from human PB mononuclear cells ($n = 2$, \square). Their cytotoxic activity was tested against the NK-sensitive K562 target cell line for 4 hours with an enzymatic LDH release assay. (B) NOD/SCID mice were injected subcutaneously with K562 cells (1×10^5) alone (\blacksquare) or 1 day later by NK cells (2.5×10^5) from human PB (\square) or generated in vitro from NOD/SCID BM (\blacksquare). NOD/SCID mice that had undergone transplantation with CB CD34⁺ cells were injected with human IL-15 and FL for 7 days and were inoculated with K562 cells 1 day later (\square). Tumor size is expressed as mean \pm SEM areas of 2 to 4 animals per treatment group. Significant differences compared with the group without NK cells are marked (* $P < .05$; ** $P < .005$).

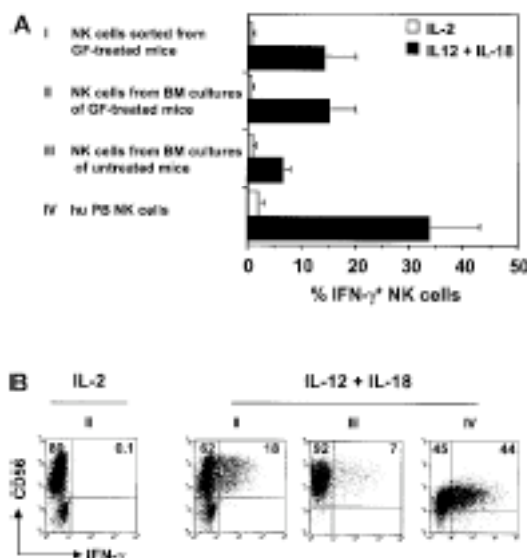


Figure 5. IFN- γ production by NK cells from NOD/SCID mice. (A) FACS sorted-purified CD56⁺ NK cells from BM of growth factor (GF)-treated NOD/SCID mice (I; $n = 3$), NK cells generated in vitro within 4 weeks from BM of GF-treated (II; $n = 3$) and untreated control (III; $n = 5$) NOD/SCID mice, and purified NK cells isolated from human PB mononuclear cells (IV; $n = 2$) were stimulated with medium containing IL-2 (\square) or IL-12 and IL-18 (\blacksquare) for 35 hours. Synthesis of IFN- γ was measured by intracellular FACS analysis, and the mean \pm SEM percentages of IFN- γ ⁺ NK cells are shown. (B) Representative FACS pictures of IFN- γ -producing NK cells generated in culture I, II, and IV are shown. Numbers represent the percentages of total cells in culture.

cells. The well-pronounced cytotoxic properties of human NK cells of NOD/SCID origin generated in vitro are in accord with the high levels of activatory receptor NKp46 expressed by these cells (Figure 3C).³³ To study the cytotoxic effect of human NK cells in vivo, NOD/SCID mice were inoculated subcutaneously with K562 cells and challenged intravenously 1 day later with NOD/SCID-BM-derived NK cells that showed strong cytotoxicity in vitro. Tumor growth, apparent 3 to 4 weeks after K562 injection, was significantly (60%-70%) suppressed by adoptively transferred NK cells (Figure 6B). This retardation of tumor growth was comparable to the effect of IL-2-stimulated NK cells obtained from human PB. We also inoculated K562 cells into NOD/SCID mice that had received transplanted CB CD34⁺ cells and had been injected with IL-15 and FL for 7 days. Under these conditions, which led to the generation of endogenous human NK cells, a marked reduction (up to 50%) of tumor growth was observed (Figure 6B). A possibly lower yield of NK cells generated in vivo may account for a lesser antitumor effect than a controlled number of cotransplanted NK cells. Based on these results, we conclude that human NK cells

derived from NOD/SCID mice are functional with respect to cytotoxicity against HLA class I-deficient targets *in vitro* and *in vivo*.

Discussion

The NOD/SCID mouse transplantation model has been widely used to study the biology of human hematopoietic stem cells of diverse ontologic origin.^{34,35} These studies indicate that the BM microenvironment of NOD/SCID mice supports the survival and multilineage development of human hematopoietic progenitors but that it can be selective with regard to the maturation of individual blood cell lineages. Within the lymphoid compartment, the B-cell lineage develops most efficiently.^{30,37} On the other hand, human T and NK cells have never been reproducibly detected, and only the transplantation of human thymic tissue or the use of IL-2R-blocking antibodies allowed T lymphopoiesis *in vivo*.^{38,39} In this study, we identified CD34⁺CD7⁺ and CD34⁻CD7⁺ NK progenitor cells in BM and spleen of NOD/SCID mice transplanted with CB-CD34⁺ cells, and we achieved NK cell differentiation after *in vivo* administration of human cytokines. The establishment of human NK cell development in NOD/SCID mice provides for the first time an *in vivo* system to study the mechanisms governing human NK cell differentiation and function.

Several studies have demonstrated that lineage development of the human graft in NOD/SCID mice can be modulated by the administration of human cytokines. The effects seen *in vivo* often did not reproduce those predicted from *in vitro* cultures, such as a decrease in B-cell lineage development in mice receiving FL and IL-7²⁸ or an inhibition of platelet production by a growth factor combination known to support megakaryopoiesis from cultured progenitors.⁴⁰ In contrast, all the cytokine combinations used in our study—namely IL-15/FL, IL-15/FL/SCF, IL-15/FL/IL-12, and IL-15/FL/SCF/MGDF/IL-2—enabled *in vivo* maturation of NK cells, thus recapitulating the previously reported effects of these cytokines on the generation of NK cells from cultured CD34⁺ progenitors. Previous studies identified IL-15 as the crucial factor for NK cell development, along with FL and SCF as cytokines that increase the frequency of NK cell precursors through the up-regulation of expression of the IL-15R complex.^{3,5} With human NK cells differentiated *in vitro* from CD34⁺ progenitors, a functional redundancy of FL and SCF on IL-15 responsiveness has been reported.³ Accordingly, the combination of IL-15 and FL was found to be sufficient to induce NK cells in NOD/SCID mice. Similarly, the number and phenotype of the NK cells did not differ depending on the cytokines used to generate them, though subtle differences of additional parameters cannot be excluded. Cytokine administration was associated with a moderate reduction in cellularity and with the level of human cell engraftment in BM and spleens. This is in agreement with previous studies,^{38,40,41} though the opposite effect was also seen in mice receiving only limited doses of stem cells.⁴² A rapid and nearly total disappearance of murine and human cells in NOD/SCID BM was observed when IL-12 was combined with IL-15 and FL. This is perhaps associated with the activation of newly formed NK cells given that an IL-12-dependent release of IFN- γ by NK cells, resulting in the suppression of hematopoiesis in the BM, has been postulated in humans and mice.^{25,26}

In growth-factor-untreated mice, we detected human CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁺CD7⁺, and CD34⁻CD7⁺ cell populations, all of which responded to IL-15, FL, and SCF *in vitro* and gave rise to CD56⁺ NK cells. Judging from the kinetics of

CD56⁺ cell generation *in vitro*, these populations may represent early, intermediate, and late stages in NK cell development. These NK cell precursors were found as late as 5 months after transplantation, indicating that they can persist for a long time in the NOD/SCID microenvironment. Similarly, undifferentiated, common lymphomyeloid CD34⁺CD38⁻CD19⁻ progenitors capable of generating human B, T, NK, and granulomonocytic cells under appropriate culture conditions *in vitro* were maintained over the long-term in NOD/SCID mice that underwent transplantation.⁴³ Altogether, these results indicate that the microenvironment of chimeric NOD/SCID mice harbors human NK progenitors, but an insufficient concentration or lack of cross-reactivity of endogenous murine NK cell-promoting cytokines, in particular IL-15, prevents progression to mature CD56⁺ NK cells above the background levels observed in untreated control NOD/SCID mice.

Human PB NK cells can be divided into 2 subsets, according to the expression of cell surface markers and functional characteristics.^{44,45} Approximately 10% of all NK cells are CD56^{dim}CD16^{-low}, and they produce abundant cytokines after activation. The prominent CD56^{dim}CD16^{low} subset, representing at least 90% of NK cells, secretes less IFN- γ but is more naturally cytotoxic. This phenotypic distinction was not apparent in the NK cell population that developed in NOD/SCID mice after growth factor injections. CD16 was expressed at various surface densities with a large proportion of NK cells being CD16^{-low}. The fact that CD56^{dim}CD16^{low} cells were not present as a predominant NK cell population in growth-factor-treated NOD/SCID mice suggests that the development of this subset requires the presence of other, possibly unknown, cytokines or that cellular selection is important and cannot be mimicked in the NOD/SCID mouse microenvironment. Despite this difference in phenotypic characteristic, human NK cells of NOD/SCID origin produce IFN- γ , are strongly cytotoxic against HLA class I-deficient K562 targets *in vitro*, and are capable of reducing K562 erythroleukemia tumor formation *in vivo*, thus functionally resembling human PB NK cells.

Although inhibitory and activatory receptors have defined functions in mature PB NK cells, their roles in developing immature NK cells is unclear. In growth-factor-treated NOD/SCID mice, we consistently found fewer human NK cells expressing the major triggering receptor NKP46 than CD16⁺ NK cells. This is different from NK cells generated *in vitro* from CD34⁺ cells, in which NKP46 is expressed by most CD56⁺ cells and CD16 by only a small proportion. Likewise, 2 other groups also observed NKP46 preceding CD16 expression during NK cell differentiation *in vitro*.^{47,48} The NOD/SCID-derived NK cells acquired the NKP46^{dim} phenotype only after isolation of the progenitors and after further culture. The mechanism that controls the level of NKP46 expression during NK cell development is unknown. Noteworthy, however, is that the surface density of NKP46 on mature NK cells from human PB is heterogeneous, with different donors harboring different proportions of NKP46^{high} and NKP46^{low} cells that display high and low cytotoxic activity, respectively.³³ Contrary to the NKP46 activatory receptor, the inhibitory receptors CD158a, CD158b, and NKB1 were not detected on NOD/SCID-derived human NK cells after culture. This may result in the absence of inhibitory signals and may enhance the killing capacity of NK cells against neoplastic targets, as shown by the important graft-versus-leukemia effect in haploidentical transplantation.¹⁷

NK cells, through the production of immunoregulatory cytokines and cytotoxic effects, are candidates for immunotherapy of transformed or infected tissues. It remains to be seen whether

adoptively transferred NK cells of NOD/SCID origin that recognized and killed HLA-deficient targets can also suppress the growth of primary human tumors. Notably, cytokines that boost the maturation of NK cells in chimeric NOD/SCID mice have been shown to generate potent antitumor responses. Treatment of tumor-bearing mice with FL leads to tumor regression through the activation of dendritic and NK cells.⁴⁹ IL-15 activates T and NK cells, and preclinical studies show enhanced protection against viral challenge and a potential for IL-15 as a tumor vaccine adjuvant.^{50,51} Interestingly, we recently found that IL-15 up-regulates FL expression and that after stem cell transplantation, FL levels are strongly increased,^{52,53} suggesting that these 2 growth factors contribute to fast recovery of NK cells in patients who have undergone transplantation.¹⁷ Ongoing discoveries of NK-specific receptors and increasing knowledge about their respective ligands⁵⁴ provide the means to modulate the responses of mature NK cells,

using either specific antibodies⁵⁵ or genetic modification. NOD/SCID mice with transplanted hematopoietic progenitors represent an important model for evaluating the immunotherapeutic efficacy of human NK cells and for investigation of the factors and signals orchestrating formation of the NK cell compartment in humans.

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VI. DISCUSSION

1. **Lentiviral transduction of NK cell lines, primary NK cells and hematopoietic progenitor cells**

The effector functions of NK cells are regulated by the complex interaction of activating and inhibitory receptors [1]. This tightly balanced regulation of NK cell activity offers the chance to manipulate the counteracting signalling of activating and inhibitory receptors on NK cells. In particular, the possibility to shift the receptor balance on NK cells towards a status that favours the activating signals would augment NK cell anti-tumor activity and thus would serve as an attractive approach in cellular immunotherapy.

Direct indication supporting the concept that a modulation of the receptor balance can enhance NK cell anti-tumor responses was provided by reports on allogeneic hematopoietic stem cell transplantation (HSCT) in the management of hematological malignancies. Ruggeri et al. [103, 106] could show that NK cells play an important role in the elimination of residual leukemic blasts after transplantation. A favourable prognosis was observed in AML patients receiving a haploidentical stem cell graft with a mismatch between inhibitory killer cell immunoglobulin-like receptors (KIRs) in the graft and the patients' HLA class I molecules (KIR-ligands). The activation and anti-tumor activity of donor-derived NK cells was favoured due to the lack of inhibition. Since NK cells do not contribute to the development of graft versus host disease (GvHD) after allogeneic transplantation [135], these pioneering studies on NK cell alloreactivity upon KIR-ligand mismatch suggest the adoptive transfer of NK cells to obtain a graft versus leukemia (GvL) effect devoid of the deleterious GvHD reactions. Indeed, pre-clinical studies were initiated with alloreactive NK cells given as donor lymphocyte infusions ("NK-DLI") in haploidentical transplantations to prevent leukemia relapse or to combat recurrent disease [137] [138].

The feasibility to manipulate the receptor balance in favour of NK cell activation was proven in a recent study in a murine model of acute leukemia. The blockade of interaction between KIRs on NK cells and their MHC class I ligands on target cells by the treatment with F(ab)₂ fragments of anti-KIR monoclonal antibodies protected mice from leukemic death. The same effect was also achieved upon the adoptive transfer of IL-2-activated NK cells treated with anti-KIR F(ab)₂

fragments *ex vivo* [159]. However, given the higher complexity in the human system, where subsets of NK cells express different inhibitory receptors for self-HLA molecules, only the simultaneous blockade of several receptors may provide a sufficient anti-leukemic effect.

The activating receptor NKG2D and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 have emerged as major triggering receptors involved in the HLA-independent lysis of various tumor cells [27]. We focused on NKp46, which is expressed by all human NK cells irrespective of their state of activation. The surface density of NKp46 varies within an individuals' polyclonal NK cell population and the expression level correlates with the spontaneous natural cytotoxicity of NK clones against allogeneic or autologous target cells [42]. Taking advantage of these activating properties of NKp46, our approach was to over-express the receptor by gene transfer and, thereby, to increase the cytolytic activity of NK cells.

Only few reports on the stable genetic modification of NK cells exist so far, and most studies were performed with NK cell lines rather than with primary NK cells. The described methods include particle-mediated transfection and the retroviral transduction with "classical" murine leukemia virus (MLV)-based vectors or vaccinia virus-derived vector systems (protocols in NK cell... ,Colonna). The genetic modifications mostly aimed at the delivery of cytokine encoding genes to NK cell lines and were shown to allow the propagation of cells independent of the particular cytokine or to enhance the effector functions of modified cells [160] [161]. In this context, some studies were designed with the goal to modify effector cells for the application in cellular immunotherapy [162], but clinical trials with gene-transfected primary NK cells, NK-T cells or tumor infiltrating T-lymphocytes (TIL) were hampered by the poor efficiency of gene-transfer and the down-regulation of transgene expression [155]. A variety of reports focused on the genetic modification of NK cell lines in order to gain new insights in the biology of intracellular signalling or receptor-ligand interactions. For example, retrovirus-mediated transduction experiments showed the successful transfer of genes encoding "wild type", mutant or chimeric versions of KIRs or of NCRs to study the role of ITAMs and ITIMs in the intracellular signalling cascade [49] [163-166].

Similar to our approach some studies aimed at the over-expression of genetically engineered chimeric receptors to induce a target specific cytolytic activity of NK cell lines. Such chimeric receptors consisted of an intracellular signalling subunit, either the ITAM containing CD3 ζ -chain or the Fc ϵ RI γ -chain, which was fused to the ligand-binding extracellular domain of CD4 to form a chimeric CD4 ζ or CD4 γ receptor, respectively. The target induced cross-link of the chimeric receptors was shown to deliver an activatory signal to the modified cells and specifically directed NK cell killing towards targets carrying the HIV-derived gp120 protein, the ligand for CD4 [167-

169]. This approach to direct NK cell effector functions in a target-specific way clearly indicates that it is feasible to augment the cytolytic activity of NK cells through the transfer of genes encoding activating receptors.

Nonetheless, expression of exogenous genes in NK cells and NK cell lines has proven to be extremely difficult. In all the above-mentioned reports only low efficiencies of gene transfer were achieved. The MLV-based transduction rate of NK cell lines never exceeded 5 to 15% [49]. Moreover, despite the stable transgene-integration to the target genome is mediated by murine retroviral vectors, high rates of transcriptional silencing due to the methylation of the viral LTRs occurs [155]. Furthermore, the retroviral transduction of primary lymphocytes needs the unspecific activation of cells, which was shown to increase the susceptibility to apoptotic death of modified cells [170].

For our gene transfer experiments we used HIV-derived lentiviral vectors, which are superior to common retroviral vectors. These vectors mediate the efficient gene delivery into mature and cell cycle arrested, terminally differentiated cells, including neurons, retinal cells [171], liver cells [172, 173] or PB-derived dendritic cells [144]. Lentiviral vectors were also demonstrated to transduce activated or even resting lymphocytes including the CD56⁺ NK-T cells with high efficiency [174] [175]. Likewise, HIV-based vectors are capable to efficiently transduce human CD34⁺ hematopoietic progenitor cells in the absence of cytokine stimulation [176], as well as the human SCID repopulating cells (SRC) that are capable of long-term engraftment in NOD/SCID mice [177] [178] [176]. Bone marrow from these primary recipients could repopulate secondary mice with transduced cells confirming that genetically modified cells included primitive progenitors, possibly the “true” quiescent stem cells [179]. By the *in vitro* differentiation of transduced progenitors the lentiviral transduction provides the opportunity to generate transgenic cells of all blood cell lineages. This approach lead to the successful transfer of the green fluorescent protein (GFP) marker gene to granulocytes, monocytes, erythroid cells, dendritic cells [180, 181] as well as natural killer cells [182]. Due to this highly efficient gene delivery to stem cells and their progeny and due to the fact that the integrated provirus is less prone to transcriptional silencing, lentiviral vectors represent a promising tool in certain gene therapy approaches.

In all lentiviral transductions carried out in our studies, HIV-based vectors of the so-called “2nd generation” were used. These vectors are self-inactivating (SIN) vectors due to an almost complete deletion of the U3 region of the 3’LTR [149] and contain a posttranscriptional regulatory element of

the woodchuck hepatitis virus (WPRE) that enhances transgene expression [150]. We used single-gene transfer vectors (“pLox-vectors”) encoding either the gene for NKp46 or for the GFP marker, as well as bicistronic constructs (“pWP-vectors”), which contained both the NKp46 and the GFP genes. The bicistronic vectors additionally carried the nuclear translocation regulatory element cPPT/CTS and in all vectors transcription was driven by the EF1-alpha promoter either in its long, intron-containing (in the pLox-vectors) or short intron-less (in the pWP-vectors) version.

1.1 Lentivirus mediated genetic modification of cell lines

We first performed transduction experiments with the Jurkat T lymphocytic cell line and the NK cell lines NKL and NK92 in order to confirm the integrity and functionality of the different vector constructs. Since the Jurkat T cell line lacks endogenous NKp46 but expresses the CD3zeta signalling subunit that serves as the adaptor protein of NKp46 in NK cells, Jurkat cells were chosen as a model system to investigate expression and signalling capacity of the transgenic NKp46 protein. NK92 and NKL cell lines express endogenous NKp46 at very low levels, which should allow to study the consequences of the receptor over-expression for NK cell effector functions.

Our results showed that the Jurkat cell line is highly susceptible for the transduction with all GFP encoding vector constructs including the bicistronic vectors. Transduction rates of virtually 100% were achieved using concentrated virus even at a low MOI (<10). In contrast, expression of the NKp46 receptor in transduced Jurkat cells was consistently less efficient. When the single-gene vector pLox-NKp46 was used between 40 and 60% of cells expressed the transgene. Also transduction with bicistronic vectors resulted in the NKp46 expression in not more than 80% of cells. It remains to be clarified if the observed failure of NKp46 expression in a subpopulation of transduced Jurkat cells is due to post-transcriptional modifications of the vector mRNA or to a post-translational phenomenon affecting the protein stability or its export to the cell surface. A limiting expression of the adaptor molecules CD3zeta and FcεRIγ is most probably not the reason since it was demonstrated that NKp46 surface expression is independent of the signalling molecules [40]. In case of the bicistronic vectors, splicing events and unspecified post-transcriptional modifications of the vector mRNA that may impair the expression of the upstream NKp46 gene cannot be ruled out but seem to be unlikely, since usually the expression of the downstream gene is affected in such multigene transfer vectors [183].

Nevertheless, the combination of FACS-sorter purification of transduced cells together with single-cell cloning resulted in the generation of clones of the Jurkat cell line that homogeneously displayed the transgenic NKp46 receptor at the cell surface.

The NK cell lines NK92 and NKL showed a very low susceptibility to the transduction with all GFP-containing vector constructs tested. Even at an MOI of 20-30, poor efficiencies between 1 to 3% were measured. The reason for this low susceptibility of NK92 and NKL compared to the T Jurkat cell line remains elusive but our results are in line with published data indicating that NK cell lines are refractory to gene transfer [49]. Despite the low transduction efficiency in NKL and NK92, we were able to generate 100% transgenic NK cell lines by the FACS sorter based enrichment of cells transduced with the NKp46 encoding bicistronic vector. Importantly, long-term culture of transgenic Jurkat cells and the two NK cell lines revealed the stable and sustained expression of the GFP marker as well as of the transgenic NKp46 receptor over a time period of at least 10 weeks. NKp46 expression was confirmed by western blot analysis and RT-PCR technique in Jurkat cells transduced either with the single-gene or the bicistronic vectors. For both vectors the sustained transgene expression at equal high levels indicates that there is no difference in the activity of both types of EF1-alpha promoters ("short" and "long" version).

Transductions performed with NK92 and NKL using the bicistronic pWP-vectors revealed a high initial efficiency of up to 45% GFP+ cells on day 2 post infection, which rapidly declined within the following few days. This phenomenon was not observed with the pLox-vectors and most probably reflected a "pseudotransduction" which might be due to the cPPT/CTS regulatory element present in the pWP constructs. Interestingly, this phenomenon of pseudotransduction was not observed in the Jurkat cell line but was also seen with primary NK cells (see below). Thus, NK cells may allow the infection and efficient nuclear translocation of vector DNA but remain refractory to stable genetic modification. In this context it needs to be elucidated whether the low transduction efficiency observed in NK cell lines and primary NK cells is due to mechanisms that minimize the transgene integration or due to mechanisms that promote the silencing of integrated vector DNA.

We chose several experimental approaches to investigate the functional integrity of the transgenic NKp46 receptor. The upregulation of the activation marker CD69 and the release of intracellular Ca²⁺ ions were measured in transduced Jurkat cells upon the monoclonal antibody mediated crosslink of the transgenic receptor. We also measured the release of IFN- γ by transgenic NKL and NK92 cells in response to the engagement by anti-NKp46. Further, we analysed the cytotoxic

activity of the transduced NK cell lines by anti-NKp46 mediated “re-directed” lysis of the NK cell-resistant murine target cell line P815.

Except for the weakly enhanced specific intracellular Ca^{2+} release in Jurkat cells, all other assays did not reveal any substantial effects upon the engagement of the transgenic NKp46 receptor neither in Jurkat cells nor in NK cell lines. Thus, our main conclusion from the functional assays is that the transgenic NKp46 receptor fails to transmit an activating signal upon ligation. One explanation for the lack of activity of the exogenous NKp46 receptor can be that the N-terminal FLAG tag on the receptor may interfere with the antibody-mediated ligation of the receptor. Alternatively, it is possible that the tag-epitope may influence the steric properties of the receptor thus interfering with an association with the adaptor proteins. The apparent although weak response of transgenic Jurkat cells as measured by the intracellular Ca^{2+} release, would be in line with this assumption.

Another possible explanation might be that the adaptor proteins necessary for transmission of the intracellular signalling are missing or limited in their accessibility to NKp46 in the transduced cell lines. Since the surface expression of NKp46 was demonstrated to be independent of the adaptor proteins [40] it is indeed possible that NKp46 is expressed at the surface but is functional inert due to the lack of associated adaptor molecules. Although we found CD3zeta and FcεRIγ mRNAs in the NK cell lines and the Jurkat clones, the amounts of the signalling subunits may be insufficient for the function of exogenous receptors. If so, a low expression of adaptor proteins could explain the lack of activation in Jurkat cells or the failure to induce cytokine release or the redirected killing of anti-NKp46 coated P815 targets by the transgenic NK cell lines.

However, in a recent report on the retroviral transduction of NK92 the successful transfer of FLAG-tagged “wild-type” NKp44 receptor and mutant versions of the NCR was shown [49]. The authors could validate that the surface expression of NKp44 needed the co-expression of DAP12 adaptor molecules and that the transgenic receptor was able to deliver triggering signals through the association with endogenous DAP12. The anti-NKp44 mediated re-directed lysis of otherwise resistant target cells and the upregulation of activation marker by the modified NK92 cell line clearly indicated that the approach to express exogenous NCRs, such as NKp44, is feasible.

Altogether, our results show that the lentiviral vector system is suitable to transfer the cDNA for the natural cytotoxicity receptor NKp46 with varying efficiency and leads to a stable transgene expression in different cell lines. Even though the lentiviral vectors showed a limited capacity to transduce the NK cell lines NK92 and NKL, the enrichment of transgenic cells with a sustained long-term transgene expression was possible. The functional data, however, strongly suggest that

the exogenous NKp46 receptor is not functional, most probably due to impairment in its signalling capacity by as yet undefined mechanism.

1.2 Lentivirus mediated genetic modification of primary NK cells

Lentiviral transductions of primary NK cells were performed either with freshly isolated NK cells at the day of isolation or with NK cells that were cultured and activated with IL-2 and PHA (“restimulated”) for 14 to 20 days. For both stages of cell activation the transduction efficiencies were equally low either with the single-gene pLox-vector or with the bicistronic pWP-vector. Even at an MOI of 20 the GFP marker was expressed by 3 to 5% of cells, indicating similarly low susceptibility to lentiviral gene transfer as with NK92 and NKL. In addition, similar to the NK cell lines, high rates of pseudotransduction in primary NK cells were observed with the bicistronic vector. Restimulated and cultured primary NK cells displayed a GFP expression on day 2 post infection in up to 80% of the cells, which rapidly dropped to levels equal or below that obtained with single gene vectors.

For the GFP encoding pLox-vector we could show that the proportion of transduced cells was maintained through the process of *in vitro* restimulation, although there was a tendency of losing the transgene expressing cells upon repeated stimulations. In addition, such cultures often were over-grown by contaminating residual CD3+ T and NK-T cells. These limitations were circumvented through the enrichment of transduced CD56+ CD3- NK cells by FACS-sorting, which resulted in the generation of pure populations of GFP-expressing NK cells suitable for the subsequent expansion. Taking into account that the process of restimulation usually leads to cell expansions in the range of 1000- to 2000-fold from a starting population of around 3×10^5 cells, this approach represents a highly efficient way to generate large amounts of transgenic NK cells.

Recent results from our lab showed that infections of freshly isolated NK cells that were stimulated for two days with IL-2 resulted in 4 to 5 times higher transduction efficiencies with up to 30% transgenic cells. The reason for this increased susceptibility to gene-transfer remains elusive. In particular, it is surprising that restimulated NK cells, which represent highly proliferating cells, are more refractory to gene transfer than NK cells activated with IL-2 only. This lower susceptibility is difficult to explain but might be caused by the PHA-dependent expansion with its unspecific effects

on cell activation. Analogous to the results achieved with cultured NK cells, cells transduced two days after isolation maintained the initial transduction efficiency during the process of restimulation.

For the gene-transfer of the NKp46 receptor to freshly isolated and IL-2 pre-activated NK cells we used the bicistronic transfer vector only; no infections were done with NKp46-encoding single-gene vectors. The enrichment of transgenic cells by FACS sorting of restimulated cultures resulted in NK cell populations that displayed GFP expression in almost 100% of the cells. In contrast to what we observed with cell lines, no exogenous NKp46 surface expression was detectable on the primary cells. Interestingly, these transduced NK cells had progressively downmodulated endogenous NKp46 during expansion, while the GFP expression remained stable even after the third consecutive restimulation. Whether the low level of endogenous NKp46 is in any relation to the absent surface expression of the transgene remains uncertain. It is particularly of interest, whether the same mechanisms which are responsible for the down regulation of endogenous NKp46, could have influenced the transgenic receptor. Mechanism limiting the NKp46 expression through the regulation of the CD3zeta and FcεRIγ adaptor proteins can be ruled out, since RT-PCR analysis showed the transcription of both molecules in transduced NK cells and since the surface expression of NKp46 was shown to be independent of the expression of adaptor proteins in NK cells [40]. Instead, it might be possible that an unspecified interference of vector mRNA with endogenous transcripts occurs, which in turn would affect both the expression of the endogenous as well as that of the transgenic receptor.

Despite lacking cell surface expression of the transgene encoded NKp46, RT-PCR analysis of transduced NK cells confirmed the presence of the bicistronic transcriptional unit including the mRNA sequence encoding the exogenous receptor. Thus, post-transcriptional modifications of the bicistronic vector mRNA affecting the up-stream gene only, interference of the vector-encoded preprotrypsin leader sequence with protein transport-processes or the instability of the exogenous NKp46 protein in primary cells may result in the observed lack of its surface expression. In favour of these possibilities is the observation that $\gamma\delta$ T cells transduced with the bicistronic vector also fail to express NKp46 (recent data; not included in the thesis). In this case the possibility of an interference of endogenous mRNA or its regulatory elements with vector transcripts can be ruled out since $\gamma\delta$ T cells lack endogenous NKp46.

In an alternative approach to express exogenous NKp46 in primary NK cells, we took advantage of the pseudotransduction achieved with the bicistronic vectors, which led to a high but transient transgene expression. This short-term effect was used to restore the NKp46 surface expression on

polyclonal primary NK cell populations that had almost completely down-modulated NKp46 expression upon the repeated PHA-dependent cell expansion. This approach allowed us to analyse the cytolytic potential of modified NK cells and to assess the functional activity of the exogenous NKp46 receptor in primary cells.

Our results revealed that the transient transduction of NKp46 did not interfere with the efficient cytotoxicity of different NK cell sensitive target cells. However, as observed for the transduced NK92 and NKL cell lines, the engagement of the transgenic receptor could not mediate any NK cell effector functions. In particular, the surface expression of exogenous NKp46 did not correlate to an enhanced cytotoxicity against target cells expressing the putative NKp46-ligand(s), (as identified through the binding of soluble NKp46 molecules). More striking, the fact that anti-NKp46 mAbs did not trigger the “redirected” lysis of the murine P815 target cell line, again strongly suggests that the exogenous receptor is impaired in its capacity to transmit any activating signals.

1.3 Lentiviral transduction of cord blood derived CD34+ hematopoietic progenitor cells

Our second approach to generate transgenic primary NK cells was to apply the lentiviral gene transfer to hematopoietic progenitor cells followed by *in vitro* differentiation towards the NK cell lineage.

CB derived CD34+ progenitors were transduced either with the pLox-vector or the bicistronic pWP-vector at an MOI of 20-30 and were immediately subjected to the differentiation culture for 6 to 8 weeks. The transduction efficiencies were determined early after infection and the maintenance of transgene expression together with the proportion of developing NK cells were monitored throughout the process of differentiation. Transductions with the GFP-encoding single-gene vectors revealed an efficiency of gene transfer that was on average $16,8 \pm 9,9\%$, which is within the broad range of results reported for lentiviral vectors that carry the EF1alpha promoter and are devoid of the cPPT/CTS regulatory element [181, 184]. In certain reports describing remarkable high rates of more than 90% gene transfer efficiency to CD34+ progenitors, transduction protocols involved multiple infections at exceedingly high MOIs with viral titers that were far beyond those we could produce [182] [185].

One of the most important findings in the transduction of progenitor cells with the pLox-vectors was that during *in vitro* differentiation the percentage of GFP+ cells was maintained at the initial

level and that the proportion of transgene expressing cells was stable within the population of developing NK cells. This is in accordance to several reports that describe a stable, multilineage transgene expression upon the *in vitro* differentiation of transduced progenitor cells [180, 181, 185, 186] and indicates that neither the lentiviral transduction per se nor the GFP expression interfered with the process of *in vitro* differentiation.

In addition, this *in vitro* differentiation approach turned out to represent a very efficient way to generate transgenic NK cells. Differentiation cultures were usually started from 2×10^5 progenitors with an average content of CD56⁺CD3⁻ NK cells that reached approximately 40% at week 4 of *in vitro* culture. Thus, given a roughly 350-fold mean expansion rate after 4 weeks, this corresponds to an average output of about 30×10^6 NK cells. Assuming the proportion of transgenic cells to be 20%, this would correspond to an absolute amount of at least 6×10^6 transduced NK cells. This high output of transgenic NK cells could be substantially increased by the FACS sorting of transduced progenitor cells on day 4 post infection, which resulted in the development of almost pure transgenic NK cell populations during the *in vitro* differentiation of CD34⁺ progenitors.

We further could demonstrate that NK cell *in vitro* differentiation cultures can be expanded with PHA in the presence of IL-2 at high rates comparable to those achieved in the restimulation of PB NK cells. However, in contrast to primary transgenic PB NK cells where the content of transduced cells was usually stable during restimulation, the differentiation cultures had a far higher tendency to loose the transgenic cells upon PHA-dependent expansion. Therefore, the FACS-sorting of transduced progenitors prior to the restimulation was necessary to maintain the transgenic cells and usually resulted in a high yield of nearly 100% transgenic NK cells.

However, this promising approach developed using the GFP marker gene was not applicable to generate NK cells transgenic for the NKp46 receptor. Transgene expression was neither detectable in differentiation cultures transduced with the single-gene pLox-vector nor in cultures transduced with the bicistronic pWP-vector. For both types of vectors distinct explanations can be suggested for the observed failure of NKp46 expression.

For transductions with the pLox-NKp46 virus we estimated the viral titers based on the average yield achieved with the GFP-encoding vectors, which possibly led to transductions below the intended MOI of 20 to 30. This may have resulted in a content of transgenic cells that was undetectable by FACS analysis, even though PCR analysis of genomic DNA and RT-PCR on whole

differentiation cultures revealed the integration and presence of transcripts of exogenous NKp46 for at least six weeks during culture.

An alternative explanation is that the transgene may interfere with the *in vitro* differentiation of NK cells, meaning that the exogenous NKp46 protein might impair the development of early NK cell progenitors. Such a suppressive, or even apoptotic effect would lead to the loss of transgenic NK cell progenitors. This hypothesis is supported by results obtained in transductions performed with the bicistronic vector, which resulted in only transient transgene expression which very rapidly decreased to low levels. This might indicate the progressive loss of transduced progenitor cells, even though it also can be contributed to pseudotransduction of progenitors as observed for cell lines and primary NK cells. In addition, we could demonstrate that differentiation cultures starting from FACS-sorter enriched transduced progenitors resulted in almost pure GFP-expressing populations that were mainly of the myeloid lineage with an NK cell content below 1%. This low NK cell content indicates the disappearance of transduced progenitors committed to the NK cell lineage, which resulted in an almost complete suppression of NK cell development. However, although in the remaining myeloid fraction of the differentiation culture no exogenous NKp46 expression was expected, the fact that all GFP+ cells were negative for NKp46, implies instead the presence of additional, yet unspecified mechanisms that suppress transgene expression in an equivalent way as observed in primary NK cells or $\gamma\delta$ T cells.

Finally, the lack of NKp46 expression upon transduction with the pWP-vectors can be explained by a differential activity of the short version of the EF1-alpha promoter in NK cell progenitors and mature NK cells. This conclusion was drawn from the fact that in differentiation cultures transduced with the “empty”, GFP-encoding control-vector (“short” EF1-alpha) the GFP expression in the developing NK cell population was markedly lower compared to the expression in the non-NK cell population. This effect was not observed in mature PB-derived NK cells where the short EF1-alpha promoter was shown to efficiently drive the GFP expression. In contrast, in all pLox-vector transduced cultures (“long” EF1-alpha) the content of GFP+ NK cells was always as high as the overall content of transgenic cells.

All these putative mechanisms may independently impair the expression of NKp46 in transduced progenitor cells, but most probably act in a synergistic way, resulting in an almost undetectable transgene expression.

1.4 Conclusions

Our results demonstrate that lentiviral vectors are suitable to obtain stably transduced NK cell lines or primary NK cells. Transgenic PB-derived NK cells can be expanded to high numbers without losing the expression of the GFP marker, thus indicating the possibility to use genetically modified and expanded NK cells for the adoptive transfer in the immunotherapy of hematological malignancies.

We further demonstrated that GFP⁺ primary NK cells can be generated by the *in vitro* differentiation of lentiviral transduced CB-derived hematopoietic progenitors. This approach represents a highly efficient method to produce large amounts of modified NK cells, in particular in combination to FACS-sorter enrichment of transduced progenitor cells together with the PHA-dependent expansion of *in vitro* generated transgenic NK cells. This approach gives the opportunity to use genetically modified progenitors in hematopoietic stem cell transplantations for the treatment of acute leukemias. Since donor-type NK cells develop rapidly in the early phase after transplantation the over-expression of tumor-specific activatory receptors would direct NK cell effector functions specifically towards the diseased cells, thereby contributing to GvL activity against residual malignant cells.

However, all attempts to genetically modify primary or *in vitro* generated NK cells to overexpress the NKp46 receptor revealed two major constraints for the transfer of the NKp46-encoding gene.

First, although a sustained expression was achieved in NK cell lines, no stable surface expression of transgenic NKp46 could be detected, neither in hematopoietic progenitor cells of differentiation cultures nor in primary PB-derived mature NK cells. Second, the transgenic NKp46 receptor we used turned out to be functional defective or at least impaired in its signalling capacity, without the ability to trigger cytotoxic responses. This failure in the induction of cell activation and NK cell effector functions was observed in stably transduced Jurkat cells or NK cell lines, as well as in transiently transduced primary NK cells.

Taken together, the stable lentivirus-mediated expression of exogenous NKp46 receptor is limited to cell lines but for yet undefined reasons seems to be not feasible in primary cells. Moreover, the lack of transgene-mediated activation in NK cell lines as well as in primary NK cells may be due to different counteracting effects that most probably interfere with each other. Some of these interfering effects can be directly attributed to the cDNA construct we used, like the N-terminal

FLAG tag, the signal peptide that is not NKp46 specific or the IRES element and thus imply the re-cloning of the vector using the original leader sequence in a backbone that is devoid of additional elements. On the other hand, if the failure to transmit an activation signal may prove to be related to a limited availability of the adaptor proteins, the co-transduction of NK cells with cDNA for the corresponding CD3zeta and FcεRIγ subunits should be considered.

Alternatively, the generation of chimeric receptors represent a reasonable approach to induce an NKp46 specific triggering. Chimeric receptors are well established in the re-direction of T cell responses [155] and were also described as an approach to direct the cytolytic activity of transgenic NK cells towards an HIV-derived epitope [169]. Such a chimera would consist of the extracellular part of NKp46 that directs the specificity towards the tumor ligands fused to the signalling components of the CD3zeta- or FcεRIγ chains.

2. Characterization and adoptive transfer of autologous AML-derived NK cells for a cellular immunotherapy of leukemia

Acute myeloid leukemia is characterized by a poor long-term outcome following conventional treatment with high-dose chemotherapy alone or in combination to HSCT. In the majority of AML patients, even a complete remission is associated with a minimal residual disease that is prone to high incidence of relapse with a rapid progression. Together with the fact that a substantial proportion of patients, in particular elderly or in a bad overall condition, cannot undergo chemotherapy or transplantation, new approaches in the management of acute leukemia are clearly of need. Recent studies on the role of NK cells in the immunosurveillance of hematological malignancies point towards the use of NK cells as an immunotherapeutic tool for the treatment of AML. The anti-leukemic activity provided by alloreactive NK cells in the context of HSCT [187] and the finding that a low or lacking anti-tumor reactivity of autologous NK cells can be correlated to the probability of leukemia relapse in AML patients [89, 90] suggest an adoptive transfer of allogeneic or autologous NK cells as a new strategy in leukemia immunotherapy.

In AML several mechanisms can be hypothesized to explain that malignant cells escape the immune surveillance by cytotoxic effector cells. Leukemic blasts were found to preferentially down-regulate the expression of HLA class I molecules that are associated with the recognition and lysis by

cytotoxic T cells, but keep those that mediate NK cell inhibition [91] [157]. Malignant cells may also escape NK cell attack by the *in vivo* selection of leukemia-initiating progenitor cells, which lack ligands for NK cell triggering receptors [34] or display a certain resistance to NK cell lysis such as an impairment in perforin binding [93]. Alternatively, the failure of NK cells to control leukemia might be due to the loss or decreased expression of activating receptors involved in the recognition of tumor targets [88].

To investigate the mechanisms of impaired recognition and lysis of leukemic blasts, we isolated NK cells from AML patients (AML-NK) and activated and expanded them *in vitro*. For the phenotypic and functional characterization of AML-NK cells, our studies focused on the expression of the activating receptors NKG2D and the NCR NKp46 and determined the cytotoxic activity of NK cells against the autologous blasts *in vitro* and *in vivo* in the NOD/SCID mouse transplantation model.

2.1 Isolation, expansion and phenotypic characterization of AML-derived NK cells

We isolated peripheral blood NK cells from 14 AML patients that were either newly diagnosed or had a recurrent disease. The AML diagnosis at clinical presentation included the sub-types M1, M2, M4 and M5 as well as not further categorized secondary AML. FACS based analysis revealed the strongly diminished content of NK cells in AML patients compared to healthy controls. At diagnosis NK cells accounted for less than 1% of PB MNCs, corresponding to an average 4-fold reduction per leukocyte content that was even 10-fold below normal levels when calculated per total MNCs. However, in absolute numbers NK cells in AML PB was in a normal range arguing that the disease did not affect the NK cell development. As a consequence of this reduction and due to the high blast content of 37-94% (per MNCs), the absolute amount of NK cells that could be recovered from patient samples was poor. For all patients the total yield of CD3-CD56+ NK cells was in a range of $0,2 - 3,0 \times 10^5$ cells. Thus, with regard to the low numbers that were far below the amount required for functional assays, the NK cells were immediately subjected to *in vitro* restimulation with PHA, IL-2 and irradiated allogeneic PB MNCs. This procedure led to the efficient expansion of NK cells equal to that observed for control-NK cells with an 1000 to 2000-fold increase in total cell numbers, a feature which was also observed with NK cells in remission [140]. Thus, unlike in

chronic myeloid leukemia, in which the proliferative capacity of NK cells decreases with the disease progression [80, 83] AML-NK cells can be expanded *in vitro* with high efficacy.

FACS analysis of the surface expression of different NK cell and lymphocyte marker revealed that the phenotype of AML-NK cells in polyclonal expansion cultures closely resembled that of control-NK cells. The restimulated NK cells acquired a uniform CD56^{bright}/CD16^{bright} phenotype and displayed comparable distribution patterns of the subsets that expressed CD158a and -b, NK-B1 or CD161.

The NKp46 expression on freshly isolated or restimulated polyclonal NK cell populations has been described to be heterogeneous. Polyclonal NK cell populations either display a NKp46 phenotype that is uniformly bright or dim or NKp46 expression is “bimodal”, representing a mixture of NK cell clones of either phenotype [42]. Our results are in accordance to published data since we could detect each phenotype in freshly isolated NK cell populations of healthy donors as well as in AML patients. We demonstrated that the surface expression levels of NKp46 and NKG2D in expanded AML-NK cell cultures did not differ from control-NK cells. In addition, we could show that the average expression level of NKp46, defined by the mean fluorescence intensity ratio (MFIR), remained at a constant level upon the *in vitro* expansion. The MFIR of NKp46 was 8,3 on freshly isolated AML-NK cells and 6,9 after expansion, or 6,8 and 8,7 in donor-NK cells, respectively. In contrast, the expression levels of NKG2D were clearly upregulated following the culture in AML-NK cells (MFIR 6,4 to 27,0) as well as in donor NK cells (MFIR 10,0 to 27,1).

Interestingly, there has been a tendency of lower expression of NKG2D in fresh patients' PB (MFIR 6,4 vs. 10,0; $P < 0,05$), which might be influenced by ligands shed from AML blasts in analogy to the downregulation of NKG2D in T cells by MIC ligands released from solid tumors [98],

The surface expression of NKp46 showed a certain dynamic during PHA-dependent restimulation, with a transient down modulation of NKp46 and its recovery to initial levels. In some polyclonal NK cell cultures the repeated restimulation was accompanied with the progressive loss of NKp46 surface expression to almost undetectable levels. This phenomenon was restricted to NKp46 since the surface expression of NKG2D was always upregulated and sustained at high levels without any major changes even upon several consecutive cycles of restimulation. It remains to be clarified if this loss of NKp46 expression is due to the down-regulation of the receptor in the whole population or whether this reflects a bias of the polyclonal population to the preferential expansion of NKp46^{dim} cells. In line with the latter is the observation that some polyclonal NK cell populations changed

from a uniform bright phenotype to a bimodal distribution and finally consisted of cells with a dim or almost negative NKp46 expression.

A negative or “dull” NCR expression in AML-NK cells upon the PHA-dependent *in vitro* expansion was described previously [88]. The authors reported a defective NCR expression in 16 out of 18 AML patients, and could correlate this phenomenon to the observed failure in cytotoxicity of autologous blasts. A similar loss or the tumor-mediated downmodulation of triggering NK cell receptors was recently demonstrated as a mechanism of tumor escape in epithelial tumors [98]. However, since we observed the same NKp46 down-regulation in NK cells derived from healthy donors, this phenomenon cannot be attributed to an AML-inherent defect in NK cell function.

With regard to the putative therapeutic application of expanded NK cells it would be of major importance to define culture conditions that are not accompanied by the loss of activating receptors, but, opposite, would allow to increase NK cell effector functions through the up-regulation of adhesion molecules, co-receptors or triggering receptors. Therefore, we investigated the influence of several cytokine combinations and their synergy to the IL-2- and PHA-dependent expansion with the goal to enhance surface expression of NKp46 or NKG2D.

The effect of cytokines on the expression of NK cell receptors has not been studied extensively. Earlier studies suggested that the expression density of most activating receptors is stable and not influenced by IL-2, IL-12 or IL-15 [188]. On the other hand, it is known that activation with IL-2 induces the expression of the natural cytotoxicity receptor NKp44, which is absent on resting NK cells [48]. Similarly, expression levels of human NKG2D are upregulated in response to IL-2 and IL-15 in NK cells [189] as well as in intraepithelial CD8+ $\alpha\beta$ + T lymphocytes [190].

We have extended these studies by using cytokines that are known to modulate homeostasis and the function of NK cells, namely IL-12, IL-15 and IL-21, and used various combinations of these cytokines along with IL-2. Our results demonstrated that these cytokines did not enhance the proliferative capacity of AML- and control-NK cells in response to IL-2 and none of the cytokine combinations could further increase the surface expression of NKp46. In contrast, the expression of NKG2D was clearly suppressed in cultures that contained IL-12 and IL-21 either singly or in combination. This in turn offered the possibility to perform functional assays to determine the consequences of receptor down modulation in a system where the NKG2D receptor-ligands interaction is well defined.

Taken together, our results show that AML-NK cells do not differ from healthy control-NK cells; even though reduced as a cell compartment, they retain a proliferative capacity that allows the *in vitro* expansion to high cell numbers and they display the same expression pattern of the major activating receptors.

2.2 Functional properties of expanded AML-NK cells

In addition to the phenotypic characterization of *in vitro* expanded AML-NK cells we determined their functional properties. The functional activities of NK cells can be defined according to their role as effector cells of the innate immunity. NK cells constitutively express several receptors for monokines and chemokines and rapidly produce a variety of cytokines such as IFN γ , IL-10, IL-13, TNF- β , TNF- α or GM-CSF in response to stimulation. This immunoregulatory role is attributed to the less abundant CD56^{bright}/CD16^{dim} subset of NK cells, whereas the majority of PB NK cells are CD56^{dim}, express high levels of CD16 and have a more important role in cytotoxicity [7]. We showed that restimulated NK cells express uniform levels of CD56 and CD16 and therefore can no longer be classified into functional subtypes. We examined the potential of expanded AML-NK cells to produce IFN- γ in response to IL-12 and IL-18 and analysed their cytolytic capacity against the NK cell sensitive target cell line K562. Intracellular FACS analysis revealed that a high proportion of AML-NK cells displayed the ability to produce IFN- γ , analogous to that of control-NK cells. Similarly, previous studies documented the ability of AML-NK cells in remission to produce IFN- γ and TNF- α after polyclonal activation [90, 140]. We also determined the cytolytic activity against the K562 target cell line in ⁵¹chromium release assays at different effector to target ratios and demonstrated that AML-NK cells were equally cytolytic as control-NK cells.

From these results we conclude that cultured AML-NK have a normal ability to produce proinflammatory cytokines and have preserved their cytolytic activity throughout the process of *in vitro* expansion.

In AML it was shown that the capacity to recognize and kill autologous malignant leukemic cells is generally poor [88, 90, 191]. However, the cytolytic activity of patient-derived NK cells against tumor cell lines could be correlated to the duration of remission [86], indicating the importance of NK cells in the immunosurveillance of acute leukemia. A recent study could reveal an association

of NK cell-mediated *in vitro* activity against autologous leukemic cells with the clinical outcome in AML and ALL patients [89]. The loss of tumor-reactive NK cells in one patient was followed by leukemia relapse, whereas in another patient treatment with IFN- α could revert the relapse to complete remission, which was accompanied by the regain of NK cell activity against autologous blasts. These results were confirmed on a larger group of 25 AML and ALL patients after chemotherapy or autologous HSCT, where a low or absent activity was shown to be predictive of leukemia relapse [90]. Thus, the data imply that the failure of NK cells to recognize and lyse autologous leukemic blasts may contribute to the high incidence of relapse in AML. Earlier studies on the NK cell activity at diagnosis found that leukemic blasts are resistant to autologous killing, and concluded that patient-derived NK cells were functionally defective [87]. Likewise, in a recent report the failure of AML-derived NK cells of lysing autologous leukemic cells was attributed to an insufficient interaction between NK cell activating receptors and their ligands on the blasts [88]. This may either be due to the defective expression of triggering receptors on NK cells or to the low density of the corresponding ligands on the tumors.

On the other hand, the fact that NK cells from AML patients in complete remission after chemotherapy or after autologous HSCT exert a substantial anti-leukemic activity *in vitro*, justifies the expansion of patient-derived NK cells for a putative application in cellular immunotherapy. A recent study demonstrated that NK cells that were collected at remission, displayed a certain *in vitro* activity against the autologous leukemic blasts after activation and *in vitro* expansion [140]. Accordingly, we isolated patient-derived NK cells at diagnosis or at disease relapse, and determined the cytotoxic activity of *in vitro* expanded cells against autologous blasts. The main goal was to define whether AML-NK cells are characterized by an inherent deficiency in target cell recognition that may contribute to the escape of leukemic blasts from NK cell surveillance.

The analysis of the cytolytic activity of expanded NK cells from 5 patients revealed that the spontaneous lysis of autologous blasts was very low. In 4 out of 5 patients the specific lysis at the E:T ratio of 10 was below 10%. Importantly, the addition of anti-HLA class I monoclonal antibodies, which interrupts the KIR-HLA interactions and thereby blocks inhibitory signalling, induced a substantial level of cytotoxicity that ranged from 40 to 70% specific lysis.

Thus, our results demonstrate that *in vitro* expanded AML-NK cells are capable to recognize and efficiently lyse autologous leukemic blasts, through the interaction of activating receptors with their ligands. These findings indicate that no intrinsic resistance in the leukemic blasts exists although NK cell activation was dominated by the HLA class I mediated inhibition as demonstrated by the

addition of HLA-masking mAbs. This strong NK cell inhibition represented by a low spontaneous cytotoxicity of leukemic blasts was also observed when allogeneic control-NK or allogeneic AML-derived NK cells were used as effectors, but rather unexpected since the high probability of KIR-HLA class I mismatches in the allogeneic set-up should favour the opposite effect of an increased susceptibility to cytotoxicity.

However, in one out of the five analysed patients, AML-NK cells had a fairly high spontaneous cytotoxic activity against the autologous blasts with a specific lysis of up to 30% at the E:T ratio 10. Even though the HLA class I expression was within the range usually measured in AML, the addition of HLA class I masking antibodies could only marginally increase the lysis by autologous NK cells. Therefore, we suggest that the elevated expression of ligands for the activating receptor NKG2D and the NCRs, NKp46 and NKp30, by the blasts may account for the observed susceptibility to cytotoxicity. The blasts of this particular patient belong to the AML subtype M5, which has been related to an increased level of NKG2D- and NCR-ligand expression. Indeed, according to recent studies in our laboratory, acute leukemias of the subtypes M1 to M4 were found to express low levels of triggering ligands, whereas the M5 and M7 subtypes of AML, which affect more mature progenitors, displayed higher ligand levels [192].

In conclusion, our data implicate that a low density of NKG2D and NCR ligands, due to the incomplete process of myeloid lineage maturation or ligand shedding [34], together with the abundance of HLA class I molecules, are compromising the recognition of blasts by NK cells.

2.3 The NOD/SCID transplantation model and immunotherapeutic consequences

One of the important issues of the present study was to explore the potential of activated and *in vitro* expanded NK cells from AML patients for an adoptive transfer in the cellular immunotherapy of leukemia. In this context we used the NOD/SCID transplantation mouse model which have previously been employed to demonstrate the cytotoxic potential of human haploidentical NK cells against CML blasts [106], to investigate the *in vivo* activity of activated and expanded AML-NK cells. First we could demonstrate that adoptively transferred AML-NK cells were able to exert an anti-tumor activity in K562 erythroleukemia engrafted NOD/SCID mice. Mice were inoculated subcutaneously with K562 cells and injected one day later with AML-NK cells. Our results showed that in mice that received the adoptively transferred AML-NK cells the tumor formation was significantly suppressed over a time period of four weeks. This effect was most probably due to an NK cell target interaction in an early phase after the adoptive transfer since we could not detect any

infiltration of the tumors with human NK cells. This is in line with a recent report demonstrating that human NK cells injected into the circulation of NOD/SCID mice accumulate in the BM and spleen within the first 24 hours post transplantation but were not anymore detectable after 72 hours [193]. Thus, in this case the suppressive effect of NK cells on tumor formation might be due to a rapid purge of tumor initiating cells.

We further extended the NOD/SCID *in vivo* model to the transplantation of human primary AML blasts. 10×10^6 AML blasts were injected, and the engraftment was determined on week 4 to 8 in the peripheral blood or in aspirated bone marrow samples. In 5 out of 7 patient samples the transplantation resulted in an engraftment with human leukemia, with an average of 27% of human cells detected in the BM of recipient mice on week 8 post transplant. These values are in accordance to previously reported results on the transplantation of AML into NOD/SCID mice with an average engraftment of 13% for 70% of the more than 60 different samples [194]. In this particular report the failure of engraftment in mice was also found to correlate to the FAB-type and the cytogenetic status of AML blasts.

Transplanted NOD/SCID mice that showed a substantial engraftment with human leukemia received 3 to 5×10^6 *in vitro* activated and expanded autologous AML-NK cells along with IL-2 and IL-15 in order to support the viability and to maintain the activation status of the infused NK cells. Since only a short-term retention of the transferred NK cells in the mice was to be expected, we analysed the mice one week after the adoptive transfer of NK cells.

Our results demonstrate that all mice that received AML-NK cells showed a significant overall reduction of tumor load in the BM. The content of human leukemic blasts of an average of about 30% before NK cell treatment was significantly reduced to about 10%, corresponding to an overall reduction of tumor-load of roughly 70%. In contrast, the content of blasts in the control-group, which did not receive NK cells, was on average 16% and increased to about 18%, irrespective of the administration of IL-2 and IL-15.

In conclusion, we could demonstrate that AML-NK cells display an anti-leukemic effect against autologous blasts *in vivo* in leukemia-repopulated NOD/SCID mice. This *in vivo* activity was observed despite the inhibitory signalling mediated by the interaction of HLA class I and the KIRs. One possible explanation for the reduction of tumor load is that NK cell target interactions induced apoptosis, which act through an HLA class I independent mechanism. Another possibility is that the murine BM microenvironment influenced the transplanted leukemic blasts in a way that rendered them more susceptible to NK cell lysis. This would imply that AML blasts may undergo a change in expression of HLA class I molecules or of ligands for activating NK cell receptors. Indeed, we

found an up to 5-fold upregulation of ligands for NKG2D and NCR receptors on leukemic blast that were recovered from the murine BM. In addition, recent results in our laboratory demonstrate that the exposure of AML blasts to myeloid-specific growth factors upregulated expression of activating ligands and increased the susceptibility to NK-mediated killing [192]. The importance of triggering ligands for the susceptibility to NK-mediated killing has been further demonstrated by studies in a mouse tumor model, in which ectopic expression of murine NKG2D ligands resulted in efficient NK- and T cell-dependent rejection of the tumor [38].

Therefore, we hypothesize that growth factors produced in the murine BM may have an influence on the ligand expression by the transplanted AML blasts, which is substantial to counteract the inhibitory signalling through HLA class I expression.

The possibility of substantial *ex vivo* expansion of highly cytotoxic AML-NK cells implicates their usefulness as cellular therapeutics for the clearance of autologous leukemia. Infusions of donor-derived NK cells, including NK cells activated by 2 week-long treatment with IL-2 *in vitro*, have been reported as safe effective in increasing the donor chimerism in the transplanted patients [195, 196]. Given the importance of activating ligand-receptor interactions for the tumor recognition process, clinical use of low dose of IL-2 [197] and IL-15 [198] to upregulate the receptors and support the maintenance of adoptively transferred NK cells along with administration of myeloid growth factors to upregulate the respective ligands may be beneficial in enhancing the effectiveness of leukemia therapy with *ex vivo* IL-2-activated autologous NK cells. Moreover, the possibility to enhance the expression of activating NK cell receptors by lentiviral-mediated genetic modification of *ex vivo* expanded AML-NK cells still represents an option to optimize such immunotherapeutic interventions.

Even though the mechanism of blast susceptibility to autologous NK cell-mediated lysis requires further clarification, the strong reduction of tumor load upon the adoptive transfer of the autologous AML-NK cells to NOD/SCID mice is promising and may be important for the design and future progress in clinical immunotherapeutic treatment strategies based on infusions of autologous NK cells.

VII. REFERENCES

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VIII. CURRICULUM VITAE / PUBLICATIONS

Curriculum vitae:

Name and Address:	Uwe Siegler Hebelstr. 29 D 79688 Hausen i.W. Germany
Date of birth	08.12.1967
Citizenship:	German
Education:	
1974-1978	Primary school Grundschule Schopfheim-Wiechs, (D)
1978-1987	High school at Theodor-Heuss Gymnasium Schopfheim, (D); Abitur June 1987
1987-1988	Military service
Nov. 1989	Matriculation at the University of Basel; Studies of Chemistry and Biology II at the Faculty of Natural Sciences, University of Basel
1992-1998	Studies of Biology I at the Faculty of Natural Sciences, University of Basel
Nov. 1996 – Feb. 1998	Diploma in Biology at the Swiss Tropical Institute, in the Laboratory of Molecular Immunology, Basel Title Msc thesis: “Clonal analysis of the T-cell response against the malaria peptide vaccine Spf66” (under supervision of PD Dr. G. Pluschke)
Jan. 2000 – Oct. 2004	PhD thesis under the supervision of Prof. A. Wodnar-Filipowicz, Laboratory of Experimental Hematology, Department of Research, University Hospital Basel;

List of Academic Teachers:

Prof. Dr. med. M.M. Burger

Prof. Dr. phil T.A. Bickle

Prof. Dr. med. A.A. Gratwohl

Prof. Dr. phil. T. Hohn

Prof. Dr. phil. N. Hynes

Prof. Dr. med. G. Holländer

Prof. Dr. med. Chr. Moroni

Prof. Dr. P. Philippsen

Prof. Dr. med. Chr. Rochlitz

Prof. Dr. A.G. Rolink

Prof. Dr. med. R.C. Skoda

Prof. Dr. phil. N. Weiss

Prof. Dr. phil. A. Wodnar-Filipowicz

Publications:

1) Activated natural killer cells from patients with acute myeloide leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice

Siegler U, Kalberer CP, Nowbakht P, Sendelov S, Meyer-Monard S, Wodnar-Filipowicz A.
Leukemia, submitted 2005

2) Human NK cell development in NOD/SCID mice receiving grafts of cord blood CD34+ cells.

Kalberer CP, Siegler U, Wodnar-Filipowicz A.
Blood. 2003 Jul 1;102(1):127-35.

3) Amino acid dimorphism and parasite immune evasion: cellular immune responses to a promiscuous epitope of Plasmodium falciparum merozoite surface protein 1 displaying dimorphic amino acid polymorphism are highly constrained.

Daubenberger CA, Nickel B, Ciatto C, Grutter MG, Poltl-Frank F, Rossi L, Siegler U, Robinson J, Kashala O, Patarroyo ME, Pluschke G.
Eur J Immunol. 2002 Dec;32(12):3667-77.

4) Herpesvirus saimiri transformed T cells and peripheral blood mononuclear cells restimulate identical antigen-specific human T cell clones.

Daubenberger CA, Nickel B, Hubner B, Siegler U, Meinl E, Pluschke G.
J Immunol Methods. 2001 Aug 1;254(1-2):99-108.

Oral presentations / posters:

1) Cytotoxic potential and anti-leukemic effect of natural killer cells from AML patients against autologous leukemic blasts in vitro and in vivo after adoptive transfer to NOD/SCID mice

Siegler U, Kalberer CP, Nowbakht P, Meyer-Monard S, Tichelli A and Wodnar-Filipowicz A.
EHA Geneva 2004; *oral presentation / abstract*

2) Anti-leukemic effect of autologous natural killer cells from AML patients after adoptive transfer to NOD/SCID mice

Siegler U, Kalberer CP, Nowbakht P, Meyer-Monard S, Tichelli A and Wodnar-Filipowicz A.
Swiss Society of Hematology
(SSH) Lausanne 2004, *oral presentation*

3) Analysis, expansion and genetic modification of cytotoxic natural killer cells from AML patients

Siegler U, Kalberer CP, Nowbakht P and Wodnar-Filipowicz A.
Annual meeting of the German, Swiss and Austrian Societies of Hematology and Oncology, Basel 2003; *poster*

4) Anti-tumor activity of human NK cells generated in NOD/SCID mice from cord blood CD34+ progenitor cells

Kalberer CP, Siegler U and Wodnar-Filipowicz A.
Swiss Society of Hematology (SSH) Basel 2003, *oral presentation / abstract*

5) Highly efficient and stable genetic modification of human NK cells derived from transgene-expressing hematopoietic progenitor cells transduced with lentiviral vectors

Siegler U, Kalberer CP and Wodnar-Filipowicz A.
SSH Geneva 2002; *oral presentation*

6) Human natural killer cell development in xenografted NOD/SCID mice is promoted by human interleukin 15, flt3 ligand and stem cell factor

Kalberer CP, Siegler U and Wodnar-Filipowicz A.

3rd Stem Cell Gene Therapy Conference, Rockville, MD USA 2002; *poster*

7) Development of cytotoxic natural killer cells overexpressing activatory receptors as a potential immunotherapy tool

Siegler U, Kalberer CP, Luther-Wyrsh A, Nissen C & Wodnar-Filipowicz A.

Int. Symposium on Cellular Therapy, Regensburg (D) 2001; *poster*

8) Development of cytotoxic natural killer (NK) cells from human hematopoietic progenitors in vitro and in vivo in NOD/SCID mice

Kalberer CP, Luther-Wyrsh A, Siegler U, Nissen C and Wodnar-Filipowicz A.

SSH Lausanne 2001; *oral presentation*

9) Development of natural killer (NK) cells from human cord blood hematopoietic progenitors in NOD/SCID mice

Kalberer CP, Luther-Wyrsh A, Siegler U, Nissen C and Wodnar-Filipowicz A.

EHA Frankfurt 2001; *poster*

10) Long-term transgene expression in natural killer cells generated from lentivirally transduced CD34+ progenitor cells

Kalberer CP, Siegler U, Luther-Wyrsh A, Nissen C and Wodnar-Filipowicz A.

ESGT Turkey 2001; *poster*

11) Natural cytotoxicity receptor NKp46 is expressed at low levels on natural killer cells generated in vitro from CD34+ cord blood cells and in vivo in NOD/SCID mice reconstituted with human hematopoietic cells

Kalberer CP, Siegler U, Colonna M, Nissen C and Wodnar-Filipowicz A.

ASH 2001; *poster*