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EXPANSION AND GENETIC MODIFICATION OF HUMAN NATURAL KILLER CELLS FOR ADOPTIVE IMMUNOTHERAPY OF CANCER

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For Mom and Dad…
Annem ve Babam için…

ABSTRACT

A century after the initial proposition that the immune system has the capacity to fight against tumors, evading destruction by immune cells is now well recognized as a hallmark of cancer. Recent decades have witnessed extraordinary improvements in the use of immunotherapy against malignancies and adoptive transfer of Natural Killer (NK) cells stands among promising tools in the fight against cancer. Clinical studies have demonstrated the anti-tumor responses generated by NK cells both in the autologous and allogeneic settings in various cancers. Direct adoptive transfer, *ex vivo* activation and/or expansion, as well as genetic modification of NK cells aspire novel improvements to current immunotherapy strategies. As such interventions develop, the quest for better preparation of NK cell based therapies continues.

This thesis, primarily investigates the feasibility and potential of *ex vivo* expanded NK cells for cancer immunotherapy. Our results produced a system that has the capacity to expand polyclonal and highly cytotoxic NK cells showing selective anti-tumor activity. Protocols for expansion of these cells from healthy donors and patients with Multiple Myeloma (MM) using current Good Manufacturing Practice (cGMP)-compliant methods have been optimized in conventional cell culture systems as well as automated bioreactors. The elevated cytotoxic activity of expanded NK cells against autologous tumor cells, along with detailed analysis of phenotypic changes during the expansion process has subsequently shifted attention to the interaction between NK and tumor cells.

Both as a basic method to identify these interactions, and as part of further plans to use genetically retargeted NK cells in cancer immunotherapy, we have investigated methods for efficient lentiviral genetic modification of NK cells. This study has resulted in an optimized stimulation and genetic modification process for NK cells that greatly enhances viral gene delivery. Along with NK cell stimulating cytokines, an inhibitor of innate immune receptor signaling that blocks the intracellular detection of viral RNA introduced by the vector was successfully utilized to enhance gene transfer efficiency, also constituting a proof-of-concept for various other gene therapy approaches.

Taken together, the work presented in this thesis aims to bring us closer to optimal *ex vivo* manipulation of NK cells for immunotherapy. Clinical trials with the long-term expanded NK cells as well as further preclinical development of NK cell genetic modification processes are warranted.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by using their Roman numerals:

I. Alici E, **SUTLU T**, Bjorkstrand B, Gilljam M, Stellan B, Nahi H, Quezada HC, Gahrton G, Ljunggren HG, and Dilber MS. Autologous anti-tumor activity by NK cells expanded from myeloma patients using GMP-compliant components.
Blood. 2008 Mar 15;111(6):3155-62.

II. **SUTLU T**, Stellan B, Gilljam M, Quezada HC, Nahi H, Gahrton G and Alici E. Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor.
Cytotherapy. 2010 Dec;12(8):1044-55.

III. **SUTLU T,** Gilljam M, Stellan B and Alici E. Inhibition of intracellular anti-viral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy.
Manuscript submitted.

Text from following review and response papers have been used for writing of the Introduction section of this thesis:

- **SUTLU T,** Alici E. *Ex vivo* expansion of natural killer cells: a question of function. *Cytotherapy. 2011 Jul;13(6):767-8.*

- Georgoudaki AM, **SUTLU T**, Alici E. Suicide gene therapy for graft-versus-host disease. *Immunotherapy. 2010 Jul;2(4):521-37*

- **SUTLU T**, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects.
J Intern Med. 2009 Aug;266(2):154-81

Related publications outside the thesis:

- Barkholt L, Alici E**,** Conrad R, **SUTLU T**, Gilljam M, Stellan B, Christensson B, Guven H, Björkström NK, Söderdahl G, Cederlund K, Kimby E, Aschan J, Ringdén O, Ljunggren HG, Dilber MS. Safety analysis of an ex-vivo expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study.
Immunotherapy. 2009 Sep;1(5):753-64
- Alici E, **SUTLU T**, Dilber MS. Retroviral gene transfer into primary human natural killer cells.
Methods Mol Biol. 2009;506:127-37.
- **SUTLU T**., Alici E, Jansson M, Wallblom A, Dilber MS, Gahrton G, Nahi H. The prognostic significance of 8p21 deletion in multiple myeloma.
Brit J Haematol. 2009 Jan;144(2):266-8.
- Alici E, Konstantinidis KV, **SUTLU T**, Aints A, Gahrton G, Ljunggren HG, Dilber MS. Anti-myeloma activity of endogenous and adoptively transferred activated natural killer cells in experimental multiple myeloma model.
Exp Hematol. 2007 Dec;35(12):1839-46.

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LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin-D
ADCC	Antibody-dependent cellular cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ASCT	Autologous stem cell transplantation
BIV	Bovine immunodeficiency virus
BLV	Bovine leukemia virus
BM	Bone marrow
BMT	Bone marrow transplantation
BrCa	Breast cancer
CD	Cluster of differentiation
CIK	Cytokine induced killer
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CR	Complete remission
CRC	Colorectal carcinoma
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
ds	Double stranded
EIAV	Equine infectious anemia virus
env	Envelope
FIV	Feline immunodeficiency virus
GALV	Gibbon ape leukemia virus
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GMP	Good manufacturing practice
GOI	Gene of interest
GvHD	Graft-versus-host disease
Hb	Hemoglobin
HCC	Hepatocellular carcinoma
HDT	High-dose chemotherapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
HTLV	Human T cell leukemia virus
IFN-	Interferon-
Ig	Immunoglobulin
IL-	Interleukin-
iPSC	Induced pluripotent stem cell
IRES	Internal ribosomal entry site
IMiDs	Immunomodulatory drugs
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer-cell immunoglobulin-like receptor
LAK	Lymphokine-activated killer
LGL	Large granular lymphocyte
LTR	Long terminal repeat
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MLV	Murine leukemia virus
MM	Multiple myeloma
MMTV	Mouse mammary tumor virus
MIP-1	Macrophage inflammatory protein-1
MOI	Multiplicity of infection
MRD	Minimal residual disease
NB	Neuroblastoma
NCR	Natural cytotoxicity receptor
OCL	Osteoclast
PBMC	Peripheral blood mononuclear cell
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PD-1	Programmed death receptor-1
PD-L1	Programmed death receptor ligand-1
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PPT	Polypurine tract
PR	Partial remission/response
PRE	Post-transcriptional regulatory element
PG	Prostaglandin
RANK	Receptor activator of nuclear factor κ-B
RCC	Renal cell carcinoma
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
SCID	Severe combined immunodeficiency
SCT	Stem cell transplantation
SD	Stable disease
SFFV	Spleen focus forming virus
shRNA	Short hairpin ribonucleic acid
SIV	Simian immunodeficiency virus
SNV	Spleen necrosis virus
ss	Single stranded
SV40	Simian virus 40
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-	Tumor necrosis factor-
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
WBC	White blood cell
VSV	Vesicular stomatitis virus
β2M	Beta-2-microglobulin

1 INTRODUCTION

Immunology as a scientific discipline is generally accepted to begin with Edward Jenner's discovery of the smallpox vaccine in 1796. Jenner used inoculations with the non-lethal cowpox virus, which also induced immunity against smallpox. Actually, the process of variolation (deliberate infection with smallpox) was already in practice outside Europe and was first imported into Europe around 1718 by Lady Mary Wortley Montagu who had seen it being practiced by physicians in Istanbul, where her husband served as the British ambassador to the Ottoman Empire[1]. The main observation at that time was that once a person recovered from smallpox (or similar symptoms produced by variolation), they did not get the disease again, or got it in a very mild form. The search for the mechanisms behind this phenomenon has evolved into the science of immunology and today we have a much better understanding of the immune system.

Traditionally, the immune system is divided into two arms: adaptive and innate immunity, both of which have cell-mediated and humoral defense mechanisms to protect the body from foreign pathogens. Considered as the first line of defense, the innate immune system is believed to precede adaptive immunity in the evolution of the immune system[2]. Since their discovery, natural killer (NK) cells have been considered characteristically more innate than adaptive because of their ability to respond against target cells in the absence of prior sensitization. However, the definitions of "innate" and "adaptive" have been blurred by recent findings showing adaptive immune features in NK cells[3], which develop from a common progenitor that also gives rise to T and B cells[4,5], constituting the third major lineage of lymphocytes.

1.1 NATURAL KILLER CELLS

Initially regarded as an "experimental artifact" in T cell cytotoxicity assays, NK cells were first discovered in mice more than 35 years ago by Rolf Kiessling and Eva Klein, who also named them natural killer cells[6,7] and in parallel by Herberman and colleagues[8,9]. Human NK cells were initially described as non-adherent, non-phagocytic, $F_c\gamma R^+$, large granular lymphocytes (LGL)[10]. Later it was appreciated that not only NK cells shared the LGL phenotype and that some NK cells displayed normal small lymphocyte morphology, depending on their activation status[11]. This made it difficult to detect NK cells just by size and morphology. The identification of the NKR-P1[12], and NK1.1[13] made it possible to define murine NK cells roughly as $NK1.1^+$ TCR^- sIg^- $CD16^+$. Today, human NK cells are defined as $CD3^-CD56^+$ lymphocytes. They comprise approximately 10-15% of all circulating lymphocytes and are also found in tissues, including the liver, peritoneal cavity and placenta. Following activation by cytokines, resting NK cells that circulate in the blood, are capable of extravasation and infiltration into most tissues that contain pathogen-infected or malignant cells[14-16].

Initially it was not clear how NK cells distinguished target cells they should kill from those that they should spare. When Klas Kärre summarized his and other people's work for his doctoral thesis, he found a common denominator not about what was

commonly expressed on target cells but about what was commonly missing. This lead to the formulation of the *missing-self* hypothesis, where he suggested that NK cells kill target cells lacking expression of self MHC class-I molecules although the mechanism was unclear[17,18] at the time (Figure 1). This model was later confirmed by the discovery of inhibitory receptors on NK cells.

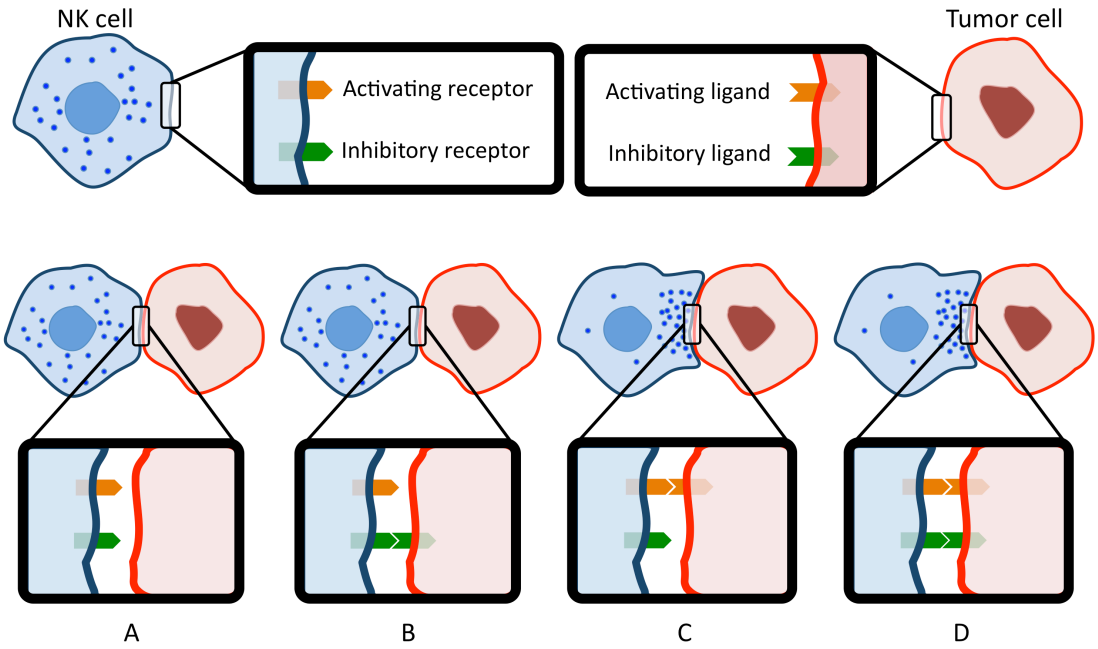


Figure 1. The recognition of tumor cells by NK cells. The figure presents four hypothetical scenarios for the encounter of an NK cell and a tumor cell. (A) Although the tumor cell does not express any inhibitory ligands, it cannot be killed by the NK cell because it also lacks the expression of any activating ligands. This target is practically invisible to the NK cell and no recognition takes place. (B) The tumor cell expresses ligands for inhibitory receptors whereas it lacks ligands for activating receptors. The NK cell recognizes the inhibitory ligands; therefore, no killing takes place. (C) The tumor cell has significantly downregulated or absent expression of inhibitory ligands along with sufficient expression of activating ligands. *Missing-self* recognition takes place and the target is killed. (D) The tumor cell expresses significant levels of both inhibitory and activating ligands. The NK cells recognize both types of ligands and the outcome of this interaction is determined by the balance of inhibitory and activating signals.

Human NK cells are conventionally separated into two subsets based on their CD56 expression. This separation is not just phenotypic but rather has many functional outcomes. The majority (~90%) of human NK cells have low-density expression of CD56 ($CD56^{dim}$), whereas ~10% of NK cells are $CD56^{bright}$. Early functional studies of these subsets revealed that the $CD56^{dim}$ cells are more cytotoxic[19]. However, there are a number of other cell-surface markers that confer unique phenotypic and functional properties to $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. The $CD56^{bright}$ subset is shown to exclusively express the IL-2 receptor α chain (IL-2Rα or CD25) while they lack or express only at very low levels the $F_C\gamma$RIII (CD16). On the other hand, the $CD56^{dim}$ subset has high expression of CD16 and lacks CD25 expression. These properties assign very different roles to the different subsets with regards to antibody dependent cellular cytotoxicity (ADCC) and response to IL-2 stimulation. In addition to distinct expression of adhesion molecules and cytokine receptors, $CD56^{bright}$ NK cells have the capacity to produce high levels of immunoregulatory cytokines, but have low-level expression of killer-cell immunoglobulin-like receptors (KIRs) and are poorly cytotoxic.

By contrast, $CD56^{dim}$ NK cells appear to produce low levels of cytokines but have high-level expression of KIRs and are potent cytotoxic effector cells. Such evidence suggests that the $CD56^{bright}$ and $CD56^{dim}$ subsets are distinct lymphocytes with unique roles in the immune system. Thus, studies of the biology of human NK cells are eventually approaching NK cells as separate $CD56^{bright}$ and $CD56^{dim}$ subsets rather than a homogenous population.

As the name implies, NK cells can kill without prior sensitization, but they are also potent producers of various cytokines, including IFN-γ, TNF-α, GM-CSF and IL-3[20]. Therefore NK cells are also believed to function as regulatory cells in the immune system, influencing other cells and responses and acting as a link between the innate and adaptive immune responses. For example, NK cells participate in the development of an autoimmune disease, myasthenia gravis, by regulating both the autoreactive T and B cells through IFN-γ production[21]. Moreover, depletion of NK cells in C57Bl/6 mice leads to increased engraftment of neuroblastoma (NB) xenografts mainly due to dysregulation of Th1 oriented B cell responses[22]. These data prove the significant impact of NK cells on adaptive immune responses. Other studies have also shown a close interaction between NK cells and dendritic cells (DC)[23]. In addition to their role as the initiators of antigen specific responses, DCs have also been shown to support the activity of NK cells[24], while reciprocally, cytokine-preactivated NK cells have been shown to activate DCs and induce their maturation and cytokine production[25-27].

1.2 NK CELL RECEPTORS

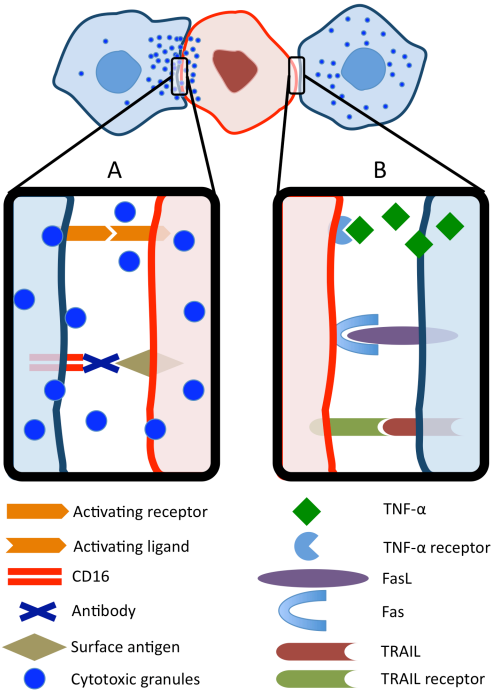
NK cell cytotoxicity is partially the result of a complex interaction between the inhibitory and activating signals coming from surface receptors[28]. Table 1 provides a selection of human NK cell activating and inhibitory receptors identified so far. Upon recognition of the ligands on the target cell surface by activating NK cell receptors, various intracellular signaling pathways drive NK cells towards cytotoxic action and this results in target cell lysis[29].

Table 1: NK cell receptors

CD	Alternative name	Type of signal	Ligand	Distribution on NK cells
CD2	LFA-2	Activation	CD58 (LFA-3)	All
CD7	LEU-9	Activation	SECTM1, Galectin	All
CD11a	LFA-1	Activation	ICAM-1,-2,-3,-4,-5	All
CD11b	Mac-1	Activation	ICAM-1, Fibrinogen	All
CD16	FcγRIII	Activation	IgG	Mainly $CD56^{dim}$
CD44	Hyalunorate receptor	Activation	Hyalouronan	All
CD59	Protectin	Activation	C8, C9	All
CD69	CLEC2C	Activation	Unknown	Activated
CD85j	ILT-2 (LIR-1)	Inhibition	HLA-A, -B, -G	Subset
CD94/CD159a	CD94/NKG2A	Inhibition	HLA-E	Most
CD94/CD159c	CD94/NKG2C	Activation	HLA-E	Most
CD96	TACTILE	Activation	CD155	Activated, Low on resting
CD160	BY55	Activation	HLA-C	All
CD161	NKR-P1	Activation/Inhibition	LLT1	Subset
CD223	Lag3	Activation	HLA Class II	Activated
CD226	DNAM-1	Activation	CD112, CD155	All
CD244	2B4	Activation/Inhibition	CD48	All
CD305	LAIR-1	Inhibition	Collagen	All
CD314	NKG2D	Activation	MICA, MICB, ULB1-4	All
CD319	CRACC	Activation	CRACC	Mature NK cells
CD328	Siglec-7	Inhibition	Sialic acid	All
CD329	Siglec-9	Inhibition	Sialic acid	Subsets
CD335	NKp46	Activation	Viral hemagglutinins	All
CD336	NKp44	Activation	Viral hemagglutinins	Activated
CD337	NKp30	Activation	Viral hemagglutinins	All
Various	KIR2DS, KIR3DS	Activation	HLA Class I	Subsets
Various	KIR2DL, KIR3DL	Inhibition	HLA Class I	Subsets
-	NKp65	Activation	KACL	Most
-	NKp80	Activation	AICL	All
-	NTB-A	Activation	NTB-A	All
-	KLRG1	Inhibition	E-,N-,P-cadherin	All

However, these processes are tightly controlled by a group of inhibitory receptors. These receptors act as negative regulators of NK cytotoxicity and inhibit the action of NK cells against "self" targets. An important group of this type of receptors is the killer-cell immunoglobulin-like receptors (KIRs), which are mainly specific for self MHC Class-I molecules. If the target cell is recognized by inhibitory KIRs, which means, it has sufficient amount of self MHC Class-I molecules on the cell surface, an inhibitory signal stops the action of cytotoxic pathways triggered by activating receptors[30,31]. KIRs are type I (extracellular amino terminus) membrane proteins that contain either two or three extracellular Ig-like domains[32] and are designated KIR2D or KIR3D, respectively. The cytoplasmic domains of the KIRs can be either short (S) or long (L), roughly corresponding to their function as either activating or inhibitory receptors respectively. Members of the KIR family recognize HLA-A, HLA-B and HLA-C alleles, and KIR2DL4 recognizes HLA-G[33]. The KIRs are clonally distributed on NK cells, which ensures that even the loss of a single HLA allele (a common event in tumorigenesis and viral infections) can be detected by a pool of NK cells[33,34].

The activating side of the balance also includes a series of different receptors. The main activating receptor group is the natural cytotoxicity receptors (NCRs)[29] and it is believed that the main control over the NK cell activating pathways is regulated by these receptors. Currently there are three different NCRs identified: NKp30[35], NKp44[36] and NKp46[37]. NKp30 and NKp46 are expressed both in activated and non-activated NK cells whereas NKp44 expression is restricted to activated NK cells. Most activating receptors do not directly signal through their cytoplasmic tail, but instead associate non-covalently with other molecules containing immunoreceptor tyrosine-based activation motifs (ITAM) that serve as the signal transducing proteins. NKp30 and NKp46 are couples with CD3ζ whereas NKp44 is coupled with DAP12. NK cell activation has been studied extensively in recent years and is discussed elsewhere[38,39].



NK cells have been described as large granular lymphocytes and their granularity is their means for target cell killing (Figure 2). These granules contain perforin and granzyme B[40] and both are postulated to bind the target surface as part of a single macromolecular complex[41].

Figure 2. Mechanisms of NK cell cytotoxicity. The cytotoxicity of NK cells is carried out by two main mechanisms. The first mechanism is granule-dependent cytotoxicity (A) where upon triggering by activating receptors or the F_c receptor (CD16), the cytotoxic granules in the cytosol of the NK cell are polarized towards the immunological synapse and the contents (mainly perforin and granzyme B) are unleashed upon the target cell by exocytosis. The second mechanism is the triggering of apoptosis pathways in the target cell via stimulation of death receptors (B) on the target cell surface by TRAIL or Fas ligand expressed on the NK cell surface as well as secretion of TNF-α.

When an NK cell is killing a target cell, perforin and granzyme B are released; granzyme enters the target cell and mediates apoptosis while perforin disrupts endosomal trafficking[42,43]. NK cells can also express FasL and TNF-related apoptosis-inducing ligand (TRAIL), which are both members of the TNF family and have been shown to induce target cell apoptosis when they bind their receptors on target cells[44,45]. TNF-α has also been suggested to mediate activation-induced cell death by NK cells[46].

Unlike T cells, NK cells don't express a unique, antigen specific receptor. A common strategy to target NK cells to tumors specifically is by making use of their ADCC capabilities *in vivo*. ADCC by NK cells is mediated through binding of immunoglobulin complexes or antibody-coated targets to the F_c receptor CD16. Antigen density, structure, and specificity of Fc binding are the critical components for efficient induction of ADCC[47]. Several isotypes of murine monoclonal antibodies (IgG1, IgG2a, IgG2b, IgG3)[48,49] have also been shown to trigger ADCC in NK cells. A comprehensive review regarding monoclonal antibody-based targeted therapy is discussed elsewhere[50].

Since virtually all ADCC activity in PBMCs is mediated by NK cells[51-53], it is important to determine how many target cells an NK cell can kill before it must refresh to continue. Bhat and Watzl reported that IL-2-activated NK cells can engage and kill 4 target cells in 16 h; after this time the cells appear to be exhausted, with reductions in available perforin and granzyme B which is reversible by IL-2 treatment[54].

1.3 NK CELLS IN CANCER

The development of any malignancy is under close surveillance by NK cells as well as other members of the immune system. Nevertheless, malignant cells obtain means to escape from the immune system and proliferate. General mechanisms include overwhelming of the immune system by the rapid growth of the tumor, inaccessibility of the tumor owing to defective vascularisation, its large dimension or its localization in immune-privileged sites and resistance to the Fas- or perforin-mediated apoptosis. The expression of FasL by tumor cells as a counterattack strategy against immune effectors such as T cells and NK cells is also common[55-57]. Additionally, the defective expression of activating receptors and various intracellular signaling molecules by T cells and NK cells in cancer patients has been observed and reported to correlate with disease progression[58]. It has also been shown that malignant cells secrete immunosuppressive factors that inhibit T and NK cell proliferation and function[59,60].

Studies on patients with AML have convincingly demonstrated the existence of an NCR^{dull} phenotype in NK cells and more interestingly that the *in vitro* co-culture of NK cells and tumor cells also result in the induction of this defective phenotype[61]. Moreover, recent data from animal studies has also confirmed that tumor growth imposes a dysregulation of hematopoiesis especially in the lymphoid compartment[62]. As a result of all these events, defective immunity secondary to tumor development has been a well-established phenomenon[63] and evading destruction by immune cells has been recognized as an emerging hallmark of cancer[64]. Table 2 presents a selection of previously defined NK cell abnormalities in cancer patients.

Table 2: NK cell abnormalities in cancer patients

Abnormality	Disease
Decreased cytotoxic activity of NK cells	Non-small cell lung cancer[65], Hepatocellular carcinoma[66,67], Stage IV rectal cancer[68], Head and neck cancer[69,70], Breast cancer[69-71], Cervical carcinoma[72], Squamous cell carcinoma of the penis[73], Bronchogenic carcinoma[74], Ovarian cancer[75], AML[76], ALL[76,77], CLL[78], CML[79], MM[80]
Defective expression of activating receptors	Hepatocellular carcinoma[66], Metastatic melanoma[81], AML[82], CLL[83], MM[84,85]
Defective NK cell proliferation	Metastatic renal cell carcinoma[86], Nasopharyngeal cancer[87], CML[88]
Increased number of $CD56^{bright}$ NK cells	Head and neck cancer[69], Breast cancer[69]
Defective expression of intracellular signalling molecules	Cervical cancer[89], Colorectal cancer[90], Ovarian cancer[91], Prostate cancer[92], AML[93], CML[93]
Decreased NK cell counts	Nasopharyngeal cancer[87], CML[88], Hepatocellular carcinoma[61]
Increased NK cell counts	CLL[94], MM[80]
Defective cytokine production	AML[76], ALL[76,77], CML[95], B-CLL[96]

1.3.1 NK cells in Multiple Myeloma

Multiple myeloma (MM) is a malignancy of plasma cells that is often asymptomatic in early stages. The main clinical symptoms of the disease are related to the

accumulation of malignant plasma cells, followed eventually by bone destruction and subsequent hypercalcemia, bone marrow failure, anemia, renal failure and an increased risk of infection due to immune failure. Patients primarily present with serious bone pain and fatigue related to anemia as well as recurrent infectious disease. The occurrence of a monoclonal immunoglobulin (M-component) in serum and light Ig chains in the urine, resulting from the sustained Ig production of the malignant plasma cells, is an important diagnostic tool. MM accounts for approximately 2% of all cancer deaths and 20% of deaths caused by hematological malignancies[97]. Factors that predict survival in MM such as β2-microglobulin (β2M), creatinine and hemoglobin (Hb) levels have been well-defined[98,99]. Furthermore, the occurrence of various chromosomal abnormalities among the malignant cells have been shown to have an impact on prognosis[100-102]. The incidence of MM in Europe is 4.5-6.0/100 000/year with a median age at diagnosis of between 63 and 70 years while the mortality is 4.1/100 000/year[103,104].

The level of cyclin D1, D2 or D3 expression in all MM cells is significantly higher than in normal BM plasma cells[105]. This makes the myeloma cells more sensitive to proliferative stimuli from the BM microenvironment[106] resulting in selective proliferation of tumor cells that produce osteolytic factors including RANK ligand and large amounts of MIP-1α as well as immunosuppressive factors such as IL-10. Approximately 70% of MM patients have elevated levels of MIP-1α in their BM plasma[107] which directly stimulates osteoclast (OCL) precursors to differentiate into bone resorbing OCL[108,109], resulting in elevated rate of bone destruction. Also, adhesive interactions between myeloma cells and BM stromal cells induce increased production of RANKL and IL-6 by stromal cells and in this way increase OCL formation[110]. Besides, MM cells have also been shown to produce DKK1 that inhibits the WNT pathway which is critical for osteoblast differentiation[111]. Altogether, these changes in the BM microenvironment lead to the development of a tumor that will cause irreversible damage to bones and induce formation of osteolytic lesions.

Allogeneic stem cell transplantation[112,113] might be curative for a small group of eligible patients, but the common treatment of choice for patients under 60 – 65 years of age has been high-dose chemotherapy (HDT) followed by autologous stem cell transplantation (ASCT)[114]. Although ASCT is still considered a golden standard for treatment of MM patients younger than 65 years of age, mainly based on two prospective trials [115,116], some doubt remains about which induction regimen should be used, whether single or tandem ASCT should be employed and whether melphalan should be used alone or in combination with other drugs as high dose treatment (HDT) [117]. Yet, approximately only one-third of all patients with MM live longer than 5 years. On the other hand, recent years have witnessed a significant increase in the survival rates for MM patients due to the introduction of combination therapies including proteasome inhibitors such as bortezomib and immunomodulatory drugs (IMiDs) such as thalidomide, and lenalidomide[118].

Despite the rapid development of new agents, MM continues to be an incurable disease with a fatal outcome in the majority of patients, especially those in advanced

stages. Thus, novel therapeutic modalities such as immunotherapy warrant exploration in an attempt to increase life expectancy[119]. Yet, the impaired immune system in MM patients is evident in their well-recognized susceptibility to infectious complications[120]. Previous reports have convincingly demonstrated that while NK cells are functional in MGUS (monoclonal gammopathy of undetermined significance, a premalignant condition resembling MM), and to some extent in early stages of MM, further progression of the disease is accompanied by a serious decline in NK cell function[121-125]. In the autologous setting, this marked NK cell defects must be overcome for successful induction of an anti-MM response by the patient's own NK cells.

Recent evidence suggests that the underlying dysfunction of the immune system in MM patients originate, at least in part, from dendritic cells[126] or regulatory T cells[127,128]. Moreover, the secretion of TGF-beta, IL-10, IL-6 and prostaglandin E2 (PGE2) by the tumor microenvironment negatively affects NK cell function while activation of signaling molecules such as STAT3 promotes MM cell growth and suppresses NK cell function[129-131]. Expression of the IL-15 receptor and autocrine stimulation of MM cells by IL-15 production can also be considered as a factor negatively affecting NK cells since this might result in the sequestration of IL-15 by MM cells which would otherwise be used for NK cell survival and activation[132]. Additionally, MM cells are shown to utilize suppression of DNAM-1 ligand expression[133] and Fas downregulation[134,135] as mechanisms of immune escape.

Although previous reports suggest that cytokine activation of NK cells may lead to a better recognition of MM cells[136,137], MM cells are considered to be resistant to lysis by resting and short term activated autologous NK cells[138-140]. This resistance has been explained by impaired NK cytotoxicity[124,141] and increased levels of soluble IL-2 receptors[142] in MM patients as well as decreased expression of activating receptors compared to those in healthy controls[84]. Moreover, the high-dose secretion of M-component may also directly effect NK cell cytotoxicity[80,141,143,144]. MM cells have also been shown to express programmed death receptor ligand-1 (PD-L1) which upon interaction with the programmed death receptor-1 (PD-1) on NK or T cells, may suppress adaptive and innate immune responses against MM[145,146].

1.4 NK CELLS IN CANCER IMMUNOTHERAPY

In 1909, Paul Erlich was the first to propose in theory that the immune system had the potential to fight against tumors[147]. Although it could not be confirmed at that time, due to the lack of knowledge on the cellular and molecular details of the immune system, half a century later, Thomas and Burnet put forward the "cancer immunosurveillance" theory[148]. While this theory was seriously challenged in the first years, it has stood the test of time and been validated, reaffirming the feasibility of mobilizing the immune system to fight tumors[149]. Today, successful applications of cancer immunotherapy cover a broad base from the use of monoclonal antibodies or recombinant cytokines to adoptive transfer of donor lymphocytes in order to trigger a graft-versus-tumor effect[150].

Figure 3 presents an overview of current and future approaches to NK cell-based immunotherapeutic strategies in the treatment of cancer. A critical prerequisite for efficient NK cell-based immunotherapy seems to be the reduction of the tumor mass by surgical removal, chemotherapy or radiotherapy in order to give the effector cells a numerical advantage. The yellow shaded upper left panel represents the *in vivo* modulation of NK cell activity against tumor via (A) stimulation with cytokines and/or (B) infusion of tumor-specific monoclonal antibodies in order to trigger an ADCC response. The green shaded lower left panel and the gray shaded right panel present approaches for adoptive transfer of autologous or allogeneic NK cells respectively. Autologous or donor NK cells can be transferred after (C & H) *ex vivo* short-term activation, (D & G) *ex vivo* long-term activation and expansion or (E & F) genetic modification. Infusion of (I) purified unstimulated donor NK cells is also under investigation.

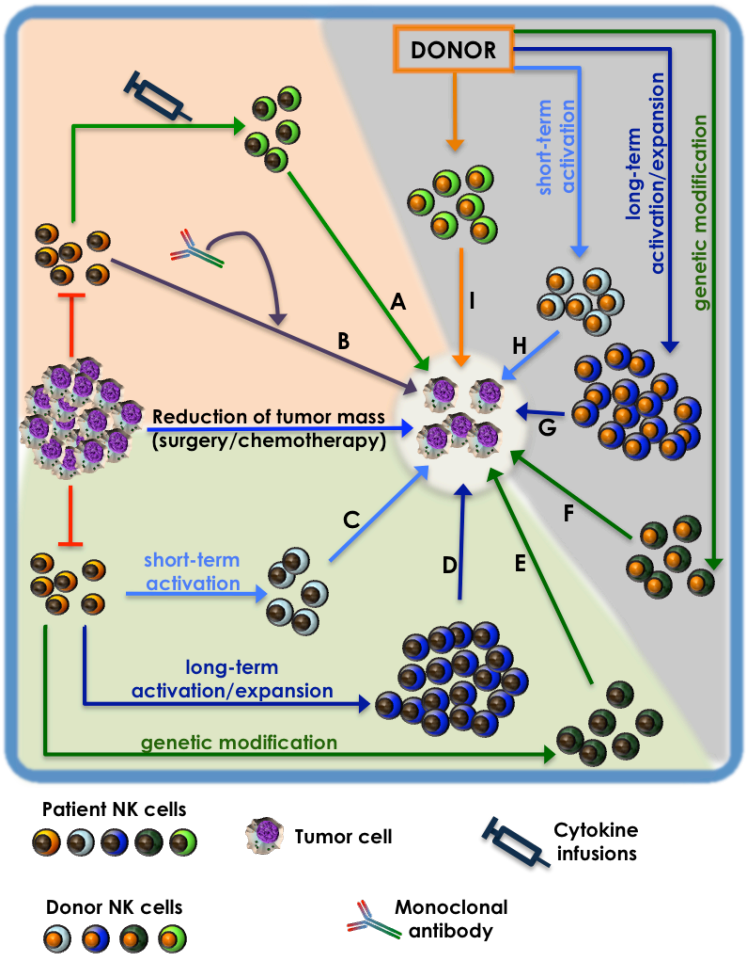


Figure 3: Natural killer cell immunotherapy in cancer

1.4.1 Modulation of endogenous NK cell activity

1.4.1.1 IL-2 alone

The cDNA encoding the human IL-2 gene was cloned in 1983[151] after a long search starting in 1965 for the soluble factors in lymphocyte conditioned media that could sustain the proliferation of T cells in culture[152,153]. It is now well known that IL-2 affects many types of cells in the immune system including cytotoxic T cells, helper T cells, regulatory T cells, B cells and NK cells. Currently, there are three distinct chains of the IL-2 receptor identified; the α (CD25), β (CD122) and γ (CD132) chains. The γ chain is shared among various cytokine receptors (IL-4, IL-7, IL-9, IL-13, IL-15, IL-21), thus named the common γ chain and it is essential for lymphoid development[154]. The β chain is shared between IL-2 and IL-15 receptors[155,156]. The β and γ chains come together to form the intermediate affinity IL-2/15 receptor. The distinction between the high affinity receptors for IL-2 and IL-15 comes with the α chains. The IL-2Rα chain alone is regarded as the low affinity receptor and is believed to lack the capacity to deliver intracellular signals due to its short intracellular tail[157]. However, when the α chain forms a complex with the β and γ chains, the high affinity IL-2 receptor is formed. The co-expression of all three chains is confined to regulatory T cells, $CD56^{bright}$ NK cells as well as activated conventional $CD4^+$ and $CD8^+$ T cells[158]. Thus, these cells are expected to give a better response to the presence low dose IL-2.

It has been well defined that IL-2 activation of NK cells can result in cytotoxic activity against targets that were previously NK-resistant[159-161]. Early reports of IL-2 based treatment on animal models have established a solid basis for the efficiency of this approach for cancer immunotherapy in many different settings[162-170]. Although cytotoxic T cells have been the primary point of interest, especially during the early phases of IL-2 use, the antitumor response triggered by IL-2 were frequently attributable to NK cells[171-175]. Likewise, our group has demonstrated in a syngeneic murine model of MM that NK cells are the primary mediators of IL-2 induced tumor rejection[175].

In the clinical setting, the pioneering works of Rosenberg *et al.*[176,177], which have demonstrated the potent immunostimulatory effect of IL-2 in advanced cancer patients, resulted in a great interest for the use of cytokines and immune effector cells for the treatment of cancer. Further reports have shown that IL-2 treatment results in *in vivo* activation of NK cell cytotoxicity[178] and this effect is dependent on the dose and schedule of IL-2 administration[179]. It has also been observed that IL-2 treatment of some cancer patients receiving a T cell depleted allogeneic BMT was well tolerated, decreased relapse risk and increased survival compared to those not receiving IL-2[180]. Since then, such an approach of stimulating endogenous NK cells with cytokines in an attempt to promote *in vivo* killing of tumor cells have been used by many investigators.

IL-2 has received FDA approval for the treatment of metastatic renal cell carcinoma (RCC) in 1992 based on its ability to induce an objective response rate of 15-20%[181]. It has also been demonstrated in RCC patients undergoing IL-2 based therapy and

nephrectomy, that a higher percentage of circulating NK cells is a predictor of response[182].

The use of IL-2 alone has been attempted in many other tumor types, mostly as an adjuvant to chemotherapy or stem cell transplantation (SCT). Treatment of patients with breast cancer and lymphoma using IL-2 was shown to significantly increase number of circulating NK cells and their cytotoxicity against NK resistant breast cancer and lymphoma cell lines[183]. Many other similar studies on immunostimulation in cancer patients have made similar observations where IL-2 infusions induce an increase in white blood cell counts, increase in circulating T cells and mostly $CD56^{bright}$ NK cells, elevated cytotoxic activity of NK cells[184-186]. Yet, such studies have primarily shown only temporary responses leading to eventual tumor relapse and no survival improvement.

The use of IL-2 for inducing NK cell-mediated killing of tumors has also been a popular approach in hematological malignancies. IL-2 has been shown to provide stimulation of PBMCs for killing of multiple MM cells[187]. Later studies have proved that NK cells have an effective cytotoxic activity against MM cell lines and tumor cells from MM patients[136]. Our group has demonstrated (PAPER I) that NK cells from MM patients can be expanded *ex vivo* using GMP-compliant components, and they show high cytotoxic activity against autologous MM cells while retaining their tolerance against normal cells of the patient[188]. Other researchers have also shown that HLA Class I molecules, NCRs and NKG2D take part in the recognition of myeloma cells by autologous and allogeneic NK cells[85,137]. Likewise, NK cells from AML patients in remission have also been expanded *ex vivo* and showed cytotoxic activity against allogeneic and autologous AML blasts, which could be further enhanced by IL-2[189]. In a clinical AML study, where IL-2 was used alone as 14-day cycles of low-dose IL-2, for *in vivo* expansion of NK cells, followed by 3 day higher doses aimed to induce cytotoxicity of *in vivo* expanded NK cells, no prolongation of disease-free or overall survival was seen and the authors concluded that low-dose IL-2 maintenance immunotherapy alone is not a successful strategy to treat older AML patients[190].

Overall, data from the reports mentioned above demonstrates that although promising outcomes have been observed, low-dose IL-2 treatment is not the optimal strategy for most indications. In most cases, low-dose IL-2 administration (picomolar serum concentrations), leads to specific expansion of the $CD56^{bright}$ NK cell subset[157]. As mentioned above, within the NK cell population, the IL-2Rα (CD25) that confers high affinity for IL-2 is uniquely expressed by $CD56^{bright}$ cells[191], which could explain their selective expansion in response to low-dose IL-2. Likewise, the *in vivo* expansion of another CD25 expressing regulatory cell subset; T_{reg} cells could also overwhelm and/or suppress the antitumor activity. The potential of T_{reg} cells to dampen NK cell-mediated antitumor responses has primarily been suggested in a murine leukemia model[192]. The effect of T_{reg} cells in cancer immunotherapy has now been better recognized[193,194] and attempts to circumvent such suppression are underway[195]. Moreover, recent advances in the engineering of novel cytokines based on IL-2 that

attempt to override the need for CD25 engagement during IL-2 signaling stand out as promising approaches to current immunotherapy modalities[196].

1.4.1.2 IL-2 in combination with other factors

Studies have shown that IL-2, IL-12 and IL-15 stimulate NK cell cytotoxicity *in vitro* and show synergy when used in combination[197,198]. Such cytokines have been widely used for *in vitro* studies in order to define requirements of NK cell activation that could potentially be used in cancer immunotherapy. As the β and γ chains of IL-2 and IL-15 receptors are shared, the intracellular signaling pathways triggered by these cytokines overlap to a certain extent[199]. Moreover, IL-2/15 engagement also results in activation of NF-κB[200] and the anti-apoptotic protein BCL-2[199] confirming the close relationship between IL-2 and IL-15 derived signals. The selective modulation of protein kinase C isoforms has also been reported to take part in NK cell activation and is suggested to be a common mechanism used by IL-12, IL-2 and IL-15[201]

The IL-2Rα chain has not been acknowledged to deliver any intracellular signals yet. However, the IL-15Rα chain has been shown to signal independently from the β/γ heterodimer, which in part explains the different effects of the two cytokines[202,203]. .

IL-12 differs from these two cytokines due to the fact that IL-12 receptor lacks the common γ chain. The IL-12 receptor is composed of two subunits: IL-12Rβ1 and IL-12Rβ2[204,205]. Studies have shown that IL-2 activated PBMCs displayed increased cytotoxic activity against autologous primary lung cancer cells, which could be further augmented with the addition of IL-12[206]. This may be explained by the observation that IL-2 upregulates the expression of IL-12Rβ1 and IL-12Rβ2[207]. The increased expression of IL-12R on NK cells in patients treated with low dose IL-2 also confirms this explanation[207]. NK cells have been shown to kill NB cell lines when activated with IL-2 and/or IL-12[208] and molecules such as LFA-3 and ICAM-1 are suggested as important modulators of the susceptibility of NB cells to NK cell mediated killing[209].

IL-21, which was primarily described in 2000[210], is a class I cytokine that has significant homology with IL-2 and IL-15. Depending on the cell type, signaling through the IL-21 receptor can activate the different downstream targets[211-213]. IL-21 alone promotes functional maturation[214] and survival of NK cells but does not induce proliferation, while it synergizes with IL-2 and IL-15 for induction of NK cell expansion *in vitro*[215] and modulation of surface receptor expression such as upregulation of NKG2A, CD25, CD86 and CD69[216]. It has also been shown that membrane-bound IL-21 (mbIL-21) performs better than mbIL-15 for *ex vivo* expansion of NK cells[217]. Other studies have suggested IL-21 might have differential effects on $CD56^{bright}$ and $CD56^{dim}$ subsets[218] but consensus prevails about induction of IFN-gamma production and ADCC activity[96,219]. Clinical studies have shown that infusion of IL-21 activates CD8+ T cells and NK cells and has an anti-tumor effect in melanoma and RCC[220-223]. It has also been suggested that unlike IL-2, IL-21 does not drive the proliferation of Treg cells and might be used as an alternative to IL-2[224]

A major hurdle in tumor immunotherapy has been the various mechanisms by which tumors induce dysfunction or tolerance of local immune cells[63]. The

immunosuppressive effect of reactive oxygen species (ROS) derived from tumor cells, tumor-associated macrophages, or monocytes activated by cytokine therapy has been well defined and addressed in various tumors[63,225]. Monocyte/macrophage derived ROS have been shown to induce apoptosis and anergy to IL-2 activation in T cells and NK cells[226]. Affirmatively, supplementation with the anti-oxidant vitamin E upregulates NKG2D expression and enhances NK cell function in CRC patients[227]. Other researchers have tried combination of IL-2 with histamine, an inhibitor of ROS synthesis in monocytes/macrophages[228], in order to counteract the negative effect of ROS originating mainly from the expanding monocyte/macrophage population. In a randomized phase II trial where 63 patients with metastatic RCC were treated with IL-2 and histamine[229], addition of histamine resulted in a decrease of monocyte expansion and number of intratumoral macrophages as well as an increase in the number of tumor-infiltrating NK cells and $CD8^+$ T cells. The same study also demonstrated a likely correlation between the number of circulating NK cells and cytotoxicity. Likewise, histamine has also been shown to synergize with IL-2[230] and reverse the inhibition of NK cell cytotoxicity against heterologous AML blasts by myeloid-derived ROS[231]. Hellstrand *et al.* have reported treatment of 22 AML patients (mean age 59) with IL-2 and histamine. The treatment was well tolerated and showed an impressive survival benefit[232], which stands out as a promising finding to counterweigh the ineffectiveness of IL-2 treatment in certain settings. A more recent report of a phase III trial with this approach including 320 patients has convincingly shown that the use of IL-2 along with histamine significantly improves leukemia-free-survival of AML patients in remission by reducing the risk of relapse[233,234] and the treatment has now been approved in EU for AML patients in first CR.

Other investigators have combined the use of IL-2 and IFN-α in the clinical setting. It has been demonstrated that perioperative treatment with IFN-α significantly increases NK cell activity although the number of cells are decreased[235]. Atzpodien *et al.* infused IL-2 along with interferon-α to 47 patients with various malignancies. They have observed a significant relation between the increase of circulating NK cell numbers ($CD56^+$) and the response[236]. Molto *et al.* have observed in 8 metastatic RCC patients undergoing immunotherapy with low dose s.c. IFN-α-2b and IL-2 following radical nephrectomy, that patients who achieved complete or partial responses had higher NK cell cytotoxic activity than those who remained in progression.[237] In another study by Moroni *et al.* 25 patients with metastatic RCC were treated with low-dose IL-2 and IFN-α. 6/25 had objective response (CR or PR) and 12/24 had stable disease[238]. In a similar study, low-dose IL-2 for 5 days per week and IFN-α twice weekly for 4 consecutive weeks was applied to 27 patients with advanced renal cell carcinoma. A significant increase in total lymphocytes, eosinophils, CD25+ cells and NK cells was observed. Within the NK cells, the $CD56^{bright}$ population had a higher expansion rate. Yet, no clinical benefit was observed.[239]

In a study combining IL-2, IFN-α and histamine, mononuclear cells in peripheral blood and tumor biopsies from 13 patients with metastatic malignant melanoma were followed and a trend towards a gradual increase in the absolute number of circulating NK cells in patients maintaining stable disease during the therapy was observed. The

extent of leukocyte infiltration in tumor tissues prior to treatment correlated with the response. Additional NK cell infiltration during treatment was seen only in responding patients.[240]

Taken together, the overall experience from supporting IL-2 treatment with secondary factors seems to be promising. It is obvious that if the results from these studies are evaluated carefully in the design of future clinical trials, such combination approaches may provide a solid basis for the use of IL-2 for stimulation of endogenous NK cells against tumors in many different indications.

1.4.2 Adoptive transfer of NK cells

1.4.2.1 Sources of NK cells for adoptive transfer

In the clinical setting, the number, purity, and state of proliferation/activation of NK cells to be used, are key factors to consider. Regarding purification, single step GMP protocols to deplete $CD4^+/CD8^+$ $\alpha\beta$T cells are possible and result in passive enrichment of innate lymphocytes such as NK cells and $\gamma\delta$T cells which seem to preserve their proliferative and cytotoxic capacity[241]. Simple purification of NK cells by a single-step or 2-step procedure may be enough for most applications. Leung *et al.*[242] have demonstrated that donor NK cells, purified by a clinical-scale two-step immunomagnetic separation method, had normal expression of cell surface markers, intracellular cytokines, perforin and granzyme B. Table 3 summarizes a selection of reports that study the isolation of clinical grade NK cells for adoptive immunotherapy.

Table 3: Examples of clinical scale NK cell purification approaches

Report	Strategy	n	Purity (%)	Recovery (%)	Viability (%)	CD3 (%)	CD14 (%)	CD19 (%)
Iyengar[243]	CliniMACS CD3 depletion CliniMACS CD56 enrichment	12	91	49	NR	0.1	7.7	0.2
Passweg[244]	CliniMACS CD3 depletion CliniMACS CD56 enrichment	6	97	35	NR	<0.01	NR	NR
Lang[245]	CliniMACS CD56 enrichment DynaBeads CD3 depletion	4	99	42	>90	NR	NR	NR
McKenna[246]	CliniMACS CD3 depletion	36	38	79	86	1	31	26
McKenna[246]	CliniMACS CD3 depletion CliniMACS CD56 enrichment	13	90	19	85	0.21	5	0.67
Koehl[247]	2X CliniMACS CD3 depletion CliniMACS CD56 enrichment	11	95	33	95	0.01	5	NR
Frohn[248]	SuperMACS NK cell selection	10	85	50	NR	1	8.5	0.3

The actual obstacle in clinical studies with adoptive transfer of NK cells originates from the fact they are normally present only in low numbers in PBMCs and effector cell preparations such as lymphokine activated killer (LAK) cells. Obtaining a large number of NK cells is an influential albeit difficult task that underlies the most significant challenge for the development of successful NK cell adoptive transfer protocols. Thus, many researchers have worked on the expansion of NK cells. Table 4 summarizes a selection of studies reporting successful *ex vivo* expansion of NK cells for adoptive immunotherapy applications. Some of these NK cell-based products have already been used in the clinic and will be discussed in more detail.

Table 4: *Ex vivo* NK cell expansion

Study	n	Start material	Medium	Serum	Feeder cells	Addition	Time (Days)	System	NK Fold expansion	Purity (% NK)	Cytotoxicity
Escudier[249] and Hercend[250]	22 RCC patients	CD3 depleted non-adherent PBMCs	DMEM	8% HS	LAZ 388	200 U/ml IL-2, 2 mM L-glutamine, 1mM sodium pyruvate, 0.2% NaOH, 100 U/ml penicillin, 0.1 mg/ml streptomycin	13-21	V-bottom microplates	55	>90	Cytotoxic against Daudi
Miller[251]	4 donors	CD5 and CD8 depleted PBSCs	RPMI-1640	10% HS	No	1000 U/ml IL-2, 2 mM L-glutamine, 1000 U/ml penicillin, 100 U/ml streptomycin	21	Polystyrene Cell Factories Teflon bags Polyelofin bags	33 21 12	88	Cytotoxic against K562 and Raji
Lister[252]	11 lymphoma 1 BrCa patients	Adherent activated NK cells	RPMI-1640	10% HS	Allogeneic MNC	6000 U/ml IL-2	14-18	Flasks	31	85	Cytotoxic against Daudi
Pierson[253]	7 donors	CD5 and CD8 depleted PBMCs	2:1 DMEM:Ham's F12-based NK medium	10% HS	No	1000 U/ml IL-2, 20 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 mg/ml L-ascorbic acid, 5 μg/L sodium selenite, 100 U/ml penicillin and streptomycin	33	Stirred-tank bioreactor (n=1) Spinner flasks (n=3) 24-well plates (n=3)	352 120 51	96 96 95	Cytotoxic against K562 and Raji
Carlens[254]	7 donors	Total PBMCs	CellGro SCGM	5% HS	No	500 U/ml IL-2, 10 ng/ml OKT3	21	Flasks	~700	55	Cytotoxic against K562
Luhm[255]	37 donors	NK cells purified with MACS NK cell kit	X-VIVO 20	No	Allogeneic MNC	100 U/ml IL-2, 10 U/ml IL-15, 100 μg/ml PHA, 1 μmol/ml ionomycin	14-21	Teflon bags	100	92	Cytotoxic against Daudi, U266, NCI cell lines but not K562
Torelli[189]	13 AML patients	Non-adherent PBMCs	RPMI-1640	10% FBS	RPMI 8866	50 U/ml IL-2	10-12	24-well plates	35	80	Cytotoxic against allogeneic and autologous AML blasts
Guven[256]	6 B-CLL patients	Total PBMCs	CellGro SCGM	5% HS	No	500 U/ml IL-2, 10 ng/ml OKT3	21	Flasks	243	74	Cytotoxic against K562
Klingemann[257]	5 donors	CD56 enriched PBMCs	X-VIVO 10	10% HS	No	200 mM L-glutamine, 500 U/ml IL-2, 10 ng/ml IL-15	14	NR	5-20	NR	Cytotoxic against K562 and Raji
Ishikawa[258]	9 glioma patients	Total PBMCs	RHAMα	5% AP	HFWT	200 U/ml IL-2	6-7	24-well plates	NR	86	Cytotoxic against HFWT
Koehl[247]	15 donors	See table 3	X-VIVO 10	5% FFP	No	1000 U/ml IL-2	10-14	Flasks, Teflon bags	5	NR	Cytotoxic against K562 and allogeneic primary leukemia cells
Berg[259]	9 donors	NK cells purified with 2-step MACS	X-VIVO 20	10% HS	EBV-TM-LCL	500 U/ml IL-2, 2 mM GLUTAMAX-I at 6.5 % CO_2	28	Flasks or Baxter 300 ml bags	815-3267	99	Cytotoxic against K562 and RCC cell lines
Fujisaki[260]	62 donors	Total PBMCs	RPMI-1640 or CellGro SCGM	10%FBS	K562-mb15-41BBL	10 U/ml IL-2	7-14	Flasks or Teflon bags	21.6 (f) 90.5 (b)	96.8 (f) 83.1 (b)	Cytotoxic against K562, U937, HL60, KG1 and prim. AML blasts
Torelli[261]	26 ALL patients	Non-adherent PBMCs	RPMI-1640	10% FBS	RPMI 8866	50 U/ml IL-2	10-12	24-well plates	35-45	90	Cytotoxic against autologous ALL blasts

Our group has previously described a novel method comprising GMP-compliant components that facilitates expansion of high numbers of polyclonal NK cells in cell culture flasks using PBMCs from healthy donors [254], as well as patients with B-CLL [256] and MM[188] (PAPER I). These cells were shown to exert specific cytotoxic activity against fresh human tumor cells *in vitro* [188] and in experimental models of human tumors [262] which opens up the possibility to be evaluated in clinical settings. Concurrently, we have successfully completed the safety evaluation of this cell product in an allogeneic setting in a phase I clinical trial with cancer patients[263]. In PAPER II, we aimed to comparatively evaluate the use of cell culture flasks, bags and a bioreactor for the *ex vivo* expansion of NK cells originating from bulk PBMCs of healthy donors and MM patients. Our results have demonstrated the feasibility of producing clinical-grade effector cells in closed automated systems under GMP conditions.

It is evident that not every NK cell expansion protocol and not every different donor yields expanded cells with similar phenotype. Factors such as distribution of KIR expressing populations and expression of other activating and inhibitory receptors may be of importance and need to be checked thoroughly. The level of CD16 expression in the final product should also be optimized in order to make way for possible future use of expanded NK cells along with tumor-targeting antibody infusions. Keeping in mind that such differences in NK cell phenotype due to the expansion method used might have a significant effect on the clinical applicability and efficiency, the ultimate answers lie in testing these cells in clinical trials. Such clinical experience is warranted to give a conclusive answer to which approach will be the most suitable to induce a graft-vs-tumor effect with a minimum of adverse events.

1.4.2.2 Adoptive transfer of autologous NK cells

Adoptive transfer studies with NK cells have proven to be efficient in various animal models[264]. We have also reported successful NK cell adoptive immunotherapy of MM in a syngeneic immunocompetent mouse model[175]. Additionally, it has also been demonstrated that activated NK cells from AML patients are cytotoxic against autologous AML blasts *in vivo* in a NOD/SCID model[265]. Supported by the achievements of adoptively transferred NK cells in experimental tumor models, various groups have evaluated the adoptive transfer of autologous NK cells for cancer immunotherapy in the clinical setting. Table 5 presents a selection of clinical trials with infusion of autologous NK cells.

The data on infusion of autologous NK cells reveal that it may be more likely to see a clinical benefit with long-term *ex vivo* activated NK cells. Corroborating this hypothesis, we have observed that long-term expansion and activation of autologous NK cells from MM patients provide significantly superior cytotoxic activity against autologous tumor cells when compared to short-term activated autologous NK cells[188] (PAPER I). Moreover, the high level of CD25 expression in long-term expanded NK cells[255,266], as compared to endogenous $CD56^{dim}$ or $CD56^{bright}$ NK cells and short-term activated NK cells may provide a higher benefit from subsequent IL-2 administration to the patients.

Table 5: Clinical trials with infusion of autologous NK cells

Study	Escudier[249]	Lister[252]	deMagalhaes-Silverman[267]	Burns[184]	Krause[268]	Ishikawa[258]	Milani[269]
Year	1994	1995	2000	2003	2004	2004	2009
Patients	10 metastatic RCC that previously had PR to IL-2 therapy	10 relapsed lymphoma 1 metastatic BrCa	5 metastatic BrCa	20 Relapsed lymphoma 14 metastatic BrCa	11 metastatic CRC 1 NSCLC	9 recurrent glioma	1 colon adenocarcinoma
NK preparation	CD3 MACS or Immunorosette depleted PBMCs cultured on LAZ388 and irradiated allogeneic PBMC feeders in DMEM with 8% HS and 200 U/ml IL-2 for 13-21 days	*In vivo* IL-2 primed (3x105 U/m2/day for 6 days) leukapheresis, followed by 14-18 day culture of adherent PBMCs with irradiated allogeneic feeders using RPMI1640 with 10% HS and 6000 U/ml IL-2	*In vivo* IL-2 primed (3x105 U/m2/day for 6 days) leukapheresis followed by 14 day culture of adherent PBMCs with irradiated allogeneic feeders using RPMI1640 with 10% HS and 6000 U/ml IL-2	Overnight activation in AIM-V with 1000 U/ml IL-2	PBMCs cultured in X-VIVO 20 with 100 U/ml IL-2 and Hsp-70 peptide (2ug/ml) for 4 days	PBMCs cultured with HFWT feeders in RHAM-α medium with 5% autologous plasma and 200 U/ml IL-2 for 14 days	PBMCs cultured in CellGro SCGM with 100 U/ml IL-2 and Hsp-70 peptide for 4 days
Purity	>90% except 1 patient (33%)	Mean: 85% (Range: 64-98%)	Mean: 83.2% (Range: 67-93%)	NR	Mean: 14% (Range: 8-20%)	85.9% +-9.6%	Mean: 22.4% (Range: 16-25)
Dose	Mean: $5.8x10^9$ total cells (Range: $1.8\text{-}15.1x10^9$)	$6.8x10^8\text{-}4x10^{10}$ total cells	Mean: $3.97x10^9$ total cells (Range: $1.55\text{-}9.1x10^9$)	Cohort 1: $4x10^7$ cells/kg Cohort 2: $8x10^7$ cells/kg Cohort 3: $0.33\text{-}2.09x10^8$ cells/kg	$0.1\text{-}1.5x10^9$ NK cells	i.c. ($0.4\text{-}2.3x10^9$ cells) i.v. ($0.2\text{-}6.5x10^9$ cells)	Mean: $1.48x10^9$ NK cells Range: ($0.9\text{-}1.9x10^9$)
Schedule	2 infusions with a mean interval of 74 days	Single infusion at day 2 after auto transplant	Single infusion at day 2 after auto transplant	2 infusions at day 29 and 43	Up to 5 infusions per patient, with 1 day intervals	Up to 3 infusions per patient	6 infusions monthly, 3 months of break and 3 more infusions monthly
IL-2 infusions	$1.6\text{-}10x10^6$ U/m^2/day IL-2 for 2 days, 5 cycles with 1 week intervals	$2x10^6$ U/m^2/day IL-2 for 4 days starting at cell infusion followed by $3X10^5$ U/m^2/day IL-2 for 90 days	$2x10^6$ U/m^2/day IL-2 for 4 days	$0.25\text{-}1.75x10^6$ U/m^2/day IL-2 during 56 days	None	None	None
Clinical Results	- Safe - All patients improved 4 CR, 1 PR	- Safe - Increased NK cell numbers and activity in peripheral blood	- Safe - No clinical effect	- Safe - No improvement of survival	- Safe - Upregulated CD94 expression and cytotoxic activity of circulating NK cells - 1 SD	- Safe - Partially effective (3 PR, 2 MR, 4 NC, 7 PD)	- Safe - No change in circulating lymphocyte subsets - Autologous anti-tumor activity by NK cells

PBMC; peripheral blood mononuclear cells, RCC; renal cell carcinoma, BrCa; breast cancer, NSCLC; non-small cell lung cance, MACS; magnetic-activated cell sorting, HS; human serum, NR; not reported, i.c.; intracranial, i.v.; intravenous, CR; complete response, PR; partial response, MR; minimal response, NC; no change, PD; progressive disease

1.4.2.3 Adoptive transfer of allogeneic NK cells

The use of allogeneic NK cells is tentatively alluring, given the current comprehensions of NK cell regulation. A provisional prerequisite for NK cell alloreactivity is that the recipient lacks one or more KIR ligands present in the donor. The donor may in such situations have NK cells that express inhibitory KIR for which there is no ligand on recipient cells. Therefore, a KIR ligand-mismatched donor is likely to give the best chances for clinical response[270-272]. In certain donor-recipient combinations, chances for *missing-self* may prevail, providing better possibilities for anti-tumor reactivity. Within the setting of NK cell-based immunotherapy, the KIR-ligand mismatch phenomenon has attracted great attention[273] after the ground-breaking retrospective analysis of haplotype mismatched hematopoietic stem cell transplants by Ruggeri *et al.* revealed delayed relapse, better engraftment and protection from GvHD in leukemia patients[271]. Later studies have shown that NK cells from healthy donors and cancer patients show higher cytotoxic activity against various KIR-ligand mismatched tumor cell lines when compared to KIR-ligand matched targets[274]. On the other hand, different prospective studies have suggested that development of NK cells after haploidentical transplantation is hampered and these cells have phenotypic and functional deficiencies[275,276]. Table 6 presents a selection of clinical trials with infusion of allogeneic NK cells[244,272,277-285].

In an interesting recent study by Shi *et al.* 10 patients with MM were treated with haploidentical NK cells before ASCT. The allogeneic NK cells persisted in the periphery of the patient reaching a maximum at around 7 days and eventually fading away until they were undetectable by day 14. The increasing donor chimerism up to day 7 suggests a confirmation for Miller *et al.*'s observation of *in vivo* expansion. Encouragingly, a complete remission rate of 50% was reported. Although the allogeneic NK cells were not detectable after day 14, the efficiency of this approach may suggest that even without long-term engraftment, a clinical benefit for the patients can be observed. This brings the question about the *in vivo* fate of adoptively transferred allogeneic NK cells. Brand *et al.* conducted a study where the allogeneic NK cells are followed by using PCR and radioactive labeling. Infused cells could be detected in circulation by PCR for up to 3 days. NK cells radiolabeled led by ^{111}In were followed by scintigraphy and were detectable for up to 6 days. A distribution over the whole body, with preference for liver, spleen, and bone marrow, was observed after a short initial uptake in the lungs. A total of 2/4 evaluable metastases showed a clear accumulation of transfused NK cells[286].

Another approach in NK cell-based tumor immunotherapy is the use of the cell line NK-92[287], which can be consistently grown under GMP conditions. This cell line does not express any KIR but still has a broad spectrum of activating receptors[288]. Following preclinical mouse studies[289,290] and *ex vivo* applications such as purging of leukemia, lymphoma and CML[291,292], NK-92 cell line has also been used as direct infusions to patients.[293,294] Data from these trials suggest that infusion of NK-92 may be safe and potentially beneficial.

Table 6: Clinical trials with infusion of allogeneic NK cells

Study	Koehl	Passweg	Miller	Shi	Barkholt	Huenecke
Year	2004	2004	2005	2008	2009	2010
Patients	2 ALL 1 AML	4 AML 1 CML	10 metastatic melanoma, 13 metastatic RCC 1 refractory HD 19 poor-prognosis AML	10 relapsed MM	1 CRC 1 HCC 2 RCC 1 CLL	2 NB
KIR mismatch	3/3 patients	3/5 patients	4/19 AML patients	10/10 patients	1/5 patients	NR
NK preparation	2-step MACS cultured in X-vivo 10 with 10% FFP and 1000 U/ml IL-2 for 14 days	2-step MACS	CD3 MACS depleted PBMCs incubated overnight in X-VIVO 15 with 1000 U/ml IL-2	CD3 MACS depleted PBMCs, incubated overnight in AIM-V with 2% HS and 300 U/ml IL-2	3 week expansion of mixed PBMC in CellGro SCGM with 5% HS, 10 ng/ml OKT3 and 500 U/ml IL-2	2-step MACS cultured 2 weeks in X-VIVO 10 with 5% FFP and 1000 U/ml IL-2
Purity	Median: 95%	Median: 97.35% Range: (77.9-98.9%)	Mean: 40% (Range: 18-68%)	NR	NR	>95%
Dose	Mean: $11.9x10^6$ cells/kg (Range: $3.3\text{-}29.5x10^6$)	Median: $0.93x10^7$ cells/kg (Range: $0.21\text{-}1.41x10^7$)	$2x10^7$ cells/kg	Mean: $15.2x10^6$ NK cells/kg (Range: $2.7\text{-}92x10^6$)	Escalating doses from $1X10^6$/kg to $40x10^6$ NK cells/kg	$7.8\text{-}45.1x10^6$ cells/kg
Schedule	Infusions at day +1, or day +2 post transplantation followed by 2 additional infusions every 4-6 weeks	2 infusions with 2 months interval	Single infusion	2 infusions at day 0 and day 2, followed by delayed autologous SC graft on day 14	3 infusions, 1 month intervals between infusions	2 infusions, at day 35-40 and day 100-130 post allo transplant
IL-2 infusions	None	None	$1.75x10^6$ U/m^2/day IL-2 for 14 days starting at NK cell infusion, last 14 AML patients: $10x10^6$ U IL-2, 3 times weekly	$3x10^6$ U IL-2 daily from day 1 to day 11	$6x10^6$ U/m^2/day IL-2 in 3 patients	None
Clinical Results	- Safe - All patients engrafted - 2 patients showed signs of response	- Safe - 2/5 increased donor chimerism - 1/5 stable donor chimerism	- Safe - *in vivo* expansion of NK cells when preceded with HDT - Remission in 5/19 AML patients	- Safe - Donor NK cells persisted up to 2 weeks - Increased level of serum IL-15 - No significant improvement of response compared to ASCT	- Safe - HCC patient showing signs of response	- Safe - Decreased NK cell counts in peripheral blood - Immediate decrease of $CD56^{bright}$ CD16- NK cells from circulation, followed by peaking at 24 hours post-infusion

PBMC; peripheral blood mononuclear cells, ALL; acute lymphoblastic leukemia, AML; acute myeloid leukemia, CML; chronic myelogenous leukemia, CRC; colorectal cancer, HCC; hepatocellular cancer, RCC; renal cell carcinoma, CLL, chronic lymphocytic leukemia, NB; neuroblastoma, KIR; killer-cell immunoglobulin-like receptor; MACS; magnetic-activated cell sorting, FFP; fresh-frozen plasma, HS; human serum, NR; not reported, HDT; high-dose therapy.

Table 6: Clinical trials with infusion of allogeneic NK cells, continued

Study	Rubnitz	Rizzieri	Brehm	Nguyen	Curti
Year	2010	2010	2011	2011	2011
Patients	10 MRD-negative AML	30 patients with myeloid and lymphoid malignancies	5 AML 5 ALL 4 NB 1 RMS 1 Hodgkin	1 AML	13 AML
KIR mismatch	9/10 patients	14/30 patients 6/6 HLA matched, 16/30 patients 3-5/6 HLA-matched	16/16	1/1 patient	13/13 patients
NK preparation	2-step MACS	CD56 MACS enrichment	Group 1: 2-step MACS Group 2: 2-step MACS cultured in X-VIVO 10 with 5% FFP and 1000 U/ml IL-2 for 9-14 days	CD3 MACS depletion	2-step MACS
Purity	NR	Median: 96% (Range: 87-100%)	Median 95% (Range: 84.4-98.6%)	NR	Median: 93.5% (Mean: 66.4-99.2%)
Dose	Mean: $29x10^6$ NK cells/kg (Range: $5.2\text{-}80.9x10^6$)	Median: $10.6x10^6$ NK cells/kg	Group 1: Median: $13.1x10^6$ cells/kg (range: $3.2\text{-}38.3x10^6$) Group 2: Median $14.6x10^6$ cells/kg (range: $6.0\text{-}45.1x10^6$)	$3x10^7$ cells/kg	Median: $2.74x10^6$ cells/kg (Range: $1.11\text{-}5x10^6$)
Schedule	Single infusion	Infusion 6-8 weeks post allo transplant, up to two more infusions with 8-week intervals	3 infusions at day +3, day +40 and day +100 post transplant	Single infusion at day 136 post allo transplant	Single infusion
IL-2 infusions	$1x10^6/m^2$/day for 2 days	No	No	$10x10^6$ U/week for 60 days starting at NK cell infusion	$10x10^6$ U/day IL-2 3 times a week for 2 weeks
Clinical Results	- Safe - All patients had engraftment of NK cells - One patient had detectable NK cells in circulation up to 189 - Expansion of alloreactive NK cells in 9/9 patients - 2-year EFS 100%	- Safe - 14 aGVHD - 42-43% 1-year OS predicted for both groups compared to 31% with T cell depleted DLI	-Safe - Infusion of IL-2 stimulated NK cells (but not unstimulated NK cells) induces decrease of circulating NK cells monocytes, dendritic cells and eosinophils for up to 24h	-Safe - CR - *in vivo* expansion of alloreactive NK cells, relapse on Day 80	- Safe - Increased IL-15 serum levels due to Flu/Cy preparative regimen - Alloreactive NK cells detected in circulation - 1/5 patients with active disease achieved CR - 2/2 patients in molecular relapse achieved CR - 3/6 patients with CR are disease-free

MRD; minimal residual disease, AML; acute myeloid leukemia, ALL; acute lymphoblastic leukemia, NB; neuroblastoma, RMS; Rhabdomyosarcoma, HLA; human leukocyte antigen; MACS; magnetic-activated cell sorting, FFP; fresh frozen plasma, NR; not reported, EFS; event-free survival, aGVHD; acute graft-versus-host disease, OS; overall survival, DLI; donor lymphocyte infusion, CR; complete response, Flu/Cy; fludarabine/cyclophosphamide.

1.5 GENETICALLY MODIFIED NK CELLS IN CANCER IMMUNOTHERAPY

1.5.1 Gene therapy

Gene therapy defines a broad set of applications that are based on the transfer of genetic material (DNA or RNA) into target cells in order to prevent or treat a disease. Both the purpose and the method of nucleic acid delivery vary greatly depending on the application. The most classical examples of gene therapy include the introduction of a functional gene into the cell, in cases where the cellular copies of the gene are non-functional due to mutations, causing what is called a monogenic disorder. In such cases, introduction of a healthy copy of the gene is expected to result in the expression of a functional protein and compensate for the loss of function caused by the mutations. The revolutionary idea here is to treat disease by the administration of genetic material, which will produce the necessary amount of protein in order to correct the condition. Therefore, instead of treating symptoms or replacing the lost function with a drug, gene therapy offers a one-shot treatment for curing the underlying pathophysiology of the disease. In this process, only the somatic cells (and most of the time only certain tissues) and not the germ cells are the target. Therefore, such gene transfer affects only the treated individual and not the offspring.

After initial propositions[295] and early clinical investigations of gene transfer into human cells[296], on September 14, 1990, Ashanti DeSilva, a four-year old patient with adenosine deaminase deficiency became the first person in history to receive gene therapy[297] at a first-in-man trial by Blaese *et al*. Positive results from this trial have paved the way for many other gene therapy clinical trials to come, all with their ups and downs. In this seminal paper[297], the authors conclude: “Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.” While the field still is busy with fulfilling the prophecy of perfecting the many components, recent years have witnessed extraordinary developments along with failures and success stories coming one after the other[298]. Besides clinical applications, gene delivery has developed into a standard laboratory technique driving discoveries in basic research from analysis of gene function by plasmid DNA transfection all the way to development of induced pluripotent stem cells (iPSCs)[299,300] using viral delivery mechanisms.

Even from the very early days, the possibility of gene transfer into a cell has surpassed the boundaries of the classical definition of gene therapy that relies on correcting defective gene expression. As with the gene-marking study by Rosenberg *et al.*[296], *ex vivo* introduction of foreign genes to the adoptively transferred hematopoietic cells were primarily used to track genetically modified cells *in vivo*[301]. Especially in the immunotherapy field, this has sparked the interest in genetically modifying immune effector cells in order to use them as microscopic soldiers that could seek and destroy the malignant cells inside the body[302,303]. Traditionally, the focus has been on T cells,

but more and more other players of the immune system such as NK cells are stepping on the scene.

1.5.2 Overview of gene delivery vectors

The major hurdle in gene therapy is the delivery of the gene-of-interest (GOI) into the cell. When it comes to hijacking cellular defense mechanisms and carrying genetic information into the cell, nature presents us with an evolutionarily perfected carrier: viruses. The natural life cycle of a virus depends on the capability of the virus to enter a target cell and use the cellular machinery to drive the expression of viral genes. As techniques for the *in vitro* manipulation of DNA have developed, the possibility of using viruses as gene delivery mechanisms have surfaced. After the initial discovery of gene transduction into *E.coli* by bacteriophage λ[304], in 1968, Rogers and Pfuderer have extrapolated this approach to demonstrate that sequences of nucleotides can be added to the viral genome of the tobacco mosaic virus *in vitro* and the virus can be used as a vector to transmit desired information into the tobacco plant[295]. Since then, viruses have been widely used as gene delivery machines.

The problem with viruses is that they are generally pathogenic and immunogenic. Therefore, other researchers have ventured upon the use of non-viral delivery methods. Since the spontaneous uptake of nucleic acids by cells is very limited, methods of non-viral gene delivery rely upon coupling of DNA to carrier molecules covalently or non-covalently or using physical methods such as microinjection or electroporation. Therefore, methods used for the delivery of genes are conventionally divided into two: viral delivery and non-viral delivery.

In order to deliver nucleic acids into a cell, non-viral vectors have to overcome many biological barriers. First, the extracellular stability of the vector has to be ensured. This is generally done by using carrier molecules that help DNA condensation and protection from nucleases as well as the use of hydrophilic moieties for steric stabilization and surface charge shielding. Common examples include the use of protamine[305], lipids[306-308], gelatin[309] and PEGylation[306,307,309-313]. Secondly, the crossing of the cellular lipid bilayer membrane presents a major barrier. Here, general mechanisms include targeting a ligand on the cell surface that could trigger receptor-mediated endocytosis such as transferrin receptor[306,313], EGF[310], antibodies[305,307], RGD[312], etc. Once the nucleic acids are taken up, disruption of the endosome and subsequent transfer into the nucleus also needs to be assured. Agents such as PEI[310,312], and DOPE[306] as well as nuclear localization signals from various viruses are commonly used for these processes. It is quite often that the designers of non-viral vectors borrow ideas and components from viruses that have been perfected by millions of years of evolution. Non-viral vectors are further reviewed in the following citations[314,315].

Contrary to non-viral vectors, viral delivery of genes is generally highly efficient. However, using viruses for gene delivery has problems of its own. In order to get rid of the pathogenicity of the virus and ensure that the virus will not be replicating and spreading, all viral genes and sequences except those necessary for packaging of the viral genome are removed. This also opens up space inside the viral genome for

therapeutic genes to be inserted. Moreover, it is common practice to change viral promoter elements and envelope proteins for enhancing safety and ensuring the tropism of the virus to the target cell type. These approaches will be discussed in detail for lentiviral vectors in the following chapter. Table 7 presents an overview of commonly used viral vectors.

Table 7: Viruses commonly used as gene therapy vectors

Vector type	Gamma-retrovirus	Lentivirus	Adenovirus	AAV	Herpes virus	Vaccinia virus
Family	Retroviridae	Retroviridae	Adenoviridae	Parvoviridae	Herpesviridae	Poxviridae
Genome type	ssRNA (+)	ssRNA (+)	dsDNA	ssDNA	dsDNA	dsDNA
Genome size	9 kb	9 kb	39 kb	5 kb	120-200 kb	130-280 kb
Coating of the particle	Enveloped	Enveloped	Naked	Naked	Enveloped	Enveloped
Infection	Dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells
Persistence	Integrating	Integrating	Non-integrating	Very low chance of integration	Non-integrating	Non-integrating
Immunoge-nicity	Low	Low	High	Low	Low	High

1.5.3 Lentiviral vectors

Lentiviruses belong to the Retroviridae family that consists of single stranded RNA viruses with the capacity of reverse transcribing their genome into double stranded DNA, which becomes stably integrated into the host cell genome.

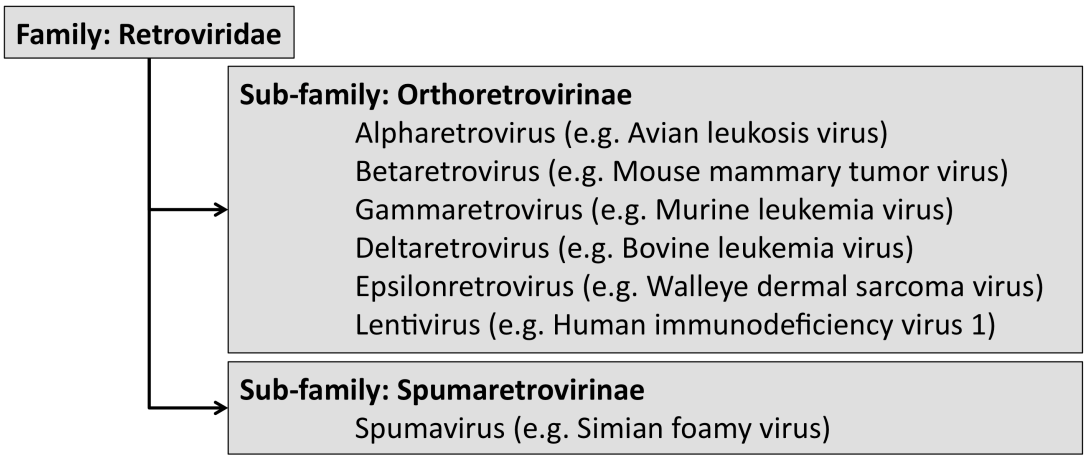


Figure 3: Classification of the Retroviridae family.

As our understanding of the biology of retroviruses have developed, rational design of vectors based on the retrovirus family has become increasingly common. Among the members of the family that have been engineered for viral vector production are the Foamy Virus[316,317], Human Immunodeficiency Virus (HIV)[318,319], Simian Immunodeficiency Virus (SIV)[320], Bovine Immunodeficiency Virus (BIV)[321], Feline Immunodeficiency Virus (FIV)[322], Equine Infectious Anemia Virus (EIAV)[323], Murine Leukemia Virus (MLV)[324,325], Bovine Leukemia Virus (BLV)[326], Rous Sarcoma Virus

(RSV)[327], Spleen Necrosis Virus (SNV)[328] and Mouse Mammary Tumor Virus (MMTV)[329].

The reverse transcribed and integrated proviral DNA of a typical simple retrovirus such as MLV is flanked by two incomplete long terminal repeats (LTR) which are normally structured into U3, R and U5 regions (Figure 4). Since transcription of the proviral DNA is initiated by the enhancer-promoter located in the 5' U3 region, the viral genomic RNA starts with R, and is followed by U5, the primer binding site (PBS) for initiation of reverse transcription, the major splice donor (SD) and the packaging and RNA dimerization signal (ψ), all located upstream of the translational start codon of gag/pol (encoding structural and replication proteins). Downstream of the gag/pol coding region the env (encoding the viral envelope glycoprotein) reading frame is found, whose expression is enabled by a splice acceptor located in pol. The 3' untranslated region of the RNA contains the polypurine tract (PPT), and the 3' incomplete LTR consisting of the 3' U3, and the 3' R region. The latter contains the polyadenylation signal and is thus followed by a polyA tail. Since the viral RNA carries a 5' cap and a 3' pA tail, it resembles a cellular mRNA. It is only due to the unique mechanism of reverse transcription that the complete LTRs are restored prior to integration of the virus into the host cell genome[330].

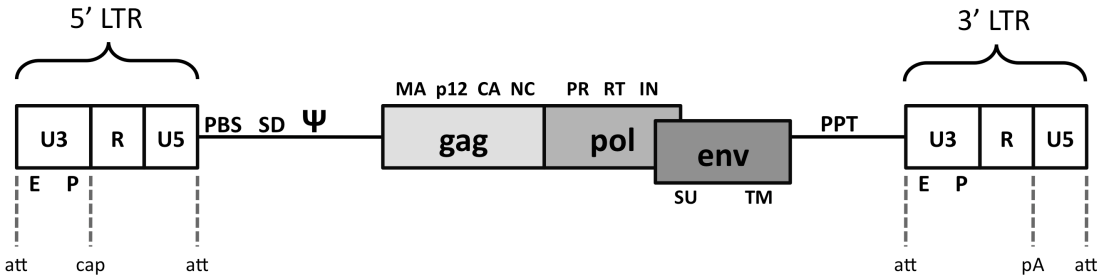


Figure 4: Genome structure of a gammaretrovirus: MLV. Indicated are the 5' and 3' long terminal repeat (LTR; open boxes) regions comprising U3, R and U5, as well as open reading frames (filled boxes) for gag, pol and envelope (env) proteins. att, attachment site; cap, 5'RNA capping site; pA, polyadenylation site; PBS, primer binding site; SD, splice donor; ψ, packaging signal; SA, splice acceptor; PPT, polypurine tract; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface; TM, transmembrane; E, enhancer; P, promoter. Figure adapted from Maetzig et al.[330].

The viral life cycle can be divided into two main phases (Figure 6). In the first phase the virus particle binds its receptor on the host cell surface (1) followed by fusion of the viral envelope to the cellular membrane (2). Once the virus is inside the cell, the capsid breaks open and with the help of the proteins packaged inside the particle reverse transcription is carried out (3). Following this, the reverse transcribed virus DNA binds integrase proteins and constitutes the pre-integration complex (PIC). The next step is transportation of the PIC into the host cell nucleus (4) and integration into the host genome (5). This step defines the major difference between lentiviruses and simple retroviruses such as gammaretrovirus. While gammaretroviruses have to wait for the disintegration of the nuclear membrane during mitosis in order to reach the host cell chromatin, lentiviruses can interact with cytoplasmic carriers and actively migrate into the nucleus without the need for nuclear membrane disintegration.

Therefore, while lentiviruses can successfully integrate into non-dividing cells, gammaretroviruses can only integrate into the host genome during cell division.

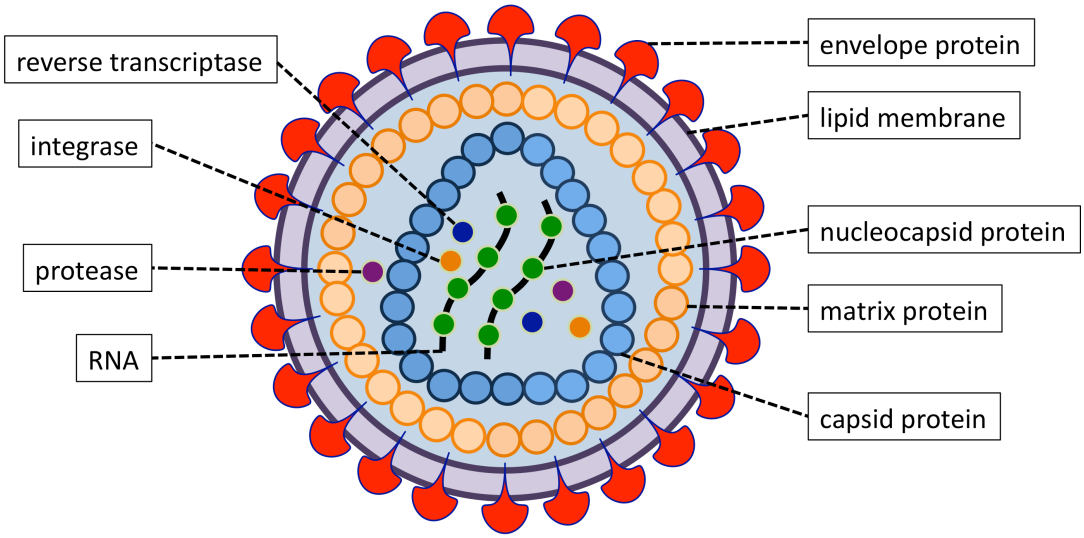


Figure 5: Structure of a simple retroviral particle

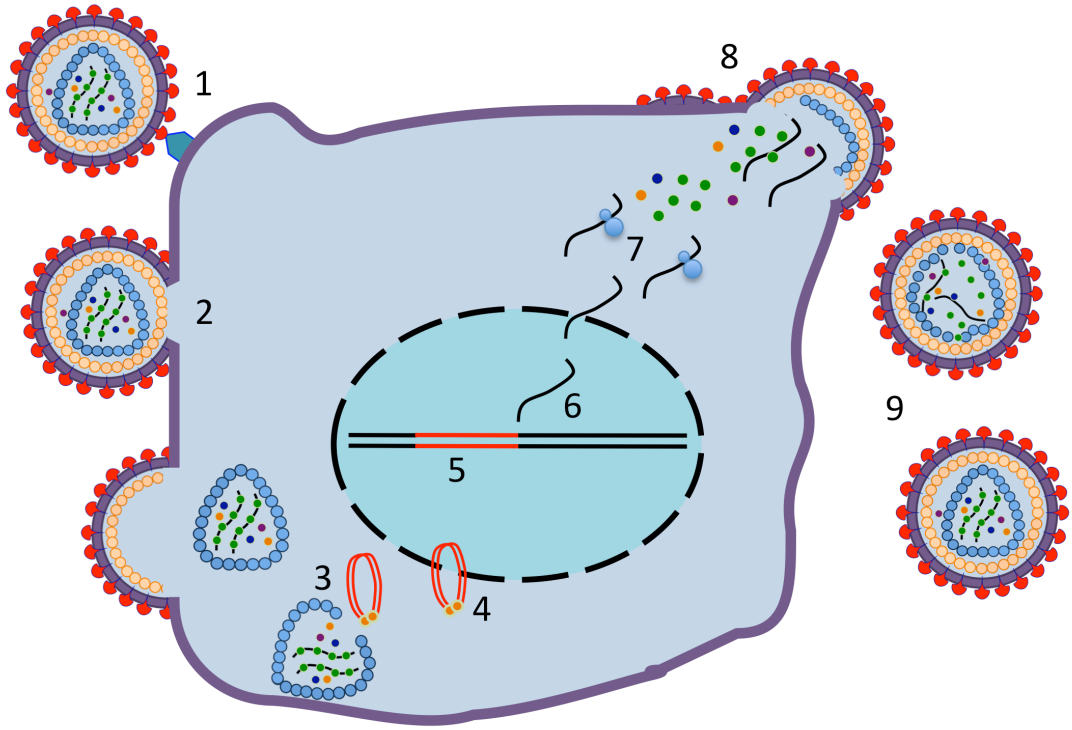


Figure 6: Life cycle of a lentivirus: HIV-1

In the second phase of the life cycle, viral genes that are now part of the host cell genome are transcribed (6) and viral proteins are expressed using the cellular machinery (7). Once all the viral proteins are expressed, assembly and budding starts on the host cell membrane (8) and new virus particles start budding off the cell, followed by maturation of the virus particle (9)

In the case of lentiviral vectors, the second phase of the life cycle is not desirable. Instead of expressing viral genes and packaging new virus particles, the expected result is the expression of the therapeutic gene. Therefore, in order to turn a virus into a viral vector, all viral elements inside the viral genome are removed and replaced with the GOI. In this case, the virus has no capacity to produce more virus particles once the cell is successfully infected. This renders the viral vector replication-incompetent, such that the particle can only infect once, increasing the safety of the procedure.

The first generation viral vectors were designed using the approach depicted in Figure 7. Basically, the whole viral genome is first cloned into a plasmid (a). Secondly, two new plasmids are derived from this one (b). In the first plasmid (called the transfer plasmid), viral genes are replaced with the gene of interest and in the second plasmid, the viral genes are present but the packaging signal is removed. When these two plasmids are co-transfected into a cell line, the viral genes are expressed from the second plasmid but the viral RNA coming from the second plasmid cannot be packaged due to the lack of a packaging signal. Instead, the viral proteins in the cell

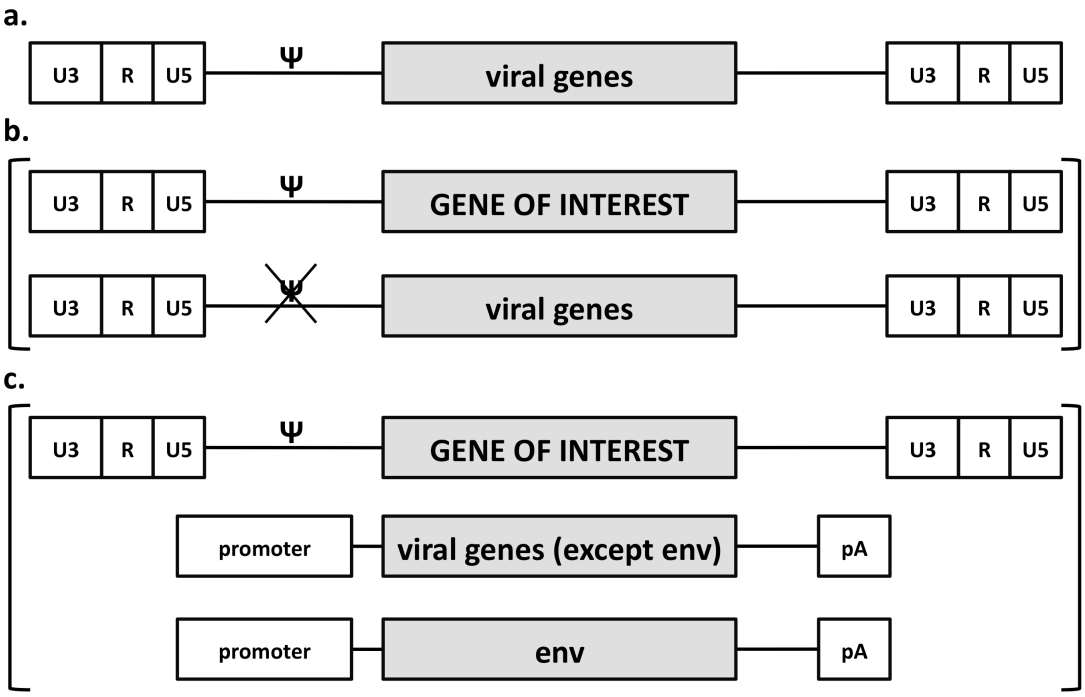


Figure 7: From virus to viral vector

can be used to package the RNA coming from the first plasmid, resulting in a virus particle that contains all the necessary components for budding, maturation and target cell infection while lacking the genes for building new virus particles. A further step from this point (c) is the removal of the envelope gene from the second plasmid and the use of a third plasmid for the env gene, which creates the possibility of using different envelope proteins for packaging the same viral genome by changing the plasmid coding for the env gene. Also, the removal of LTRs provides extra security by decreasing sequence similarity between the transfer plasmid and the packaging plasmids, therefore decreasing the risk of recombination between the plasmids during

virus production, which could result in the production of a replication-competent viral particle.

The first lentiviral gene delivery systems used replication-incompetent HIV-1 vectors to study different aspects of the viral life cycle in the early 1990s[331-335], but the key breakthrough came with the construction of vectors that, in contrast to MLV-derived ones, were capable of transducing non-dividing neurons when injected into rat brains[318]. This first lentiviral vector generation was made of three plasmids (as in Figure 7c) in which the packaging functions were provided by an env-coding plasmid and by a packaging plasmid expressing all viral genes except env under the control of a CMV promoter. The transfer vector was composed of an expression cassette framed by two wild type LTRs and bearing sequences required for viral RNA export in producing cells (the Rev-Responsive Element, RRE), genome packaging and reverse transcription. In the second generation packaging vectors, most accessory genes of HIV-1 were eliminated (vif, vpr, vpu and nef) and only Tat and Rev were retained[336], while in the third, Tat was also removed and Rev was provided on a fourth plasmid[319]. Therefore, third generation vectors are based on four plasmids instead of three, which further decreases the risk of producing replication competent lentivirus. In the case of transfer vectors, a number of modifications contributed to increase the performance of gene transfer, as for example the use of post transcriptional regulatory elements that enhance the transgene transcriptional expression, or the use of heterologous polyadenylation enhancer elements, as those derived from simian virus 40 (SV40) or β-globin, or the use of different internal promoters to express a particular GOI.

Expanding the natural tropism of the viral vector by using a different envelope glycoprotein rather than that of the original virus is a commonly used method called pseudotyping[337]. For example, in the case of HIV-1 based lentiviral vectors, the natural tropism of the viral vector would exclusively be $CD4^+$ T cells due to the specificity of HIV-1 envelope glycoproteins. Yet, the use of the envelope glycoprotein from vesicular stomatitis virus (VSV-G) enables highly efficient packaging of viral particles and broadens the tropism of the viral vector as it uses common membrane lipids as receptors[338]. Aside from VSV-G, for genetic modification of human hematopoietic cells, pseudotyping lentiviral vectors with the envelope glycoproteins of following viruses have been reported to provide an efficient approach: Venezuelan equine encephalitis virus (VEEV)[339], Measles virus (MV)[340], Feline endogenous virus (RD114)[341-344], Human T-cell leukemia virus type-1 (HTLV-1)[345] and Gibbon ape leukemia virus (GALV)[344].

Successful genetic modification is marked by persistent transgene expression throughout cellular proliferation and is retained in the progeny. Using integrating viral vectors ensures stable integration of the transgene into the target cell genome. This has generated a great deal of debate following reports of malignant transformation of cells due to random integration of the viral vector in the genome causing insertional mutagenesis[346,347]. A single random insertion of a retroviral copy may induce oncogene activation and subsequent malignant transformation of the genetically modified cells[348]. Lentiviral vectors also have the ability to insert several vector copies

into the target cells[349], which leads to a similar prediction for the risk of insertional mutagenesis[350,351]. However, one could argue about whether insertional mutagenesis is a justifiable concern in the context of genetic modification of terminally differentiated cells as compared to stem cells. It is highly likely that terminally differentiated cells will not be able to sustain tumor growth due to their finite lifespan. As the theory of cancer stem cells[352] gains momentum, confirmed by observations of tumor sustainability through endeavors of a small stem cell-like population, modification of terminally differentiated cells seems safer compared to modification of stem cells. It could be argued that although one single hit could trigger tumorigenesis at the stem cell level, it would take many more hits in a "destined-to-die" terminally differentiated cell. Moreover, current evidence suggests that mature T cells are resistant to oncogene transformation[353]. Although promising, such conclusions should be taken with caution and it should be kept in mind that malignancies of terminally differentiated cells –such as NK or T cell lymphomas- do exist.

The possibility for genetic rearrangement should be significantly lower in fully committed differentiated effector cells. Nonetheless, the risk of insertional mutagenesis associated with the use of integrating vectors needs to be further investigated and the need for development of vectors with safe integration sites, increased transduction efficacy at low multiplicity-of-infection (MOI) or stable episomal gene expression is essential. As a consequence, the choice of an appropriate vector for gene delivery as well as the targeted delivery and expression of the transgene are important issues in gene therapy settings.

1.5.4 Genetic modification of NK cells

Gene transfer into NK cells may open new possibilities for the immunotherapy of cancer in both autologous and allogeneic settings. The use of genetically modified NK cells that have been redirected to tumor targets via the introduction of either activating or chimeric antigen receptors presents a hot prospect for further clinical applications[354]. Applications of genetic modification could include various approaches from induction of proliferation/survival via cytokine gene therapy to specific targeting of NK cells to certain tissues or malignant cells. Although transient methods such as electroporation[355-357] or nucleofection[358] applications are under significant improvement, stable transduction using retroviral[359-365] or lentiviral[366-372] vectors present a greater advantage in terms of long-term effects and sustainability.

Liu *et al.* have reported transfection of the CD18 gene into a clone of the NK cell line YT-1 that lacks functional CD18 expression. They have demonstrated that upon genetic modification, the cell line restores its cytotoxic capacity against a B cell lymphoma line[373]. It has also been shown that the delivery of the IL-15 gene to NK cell lines increases proliferative rate and cytotoxic capacity[374,375]. Likewise, delivery of the IL-12 gene to mouse NK cells increased their survival capacity and *in vivo* anti-tumor activity[376].

Systemic IL-2 administration frequently causes undesirable side effects[377,378], e.g. the activation of other immune cell populations. More specifically, activated T cells

increase the chance of GvHD[379], while the stimulation of immunosuppressive T_{reg} cells is suboptimal for cancer patients[380]. In settings where IL-2 is given primarily to enhance NK activity, administration in a form that stimulates NK cells, without unwanted side effects, would be ideal. There have been various reports on IL-2 gene delivery via retroviral transduction[363] or particle mediated[381] transfection to the IL-2 dependent NK cell line NK-92. Stable transduction of the IL-2 gene increased cytotoxic activity against tumor cell lines *in vitro.* Such a modification enabled the secretion of IL-2 by the NK92 cells and saved the cells from the dependency on exogenous IL-2 supplementation. Moreover, the IL-2 transduced cells showed greater *in vivo* antitumor activity in mice[363]. Similarly, Miller *et al.* have reported that IL-2 transduced mouse NK cells sustained proliferation in the absence of exogenously supplied IL-2[382]. However, the expression of IL-2 in a secreted manner by NK cells may affect neighboring cells or have the potential to cause a systemic IL-2 effect in patients. This risk prompted us to continue investigation to seek alternative approaches for IL-2 delivery retained in NK cells in a controlled and localized manner. Our group has constructed an endoplasmic reticulum-retained IL-2 gene that is not secreted but still confines autocrine growth stimulation to NK-92 cells[365]. Such an approach may be useful for future applications where secretion of high levels of IL-2 by the adoptively transferred NK cells might cause side effects.

Another approach to genetic modification of NK cells for cancer immunotherapy is retargeting of the NK cells to tumor cells via the expression of chimeric antigen specific receptors. This is generally done by using a single-chain variable fragment receptor specific for a certain tumor-associated antigen fused to the intracellular portion of the signalling molecule CD3ζ. Such receptors have been used by many different groups and have proven to be efficiently working in NK cells. Chimeric receptors against CEA[383], CD33[384] and Her2/neu[364,385,386], have been successfully delivered to NK cell lines and were shown to increase antigen specific cytotoxic activity of NK cells both *in vitro* and *in vivo*.

These improvements have rapidly been translated to settings of primary NK cells and experimental models. Pegram *et al.* have gene modified primary mouse cells to express a chimeric receptor against Her2/neu and observed that the adoptive transfer of these cells to mice bearing $Her2^+$ tumors inhibits tumor progression *in vivo*[387]. Likewise, Kruschinski *et al.* have modified primary NK cells from human donors to express a chimeric receptor against Her2/neu and observed high level of cytotoxic activity against $Her2^+$ cell lines both *in vitro* and in xenograft models with $RAG2^{-/-}$ mice[388]. Moreover, Imai *et al.* have successfully demonstrated that NK cells from B-lineage ALL patients genetically modified to express a chimeric receptor against CD19 efficiently kill autologous leukemic cells *in vitro*[362]. Taken together, these data indicate that the adoptive transfer of chimeric antigen-specific bearing NK cells might be an efficient approach in cancer immunotherapy.

Optimization of viral genetic modification in NK cells presents a multi-faceted problem ranging from the source of NK cells to culture conditions, the choice of cytokines and critical viral elements such as envelopes or promoters and the process of viral

infection. Previous reports have included various approaches such as the use of feeder cells[362,371,388], multiple rounds of transductions[359,369,371] or co-culture with virus producing cells[363] in an attempt to ensure efficient culture and genetic modification of NK cells. However, efficiency of viral gene delivery to NK cells has always proven challenging and less efficient than other cells of the hematopoietic system. In fact, this is not to be unforeseen, since it is well established that NK cells are among the first-responders to viral infections[389] and must have been evolutionarily selected to have high endurance against a virus infection[390].

While high resistance against viral infections serves the evolutionary purpose of the NK cell, it presents a big disadvantage when it comes to genetic modification via the use of viral vectors. As with wild-type viruses, intracellular recognition of viral components by pattern recognition receptors is a possible mechanism of cellular response against viral vectors[391,392]. Although the literature is scarce regarding the activation of such responses against lentiviral vectors, it has been shown that an innate immune response against the vector can be generated by plasmacytoid DCs[393]. Such responses against lentiviral vectors have also been documented during *in vivo* studies after systemic administration of the vector, resulting type 1 IFN responses and vector clearance[394]. In PAPER III, we aimed at looking into whether these mechanisms could be factors contributing to the resistance against viral gene delivery, and whether such recognition pathways could be efficiently blocked in order to increase genetic modification efficiency.

2 AIMS OF THIS THESIS

The long-term goal of this research is to use *ex vivo* expanded and/or genetically modified human NK cells with high anti-tumor activity in adoptive immunotherapy of cancer. In order to reach this goal, we have aimed at optimizing protocols for the expansion and genetic modification of NK cells. More specifically, we have focused on:

- Investigation of the feasibility of expanding NK cells with autologous anti-tumor activity from PBMCs of patients with multiple myeloma using GMP-grade components (PAPER I)
- Optimization and validation of a clinical grade, large-scale NK cell expansion process in an automated bioreactor (PAPER II)
- Efficient lentiviral genetic modification of NK cells (PAPER III)

3 METHODOLOGY

3.1 NK CELL CULTURE AND EXPANSION

3.1.1 Expansion of NK cells in cell culture flasks (PAPERS I and II)

PBMCs were initially thawed and cultured in cell culture flasks at a concentration of $0.5x10^6$ cells/ml in CellGro SCGM serum-free medium with the addition of 5% human serum and 500 U/ml rhIL-2 (Proleukin). At the beginning of the culture, the medium was further supplemented with GMP grade monoclonal anti-CD3 antibody (OKT3) at a final concentration of 10 ng/ml. The cultures were then replenished with fresh medium containing 500 U/ml IL-2 but not OKT3, every other day throughout the culture period. Total cell numbers were assessed by staining cells with Trypan blue dye on days 0, 5–6, 9–10, 14–15, and 20 of culture. Absolute cell counts were calculated by multiplying the total number of cells by the percentage of specific subsets determined by flow cytometry. To prevent contact inhibition of cell growth, the cells were transferred to bigger flasks when necessary. The final products were evaluated for purity, viability, phenotype and cytokine secretion. Figure 8 demonstrates the experimental layout for NK cell expansion studies.

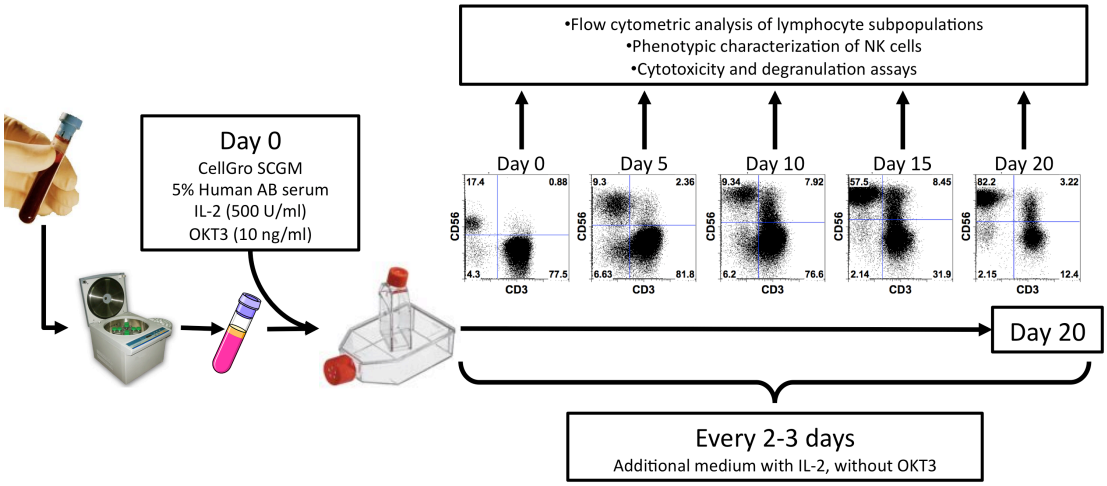


Figure 8: NK cell expansion process

3.1.2 Expansion of NK cells in bags (PAPER II)

Vuelife™ (American Fluoroseal Corporation, MD, USA) is a sterile cell culture bag made of fluorinated ethylene-propylene that is claimed to be biologically, immunologically and chemically inert. It is highly permeable to gases and optically clear. The cultures in Vuelife bags were initiated with $5x10^5$ cells/ml in 60 ml medium using 72 ml Vuelife bags. The bags were incubated in a humidified incubator at 37°C and 5% CO_2. Fresh medium was added every other day to adjust the concentration to $1x10^6$ cells/ml until day 10 and of $2x10^6$ cells/ml thereafter. Cells were split to larger bags when necessary.

3.1.3 Expansion of NK cells in bioreactor (PAPER II)

The Wave Bioreactor is a cell culture system where the cells are grown inside a temperature and CO_2 controlled disposable, sterile bag that is placed on a rocking heated platform. We have used a Wave Bioreactor System 2/10 (GE Healthcare, Somerset, NJ, USA). Our previous experience with this system has shown suboptimal efficiency when the expansion was initiated with low volumes and/or low cell doses. Yet, the amount of cells in regular peripheral blood samples from healthy donors did not allow starting the expansions directly in the bioreactor. Therefore, in initial optimization experiments we have initiated the cultures in flasks and transferred the cells into the bioreactor at around day 5 when sufficient number of cells was reached. The bioreactor cultures at this day were started with $2x10^6$ cells/ml in 800 ml. In final validation experiments, a whole unit of peripheral blood, or apheresis product from donors and MM patients were obtained and the cultures were initiated directly in bioreactors from day 0. The conditions for the bioreactor were as follows at all times: Temperature 37°C, CO_2: 5%, Airflow: 0.1, Rocking rate: 6/min, Rocking angle: 6°. The cells were sampled and counted every other day and no further feeding was done until the cell density reached $3x10^6$ cells/ml. From then on, the culture was fed with 300 ml of medium per day. When the cells reached a density of $7x10^6$ cells/ml, the feeding was increased to 500 ml/day; after $1x10^7$ cells/ml, to 750 ml/day and after $2.5x10^7$, to 1L/day.

3.1.4 Culture of NK cells for lentiviral transduction (PAPER III)

After magnetic isolation by a single-step NK cell enrichment kit, the cells were put into culture at a concentration of $1x10^6$ cells/ml in CellGro SCGM supplemented with 10% HS and 1000 U/ml rhIL-2. In indicated experiments, IL-12, IL-15 and IL-21 were used at a concentration of 20 ng/ml. The cells were kept in culture for different times before lentiviral transduction was carried out.

3.2 EVALUATION OF NK CELL MEDIATED CYTOTOXICITY

3.2.1 ^{51}Cr release assay (PAPERS I-II-III)

The cytotoxic capacities of NK cells were evaluated *in vitro* with a standard 4-hour ^{51}Cr-release assay against K562 cells. In short, K562 target cells were labeled with 100 µCi of ^{51}Cr for 1 hour at 37°C, washed twice with PBS, and resuspended in RPMI medium. A total of $3x10^4$ target cells in 100 µl RPMI medium was placed in triplicates into V-bottomed 96-well plates and incubated for 4 hours with 100 µl of effector cells at appropriate concentrations to obtain effector:target (E:T) ratios from 1:3 to 10:1. Aliquots of supernatants were counted using a Packard Cobra Auto-Gamma 5000 Series Counting System. The percentage specific ^{51}Cr release was calculated according to the formula: percent specific release= [(experimental release -spontaneous release)/(maximum release-spontaneous release)]x100.

3.2.2 Flow cytometry-based cytotoxicity assay (PAPER I)

Target cells, were labeled with TFL4 reagent from the CytoToxiLux-PLUS kit (OncoImmunin Inc., Gaithersburg, MD, USA) according to the manufacturer's

instructions. In all flow cytometry based cytotoxicity assays, $5x10^4$ labeled target cells were placed in tubes together with different amounts of effector cells to obtain effector:target ratios from 1:3 to 10:1 in a final volume of 300 µl RPMI medium and incubated at 37°C for 4 h. The cells were then washed once with PBS. Following Fc receptor blockade with IgG (1 µg/10^5 cells) on ice for 20 min to avoid antibody-dependent cellular cytotoxicity, the cells were incubated with appropriate amounts of fluorochrome conjugated mAbs against CD38 and CD138 (when autologous BM samples were used as targets) or CD34 (when magnetically separated $CD34^+$ cells were used as targets) at 4°C for 30 min. After washing with PBS, the cells were resuspended in 500 µl of PBS containing 5 µg 7-aminoactinomycin D (7-AAD; Invitrogen, Carlsbad, CA, USA) and incubated in the dark for an additional 15 min at 4°C before data acquisition by flow cytometry.

During analysis of the flow cytometry data, targets cells were isolated from the effector cells by TFL4 positivity and the percentage of live or dead cells were determined by using 7-AAD staining on this $TFL4^+$ population as a whole or with further gating on $CD38^+CD138^+$ cells (for BM samples as targets) and $CD34^+$ cells (for CD34 enriched samples as targets). Cytotoxicity was assessed according to the following formula: percent killing = [(experimental death-spontaneous death)/ (maximum death-spontaneous death)] x100.

3.3 ANALYSIS OF NK CELL DEGRANULATION

In PAPERS I-II-III, NK cells were co-incubated with K562 target cells at a ratio of 1:1 in a final volume of 200 µl in round-bottomed 96-well plates at 37°C and 5% CO_2 for 6 h. Fluorochrome-conjugated anti-CD107a mAb or the corresponding IgG1 isotype control was added at the initiation of the assay. After 1 h of coincubation, Monensin was added at a 1:100 dilution. Surface staining was done by incubating cells with anti-CD3 and anti-CD56 mAbs for 30 min at +4°C. The cells were then washed, resuspended in PBS and immediately analyzed by flow cytometry.

3.4 FLOW CYTOMETRY

All antibody stainings (PAPERS I-II-III) for flow cytometry were done according to the following protocol: The cells were washed once with PBS and incubated with appropriate amounts of antibody at 4°C for 30 min. The labeled cells were then washed with PBS and fixed in 1-4% PFA prior to data acquisition. Data acquisition was done on FACSCalibur (BD) and CyFlow ML (Partec GmbH, Munster, Germany). Data were analyzed with CellQuest Pro (BD), FloMax (Partec) and FlowJo (TreeStar Inc.) softwares.

In detailed phenotyping analysis, for each cell surface receptor analyzed, mean fluorescence intensity (MFI) values were calculated for day 0 and day 20 samples. To estimate the change in receptor expression between different samples, we calculated MFI ratios (MFI_{day20}/MFI_{day0} or $MFI_{bioreactor}/MFI_{flask}$) for each receptor. When the MFI for a sample was higher than another, the MFI ratio was higher than 1, which

indicated the relative extent of overexpression in that receptor. Likewise, an MFI ratio below 1 was interpreted as downregulation in the expression of that receptor.

The following antibodies were used during the experiments:

PAPER I:

CD2 (RPA-2.10), CD3 (UCHT-1), CD4 (SK3), CD7 (M-T701), CD8 (HIT8a), CD14 (MOP9), CD16 (3G8), CD19 (HIB19), CD25 (M-A251), CD27 (M-T271), CD38 (HIT2), CD56 (B159), CD57 (NK-1), CD161 (DX12), CD183 (3D12), CD184 (12G5), CD195 (2D7/CCR5), CD197 (1C6/CXCR3), CD226 (DX11), NKB1 (DX9), LFA-1 (HI111), CD62L (DREG56), CD69 (FN50) and CD138 (MI15) purchased from BD Biosciences, San Jose, CA, USA; CD48 (MEM102) from Biosource AB, Stockholm, Sweden; CD158B1/B2,j (GL183), CD244(2B4) (C1.7), NKG2D (ON71), NKp30 (Z25), NKp44 (Z231), NKp46 (BAB281), LIR-1 (HP-F1), Valpha24 (C15), Vbeta11 (C21) from Beckman Coulter Inc., Fullerton, CA, USA; NKG2A (131411), NKG2C (134591), KIR2DL1 (143211), KIR2DL3 (180701) from R&D Systems, Minneapolis, MN, USA.

PAPER II:

CD11a (HI111), CD3 (UCHT-1), CD7 (M-T701), CD14 (MOP9), CD16 (3G8), CD19 (HIB19), CD25 (M-A251), CD27 (M-T271), CD56 (B159), CD57 (NK-1), CD226 (DX11), NKB1 (DX9) and CD62L (DREG56) purchased from BD Biosciences, San Jose, CA, USA; CD244(2B4) (C1.7), NKG2D (ON71), NKp30 (Z25), NKp44 (Z231), NKp46 (BAB281), from Beckman Coulter Inc., Fullerton, CA, USA; NKG2A (131411), NKG2C (134591), KIR2DL1 (143211), KIR2DL3 (180701) from R&D Systems, Minneapolis, MN, USA. Other antibodies used for further characterization of the final cell product were CD38 (HIT2), CD138 (MI15) and FoxP3 (250D/C7) from BD Biosciences.

PAPER III:

CD56 (NCAM16.2), CD56 (B159), CD3 (SK7), CD3 (SP34-2), CD69 (FN50), NKp44 (P44-8.1), CD16 (3G8), CD226 (DNAM-1) (DX11), CD25 (M-A251), NKG2D (1D11) from BD Biosciences; NKG2A (Z199), CD158a,h (KIR2DL1/S1) (EB6B), CD158b1/b2,j (KIR2DL2/3/S2) (GL183), NKp30 (Z25), NKp46 (BAB281), CD244 (2B4) (C1.7) from Beckmann Coulter; CD158e1/e2 (KIR3DL1/S1) (DX9), CD62L (DREG-56) from BioLegend and CD45 (HI30) from Invitrogen.

3.5 PRODUCTION OF LENTIVIRAL VECTORS

For production of VSV-G pseudotyped lentiviral vectors, 14x106 293FT cells were plated into a poly-D-lysine coated 150 mm dish. Next day cells were transfected with 30 µg of LeGO-G2 plasmid (courtesy of Prof. Boris Fehse, University Medical Center Hamburg-Eppendorf, Hamburg, Germany), 15 µg of pMDLg/pRRE, 10 µg of pRSV-REV and 5 µg of phCMV-VSV-G using calcium phosphate transfection in the presence of 25 µM Chloroquine. 10 hours after transfection, the medium was changed and thereafter virus containing supernatant was collected every 24 hours for 2-3 days and stored in -80°C until further use. A small aliquot from each production was used to determine viral titers by transduction of 293FT cells with serially diluted amounts of virus supernatant. Figure 9 illustrates the key features of the LeGO-G2 vector.

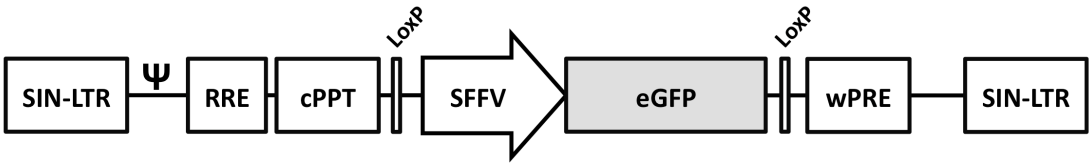


Figure 9: LeGO-G2 vector. SIN-LTR, self-inactivating-long-terminal repeat; RRE, rev-responsive element; cPPT, central polypurine tract; LoxP, loxp sites to allow for excision after introduction of CRE recombinase; SFFV, spleen focus-forming virus promoter; eGFP, enhanced green fluorescent protein coding sequence; wPRE, Woodchuck hepatitis virus post-transcriptional regulatory element.

3.6 LENTIVIRAL TRANSDUCTION OF NK CELLS

For each lentiviral transduction, $0.25x10^6$ NK cells per well were seeded in a 24-well plate and mixed with an appropriate amount of virus supernatant in the presence of 8 µg/ml of protamine sulfate or polybrene in a final volume of no more than 1 ml. The cytokines were replenished and plates were centrifuged at 1000xg for 1 hour at room temperature. After centrifugation, without removing viral supernatants, the plates were incubated at 37°C, 5% CO_2 for 4-6 hours. At the end of the incubation, a second centrifugation at 1000xg for 1 hour at room temperature was carried out, after which the supernatants were removed from the wells and 1 ml of fresh NK cell growth medium per well was added. The cells were maintained in this medium with daily addition cytokines for at least 3 days before acquisition of eGFP expression was carried out. In indicated experiments, the following inhibitors of TLR and RLR signaling were present during the transduction: 2-aminopurine, BAY11-7082, Celastrol, CLI-095, H-89, BX795, Norharmane and IRAK1/4 inhibitor.

4 RESULTS AND DISCUSSION

4.1 ANTI-TUMOR ACTIVITY OF EXPANDED NK CELLS FROM MM PATIENTS (PAPER I)

Following our results that showed the potent anti-myeloma effect of adoptively transferred IL-2 activated NK cells in an animal model[175], we have decided to investigate the feasibility of a similar approach in the human setting. Since results from animal studies have indicated a dose-dependent effect of NK cells for MM immunotherapy, we have primarily investigated whether NK cells from MM patients can be expanded *ex vivo* and whether the expansion and activation process reverses the phenotypic and functional defects in this lymphocyte population, making them efficient killers of autologous tumor cells.

To study whether NK cells from MM patients can be expanded *ex vivo* using GMP-compliant components, cultures of PBMCs from MM patients were established using a method previously reported by our group[254]. By day 20, the total cell population had expanded on average 511-fold (range: 123-1545) and, of these, NK cells had expanded on average 1625-fold (range: 502-2658). Because the expansion of NK cells was relatively higher than that of the other cell types, NK cells dominated the culture towards the end of the incubation, reaching on average 65% of the cells by day 20. Figure 10 shows the expansion kinetics of NK cells in this study. These results show that NK cells from MM patients can be efficiently expanded *ex vivo*.

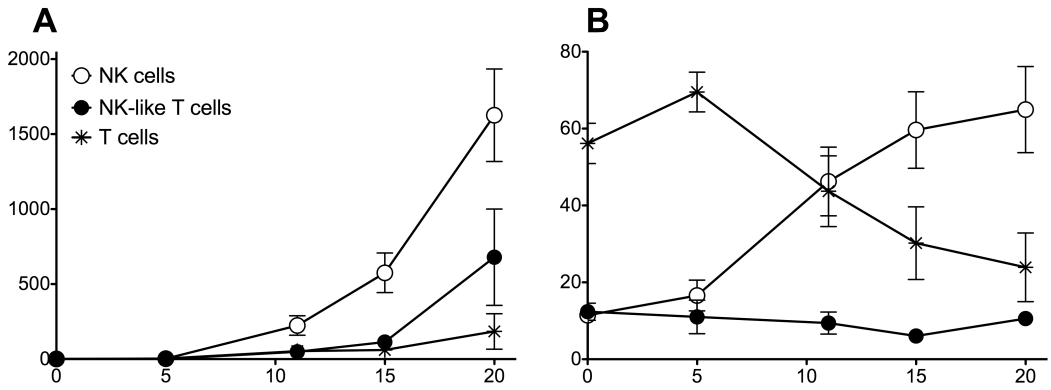


Figure 10: Expansion kinetics of NK cells from 7 newly diagnosed MM patients. (A) Fold expansion and (B) percentage of different lymphocyte subpopulations during the culture period.

As expected, cytotoxicity by the expanded cells against the standard NK cell target K562 was markedly elevated compared to effector cells from day 5 or day 0 cultures. Yet, the real challenge in this case was whether the expanded cells showed any cytotoxic activity against autologous MM cells. Our results revealed that day 20 expanded cells were highly cytotoxic against autologous MM cells whereas neither day 0 nor day 5 cells showed more than low levels of cytotoxicity (Figure 11).

In an attempt to identify the phenotypic changes associated with this cytotoxic activity, we also phenotyped the expanded NK cell population to compare the starting

material with that at day 20 of expansion. Since a balance of activating and inhibitory signals regulates NK cell function[29], optimal NK cell effector function is expected when the expression of activating NK cell receptors is adequate and not suppressed by inhibitory signals. Our results show that, following *ex vivo* expansion, NK cells undergo major phenotypic changes and upregulate the expression of many activating receptors (2B4, CD8, CD16, CD27, CD226, NKG2C, NKG2D, NKp30, NKp44 and NKp46).

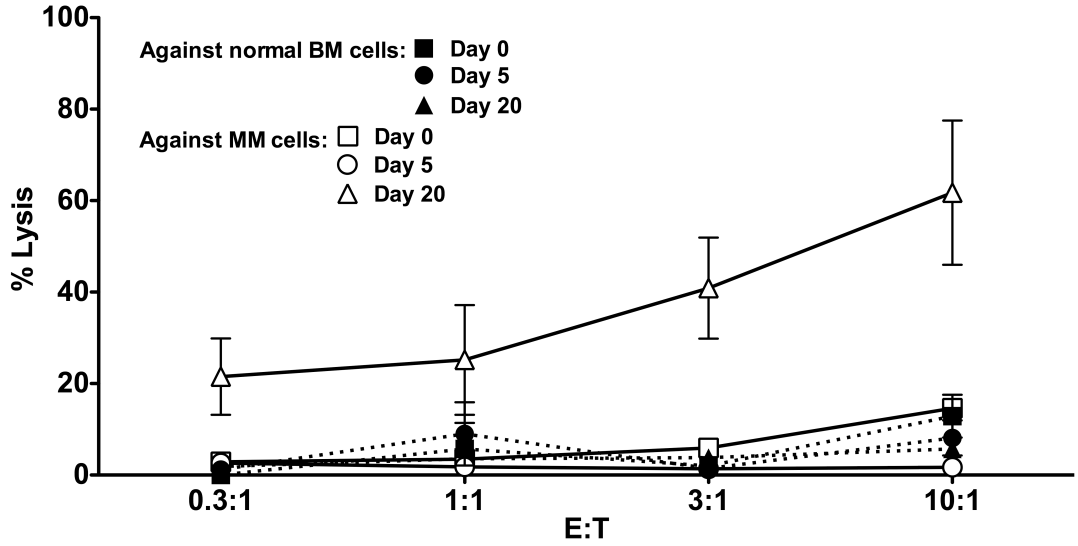


Figure 11: Cytotoxic activity of expanded cells against MM and non-MM cells of the bone marrow

To determine the relative contributions of the foregoing activating receptors, we performed cytotoxicity experiments after blocking receptors with antibodies either alone or in different combinations. We observed no specific association of NK cell cytotoxicity with a single receptor but, rather, effects were likely mediated by several receptors. This outcome indicates that several activating receptors may contribute to MM cytotoxicity in line with current knowledge of synergy among receptors for induction of cytotoxicity[395]. Presumably, recognition of tumor cells by NK cells often involves a combination of receptors that synergistically deliver activating signals. Such a phenomenon may, at least in part, explain our observations in blocking experiments and merit further investigation.

Our data also indicate that the recognition of autologous MM cells by NK cells expanded *ex vivo* involves a certain degree of specificity since we observed no cytotoxicity against non-MM cells in the BM or towards magnetically separated autologous CD34+ cells from BM and autologous PHA-blasts.

Alongside research to unravel the mechanisms responsible for the phenomena reported here, the feasibility of using autologous NK cells expanded *ex vivo* for the management of MM patients is worthy of further exploration in order to verify their clinical potential. In this context, we propose that expanded NK cells can be used as a support to ASCT for preemptive treatment of relapse and better eradication of malignant cells in MM patients. Since our results convincingly demonstrated that expanded autologous NK cells could be a potentially effective approach for MM

immunotherapy, we decided to initiate a clinical study in order to investigate this possibility. In order to be able to run this clinical trial, we need a larger scale and practical process to expand the NK cells.

4.2 LARGE-SCALE EXPANSION OF NK CELLS (PAPER II)

In this study, we have investigated the feasibility of large-scale NK cell expansion using closed systems. We have evaluated two different closed systems (cell culture bags and an automated bioreactor) in comparison to conventional cell culture flasks using PBMCs from healthy donors as well as MM patients, with the aim of optimizing an automated GMP-compatible protocol that allows large-scale production of activated NK cells to be used in the clinical trial.

Separation of NK cells or NK precursors prior to *ex vivo* culture[247,251,255,257] and/or use of feeder cell lines[258,261] have been widely used for NK cell expansion in previous reports. In this study, we did not utilize any feeder cells or separation steps but rather use bulk PBMCs for culture as we did in PAPER I, which results in a cell population enriched in NK cells that is distinct from LAK[396] and cytokine-induced killer (CIK) cells [397,398] both in terms of NK cell content and anti-tumor activity[399].

As the initiation of cultures in bags or bioreactor required a high number of cells, for the first sets of experiments, we initiated cultures in flasks and transferred to a bag or bioreactor when sufficient amount of cells was reached. The NK expansion in bags appeared impressive when compared to flask especially in three out of five donors. However, we observed that expansion in bags might result in a lower NK cell purity in the end. Next, we evaluated the use of an automated bioreactor system in comparison with expansion in flasks. Although fold expansions of NK cells were lower than flasks in four out of five donors, the percentages of these subpopulations in the final product were more comparable and correlating with flask expansion. In order to clarify if the NK cells in the final products of different systems retain the same activation status and show comparable cytotoxicity, we evaluated the cytotoxic activity of the final products against the K562 cell and observed no significant difference between the cytotoxic activities of NK cells expanded under different conditions.

After demonstrating the feasibility of using the bioreactor for the expansion process, we continued with validation of the expansion process in the bioreactor under cGMP conditions using apheresis products or whole-unit peripheral blood to initiate the culture directly in the bioreactor from day 0. PBMCs from two healthy donors and two MM patients were used for this validation process. For comparison purposes, PBMCs from the same four donors were also expanded using flasks. Results are presented in figure 12.

Although the NK cell purity was slightly lower in bioreactors, the final number of NK cells reached was sufficient to facilitate a trial for clinical testing. Moreover, we observed that when expansion was initiated in bioreactors from the beginning, the cytotoxic activity of the final product against K562 cells was remarkably higher when compared to the final product of flask expansions in 3 out of 4 donors.

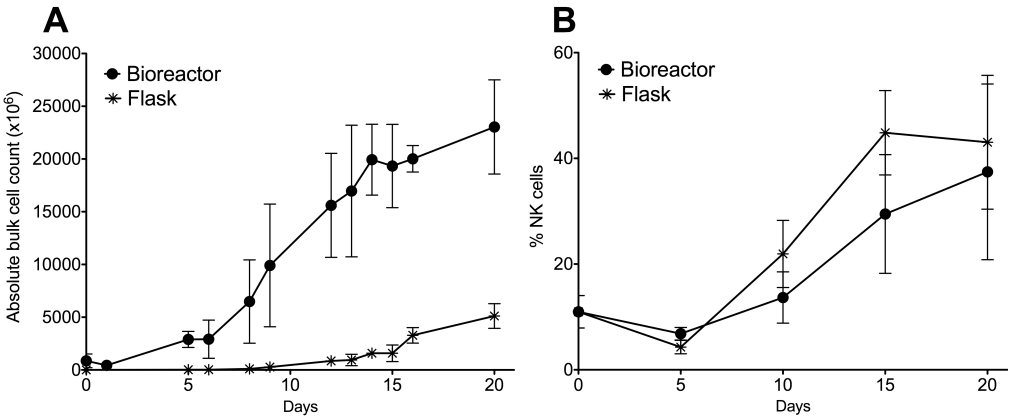


Figure 12: Expansion of NK cells in the Wave bioreactor. (A) Number of total cells and (B) percentage of NK cells in culture during the expansion period.

Correlation of receptor expression levels with the response of NK cells against K562 has revealed that CD132, CD25, CD57 and NKG2C expression levels were inversely correlated with the extent of degranulation while expression levels of NKp30, NKp44 and NKp46 were directly correlated. Observing a reverse correlation with the expression of the activating NK cell receptor NKG2C is unusual but has very little meaning in this case, as the target K562 cells are known to lack the expression of its ligand, HLA-E[400]. Statistical analysis of receptors correlating with NK cell response revealed that NKp44 both correlates positively with response, and is expressed at a significantly higher level in bioreactor products when compared to flask expansions (Figure 13).

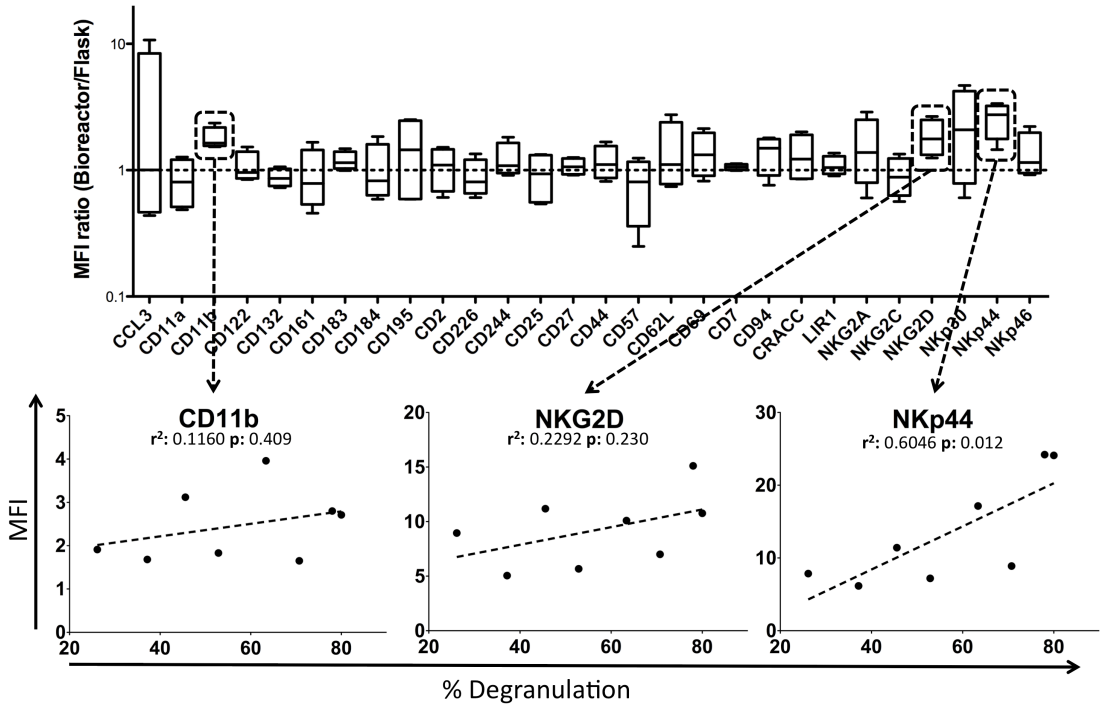


Figure 13: Phenotypic comparison of NK cells expanded in flasks or bioreactor. The bars depict the MFI ratios of receptor expression after normalization with isotype controls. Values above 1 indicate higher level of expression in bioreactor products, and values below 1 indicate lower level of expression in bioreactor products.

This can, at least in part, explain the observation of high cytotoxic capacity of the bioreactor products. Unlike the other NCRs, NKp44[36] is expressed exclusively on activated NK cells and is upregulated after *in vitro* IL-2 stimulation[401]. Therefore, in this case, it might be presenting as a surrogate marker of how well the IL-2 in the culture is being used and what the extent of activation in the NK cell population is. Thus, the elevated expression of NKp44 provides a functional significance to the expansion procedure being carried out in the bioreactor rather than conventional cell culture flasks.

In conclusion, we have optimized the expansion of clinical grade NK cells from PBMCs of healthy individuals and MM patients, using an automated bioreactor. These cells may be used for the treatment of patients with malignancies and clinical trials will help to shape the future role of NK cells in cancer immunotherapy. Having optimized the procedure for NK cell expansion in a closed-automated bioreactor using clinical grade components, we are currently at the process of translating this research into the clinic by initiating a phase I/II clinical trial to evaluate primarily safety and, to some extent, efficacy of these cells against MM after ASCT.

4.3 LENTIVIRAL GENETIC MODIFICATION OF NK CELLS (PAPER III)

Since we were not able to pinpoint the exact nature of the interaction between NK cells and MM cells in PAPER I, we decided to investigate these interactions by genetically modifying NK cells. Current approaches to characterize NK-tumor interactions rely mainly on surface phenotyping of tumor cells for a limited number of identified NK cell ligands and cytotoxicity assays in the presence of blocking antibodies against receptors on the NK cell surface. Both approaches, although widely used, present serious defects in detecting targets that could be of therapeutic significance. Phenotyping the identified ligands often results in detection of one or more NK cell ligand on the tumor targets, but the main restriction is the lack of knowledge about ligands and availability of specific antibodies for those. Even for the identified ligands that do have antibodies available, the mere detection of ligand expression on the target cell surface provides very limited information about the functional significance of a possible interaction through that ligand. In that sense, measuring the cytotoxic activity of the NK cell after blocking of the activating receptor that will engage the detected ligand proves to be more informative. However, this method has an inherent assumption that the NK cell already expresses the receptor in question. Yet, it has been repetitively observed by many researchers that malignant cells induce phenotypic aberrations on NK cells[63,402,403]. If the tumor has already succeeded to modify the phenotype of the patient's NK cells and caused the downregulation of a certain receptor, there's no information that could be gained by blocking a receptor which is not there. Therefore, genetic modification provides information that would not be possible to reach otherwise. For this purpose, we decided to optimize a lentiviral transduction protocol for primary human natural killer cells[404,405].

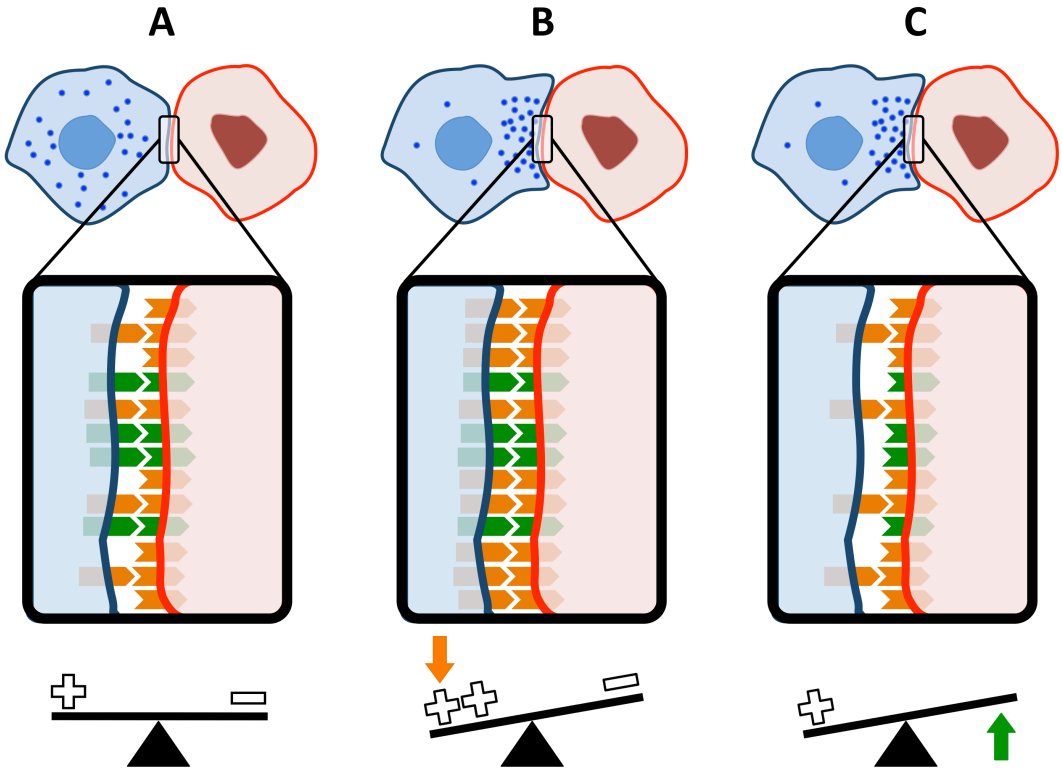


Figure 14: Rationale for genetic modification of NK cells for identifying roles of activating and inhibitory receptors in the interaction between the NK cells and the tumor cells.

Figure 14 demonstrates the rationale behind our approach. In a setting where the NK cell (blue) remains unresponsive to presented targets such as autologous tumor cells (red), a balance of activating and inhibitory signals prevails (A). It is possible to overcome this balance via genetic modification by either upregulating activating receptors (orange) on the NK cell surface (B) or downregulating inhibitory receptors (green) in order to abolish the inhibitory signalling (C). Such an approach can be used for gaining a basic understanding of the receptors involved in target cell killing or tolerance while presenting functional data regarding possible therapeutic effects of such modified cells.

However, efficiency of viral gene delivery to NK cells has always proven challenging and less efficient than other cells of the hematopoietic system. In fact, this is not to be unforeseen, since it is well established that NK cells are among the first-responders to viral infections[389] and must have been evolutionarily selected to have high endurance against a virus infection[390]. The intracellular anti-viral response of NK cells has been studied thoroughly in wild-type virus infections[406] but it has been mostly overlooked from a gene therapy point-of-view whether these responses are still active against viral vectors and have a significant effect in the resistance of NK cells to efficient viral transduction.

For this purpose, we primarily tried to establish a firm starting point by evaluating different cytokine stimulations prior to viral transduction. Among the cytokines we have tested were IL-2 and IL-15, which are commonly used for culture and activation

of NK cells, as well as IL-12 and IL-21 that have been reported previously to have a positive effect on genetic modification efficiency of NK cells[368,371]. We have observed that, of the tested cytokines, a combination of IL-2 and IL-21 is sufficient for optimal stimulation of NK cells prior to transduction (Figure 15).

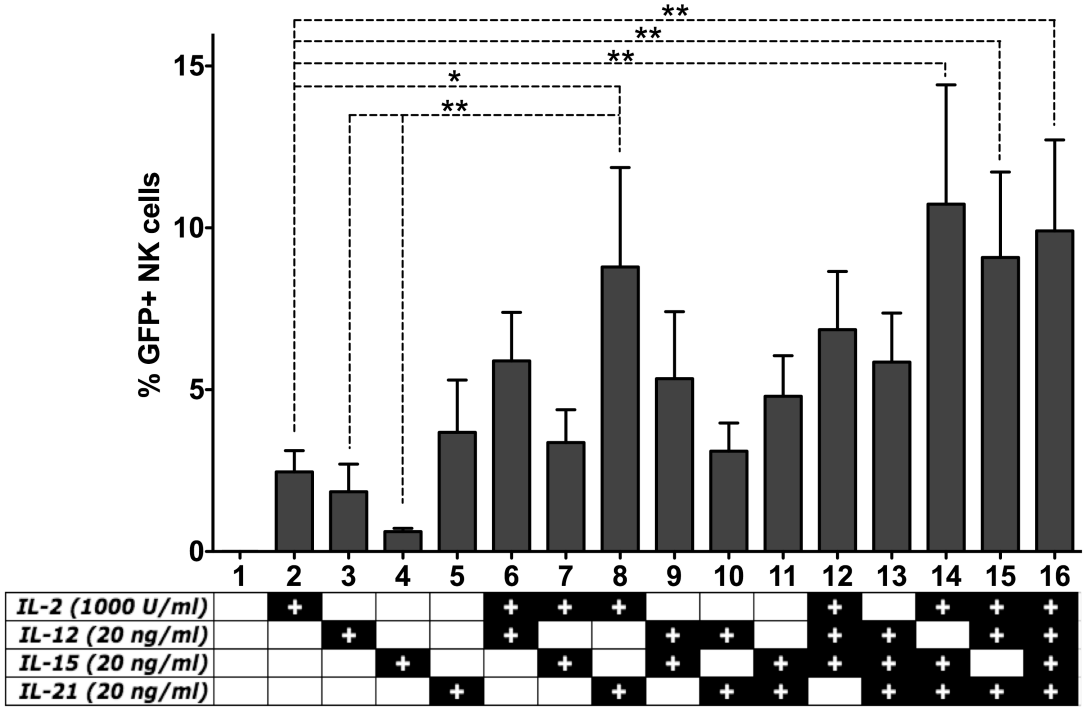


Figure 15. The effect of cytokine stimulation on lentiviral transduction efficiency. (* p<0.05;** p<0.01)

Furthermore, we have hypothesized that inhibition of innate immune receptor signaling would contribute enhanced transduction efficiency. It is well known that TLRs and RLRs play a major role in detection of viral infections and induction of an anti-viral state[407,408]. Many wild-type viruses have developed elaborate schemes to avoid detection by these receptors and increase their virulence[409]. In the case of viral vectors, the removal of various viral genes that counteract host responses but are dispensable for vector production is often preferred due to safety and practicality considerations. Inevitably, this would render viral vectors more prone to inducing strong innate responses upon target cell infection[391]. We have hypothesized that TLR or RLR mediated detection of viral vector components might activate an anti-viral response in NK cells, negatively effecting the efficiency of lentiviral transduction. In order to test this hypothesis, we have attempted to use small molecule inhibitors of TLR and RLR signaling preceding lentiviral transduction.

We have discovered that the use of BX795 at 2μM concentration dramatically increased transduction efficiency. BX795 is an inhibitor of TBK1/IKKε complex that acts as a common mediator in the signaling pathways of RIG-I, MDA-5 and TLR3[410]. Therefore, it might be possible to state that the lentiviral RNA is recognized by one or more of these receptors and an anti-viral response is triggered, which can be inhibited by the use of BX795. These results indicate that during transduction, intracellular anti-viral defense mechanisms including one or more of the receptors RIG-I, MDA-5 and TLR3 are activated and contribute significantly to the resistance of NK cells to lentiviral

genetic modification. Testing different concentrations of BX795 showed that the inhibitor has a dose-dependent effect on increasing genetic modification efficiency in NK cells (Figure 16). Although a significant effect is seen at 2μM concentration, this effect increases even more up to 6μM after which it seems to stabilize.

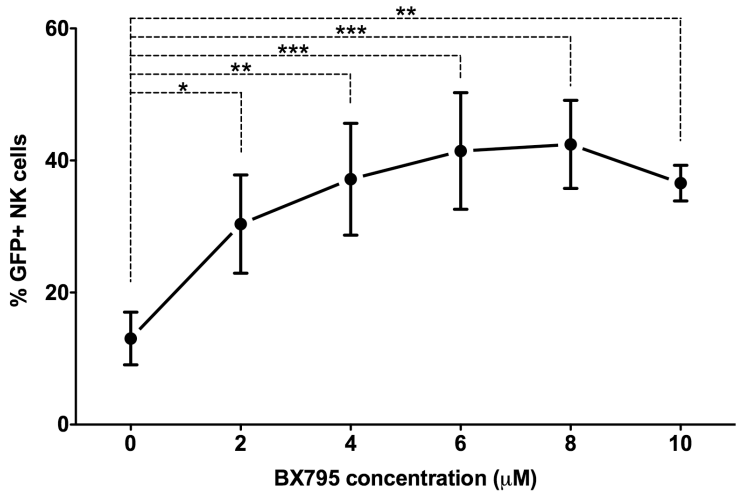


Figure 16. Dose response and of BX795 treatment. NK cells stimulated with IL-2/IL-21 for two days were transduced in the presence of various concentrations of BX795. Enhancement of transduction efficiency by BX795 is dose dependent and a concentration of 6μM is sufficient to get maximum response.

We have also investigated whether the process of genetic modification using BX795 along with IL-2/IL-21 stimulation presents any functional or phenotypic concerns regarding NK cell cytotoxic capacity. We have not observed any alteration in cellular cytotoxicity after treatment with BX795 alone or transduction in the presence of BX795.

Our results present a proof-of-principle for the feasibility of such approaches for enhancement of gene therapy applications. From our preliminary observations with other cell types, it is clear that not only NK cells but also cells of various types such as hematopoietic and mesenchymal stem cells will benefit from this approach. Further characterization of pathways involved in this response and in-depth analysis of the use of such inhibitors is warranted to improve gene therapy strategies.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work presented in this thesis is dedicated to bringing NK cell-based immunotherapeutic approaches into the clinic. We have discovered that long-term *ex vivo* expansion and activation of autologous NK cells from MM patients provides significantly superior cytotoxic activity against autologous tumor cells when compared to short-term activated autologous NK cells. Having optimized the procedure for NK cell expansion in a closed-automated bioreactor using clinical grade GMP-compliant components, we have finished all the preclinical requirements and got approval from Swedish Medicinal Products agency and the ethical committees to initiate a first-in-man phase I/II clinical trial.

A phase I/II clinical trial was designed to evaluate primarily safety and, to some extent, efficacy of these cells against MM after autologous stem cell transplantation. 12 subjects will be enrolled in this study in which patients will be offered the opportunity of receiving autologous *ex vivo* expanded NK cells after ASCT.

The objectives of this trial will be:

- To determine the safety of *ex vivo* expanded NK cell infusions for the management of MM relapse after ASCT.
- To evaluate the survival and functional capabilities of the infused product.
- To determine the efficacy of these cells to restore clinical, cytogenetic and molecular remission in patients with MM.

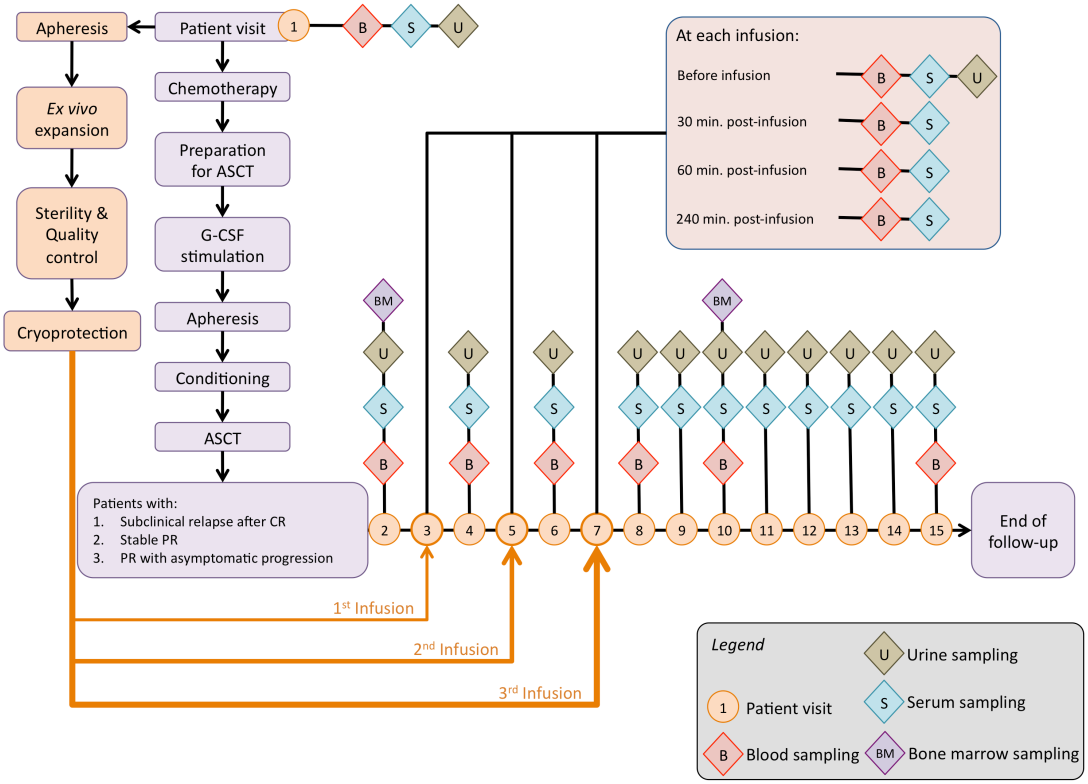


Figure 17: Design of the clinical protocol for expanded NK cell therapy in MM patients

We intend to use PBMCs and tumor samples from patients in a medium-throughput assay where the outcome of each genetic modification will be assessed. Basically, PBMCs will be used to enrich NK cells by magnetic cell separation, which will then be cultured in specialized media with minimal amount of cytokines. Next, the transduction of NK cells will be carried out by lentiviral vectors encoding genes for activating receptors or shRNAs against inhibitory receptors along with GFP or tdTomato. After transduction, the NK cells will be used in degranulation assays, where the response against target cells will be measured. Besides the regular control (NK cells transduced with a vector expressing only GFP) we will also be able to use the non-transduced cells for each transduction as an internal control and compare the degranulation of GFP+ cells and GFP- cells. The same approach will be used for genetically modifying NK cells with all intended genes and shRNAs. The experimental layout is presented in the figure 18.

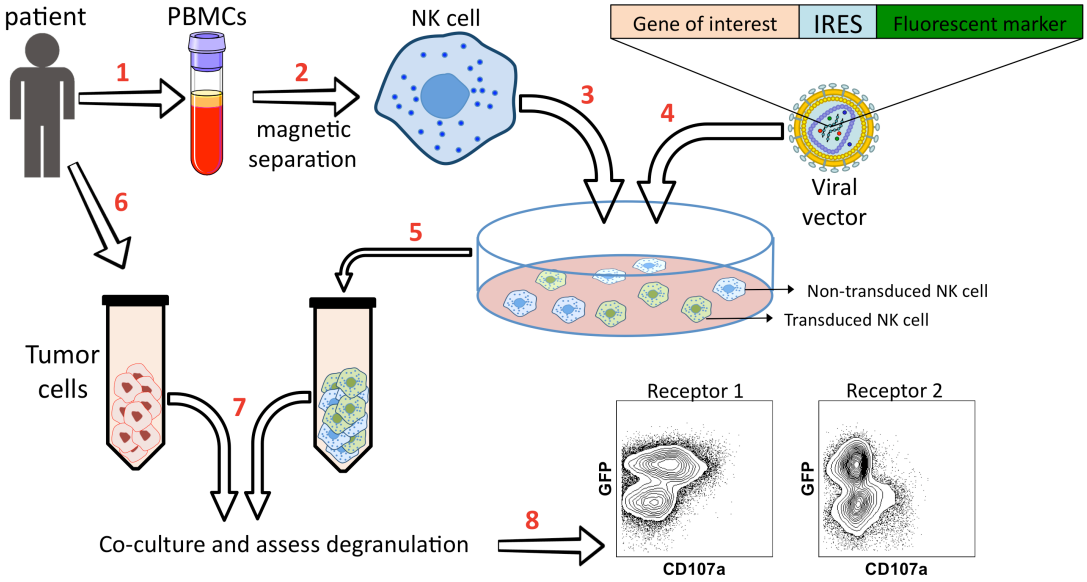


Figure 18: Scheme for genetic screening of NK/tumor interactions

In order to assess the specificity and safety of observed responses, we intend to use non-malignant autologous PBMCs as a control target while also keeping an eye on the background degranulation when there are no targets present. For each patient, NK cells will be transduced with various different activating receptors or shRNAs and response against autologous tumor cells as well as autologous PBMCs will be assessed and analyzed side-by-side. This will provide information regarding the role of each receptor in the interaction with target cells and whether that role is tumor-specific or not. Genetic modification strategies and viral vectors used in this study will also enable upregulation of a pair of receptors at a time in case certain synergistic interactions between those receptors are to be studied.

With such an approach, we will rely on neither the identity of the ligand nor the presence of receptor expression on the NK cell. Moreover, this method will allow the functional screening of activating receptors while accounting for the complex network of interactions between the NK cell and the tumor cell because it is being carried on

the patient's autologous NK cells and tumor cells. It will also provide patient-specific data regarding the significance of each receptor in a possible NK cell-based immuno-gene therapy setting. As both the character of the tumor cell population and phenotypic status of NK cells differ from patient to patient, such an approach will prove to be instrumental in developing patient-tailored cancer immunotherapy approaches based on NK cells by providing therapeutically relevant targets. Analysis of effects on interactions with non-tumor cell populations will increase this relevance even further by means of controlling the specificity of identified targets. Finally, the results will establish a firm background of knowledge for facilitation of NK cell genetic modification processes for possible applications in cancer immunotherapy.

There is a need for novel therapies against hematological malignancies and solid tumors. With recent advances in the field of cellular and molecular immunology we are facing a new area of immunotherapy. The specificities of NK cell mediated tumor cell recognition are slowly being decoded. This is crucial in order to define the factors that govern the cytolytic potential of NK cells to be used for immuno-gene therapy of hematological malignancies as well as solid tumors. The methods presented in this thesis comprise expansion of NK cells for clinical purposes and preclinical development of NK cell genetic modification processes. The next step in this road is to bring the two approaches together and develop a protocol for large-scale genetic modification of NK cells, which could facilitate clinical trials with genetically modified NK cell infusions if the results from the screening assays provide promising results.

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7 REFERENCES

1. Strathern P. A brief history of medicine: from Hippocrates to gene therapy. London: Robinson; 2005.

2. Travis J. Origins. On the origin of the immune system. Science. 2009;324:580-582.

3. Sun JC, Lanier LL. Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? Eur J Immunol. 2009;39:2059-2064.

4. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity. 1995;3:459-473.

5. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91:661-672.

6. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol. 1975;5:117-121.

7. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol. 1975;5:112-117.

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

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

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

11. Dvorak AM, Galli SJ, Marcum JA, et al. Cloned mouse cells with natural killer function and cloned suppressor T cells express ultrastructural and biochemical features not shared by cloned inducer T cells. J Exp Med. 1983;157:843-861.

12. Chambers WH, Vujanovic NL, DeLeo AB, Olszowy MW, Herberman RB, Hiserodt JC. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. J Exp Med. 1989;169:1373-1389.

13. Ryan JC, Turck J, Niemi EC, Yokoyama WM, Seaman WE. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. J Immunol. 1992;149:1631-1635.

14. Fogler WE, Volker K, McCormick KL, Watanabe M, Ortaldo JR, Wiltrout RH. NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction. J Immunol. 1996;156:4707-4714.

15. Biron CA. Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol. 1997;9:24-34.

16. Glas R, Franksson L, Une C, et al. Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype. An adaptive component of NK cell-mediated responses. J Exp Med. 2000;191:129-138.

17. Karre K. NK Cells, MHC Class I Molecules and the Missing Self. Scand J Immunol. 2002;55:221-228.

18. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.

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

20. Perussia B. Lymphokine-activated killer cells, natural killer cells and cytokines. Curr Opin Immunol. 1991;3:49-55.

21. Ishihara S, Nieda M, Kitayama J, et al. Alpha-glycosylceramides enhance the antitumor cytotoxicity of hepatic lymphocytes obtained from cancer patients by activating CD3-CD56+ NK cells in vitro. J Immunol. 2000;165:1659-1664.

22. Jensen M, Tawadros S, Sedlacek HH, Schultze JL, Berthold F. NK cell depletion diminish tumour-specific B cell responses. Immunol Lett. 2004;93:205-210.

23. Zitvogel L. Dendritic and natural killer cells cooperate in the control/switch of innate immunity. J Exp Med. 2002;195:F9-14.

24. Fernandez NC, Lozier A, Flament C, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med. 1999;5:405-411.

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

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

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

28. Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003;15:308-314.

29. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.

30. Veillette A, Latour S, Davidson D. Negative regulation of immunoreceptor signaling. Annu Rev Immunol. 2002;20:669-707.

31. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. Immunol Rev. 2008;224:70-84.

32. Biassoni R, Cantoni C, Pende D, et al. Human natural killer cell receptors and co-receptors. Immunol Rev. 2001;181:203-214.

33. Moretta L, Moretta A. Killer immunoglobulin-like receptors. Curr Opin Immunol. 2004;16:626-633.

34. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. Embo J. 2004;23:255-259.

35. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190:1505-1516.

36. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med. 1998;187:2065-2072.

37. Sivori S, Vitale M, Morelli L, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med. 1997;186:1129-1136.

38. Lanier LL. On guard--activating NK cell receptors. Nat Immunol. 2001;2:23-27.

39. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225-274.

40. Trambas CM, Griffiths GM. Delivering the kiss of death. Nat Immunol. 2003;4:399-403.

41. Metkar SS, Wang B, Aguilar-Santelises M, et al. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity. 2002;16:417-428.

42. Browne KA, Blink E, Sutton VR, Froelich CJ, Jans DA, Trapani JA. Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol. 1999;19:8604-8615.

43. Shresta S, Heusel JW, Macivor DM, Wesselschmidt RL, Russell JH, Ley TJ. Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis. Immunol Rev. 1995;146:211-221.

44. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med. 1995;181:1235-1238.

45. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med. 1998;188:2375-2380.

46. Lee RK, Spielman J, Zhao DY, Olsen KJ, Podack ER. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. J Immunol. 1996;157:1919-1925.

47. Hooijberg E, Sein JJ, van den Berk PC, Hekman A. Characterization of a series of isotype switch variants of a new CD20 monoclonal antibody. Hybridoma. 1996;15:23-31.

48. Kipps TJ, Parham P, Punt J, Herzenberg LA. Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. J Exp Med. 1985;161:1-17.

49. Ortaldo JR, Woodhouse C, Morgan AC, Herberman RB, Cheresh DA, Reisfeld R. Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. J Immunol. 1987;138:3566-3572.

50. Sharkey RM, Goldenberg DM. Targeted therapy of cancer: new prospects for antibodies and immunoconjugates. CA Cancer J Clin. 2006;56:226-243.

51. Golay J, Manganini M, Facchinetti V, et al. Rituximab-mediated antibody-dependent cellular cytotoxicity against neoplastic B cells is stimulated strongly by interleukin-2. Haematologica. 2003;88:1002-1012.

52. Dall'Ozzo S, Tartas S, Paintaud G, et al. Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. Cancer Res. 2004;64:4664-4669.

53. Fischer L, Penack O, Gentilini C, et al. The anti-lymphoma effect of antibody-mediated immunotherapy is based on an increased degranulation of peripheral blood natural killer (NK) cells. Exp Hematol. 2006;34:753-759.

54. Bhat R, Watzl C. Serial killing of tumor cells by human natural killer cells - enhancement by therapeutic antibodies. PLoS ONE. 2007;2:e326.

55. O'Connell J, Bennett MW, O'Sullivan GC, et al. Fas ligand expression in primary colon adenocarcinomas: evidence that the Fas counterattack is a prevalent mechanism of immune evasion in human colon cancer. J Pathol. 1998;186:240-246.

56. Bennett MW, O'Connell J, O'Sullivan GC, et al. The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. J Immunol. 1998;160:5669-5675.

57. Yamauchi A, Taga K, Mostowski HS, Bloom ET. Target cell-induced apoptosis of interleukin-2-activated human natural killer cells: roles of cell surface molecules and intracellular events. Blood. 1996;87:5127-5135.

58. Matsuda M, Petersson M, Lenkei R, et al. Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. Int J Cancer. 1995;61:765-772.

59. Orleans-Lindsay JK, Barber LD, Prentice HG, Lowdell MW. Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function--implications for the adoptive immunotherapy of leukaemia. Clin Exp Immunol. 2001;126:403-411.

60. Wilson EB, El-Jawhari JJ, Neilson AL, et al. Human tumour immune evasion via TGF-beta blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity. PLoS One;6:e22842.

61. Fathy A, Eldin MM, Metwally L, Eida M, Abdel-Rehim M. Diminished absolute counts of CD56dim and CD56bright natural killer cells in peripheral blood from Egyptian patients with hepatocellular carcinoma. Egypt J Immunol. 2009;16:17-25.

62. Richards J, McNally B, Fang X, Caligiuri MA, Zheng P, Liu Y. Tumor growth decreases NK and B cells as well as common lymphoid progenitor. PLoS One. 2008;3:e3180.

63. Kiessling R, Wasserman K, Horiguchi S, et al. Tumor-induced immune dysfunction. Cancer Immunol Immunother. 1999;48:353-362.

64. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell;144:646-674.

65. Carrega P, Morandi B, Costa R, et al. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. Cancer. 2008;112:863-875.

66. Jinushi M, Takehara T, Tatsumi T, et al. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. J Hepatol. 2005;43:1013-1020.

67. Taketomi A, Shimada M, Shirabe K, Kajiyama K, Gion T, Sugimachi K. Natural killer cell activity in patients with hepatocellular carcinoma: a new prognostic indicator after hepatectomy. Cancer. 1998;83:58-63.

68. Koda K, Saito N, Oda K, et al. Natural killer cell activity and distant metastasis in rectal cancers treated surgically with and without neoadjuvant chemoradiotherapy. J Am Coll Surg. 2003;197:254-260.

69. Bauernhofer T, Kuss I, Henderson B, Baum AS, Whiteside TL. Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer. Eur J Immunol. 2003;33:119-124.

70. Baskic D, Vujanovic L, Arsenijevic N, Whiteside TL, Myers EN, Vujanovic NL. Suppression of natural killer-cell and dendritic-cell apoptotic tumoricidal activity in patients with head and neck cancer. Head Neck.

71. Konjevic G, Jurisic V, Jovic V, et al. Investigation of NK cell function and their modulation in different malignancies. Immunol Res.

72. Mocchegiani E, Ciavattini A, Santarelli L, et al. Role of zinc and alpha2 macroglobulin on thymic endocrine activity and on peripheral immune efficiency (natural killer activity and interleukin 2) in cervical carcinoma. Br J Cancer. 1999;79:244-250.

73. Campos MM, de Souza MH, Pires V, Scheiner MA, Esteves EB, Ornellas AA. Clinical implications of natural killer cytotoxicity in patients with squamous cell carcinoma of the penis. Nat Immun. 1998;16:256-262.

74. LeFever AV, Funahashi A. Phenotype and function of natural killer cells in patients with bronchogenic carcinoma. Cancer Res. 1991;51:5596-5601.

75. Lutgendorf SK, Sood AK, Anderson B, et al. Social support, psychological distress, and natural killer cell activity in ovarian cancer. J Clin Oncol. 2005;23:7105-7113.

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

77. Wakiguchi H, Kubota H, Hisakawa H, Fujieda M, Kurashige T. Defective natural killer cell activity and deficient production of interferon-gamma in children with acute lymphoblastic leukemia. Acta Paediatr Jpn. 1994;36:361-365.

78. Katrinakis G, Kyriakou D, Papadaki H, Kalokyri I, Markidou F, Eliopoulos GD. Defective natural killer cell activity in B-cell chronic lymphocytic leukaemia is associated with impaired release of natural killer cytotoxic factor(s) but not of tumour necrosis factor-alpha. Acta Haematol. 1996;96:16-23.

79. Rajaram N, Tatake RJ, Advani SH, Naik SL, Gangal SG. Natural killer and lymphokine-activated killer cell functions in chronic myeloid leukemia. Cancer Immunol Immunother. 1990;31:44-48.

80. Frassanito MA, Silvestris F, Cafforio P, Silvestris N, Dammacco F. IgG M-components in active myeloma patients induce a down-regulation of natural killer cell activity. Int J Clin Lab Res. 1997;27:48-54.

81. Konjevic G, Mirjacic Martinovic K, Vuletic A, et al. Low expression of CD161 and NKG2D activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients. Clin Exp Metastasis. 2007;24:1-11.

82. Costello RT, Sivori S, Marcenaro E, et al. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood. 2002;99:3661-3667.

83. Veuillen C, Aurran-Schleinitz T, Castellano R, et al. Primary B-CLL Resistance to NK Cell Cytotoxicity can be Overcome In Vitro and In Vivo by Priming NK Cells and Monoclonal Antibody Therapy. J Clin Immunol.

84. Fauriat C, Mallet F, Olive D, Costello RT. Impaired activating receptor expression pattern in natural killer cells from patients with multiple myeloma. Leukemia. 2006;20:732-733.

85. El-Sherbiny YM, Meade JL, Holmes TD, et al. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. Cancer Res. 2007;67:8444-8449.

86. Gati A, Da Rocha S, Guerra N, et al. Analysis of the natural killer mediated immune response in metastatic renal cell carcinoma patients. Int J Cancer. 2004;109:393-401.

87. Tsukuda M, Sawaki S, Yanoma S. Suppressed cellular immunity in patients with nasopharyngeal carcinoma. J Cancer Res Clin Oncol. 1993;120:115-118.

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

89. Kono K, Ressing ME, Brandt RM, et al. Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer. Clin Cancer Res. 1996;2:1825-1828.

90. Nakagomi H, Petersson M, Magnusson I, et al. Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. Cancer Res. 1993;53:5610-5612.

91. Lai P, Rabinowich H, Crowley-Nowick PA, Bell MC, Mantovani G, Whiteside TL. Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma. Clin Cancer Res. 1996;2:161-173.

92. Healy CG, Simons JW, Carducci MA, et al. Impaired expression and function of signal-transducing zeta chains in peripheral T cells and natural killer cells in patients with prostate cancer. Cytometry. 1998;32:109-119.

93. Buggins AG, Hirst WJ, Pagliuca A, Mufti GJ. Variable expression of CD3-zeta and associated protein tyrosine kinases in lymphocytes from patients with myeloid malignancies. Br J Haematol. 1998;100:784-792.

94. Palmer S, Hanson CA, Zent CS, et al. Prognostic importance of T and NK-cells in a consecutive series of newly diagnosed patients with chronic lymphocytic leukaemia. Br J Haematol. 2008;141:607-614.

95. Chang WC, Fujimiya Y, Casteel N, Pattengale P. Natural killer cell immunodeficiency in patients with chronic myelogenous leukemia. III. Defective interleukin-2 production by T-helper and natural killer cells. Int J Cancer. 1989;43:591-597.

96. Eskelund CW, Nederby L, Thysen AH, Skovbo A, Roug AS, Hokland ME. Interleukin-21 and rituximab enhance NK cell functionality in patients with B-cell chronic lymphocytic leukaemia. Leuk Res;35:914-920.

97. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer. 2002;2:175-187.

98. Durie BG, Kyle RA, Belch A, et al. Myeloma management guidelines: a consensus report from the Scientific Advisors of the International Myeloma Foundation. Hematol J. 2003;4:379-398.

99. Chng WJ, Fonseca R. Risk stratification of patients with newly diagnosed multiple myeloma: optimizing treatment based on pretreatment characteristics. Clin Lymphoma Myeloma. 2005;6:200-207.

100. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood. 2007;109:3489-3495.

101. Nahi H, Sutlu T, Jansson M, Alici E, Gahrton G. Clinical impact of chromosomal aberrations in multiple myeloma. J Intern Med;269:137-147.

102. Sutlu T, Alici E, Jansson M, et al. The prognostic significance of 8p21 deletion in multiple myeloma. Br J Haematol. 2009;144:266-268.

103. Harousseau JL, Dreyling M. Multiple myeloma: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann Oncol. 2009;20 Suppl 4:97-99.

104. Harousseau JL, Dreyling M. Multiple myeloma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol;21 Suppl 5:v155-157.

105. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J, Jr. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood. 2005;106:296-303.

106. Roodman GD. Pathogenesis of myeloma bone disease. Blood Cells Mol Dis. 2004;32:290-292.

107. Choi SJ, Cruz JC, Craig F, et al. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. Blood. 2000;96:671-675.

108. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD. Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. Blood. 2001;97:3349-3353.

109. Uneda S, Hata H, Matsuno F, et al. Macrophage inflammatory protein-1 alpha is produced by human multiple myeloma (MM) cells and its expression correlates with bone lesions in patients with MM. Br J Haematol. 2003;120:53-55.

110. Teoh G, Anderson KC. Interaction of tumor and host cells with adhesion and extracellular matrix molecules in the development of multiple myeloma. Hematol Oncol Clin North Am. 1997;11:27-42.

111. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349:2483-2494.

112. Gahrton G, Tura S, Ljungman P, et al. Allogeneic bone marrow transplantation in multiple myeloma. European Group for Bone Marrow Transplantation. N Engl J Med. 1991;325:1267-1273.

113. Crawley C, Lalancette M, Szydlo R, et al. Outcomes for reduced-intensity allogeneic transplantation for multiple myeloma: an analysis of prognostic factors from the Chronic Leukaemia Working Party of the EBMT. Blood. 2005;105:4532-4539.

114. Bjorkstrand B, Gahrton G. High-dose treatment with autologous stem cell transplantation in multiple myeloma: past, present, and future. Semin Hematol. 2007;44:227-233.

115. Attal M, Harousseau JL, Stoppa AM, et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. N Engl J Med. 1996;335:91-97.

116. Child JA, Morgan GJ, Davies FE, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med. 2003;348:1875-1883.

117. Blade J, Rosinol L, Cibeira MT, Rovira M, Carreras E. Hematopoietic stem cell transplantation for multiple myeloma beyond 2010. Blood.

118. Anderson KC. New insights into therapeutic targets in myeloma. Hematology Am Soc Hematol Educ Program;2011:184-190.

119. Hayashi T, Hideshima T, Akiyama M, et al. Ex vivo induction of multiple myeloma-specific cytotoxic T lymphocytes. Blood. 2003;102:1435-1442.

120. Zinneman HH, Hall WH. Recurrent pneumonia in multiple myeloma and some observations on immunologic response. Ann Intern Med. 1954;41:1152-1163.

121. Osterborg A, Nilsson B, Bjorkholm M, Holm G, Mellstedt H. Natural killer cell activity in monoclonal gammopathies: relation to disease activity. Eur J Haematol. 1990;45:153-157.

122. Jurisic V, Srdic T, Konjevic G, Markovic O, Colovic M. Clinical stage-depending decrease of NK cell activity in multiple myeloma patients. Med Oncol. 2007;24:312-317.

123. De Rossi G, De Sanctis G, Bottari V, et al. Surface markers and cytotoxic activities of lymphocytes in monoclonal gammopathy of undetermined significance and untreated multiple myeloma. Increased phytohemagglutinin-induced cellular cytotoxicity and inverted helper/suppressor cell ratio are features common to both diseases. Cancer Immunol Immunother. 1987;25:133-136.

124. Ogmundsdottir HM. Natural killer cell activity in patients with multiple myeloma. Cancer Detect Prev. 1988;12:133-143.

125. Garcia-Sanz R, Gonzalez M, Orfao A, et al. Analysis of natural killer-associated antigens in peripheral blood and bone marrow of multiple myeloma patients and prognostic implications. Br J Haematol. 1996;93:81-88.

126. Brown RD, Pope B, Murray A, et al. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. Blood. 2001;98:2992-2998.

127. Prabhala RH, Neri P, Bae JE, et al. Dysfunctional T regulatory cells in multiple myeloma. Blood. 2006;107:301-304.

128. Beyer M, Kochanek M, Giese T, et al. In vivo peripheral expansion of naive CD4+CD25high FoxP3+ regulatory T cells in patients with multiple myeloma. Blood. 2006;107:3940-3949.

129. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol. 2007;138:563-579.

130. Lu ZY, Bataille R, Poubelle P, Rapp MJ, Harousseau JL, Klein B. An interleukin 1 receptor antagonist blocks the IL-1-induced IL-6 paracrine production through a prostaglandin E2-related mechanism in multiple myeloma. Stem Cells. 1995;13 Suppl 2:28-34.

131. Wang T, Niu G, Kortylewski M, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med. 2004;10:48-54.

132. Tinhofer I, Marschitz I, Henn T, Egle A, Greil R. Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma. Blood. 2000;95:610-618.

133. Soriani A, Zingoni A, Cerboni C, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. Blood. 2009;113:3503-3511.

134. Bernal M, Garrido P, Jimenez P, et al. Changes in activatory and inhibitory natural killer (NK) receptors may induce progression to multiple myeloma: implications for tumor evasion of T and NK cells. Hum Immunol. 2009;70:854-857.

135. Landowski TH, Qu N, Buyuksal I, Painter JS, Dalton WS. Mutations in the Fas antigen in patients with multiple myeloma. Blood. 1997;90:4266-4270.

136. Frohn C, Hoppner M, Schlenke P, Kirchner H, Koritke P, Luhm J. Anti-myeloma activity of natural killer lymphocytes. Br J Haematol. 2002;119:660-664.

137. Carbone E, Neri P, Mesuraca M, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. Blood. 2005;105:251-258.

138. Uchida A, Yagita M, Sugiyama H, Hoshino T, Moore M. Strong natural killer (NK) cell activity in bone marrow of myeloma patients: accelerated maturation of bone marrow NK cells and their interaction with other bone marrow cells. Int J Cancer. 1984;34:375-381.

139. Bianchi A, Omede P, Attisano C, et al. Phenotypic and functional analysis of peripheral blood lymphocytes during interferon-alpha 2b therapy in multiple myeloma patients with low tumor mass. Haematologica. 1991;76:383-388.

140. Gahrton G, Durie BG, Samson D. Multiple Myeloma and Related Disorders. London: Arnold; 2004.

141. Matsuzaki H, Kagimoto T, Oda T, Kawano F, Takatsuki K. Natural killer activity and antibody-dependent cell-mediated cytotoxicity in multiple myeloma. Jpn J Clin Oncol. 1985;15:611-617.

142. Nielsen H, Nielsen HJ, Tvede N, et al. Immune dysfunction in multiple myeloma. Reduced natural killer cell activity and increased levels of soluble interleukin-2 receptors. APMIS. 1991;99:340-346.

143. Komiyama K, Crago SS, Itoh K, Moro I, Mestecky J. Inhibition of natural killer cell activity by IgA. Cell Immunol. 1986;101:143-155.

144. Gherman M, Manciulea M, Bancu AC, Sulica A, Stanworth DR, Herberman RB. Regulation of human natural cytotoxicity by IgG--I. Characterization of the structural site on monomeric IgG responsible for inhibiting natural killer cell activity. Mol Immunol. 1987;24:743-750.

145. Benson DM, Jr., Bakan CE, Mishra A, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. Blood;116:2286-2294.

146. Ishida M, Iwai Y, Tanaka Y, et al. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. Immunol Lett. 2002;84:57-62.

147. Ehrlich P. Über den jetzigen stand der karzinomforschung. Ned Tijdschr Geneeskd. 1909;5:273-290.

148. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957;1:779-786.

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

150. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature;480:480-489.

151. Taniguchi T, Matsui H, Fujita T, et al. Structure and expression of a cloned cDNA for human interleukin-2. Nature. 1983;302:305-310.

152. Kasakura S, Lowenstein L. A factor stimulating DNA synthesis derived from the medium of leukocyte cultures. Nature. 1965;208:794-795.

153. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science. 1976;193:1007-1008.

154. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity. 1995;2:223-238.

155. Grabstein KH, Eisenman J, Shanebeck K, et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science. 1994;264:965-968.

156. Bamford RN, Grant AJ, Burton JD, et al. The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Proc Natl Acad Sci U S A. 1994;91:4940-4944.

157. Becknell B, Caligiuri MA. Interleukin-2, interleukin-15, and their roles in human natural killer cells. Adv Immunol. 2005;86:209-239.

158. Malek TR. The biology of interleukin-2. Annu Rev Immunol. 2008;26:453-479.

159. Robinson BW, Morstyn G. Natural killer (NK)-resistant human lung cancer cells are lysed by recombinant interleukin-2-activated NK cells. Cell Immunol. 1987;106:215-222.

160. Lotzova E, Savary CA, Herberman RB. Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. J Immunol. 1987;138:2718-2727.

161. Lotzova E, Savary CA, Herberman RB. Inhibition of clonogenic growth of fresh leukemia cells by unstimulated and IL-2 stimulated NK cells of normal donors. Leuk Res. 1987;11:1059-1066.

162. Bubenik J, Perlmann P, Indrova M, Simova J, Jandlova T, Neuwirt J. Growth inhibition of an MC-induced mouse sarcoma by TCGF (IL 2)-containing preparations. Preliminary report. Cancer Immunol Immunother. 1983;14:205-206.

163. Lafreniere R, Rosenberg SA. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. Cancer Res. 1985;45:3735-3741.

164. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwarz SL. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. J Exp Med. 1985;161:1169-1188.

165. Maekawa R, Matsumoto M, Kitagawa T, Harada M, Sato K. Effect of recombinant interleukin 2 (R-IL2) on in vivo growth of murine myeloma X5563. Cancer Immunol Immunother. 1986;23:25-30.

166. Thompson JA, Peace DJ, Klarnet JP, Kern DE, Greenberg PD, Cheever MA. Eradication of disseminated murine leukemia by treatment with high-dose interleukin 2. J Immunol. 1986;137:3675-3680.

167. Vaage J. Local and systemic effects during interleukin-2 therapy of mouse mammary tumors. Cancer Res. 1987;47:4296-4298.

168. Rutten VP, Klein WR, De Jong WA, et al. Local interleukin-2 therapy in bovine ocular squamous cell carcinoma. A pilot study. Cancer Immunol Immunother. 1989;30:165-169.

169. Maas RA, Van Weering DH, Dullens HF, Den Otter W. Intratumoral low-dose interleukin-2 induces rejection of distant solid tumour. Cancer Immunol Immunother. 1991;33:389-394.

170. Den Otter W, Maas RA, Koten JW, et al. Effective immunotherapy with local low doses of interleukin-2. In Vivo. 1991;5:561-565.

171. Hinuma S, Naruo K, Ootsu K, Houkan T, Shiho O, Tsukamoto K. Suppression of pulmonary tumour metastasis in mice by recombinant human interleukin-2: role of asialo GM1-positive cells. Immunology. 1987;60:173-179.

172. Whiteside TL, Chikamatsu K, Nagashima S, Okada K. Antitumor effects of cytolytic T lymphocytes (CTL) and natural killer (NK) cells in head and neck cancer. Anticancer Res. 1996;16:2357-2364.

173. Whiteside TL, Sung MW, Nagashima S, Chikamatsu K, Okada K, Vujanovic NL. Human tumor antigen-specific T lymphocytes and interleukin-2-activated natural killer cells: comparisons of antitumor effects in vitro and in vivo. Clin Cancer Res. 1998;4:1135-1145.

174. Egilmez NK, Jong YS, Iwanuma Y, et al. Cytokine immunotherapy of cancer with controlled release biodegradable microspheres in a human tumor xenograft/SCID mouse model. Cancer Immunol Immunother. 1998;46:21-24.

175. Alici E, Konstantinidis KV, Sutlu T, et al. Anti-myeloma activity of endogenous and adoptively transferred activated natural killer cells in experimental multiple myeloma model. Exp Hematol. 2007;35:1839-1846.

176. Rosenberg SA, Lotze MT, Muul LM, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med. 1985;313:1485-1492.

177. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med. 1987;316:889-897.

178. Phillips JH, Gemlo BT, Myers WW, Rayner AA, Lanier LL. In vivo and in vitro activation of natural killer cells in advanced cancer patients undergoing combined recombinant interleukin-2 and LAK cell therapy. J Clin Oncol. 1987;5:1933-1941.

179. Gratama JW, Bruin RJ, Lamers CH, et al. Activation of the immune system of cancer patients by continuous i.v. recombinant IL-2 (rIL-2) therapy is dependent on dose and schedule of rIL-2. Clin Exp Immunol. 1993;92:185-193.

180. Soiffer RJ, Murray C, Gonin R, Ritz J. Effect of low-dose interleukin-2 on disease relapse after T-cell-depleted allogeneic bone marrow transplantation. Blood. 1994;84:964-971.

181. Margolin K. Cytokine therapy in cancer. Expert Opin Biol Ther. 2008;8:1495-1505.

182. Belldegrun A, Tso CL, Kaboo R, et al. Natural immune reactivity-associated therapeutic response in patients with metastatic renal cell carcinoma receiving tumor-infiltrating lymphocytes and interleukin-2-based therapy. J Immunother Emphasis Tumor Immunol. 1996;19:149-161.

183. Miller JS, Tessmer-Tuck J, Pierson BA, et al. Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained in vivo natural killer cell activity. Biol Blood Marrow Transplant. 1997;3:34-44.

184. Burns LJ, Weisdorf DJ, DeFor TE, et al. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. Bone Marrow Transplant. 2003;32:177-186.

185. Kalwak K, Ussowicz M, Gorczynska E, et al. Immunologic effects of intermediate-dose IL-2 i.v. after autologous hematopoietic cell transplantation in pediatric solid tumors. J Interferon Cytokine Res. 2003;23:173-181.

186. Marti F, Pardo N, Peiro M, et al. Progression of natural immunity during one-year treatment of residual disease in neuroblastoma patients with high doses of interleukin-2 after autologous bone marrow transplantation. Exp Hematol. 1995;23:1445-1452.

187. Gottlieb DJ, Prentice HG, Mehta AB, et al. Malignant plasma cells are sensitive to LAK cell lysis: pre-clinical and clinical studies of interleukin 2 in the treatment of multiple myeloma. Br J Haematol. 1990;75:499-505.

188. Alici E, Sutlu T, Bjorkstrand B, et al. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. Blood. 2008;111:3155-3162.

189. Torelli GF, Guarini A, Palmieri G, et al. Expansion of cytotoxic effectors with lytic activity against autologous blasts from acute myeloid leukaemia patients in complete haematological remission. Br J Haematol. 2002;116:299-307.

190. Baer MR, George SL, Caligiuri MA, et al. Low-dose interleukin-2 immunotherapy does not improve outcome of patients age 60 years and older with acute myeloid leukemia in first complete remission: Cancer and Leukemia Group B Study 9720. J Clin Oncol. 2008;26:4934-4939.

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

192. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol. 1999;163:5211-5218.

193. Ghiringhelli F, Menard C, Martin F, Zitvogel L. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev. 2006;214:229-238.

194. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol. 2006;6:295-307.

195. Curiel TJ. Regulatory T cells and treatment of cancer. Curr Opin Immunol. 2008;20:241-246.

196. Levin AM, Bates DL, Ring AM, et al. Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. Nature.

197. Seidel MG, Freissmuth M, Pehamberger H, Micksche M. Stimulation of natural killer activity in peripheral blood lymphocytes of healthy donors and melanoma patients in vitro: synergism between interleukin (IL)-12 and IL-15 or IL-12 and IL-2. Naunyn Schmiedebergs Arch Pharmacol. 1998;358:382-389.

198. DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA, Bloom ET. IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. Cell Immunol. 1995;165:33-43.

199. Miyazaki T, Kawahara A, Fujii H, et al. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. Science. 1994;266:1045-1047.

200. McDonald PP, Russo MP, Ferrini S, Cassatella MA. Interleukin-15 (IL-15) induces NF-kappaB activation and IL-8 production in human neutrophils. Blood. 1998;92:4828-4835.

201. Vitale M, Bassini A, Secchiero P, et al. NK-active cytokines IL-2, IL-12, and IL-15 selectively modulate specific protein kinase C (PKC) isoforms in primary human NK cells. Anat Rec. 2002;266:87-92.

202. Bulfone-Pau SS, Bulanova E, Pohl T, et al. Death deflected: IL-15 inhibits TNF-alpha-mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15Ralpha chain. FASEB J. 1999;13:1575-1585.

203. Bulanova E, Budagian V, Pohl T, et al. The IL-15R alpha chain signals through association with Syk in human B cells. J Immunol. 2001;167:6292-6302.

204. Bacon CM, McVicar DW, Ortaldo JR, Rees RC, O'Shea JJ, Johnston JA. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. J Exp Med. 1995;181:399-404.

205. Bacon CM, Petricoin EF, 3rd, Ortaldo JR, et al. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci U S A. 1995;92:7307-7311.

206. Hiraki A, Kiura K, Yamane H, et al. Interleukin-12 augments cytolytic activity of peripheral blood mononuclear cells against autologous lung cancer cells in combination with IL-2. Lung Cancer. 2002;35:329-333.

207. Wang KS, Frank DA, Ritz J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. Blood. 2000;95:3183-3190.

208. Rossi AR, Pericle F, Rashleigh S, Janiec J, Djeu JY. Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. Blood. 1994;83:1323-1328.

209. Foreman NK, Rill DR, Coustan-Smith E, Douglass EC, Brenner MK. Mechanisms of selective killing of neuroblastoma cells by natural killer cells and lymphokine activated killer cells. Potential for residual disease eradication. Br J Cancer. 1993;67:933-938.

210. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc Natl Acad Sci U S A. 2000;97:11439-11444.

211. Mehta DS, Wurster AL, Grusby MJ. Biology of IL-21 and the IL-21 receptor. Immunol Rev. 2004;202:84-95.

212. Strengell M, Sareneva T, Foster D, Julkunen I, Matikainen S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. J Immunol. 2002;169:3600-3605.

213. Strengell M, Matikainen S, Siren J, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. J Immunol. 2003;170:5464-5469.

214. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. J Immunol. 2004;172:2048-2058.

215. de Rham C, Ferrari-Lacraz S, Jendly S, Schneiter G, Dayer JM, Villard J. The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. Arthritis Res Ther. 2007;9:R125.

216. Skak K, Frederiksen KS, Lundsgaard D. Interleukin-21 activates human natural killer cells and modulates their surface receptor expression. Immunology. 2008;123:575-583.

217. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One;7:e30264.

218. Wendt K, Wilk E, Buyny S, Schmidt RE, Jacobs R. Interleukin-21 differentially affects human natural killer cell subsets. Immunology. 2007;122:486-495.

219. Park YK, Shin DJ, Cho D, et al. Interleukin-21 increases direct cytotoxicity and IFN-gamma production of ex vivo expanded NK cells towards breast cancer cells. Anticancer Res;32:839-846.

220. Schmidt H, Brown J, Mouritzen U, et al. Safety and clinical effect of subcutaneous human interleukin-21 in patients with metastatic melanoma or renal cell carcinoma: a phase I trial. Clin Cancer Res;16:5312-5319.

221. Davis ID, Brady B, Kefford RF, et al. Clinical and biological efficacy of recombinant human interleukin-21 in patients with stage IV malignant melanoma without prior treatment: a phase IIa trial. Clin Cancer Res. 2009;15:2123-2129.

222. Davis ID, Skrumsager BK, Cebon J, et al. An open-label, two-arm, phase I trial of recombinant human interleukin-21 in patients with metastatic melanoma. Clin Cancer Res. 2007;13:3630-3636.

223. Curti BD. Immunomodulatory and antitumor effects of interleukin-21 in patients with renal cell carcinoma. Expert Rev Anticancer Ther. 2006;6:905-909.

224. Gowda A, Ramanunni A, Cheney C, et al. Differential effects of IL-2 and IL-21 on expansion of the CD4+ CD25+ Foxp3+ T regulatory cells with redundant roles in natural killer cell mediated antibody dependent cellular cytotoxicity in chronic lymphocytic leukemia. MAbs;2:35-41.

225. Otsuji M, Kimura Y, Aoe T, Okamoto Y, Saito T. Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. Proc Natl Acad Sci U S A. 1996;93:13119-13124.

226. Hansson M, Asea A, Ersson U, Hermodsson S, Hellstrand K. Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. J Immunol. 1996;156:42-47.

227. Hanson MG, Ozenci V, Carlsten MC, et al. A short-term dietary supplementation with high doses of vitamin E increases NK cell cytolytic activity in advanced colorectal cancer patients. Cancer Immunol Immunother. 2007;56:973-984.

228. Hansson M, Hermodsson S, Brune M, et al. Histamine protects T cells and natural killer cells against oxidative stress. J Interferon Cytokine Res. 1999;19:1135-1144.

229. Donskov F, Hokland M, Marcussen N, Torp Madsen HH, von der Maase H. Monocytes and neutrophils as 'bad guys' for the outcome of interleukin-2 with and without histamine in metastatic renal cell carcinoma--results from a randomised phase II trial. Br J Cancer. 2006;94:218-226.

230. Brune M, Hellstrand K. Remission maintenance therapy with histamine and interleukin-2 in acute myelogenous leukaemia. Br J Haematol. 1996;92:620-626.

231. Brune M, Hansson M, Mellqvist UH, Hermodsson S, Hellstrand K. NK cell-mediated killing of AML blasts: role of histamine, monocytes and reactive oxygen metabolites. Eur J Haematol. 1996;57:312-319.

232. Hellstrand K, Mellqvist UH, Wallhult E, et al. Histamine and interleukin-2 in acute myelogenous leukemia. Leuk Lymphoma. 1997;27:429-438.

233. Martner A, Thoren FB, Aurelius J, Soderholm J, Brune M, Hellstrand K. Immunotherapy with histamine dihydrochloride for the prevention of relapse in acute myeloid leukemia. Expert Rev Hematol;3:381-391.

234. Brune M, Castaigne S, Catalano J, et al. Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. Blood. 2006;108:88-96.

235. Houvenaeghel G, Bladou F, Blache JL, et al. Tolerance and feasibility of perioperative treatment with interferon-alpha 2a in advanced cancers. Int Surg. 1997;82:165-169.

236. Atzpodien J, Kirchner H, Korfer A, et al. Expansion of peripheral blood natural killer cells correlates with clinical outcome in cancer patients receiving recombinant subcutaneous interleukin-2 and interferon-alpha-2. Tumour Biol. 1993;14:354-359.

237. Molto L, Carballido J, Manzano L, et al. Immunological changes in peripheral blood mononuclear cells of patients with metastatic renal cell carcinoma after low doses of subcutaneous immunotherapy with IFN-alpha-2b and IL-2. J Immunother. 1999;22:260-267.

238. Moroni M, Porta C, De Amici M, Quaglini S, Cattabiani MA, Buzio C. Eosinophils and C4 predict clinical failure of combination immunotherapy with very low dose subcutaneous interleukin-2 and interferon in renal cell carcinoma patients. Haematologica. 2000;85:298-303.

239. Pavone L, Andrulli S, Santi R, Majori M, Buzio C. Long-term treatment with low doses of interleukin-2 and interferon-alpha: immunological effects in advanced renal cell cancer. Cancer Immunol Immunother. 2001;50:82-86.

240. Jorkov AS, Donskov F, Steiniche T, et al. Immune response in blood and tumour tissue in patients with metastatic malignant melanoma treated with IL-2, IFN alpha and histamine dihydrochloride. Anticancer Res. 2003;23:537-542.

241. Smetak M, Kimmel B, Birkmann J, et al. Clinical-scale single-step CD4(+) and CD8(+) cell depletion for donor innate lymphocyte infusion (DILI). Bone Marrow Transplant. 2008;41:643-650.

242. Leung W, Iyengar R, Leimig T, Holladay MS, Houston J, Handgretinger R. Phenotype and function of human natural killer cells purified by using a clinical-scale immunomagnetic method. Cancer Immunol Immunother. 2005;54:389-394.

243. Iyengar R, Handgretinger R, Babarin-Dorner A, et al. Purification of human natural killer cells using a clinical-scale immunomagnetic method. Cytotherapy. 2003;5:479-484.

244. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia. 2004;18:1835-1838.

245. Lang P, Pfeiffer M, Handgretinger R, et al. Clinical scale isolation of T cell-depleted CD56+ donor lymphocytes in children. Bone Marrow Transplant. 2002;29:497-502.

246. McKenna DH, Jr., Sumstad D, Bostrom N, et al. Good manufacturing practices production of natural killer cells for immunotherapy: a six-year single-institution experience. Transfusion. 2007;47:520-528.

247. Koehl U, Esser R, Zimmermann S, et al. Ex vivo expansion of highly purified NK cells for immunotherapy after haploidentical stem cell transplantation in children. Klin Padiatr. 2005;217:345-350.

248. Frohn C, Doehn C, Durek C, et al. Feasibility of the adoptive transfusion of allogenic human leukocyte antigen-matched natural killer cells in patients with renal cell carcinoma. J Immunother. 2000;23:499-504.

249. Escudier B, Farace F, Angevin E, et al. Immunotherapy with interleukin-2 (IL2) and lymphokine-activated natural killer cells: improvement of clinical responses in metastatic renal cell carcinoma patients previously treated with IL2. Eur J Cancer. 1994;30A:1078-1083.

250. Hercend T, Farace F, Baume D, et al. Immunotherapy with lymphokine-activated natural killer cells and recombinant interleukin-2: a feasibility trial in metastatic renal cell carcinoma. J Biol Response Mod. 1990;9:546-555.

251. Miller JS, Klingsporn S, Lund J, Perry EH, Verfaillie C, McGlave P. Large scale ex vivo expansion and activation of human natural killer cells for autologous therapy. Bone Marrow Transplant. 1994;14:555-562.

252. Lister J, Rybka WB, Donnenberg AD, et al. Autologous peripheral blood stem cell transplantation and adoptive immunotherapy with activated natural killer cells in the immediate posttransplant period. Clin Cancer Res. 1995;1:607-614.

253. Pierson BA, Europa AF, Hu WS, Miller JS. Production of human natural killer cells for adoptive immunotherapy using a computer-controlled stirred-tank bioreactor. J Hematother. 1996;5:475-483.

254. Carlens S, Gilljam M, Chambers BJ, et al. A new method for in vitro expansion of cytotoxic human CD3-CD56+ natural killer cells. Hum Immunol. 2001;62:1092-1098.

255. Luhm J, Brand JM, Koritke P, Hoppner M, Kirchner H, Frohn C. Large-scale generation of natural killer lymphocytes for clinical application. J Hematother Stem Cell Res. 2002;11:651-657.

256. Guven H, Gilljam M, Chambers BJ, et al. Expansion of natural killer (NK) and natural killer-like T (NKT)-cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): a potential source for cellular immunotherapy. Leukemia. 2003;17:1973-1980.

257. Klingemann HG, Martinson J. Ex vivo expansion of natural killer cells for clinical applications. Cytotherapy. 2004;6:15-22.

258. Ishikawa E, Tsuboi K, Saijo K, et al. Autologous natural killer cell therapy for human recurrent malignant glioma. Anticancer Res. 2004;24:1861-1871.

259. Berg M, Lundqvist A, McCoy P, Jr., et al. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy. 2009;11:341-355.

260. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res. 2009;69:4010-4017.

261. Torelli GF, Guarini A, Maggio R, Alfieri C, Vitale A, Foa R. Expansion of natural killer cells with lytic activity against autologous blasts from adult and pediatric acute lymphoid leukemia patients in complete hematologic remission. Haematologica. 2005;90:785-792.

262. Guimaraes F, Guven H, Donati D, et al. Evaluation of ex vivo expanded human NK cells on antileukemia activity in SCID-beige mice. Leukemia. 2006;20:833-839.

263. .

264. Basse PH, Whiteside TL, Herberman RB. Cancer immunotherapy with interleukin-2-activated natural killer cells. Mol Biotechnol. 2002;21:161-170.

265. Siegler U, Kalberer CP, Nowbakht P, Sendelov S, Meyer-Monard S, Wodnar-Filipowicz A. Activated natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. Leukemia. 2005;19:2215-2222.

266. Clausen J, Vergeiner B, Enk M, Petzer AL, Gastl G, Gunsilius E. Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. Immunobiology. 2003;207:85-93.

267. deMagalhaes-Silverman M, Donnenberg A, Lembersky B, et al. Posttransplant adoptive immunotherapy with activated natural killer cells in patients with metastatic breast cancer. J Immunother. 2000;23:154-160.

268. Krause SW, Gastpar R, Andreesen R, et al. Treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells: a clinical phase i trial. Clin Cancer Res. 2004;10:3699-3707.

269. Milani V, Stangl S, Issels R, et al. Anti-tumor activity of patient-derived NK cells after cell-based immunotherapy--a case report. J Transl Med. 2009;7:50.

270. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A. Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. Curr Opin Immunol. 2005;17:211-217.

271. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.

272. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-3057.

273. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, Velardi A. Natural killer cell alloreactivity and haplo-identical hematopoietic transplantation. Cytotherapy. 2006;8:554-558.

274. Igarashi T, Wynberg J, Srinivasan R, et al. Enhanced cytotoxicity of allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility against melanoma and renal cell carcinoma cells. Blood. 2004;104:170-177.

275. Nguyen S, Dhedin N, Vernant JP, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood. 2005;105:4135-4142.

276. Schulze A, Schirutschke H, Oelschlagel U, et al. Altered phenotype of natural killer cell subsets after haploidentical stem cell transplantation. Exp Hematol. 2008;36:378-389.

277. Koehl U, Sorensen J, Esser R, et al. IL-2 activated NK cell immunotherapy of three children after haploidentical stem cell transplantation. Blood Cells Mol Dis. 2004;33:261-266.

278. Shi J, Tricot G, Szmania S, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. Br J Haematol. 2008;143:641-653.

279. Barkholt L, Alici E, Conrad R, et al. Safety analysis of ex vivo-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study. Immunotherapy. 2009;1:753-764.

280. Huenecke S, Zimmermann SY, Kloess S, et al. IL-2-driven regulation of NK cell receptors with regard to the distribution of CD16+ and CD16- subpopulations and in vivo influence after haploidentical NK cell infusion. J Immunother;33:200-210.

281. Rubnitz JE, Inaba H, Ribeiro RC, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J Clin Oncol;28:955-959.

282. Rizzieri DA, Storms R, Chen DF, et al. Natural killer cell-enriched donor lymphocyte infusions from A 3-6/6 HLA matched family member following nonmyeloablative allogeneic stem cell transplantation. Biol Blood Marrow Transplant;16:1107-1114.

283. Brehm C, Huenecke S, Quaiser A, et al. IL-2 stimulated but not unstimulated NK cells induce selective disappearance of peripheral blood cells: concomitant results to a phase I/II study. PLoS One;6:e27351.

284. Nguyen S, Beziat V, Norol F, et al. Infusion of allogeneic natural killer cells in a patient with acute myeloid leukemia in relapse after haploidentical hematopoietic stem cell transplantation. Transfusion;51:1769-1778.

285. Curti A, Ruggeri L, D'Addio A, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood;118:3273-3279.

286. Brand JM, Meller B, Von Hof K, et al. Kinetics and organ distribution of allogeneic natural killer lymphocytes transfused into patients suffering from renal cell carcinoma. Stem Cells Dev. 2004;13:307-314.

287. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia. 1994;8:652-658.

288. Maki G, Klingemann HG, Martinson JA, Tam YK. Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J Hematother Stem Cell Res. 2001;10:369-383.

289. Yan Y, Steinherz P, Klingemann HG, et al. Antileukemia activity of a natural killer cell line against human leukemias. Clin Cancer Res. 1998;4:2859-2868.

290. Tam YK, Miyagawa B, Ho VC, Klingemann HG. Immunotherapy of malignant melanoma in a SCID mouse model using the highly cytotoxic natural killer cell line NK-92. J Hematother. 1999;8:281-290.

291. Klingemann HG, Wong E, Maki G. A cytotoxic NK-cell line (NK-92) for ex vivo purging of leukemia from blood. Biol Blood Marrow Transplant. 1996;2:68-75.

292. Maki G, Tam YK, Berkahn L, Klingemann HG. Ex vivo purging with NK-92 prior to autografting for chronic myelogenous leukemia. Bone Marrow Transplant. 2003;31:1119-1125.

293. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematother Stem Cell Res. 2001;10:535-544.

294. Arai S, Meagher R, Swearingen M, et al. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy. 2008;10:625-632.

295. Rogers S, Pfuderer P. Use of viruses as carriers of added genetic information. Nature. 1968;219:749-751.

296. Rosenberg SA, Aebersold P, Cornetta K, et al. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med. 1990;323:570-578.

297. Blaese RM, Culver KW, Miller AD, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science. 1995;270:475-480.

298. VandenDriessche T. Gene therapy delivers. Hum Gene Ther. 2009;20:1222-1223.

299. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663-676.

300. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861-872.

301. Cai Q, Rubin JT, Lotze MT. Genetically marking human cells--results of the first clinical gene transfer studies. Cancer Gene Ther. 1995;2:125-136.

302. Wei MQ, Metharom P, Ellem KA, Barth S. Search for "weapons of mass destruction" for cancer -- immuno/ gene therapy comes of age. Cell Mol Immunol. 2005;2:351-357.

303. Li CY, Huang Q, Kung HF. Cytokine and immuno-gene therapy for solid tumors. Cell Mol Immunol. 2005;2:81-91.

304. Morse ML, Lederberg EM, Lederberg J. Transduction in Escherichia Coli K-12. Genetics. 1956;41:142-156.

305. Song E, Zhu P, Lee SK, et al. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol. 2005;23:709-717.

306. Yu W, Pirollo KF, Rait A, et al. A sterically stabilized immunolipoplex for systemic administration of a therapeutic gene. Gene Ther. 2004;11:1434-1440.

307. Zhang Y, Zhang YF, Bryant J, Charles A, Boado RJ, Pardridge WM. Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. Clin Cancer Res. 2004;10:3667-3677.

308. Morrissey DV, Lockridge JA, Shaw L, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol. 2005;23:1002-1007.

309. Kaul G, Amiji M. Tumor-targeted gene delivery using poly(ethylene glycol)-modified gelatin nanoparticles: in vitro and in vivo studies. Pharm Res. 2005;22:951-961.

310. Walker GF, Fella C, Pelisek J, et al. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. Mol Ther. 2005;11:418-425.

311. Judge A, McClintock K, Phelps JR, Maclachlan I. Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. Mol Ther. 2006;13:328-337.

312. Schiffelers RM, Ansari A, Xu J, et al. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res. 2004;32:e149.

313. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. Cancer Res. 2005;65:8984-8992.

314. Pathak A, Patnaik S, Gupta KC. Recent trends in non-viral vector-mediated gene delivery. Biotechnol J. 2009;4:1559-1572.

315. Li SD, Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther. 2006;13:1313-1319.

316. Heinkelein M, Dressler M, Jarmy G, et al. Improved primate foamy virus vectors and packaging constructs. J Virol. 2002;76:3774-3783.

317. Trobridge G, Josephson N, Vassilopoulos G, Mac J, Russell DW. Improved foamy virus vectors with minimal viral sequences. Mol Ther. 2002;6:321-328.

318. Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996;272:263-267.

319. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998;72:8463-8471.

320. Negre D, Duisit G, Mangeot PE, Moullier P, Darlix JL, Cosset FL. Lentiviral vectors derived from simian immunodeficiency virus. Curr Top Microbiol Immunol. 2002;261:53-74.

321. Matukonis M, Li M, Molina RP, Paszkiet B, Kaleko M, Luo T. Development of second- and third-generation bovine immunodeficiency virus-based gene transfer systems. Hum Gene Ther. 2002;13:1293-1303.

322. Poeschla EM, Wong-Staal F, Looney DJ. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. Nat Med. 1998;4:354-357.

323. Olsen JC. Gene transfer vectors derived from equine infectious anemia virus. Gene Ther. 1998;5:1481-1487.

324. Boris-Lawrie K, Temin HM. The retroviral vector. Replication cycle and safety considerations for retrovirus-mediated gene therapy. Ann N Y Acad Sci. 1994;716:59-70; discussion 71.

325. Baum C, Schambach A, Bohne J, Galla M. Retrovirus vectors: toward the plentivirus? Mol Ther. 2006;13:1050-1063.

326. Derse D, Martarano L. Construction of a recombinant bovine leukemia virus vector for analysis of virus infectivity. J Virol. 1990;64:401-405.

327. Hughes SH. The RCAS vector system. Folia Biol (Praha). 2004;50:107-119.

328. Watanabe S, Temin HM. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5' long terminal repeat and the start of the gag gene. Proc Natl Acad Sci U S A. 1982;79:5986-5990.

329. Gunzburg WH, Salmons B. Mouse mammary tumor virus mediated transfer and expression of neomycin resistance to infected cultured cells. Virology. 1986;155:236-248.

330. Maetzig T, Galla M, Baum C, Schambach A. Gammaretroviral vectors: biology, technology and application. Viruses;3:677-713.

331. Helseth E, Kowalski M, Gabuzda D, Olshevsky U, Haseltine W, Sodroski J. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J Virol. 1990;64:2416-2420.

332. Page KA, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J Virol. 1990;64:5270-5276.

333. Poznansky M, Lever A, Bergeron L, Haseltine W, Sodroski J. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. J Virol. 1991;65:532-536.

334. Buchschacher GL, Jr., Panganiban AT. Human immunodeficiency virus vectors for inducible expression of foreign genes. J Virol. 1992;66:2731-2739.

335. Parolin C, Dorfman T, Palu G, Gottlinger H, Sodroski J. Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. J Virol. 1994;68:3888-3895.

336. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875.

337. Cronin J, Zhang XY, Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther. 2005;5:387-398.

338. Seganti L, Superti F, Girmenia C, Melucci L, Orsi N. Study of receptors for vesicular stomatitis virus in vertebrate and invertebrate cells. Microbiologica. 1986;9:259-267.

339. Kolokoltsov AA, Weaver SC, Davey RA. Efficient functional pseudotyping of oncoretroviral and lentiviral vectors by Venezuelan equine encephalitis virus envelope proteins. J Virol. 2005;79:756-763.

340. Frecha C, Costa C, Negre D, et al. Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. Blood. 2008;112:4843-4852.

341. Sandrin V, Boson B, Salmon P, et al. Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. Blood. 2002;100:823-832.

342. Hanawa H, Kelly PF, Nathwani AC, et al. Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. Mol Ther. 2002;5:242-251.

343. Zhang XY, La Russa VF, Reiser J. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. J Virol. 2004;78:1219-1229.

344. Relander T, Johansson M, Olsson K, et al. Gene transfer to repopulating human CD34+ cells using amphotropic-, GALV-, or RD114-pseudotyped HIV-1-based vectors from stable producer cells. Mol Ther. 2005;11:452-459.

345. Landau NR, Page KA, Littman DR. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J Virol. 1991;65:162-169.

346. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest. 2008;118:3132-3142.

347. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003;302:415-419.

348. Li CM, Park JH, Simonaro CM, et al. Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. Genomics. 2002;79:218-224.

349. Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. Cell. 2002;110:521-529.

350. Montini E, Cesana D, Schmidt M, et al. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol. 2006;24:687-696.

351. Montini E, Cesana D, Schmidt M, et al. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Invest. 2009;119:964-975.

352. Dick JE. Stem cell concepts renew cancer research. Blood. 2008;112:4793-4807.

353. Newrzela S, Cornils K, Li Z, et al. Resistance of mature T cells to oncogene transformation. Blood. 2008;112:2278-2286.

354. Pegram HJ, Kershaw MH, Darcy PK. Genetic modification of natural killer cells for adoptive cellular immunotherapy. Immunotherapy. 2009;1:623-630.

355. Grund EM, Muise-Helmericks RC. Cost efficient and effective gene transfer into the human natural killer cell line, NK92. J Immunol Methods. 2005;296:31-36.

356. Schoenberg K, Trompeter HI, Uhrberg M. Delivery of DNA into natural killer cells for immunotherapy. Methods Mol Biol. 2008;423:165-172.

357. Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res. 2009;33:1255-1259.

358. Trompeter HI, Weinhold S, Thiel C, Wernet P, Uhrberg M. Rapid and highly efficient gene transfer into natural killer cells by nucleofection. J Immunol Methods. 2003;274:245-256.

359. Becknell B, Trotta R, Yu J, et al. Efficient infection of human natural killer cells with an EBV/retroviral hybrid vector. J Immunol Methods. 2005;296:115-123.

360. Guven H, Konstantinidis KV, Alici E, et al. Efficient gene transfer into primary human natural killer cells by retroviral transduction. Exp Hematol. 2005;33:1320-1328.

361. Alici E, Sutlu T, Sirac Dilber M. Retroviral gene transfer into primary human natural killer cells. Methods Mol Biol. 2009;506:127-137.

362. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood. 2005;106:376-383.

363. Nagashima S, Mailliard R, Kashii Y, et al. Stable transduction of the interleukin-2 gene into human natural killer cell lines and their phenotypic and functional characterization in vitro and in vivo. Blood. 1998;91:3850-3861.

364. Uherek C, Tonn T, Uherek B, et al. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood. 2002;100:1265-1273.

365. Konstantinidis KV, Alici E, Aints A, Christensson B, Ljunggren HG, Dilber MS. Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. Exp Hematol. 2005;33:159-164.

366. Tran J, Kung SK. Lentiviral vectors mediate stable and efficient gene delivery into primary murine natural killer cells. Mol Ther. 2007;15:1331-1339.

367. Baeriswyl V, Wodnar-Filipowicz A, Kalberer CP. The effect of silencing NKG2D through RNA interference on receptor functions in interleukin-2-activated human natural killer cells. Haematologica. 2006;91:1538-1541.

368. Figueiredo C, Seltsam A, Blasczyk R. Permanent silencing of NKG2A expression for cell-based therapeutics. J Mol Med (Berl). 2009;87:199-210.

369. Karimi M, Cao TM, Baker JA, Verneris MR, Soares L, Negrin RS. Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated CD8+ T cells and NK cells. J Immunol. 2005;175:7819-7828.

370. Kung SK. Introduction of shRNAs into primary NK cells with lentivirus. Methods Mol Biol;612:233-247.

371. Micucci F, Zingoni A, Piccoli M, Frati L, Santoni A, Galandrini R. High-efficient lentiviral vector-mediated gene transfer into primary human NK cells. Exp Hematol. 2006;34:1344-1352.

372. Tran J, Mahmood S, Carlyle JR, Kung SK. Altering the specificity of NK:target cell interactions by genetic manipulation of NK receptor expression on primary mouse NK cells. Vaccine;28:3767-3772.

373. Liu JH, Wei S, Blanchard DK, Djeu JY. Restoration of lytic function in a human natural killer cell line by gene transfection. Cell Immunol. 1994;156:24-35.

374. Zhang J, Sun R, Wei H, Tian Z. Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy. Haematologica. 2004;89:338-347.

375. Jiang W, Zhang J, Tian Z. Functional characterization of interleukin-15 gene transduction into the human natural killer cell line NKL. Cytotherapy. 2008;10:265-274.

376. Goding SR, Yang Q, Knudsen KB, Potter DM, Basse PH. Cytokine gene therapy using adenovirally transduced, tumor-seeking activated natural killer cells. Hum Gene Ther. 2007;18:701-711.

377. Maas RA, Dullens HF, Den Otter W. Interleukin-2 in cancer treatment: disappointing or (still) promising? A review. Cancer Immunol Immunother. 1993;36:141-148.

378. Ardizzoni A, Bonavia M, Viale M, et al. Biologic and clinical effects of continuous infusion interleukin-2 in patients with non-small cell lung cancer. Cancer. 1994;73:1353-1360.

379. Roychowdhury S, Blaser BW, Freud AG, et al. IL-15 but not IL-2 rapidly induces lethal xenogeneic graft-versus-host disease. Blood. 2005;106:2433-2435.

380. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol. 2004;4:665-674.

381. Tam YK, Maki G, Miyagawa B, Hennemann B, Tonn T, Klingemann HG. Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. Hum Gene Ther. 1999;10:1359-1373.

382. Miller JS, Tessmer-Tuck J, Blake N, et al. Endogenous IL-2 production by natural killer cells maintains cytotoxic and proliferative capacity following retroviral-mediated gene transfer. Exp Hematol. 1997;25:1140-1148.

383. Schirrmann T, Pecher G. Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth inhibition in vivo. Cancer Gene Ther. 2002;9:390-398.

384. Schirrmann T, Pecher G. Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor. Leuk Res. 2005;29:301-306.

385. Demirtzoglou FJ, Papadopoulos S, Zografos G. Cytolytic and cytotoxic activity of a human natural killer cell line genetically modified to specifically recognize HER-2/neu overexpressing tumor cells. Immunopharmacol Immunotoxicol. 2006;28:571-590.

386. Meier R, Piert M, Piontek G, et al. Tracking of [18F]FDG-labeled natural killer cells to HER2/neu-positive tumors. Nucl Med Biol. 2008;35:579-588.

387. Pegram HJ, Jackson JT, Smyth MJ, Kershaw MH, Darcy PK. Adoptive transfer of gene-modified primary NK cells can specifically inhibit tumor progression in vivo. J Immunol. 2008;181:3449-3455.

388. Kruschinski A, Moosmann A, Poschke I, et al. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. Proc Natl Acad Sci U S A. 2008;105:17481-17486.

389. Brandstadter JD, Yang Y. Natural killer cell responses to viral infection. J Innate Immun;3:274-279.

390. Lanier LL. Evolutionary struggles between NK cells and viruses. Nat Rev Immunol. 2008;8:259-268.

391. Shayakhmetov DM, Di Paolo NC, Mossman KL. Recognition of virus infection and innate host responses to viral gene therapy vectors. Mol Ther;18:1422-1429.

392. Huang X, Yang Y. Innate immune recognition of viruses and viral vectors. Hum Gene Ther. 2009;20:293-301.

393. Rossetti M, Gregori S, Hauben E, et al. HIV-1-derived lentiviral vectors directly activate plasmacytoid dendritic cells, which in turn induce the maturation of myeloid dendritic cells. Hum Gene Ther;22:177-188.

394. Brown BD, Sitia G, Annoni A, et al. In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. Blood. 2007;109:2797-2805.

395. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood. 2006;107:159-166.

396. Ramsdell FJ, Golub SH. Generation of lymphokine-activated killer cell activity from human thymocytes. J Immunol. 1987;139:1446-1453.

397. Chan JK, Hamilton CA, Cheung MK, et al. Enhanced killing of primary ovarian cancer by retargeting autologous cytokine-induced killer cells with bispecific antibodies: a preclinical study. Clin Cancer Res. 2006;12:1859-1867.

398. Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. J Immunol. 1994;153:1687-1696.

399. Bordignon C, Carlo-Stella C, Colombo MP, et al. Cell therapy: achievements and perspectives. Haematologica. 1999;84:1110-1149.

400. Khalil-Daher I, Riteau B, Menier C, et al. Role of HLA-G versus HLA-E on NK function: HLA-G is able to inhibit NK cytolysis by itself. J Reprod Immunol. 1999;43:175-182.

401. Biassoni R, Bottino C, Cantoni C, Moretta A. Human natural killer receptors and their ligands. Curr Protoc Immunol. 2002;Chapter 14:Unit 14 10.

402. Fauriat C, Just-Landi S, Mallet F, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. Blood. 2007;109:323-330.

403. Sutlu T, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. J Intern Med. 2009;266:154-181.

404. Alici E, Sutlu T, Sirac Dilber M. Retroviral gene transfer into primary human natural killer cells. Methods in molecular biology. 2009;506:127-137.

405. Guven H, Konstantinidis KV, Alici E, et al. Efficient gene transfer into primary human natural killer cells by retroviral transduction. Experimental hematology. 2005;33:1320-1328.

406. Seya T, Kasamatsu J, Azuma M, Shime H, Matsumoto M. Natural killer cell activation secondary to innate pattern sensing. J Innate Immun;3:264-273.

407. Wilkins C, Gale M, Jr. Recognition of viruses by cytoplasmic sensors. Curr Opin Immunol;22:41-47.

408. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. Int Rev Immunol;30:16-34.

409. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol. 2008;89:1-47.

410. Clark K, Plater L, Peggie M, Cohen P. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J Biol Chem. 2009;284:14136-14146.