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THE THERAPEUTIC POTENTIAL OF *EX VIVO* EXPANDED NATURAL KILLER (NK) CELLS FOR IMMUNOTHERAPY OF CANCER

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The one who does not know but knows that he does not know, is a child, teach him,
The one who knows but does not know that he knows, is in sleep, wake him up,
The one who does not know, but does not know that he does not know, is an ignorant, avoid him,
The one who knows, and knows that he knows, is a leader, follow him.

Chinese proverb

To my family

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1 ABSTRACT

Cell and gene therapy of cancers has received much attention in the past decade. A number of applications of cancer treatment have been developed and used. One of the recent applications is immunotherapy of tumours with human natural killer (NK) cells. A major challenge to the successful application of this treatment has been the identification, expansion, and gene modification of appropriate effector cells. We therefore developed a novel expansion method that is simple, good manufacturing practice (GMP)-compatible, cost-effective (Paper I). For NK cell immunotherapy of patients with B-cell chronic lymphocytic leukaemia (B-CLL), efficient NK cell expansion was obtained using the same method (Paper II). However, NK cell expansion rates were lower in cultures from patients with progressive B-CLL, demonstrating the negative effect of disease progression on NK cell expansion. In the same study (Paper II), it was also demonstrated that samples obtained from the same patients at different time points had similar NK expansion capacity, indicating reproducibility and also the reability of the method. In addition, in both studies it was shown that half of the expanded T cells possess an NK-like T (NKT) phenotype ($CD3^+CD56^+$).

Moreover, using retroviral vector transduction, efficient gene transfer into primary human *ex vivo* expanded NK cells was demonstrated. Days 5 and 6 of expansion seem to be the best days for NK cell transduction (Paper III). In addition, the safety and the *in vivo* anti-tumour activity of human *ex vivo* expanded NK cells were evaluated against human K562 cells in SCID-beige mice (Paper IV). Mice treated with expanded NK cells had significantly prolonged survival compared to the untreated group. Finally, NK and NKT cell populations that were expanded with GMP components were infused into patients with tumors. Such killer cells with or without IL-2 injections were shown to be safe and to some extent had anti-tumor activity (Paper V).

In conclusion, these studies have shown that NK as well as NKT cell expansion is possible with such a simple, cost effective and easy-to-use method. We were able to expand and gene-modify with retroviral vectors NK cells directly from the peripheral blood of both healthy donors and patients with B-CLL and show that NK cells can eradicate tumour cells *in vivo*. This may open new possibilities for current and future cell- and gene therapy approaches.

Keywords: Natural killer (NK) cells, Natural killer-like T (NKT) cells, Gene transfer, Retrovirus, Good manufacturing practice (GMP), *Ex vivo*, *In vivo*, SCID-beige.

2 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. They are referred to in the text by their roman numerals.

I. Carlens S, Gilljam M, Chambers BJ, Aschan J, **Guven H**, Ljunggren HG, Christensson B, Dilber MS. A novel method for *in vitro* expansion of cytotoxic human $CD3^{-}CD56^{+}$ NK cells. **Human Immunology** 2001;62:1092-1098.

II. **Guven H**, Gilljam M, Chambers BJ, Ljunggren HG, Christensson B, Kimby E, Dilber MS. Expansion of natural killer (NK) and natural killer-like (NKT) cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): a potential source for immunotherapy. **Leukemia** 2003;17:1973-1980.

III. **Guven H**, Konstantinidis KV, Alici E, Aints A, Christensson B, Ljunggren HG, Dilber MS. Efficient gene transfer into primary human natural killer (NK) cells with retroviral transduction. **Experimental Hematology** 2005;33(11):1320-1328.

IV. Guimaraes F, **Guven H**, Donati D, Christensson B, Ljunggren HG, Bejarano MT, Dilber MS. Evaluation of *ex vivo* expanded human NK cells on anti-leukemia activity in SCID-beige mice. **Leukemia** 2005, resubmitted.

V. Barkholt L, **Guven H**, Alexandra Treschow, Reka Conrad, Kerstin Cederlund, Ljunggren HG, Aschan J, Christensson B, Gilljam M, Stellan B, Ringden O, Dilber MS. Donor derived natural killer (NK) and natural killer-like T (NKT) cell infusions are safe with a potential biological anti-tumour effect.
Manuscript

Publications out of the thesis

I. Aints A, Belusa R, Andersson RM, **Guven H**, Dilber MS. Enhanced ouabain resistance gene as a eukaryotic selection marker.
Human Gene Therapy 2002;13(8):969-977.

II. Aints A, **Guven H**, Gahrton G, Smith CI, Dilber MS. Mapping of herpes simplex virus-1 VP22 functional domains for inter- and subcellular protein targeting.
Gene Therapy 2001;8(14):1051-1056.

3 LIST OF ABBREVIATIONS

AFP, alpha-feto protein
AML, acute myeloid leukaemia
ALL, acute lymphocytic leukaemia
BM, bone marrow
CB, cord blood
CML, chronic myeloid leukemia
CLL, chronic lymphocytic leukaemia
CRC, colorectal cancer
CRP, serum reactive protein
CT, computed tomography
DC, dendritic cell
DLI, donor lymphocyte infusion
E:T, effector : target ratio
GFP, green fluorescent protein
GVHD, graft-versus-host disease
GVL, graft-versus-leukemia
GVT, graft-versus-tumour
HCC, hepatocellular cancer
HCV, hepatitis C virus
HLA, human leukocyte antigen
HSCT, haematopoietic stem cell transplantation
HS, human serum
IFN-γ, interferon-gamma
IL-2, interleukin 2
IL-15, interleukin 15
LAK cells, lymphokine activated killer cells
Mab, monoclonal antibody
MHC, major histocompatibility complex
NK cells, natural killer cells
NKT cells, natural killer-like T cells
OKT3, orthoclone, monoclonal antibody against CD3
PBMC, peripheral blood mononuclear cells
RCC, renal cell carcinoma
SCGM, stem cell growth medium
TCD, T-cell depleted
TNF-α, tumour necrosis factor-alpha
WBC, white blood cell count
eYFP, enhanced yellow fluorescent protein

4 GENERAL INTRODUCTION

4.1 Immunotherapy Overview

The survival of cancer patients has improved steadily with the advent of effective anticancer treatments such as chemotherapy and radiotherapy during the last century. However, the majority of patients with metastatic disease will not be cured by these measures and will eventually die of their disease. New and more effective methods of treatment are needed. The immune system is a potent force for rejecting transplanted organs or microbial pathogens. Both innate and adaptive immune responses play a role in tumour clearance. It has been estimated that only approximately 1.4% of all multicellular animal species on this planet posses an adaptive immune system (Parish et al., 1997). As a result, if the immune system controls tumour development in the 98.6% of animal species that lack adaptive immunity it stands to reason that the innate response must be involved.

In recent years, much has been discovered about the mechanisms through which the immune system recognizes and responds to tumours. Improved adjuvants are available. Means by which cancer cells overcome immunological attack can be exploited and overcome. Most importantly, the immunological control mechanisms responsible for initiating and maintaining an effective immune response against tumours and pathogens are better understood. It is now possible to manipulate immune effector cells *ex vivo* in order to induce an effective antitumour response. At the same time, it is possible to enhance other aspects of the immune system, both specific (e.g. antibody responses) and innate (natural killer (NK) cells).

An early technique used in the modern era of cancer immunotherapy was isolation of tumour-infiltrating lymphocytes (TIL) that were then expanded *ex vivo* and administered back to patients (Topalian et al., 1988; Rosenberg et al., 1988). These cells could be given together with systemic cytokines such as Interleukin-2 (IL-2) (Topalian et al., 1988; Rosenberg et al., 1988), or could be transfected with cytokine genes such as TNF (TNF/TIL human gene therapy clinical protocol, 1990; Hwu et al., 1994). A variation of this approach is to activate NK cells *ex vivo* prior to reinjection.

4.2 NK cells

NK cells are one of the cellular mediators of innate defence. They are lymphoid cells that, without the need for immunization or pre-activation, can recognize and kill aberrant cells and rapidly produce soluble factors, cytokines or chemokines that have antimicrobial effects or that prime other cells of the immune system. NK cells comprise ~15% of all circulating lymphocytes and are also found in peripheral tissues, including the liver, peritoneal cavity and placenta. Resting NK cells circulate in the blood, but, following activation by cytokines, they are capable of extravasation and infiltration into most tissues that contain pathogen-infected or malignant cells (Wiltrout et al., 1984; Fogler et al., 1996; Biron et al., 1997; Glas et al., 2000).

NK cells have a heterogeneous arsenal of surface receptors that allow them to respond to microbial products, cytokines, stress signals and inducible molecules that are expressed after target cell transformation. NK cells are therefore crucial for defence against infectious diseases and cancer.

4.3 NK cell subsets

NK cells can be divided into two subsets based on their CD56 antigen expression. The CD56 antigen is an isoform of the human neural cell adhesion molecule with unknown function on human NK cells (Lanier et al., 1989), although early studies suggested that this molecule might mediate interactions between NK cells and target cells (Nitta et al., 1989; Suzuki et al., 1991). Thus, there is no known direct functional significance of high- or low-level expression of CD56. The majority (≈ 90%) of human NK cells have low-density expression of CD56 ($CD56^{dim}$) and express high levels of Fcγ receptor III (FcγRIII, CD16), whereas ≈ 10% of NK cells are $CD56^{bright}CD16^{dim}$ or $CD56^{bright}CD16^{-}$.

Early functional studies of these subsets revealed that resting $CD56^{dim}$ cells are the more cytotoxic subsets (Lanier et al., 1986). 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 (Figure 1). Recent evidence has suggested that human NK cell subsets are distinct lymphocytes with unique roles in the innate immune response. In addition to distinct expression of adhesion molecules and cytokine receptors, the

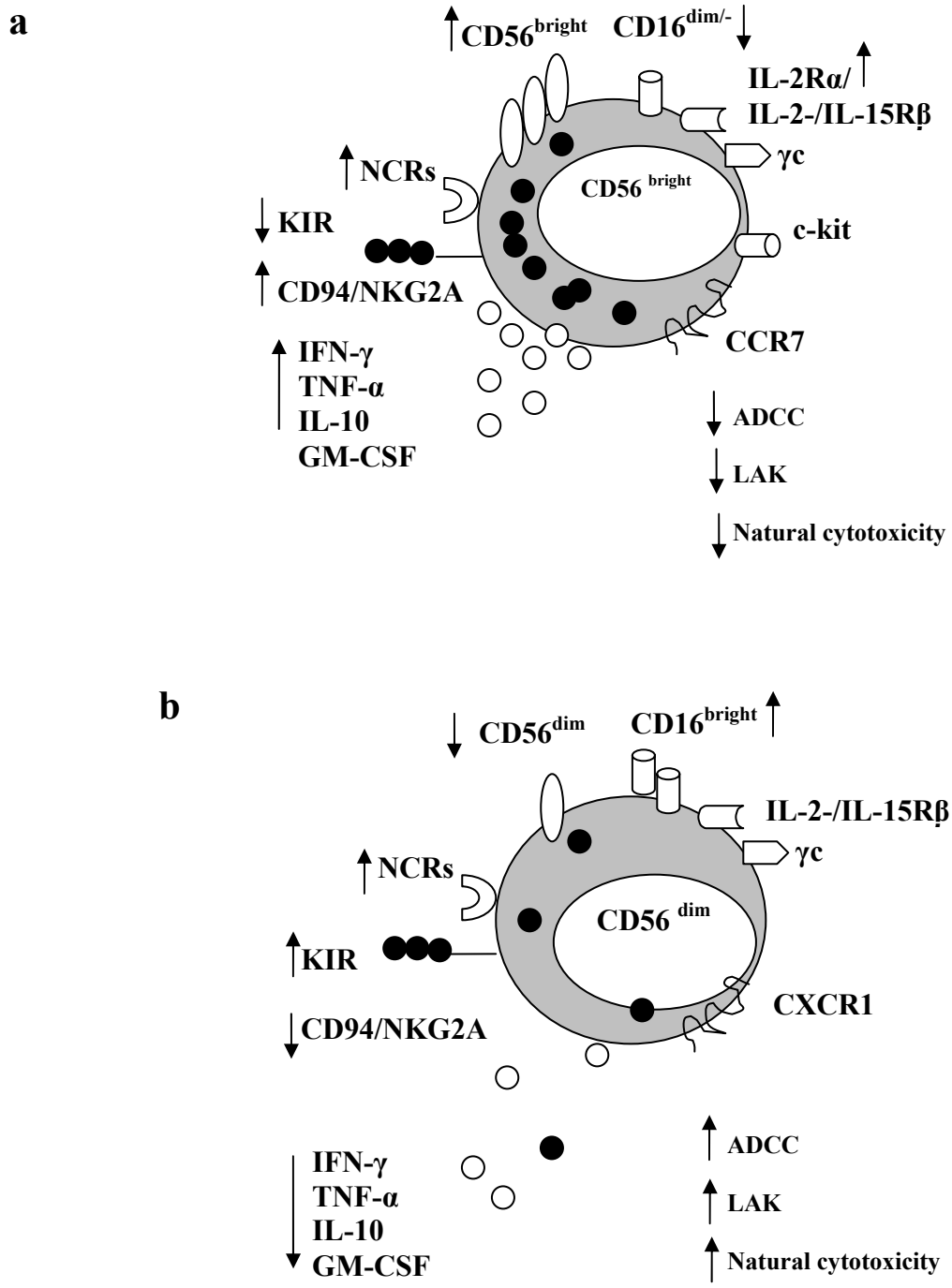

Figure 1. Outline of human NK cell subsets.

$CD56^{bright}$ NK cell has the capacity to produce high levels of immunoregulatory cytokines, but has low-level expression of killer-cell immunoglobulin-like receptors (KIRs) and is poorly cytotoxic. By contrast, the $CD56^{dim}$ NK cell appears to produce low levels of cytokines but has high-level expression of KIRs and is a potent cytotoxic effector cell. Thus, future studies of the

biology of human NK cells might approach NK cells as separate $CD56^{bright}$ and $CD56^{dim}$ subsets rather than a homogenouspopulation.

4.4 NK cell development

NK cells are derived from haematopoietic stem cells (HSCs). Bone marrow (BM) is the main site of NK cell generation in adults. The bone marrow microenvironment provides a rich source of cytokines and growth factors that can support NK cell development *in vitro* and contains stromal cells that are required for full maturation of NK cells. Early studies showed that NK cell generation from human $CD34^+$ HSCs required stromal-cell contact, whereas more-differentiated $CD34^+CD7^+$ cells could generate NK cells when cultured in IL-2 alone (Miller et al, 1994). Similarly, fetal liver $CD34^+CD38^+$ cells could generate NK cells *in vitro* using IL-15 (Jaleco et al., 1997). It was later reported that the stromal cell requirements could be replaced by early-acting cytokines such as stem cell factor (SCF) also known as c-kit ligand, fetal liver kinase 2 ligand (FLK2), also known as FMS-like tyrosine kinase 3 ligand (FLT3L) and IL-7 (Mrozek et al., 1996; Williams et al., 1997).

HSCs give rise to precursors of different haematopoietic lineages. The first step of HSC commitment generates common lymphoid progenitors (CLPs) (Kondo et al., 1997). CLPs and their immediate precursors, the earliest lymphoid progenitors (ELPs) (Igarashi et al., 2002), further differentiate to give rise to B-, T- and NK-cell-restricted precursors. Although existence of a committed natural killer cell progenitor (NKP) was assumed by analogy, definitive evidence for its existence was, until recently, lacking. Some clues were provided from studies of the generation of NK cells from HSCs *in vitro*. These studies indicated that once generated, NKPs could be driven to become NK cells under the influence of IL-2 or IL-15 alone (Mrozek et al., 1996; Williams et al., 1997). By culturing HSCs in these cytokines, a subpopulation of IL-2 receptorβ-chain (IL-2R)-expressing cells that presumably were NKPs were identified ((Mrozek et al., 1996; Williams et al., 1997). Human NKPs have not been fully characterized *ex vivo*; however, the first IL-2Rβ-expressing cells that emerge following culture of human $CD34^+$ HSCs with C-kit ligand or FLT3L have been shown to express CD38 and transcripts for IL-15R , and are negative for CD7, CD16, CD56 and several NK cell receptors (Yu et al., 1998). The molecular and cellular map of the maturation of NKPs to NK cells is largely incomplete. What is known is that during this process NK cells establish a characteristic cell surface phenotype and the capacity to elicit effector functions. Most attempts to characterize intermediates in NK-cell development have depended on markers of mature NK cells, including NK1.1, DX5 and

Ly49 in mice, and CD161, CD56, CD16 and KIRs in humans. Careful phenotypic and functional analyses of rare immature NK cells isolated from fetal liver, cord blood and bone marrow, or those that develop *in vitro* from HSCs (Mrozek et al., 1996; Rosmaraki et al., 2001, Bennett et al., 1996; Miller et al., 1994) support a linear model of NK cell maturation (Figure 2).

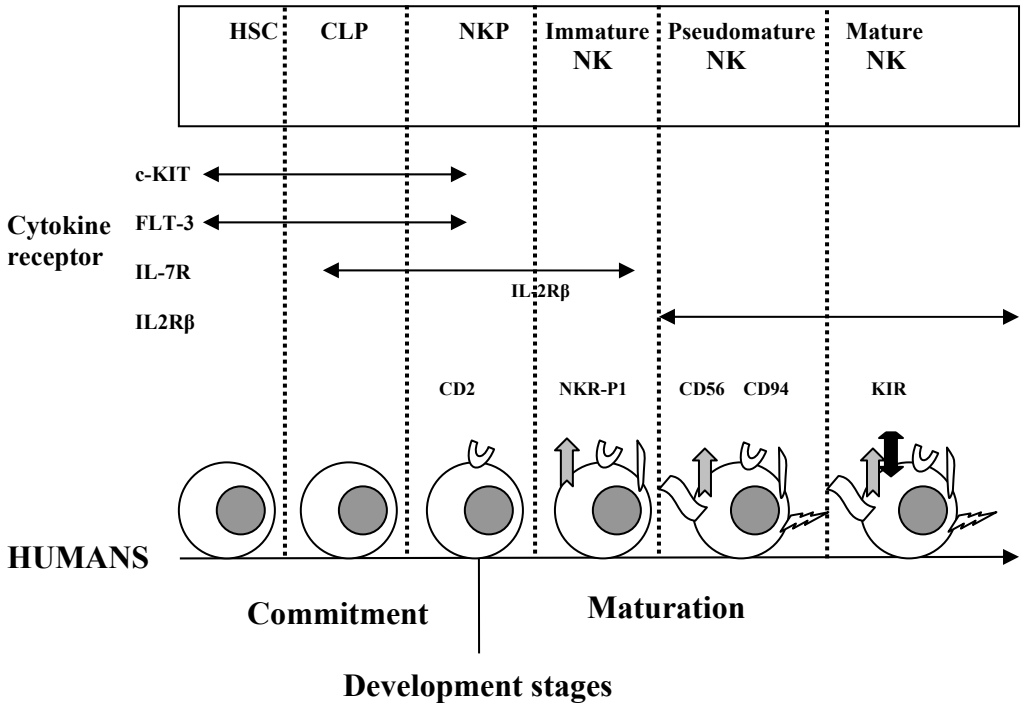

Figure 2: A working model for NK cell development (modified from Colucci et al., 2003).

4.5 NK cell recognition

Mature NK cells mainly have an ability to recognize their target cells by major histocompatibility complex (MHC) class I alleles. Initially, Ljunggren and Karre proposed the "missing-self" hypothesis (Ljunggren et al., 1990) (Figure 3). This hypothesis states that cells that express host MHC class I alleles are protected from NK cells; however, if a target cell fails to express MHC class I molecules then NK cells kill this cell. The "missing-self" hypothesis is supported by findings that tumour cells or virally infected cells that have decreased expression of MHC class I molecules become susceptible to NK cell killing (Karre et al., 1986; Tay et al., 1998). An outcome of this idea is that healthy allogeneic cells are targets for NK cells because they also lack self-MHC alleles (Yu et al., 1992).

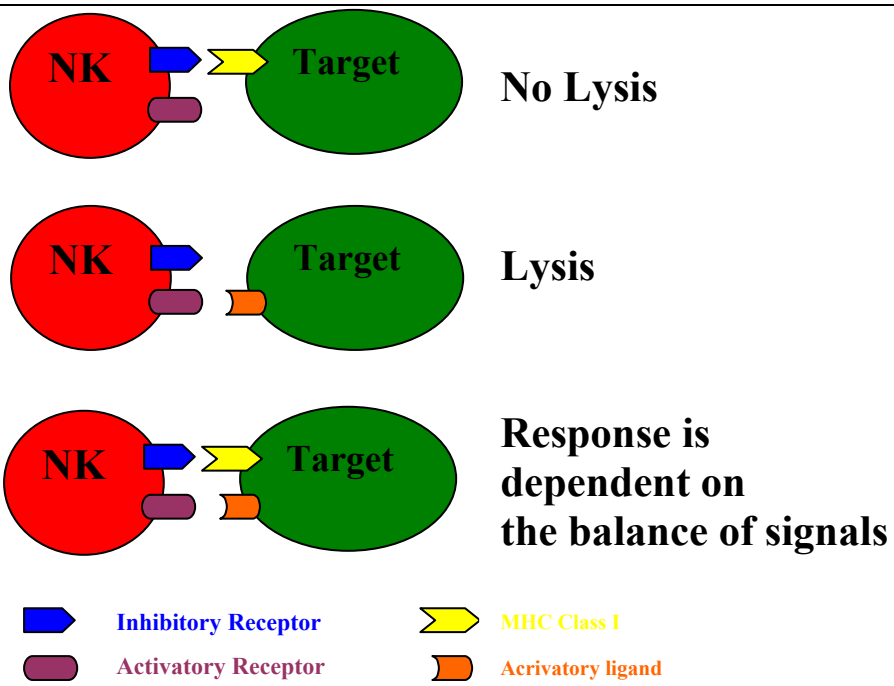

Figure 3. The 'missing-self' hypothesis.

In humans NK cells express receptors for MHC class I molecules which exert an inhibitory effect on cell-mediated cytotoxicity. These surface molecules, belonging to the immunoglobulin superfamily, have been termed killer-cell inhibitory receptors (KIRs). Two distinct KIR families have been described: a) KIRs with Ig-like domains, recognizing HLA-A, B and C alleles; and b) the CD94/NKG2A subtype, with a lectin domain, recognizing peptides related to the HLA-E class I system (Braud et al., 1998). In addition, receptors important in mediating activating signals in NK cells include the natural cytotoxicity receptors (NCRs), whose ligands are unknown, and the C-type lectin receptor NKG2D, which recognizes a number of MHC class I–like ligands that are induced or upregulated on target cells during “stress” or neoplastic transformation.

The family of receptors best described on human NK cells is the MHC Class I–specific paired activating and inhibitory KIRs. Appreciation of the function of KIR and their ligands has had important application in immunotherapy associated with mismatched HSC transplantation. Structurally, KIRs are characterized by either 2 Ig-like (KIR2D) or 3 Ig-like (KIR3D) extracellular domains that specifically recognize groups of MHC class I alleles. KIR can be further subdivided into those with long cytoplasmic tails (KIR2DL and KIR3DL) or short cytoplasmic tails (KIR2DS and KIR3DS). Although each set of paired activating and inhibitory

KIR has an identical extracellular domain and therefore binds to identical ligands, functional differences reside in alternative intracellular motifs associated with the cytoplasmic tails. The long-tailed receptors mediate an inhibitory signal due to the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domains. On the other hand, the short tailed receptors are associated with activating signals owing to their association with adaptor proteins bearing immunoreceptor tyrosine-based activating motifs (ITAM). HLA-C is the main ligand recognized by paired inhibitory and activating KIRs
(Table 1).

The paired inhibitory and activating C-type lectin receptors exist as disulfidebonded heterodimers of an invariant common subunit, CD94, linked to a distinct glycoprotein encoded by a gene of the *NKG2* family (Lazetic et al., 1996; Carretero et al., 1997; Brooks et al., 1997). CD94 is a product of a single nonpolymorphic gene and lacks a cytoplasmic domain for intrinsic signal transduction (Chang et al., 1995). The extracellular and cytoplasmic domains of NKG2 molecules determine the functional specificity of the receptor. As shown in Table 1, only CD94/ NKG2A (and its splice variant NKG2B) is inhibitory, whereas the other heterodimers of this family, including CD94/NKG2C and CD94/NKG2E (and its splice variant NKG2H) are activating receptors. The CD94/NKG2A/B and CD94/NKG2C receptors specifically recognize the nonclassic class I MHC molecule HLA-E, which is loaded with leader peptides derived from the signal sequences of classic class I MHC molecules HLA-A, -B, and -C (Braud et al., 1998; Borrego et al., 1998).

The significance of the existence of paired inhibitory and activating receptors for MHC class I remains unclear. In the cases of KIR and CD94/NKG2D receptors, the affinity of the activating receptor is lower than that of the corresponding inhibitory receptor (Vales-Gomez et al., 1998), ensuring a predominance of the inhibitory signal when both activating and inhibitory receptors recognizing HLA molecules are expressed on the same NK cells. However, only a minority of NK cell clones express both activating and inhibitory isoforms that recognize the same HLA allotypes (Valiante et al., 1997; Uhrberg et al., 1997). More commonly, NK cell clones expressing an activating receptor coexpress at least one inhibitory receptor specific for a different HLA class I allele, which can be either a KIR or a CD94/NKG2 molecule. Therefore the MHC class I–specific activating receptors may function to detect altered class I expression on cells (Biassoni et al., 2001).

Receptors			
	Inhibitory	Activating	
Killer Immunoglobulin-like receptors (KIR)	KIR2DL1	KIR2DS1	Group 2 HLA-C Asn77Lys80
	KIR2DL2	KIR2DS2	Group 1 HLA-C Ser77Asn80
	KIR2DL3		
		KIR2DL4†	HLA-G
		KIR2DS4	Unknown
	KIR2DL5	KIR2DS5	Unknown
		KIR3DS1	Unknown
	KIR3DL1		HLA-Bw4
	KIR3DL2		HLA-A3, -A11
	KIR2DL7		Unknown
C-Type lectin receptors	CD94/NKG2A/B‡	CD94/NKG2C	HLA-E (loaded with HLA-A, -B, -C leader peptides)
		CD94/NKG2B/H‡	Unknown

* Asn indicates asparagine; Lys, lysine.
† Although KIR"DL4 ha immunoreceptor tyrosine based inhibitory motifs in its long cytoplasmic tail, it is functionally an activating KIR, and upon ligation with its ligand induces cytokine secretion of Interferon-gamma without inducing cytotoxicity.
‡NKG2a/NKG2B, and NKG2B/NKG2H are splice variants.

Table 1. Paired inhibitory and activating natural killer receptors (Farag et al., 2003).

Although the activating KIR and CD95/NKG2 receptors may be important in mediating NK cytotoxicity in an MHC Class I–specific manner against MHC class I–bearing cells, other activating receptors may be important in mediating cytotoxicity against abnormal, MHC class I–deficient, or class I–negative targets. In humans, these receptors include the NCRs and the homodimeric NKG2D receptor (Bauer et al., 1999). The NCRs are Ig-like activating receptors that have been implicated in NK cell recognition and lysis of tumour cells (Pessimo et al., 1998; Pende et al., 1999; Cantoni et al., 1999). Three NCR have been described, two of which (NKp46 (Sivori et al., 1997), and NKp30 (Pende et al., 1999) are constitutively expressed on all peripheral blood NK cells. A third, NKp44, is only expressed on activated NK cells (Vitale et al., 1998). The ligands for these receptors are unknown, although target tumour cells appear to differ significantly in expression (Pende et al., 2001).

NKG2D is the best characterized activating receptor described on NK cells. The ligands for the NKG2D receptor belong to 2 distinct families, the polymorphic MHC class I chain-related (MIC) peptides, MICA and MICB, and the human cytomegalovirus UL16 binding proteins (ULBP-1, -2, and -3) (Biassoni et al., 2001; Bahram et al., 2000). These ligands are not generally expressed on normal cells, but their expression can be induced by certain noxious stimuli, infections, and neoplastic transformation.

4.6 NK cell killing

Perforin/granzyme-mediated apoptosis is the principal pathway used by NK cells to eliminate virus-infected or transformed cells (Trapani et al., 2000). Studies in perforin-deficient mice have revealed that this protein is required for most NK cell cytotoxicity (Kagi et al., 1994) (Figure 4a).

NK cells can express at least three TNF- family ligands FAS ligand (FASL), TNF and TNF-related apoptosis inducing ligand (TRAIL) which have all been shown to induce tumour cell apoptosis. Sensitivity to each of these ligands is controlled by several parameters, such as cancer-cell expression of the receptors for these ligands and intracellular protective mechanisms against apoptosis (Figure 4b). NK cells that express TRAIL kill tumour cells that are sensitive to recombinant TRAIL *in vitro* (Kayagaki et al., 1999; Takeda et al., 2001). IFN-γ-mediated TRAIL induction on NK cells was also reported (Smyth et al., 2001a). NK cell expression of FASL also contributes to the suppression of tumour growth *in vivo* (Bradley et al., 1998). Many tumour cells do not normally express FAS, but NK cells directly cause cancer cells to upregulate FAS expression and then kill them in a FAS-dependent manner (Screpanti et al., 2001).

In addition, NK cells secrete a number of effector cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-5, IL-10 and IL-13. Although NK cell secretion of IFN-γ is involved in the suppression of viruses and other pathogens (Biron, 1999), recent evidence indicates that IFN-γ also contributes to effector functions against tumour metastases and sarcoma induction (Street et al., 2001) (Fig. 4c). Cytokine activation by means of IL-2, IL-12, CD16 ligation or exposure to tumour cells is also associated with nitric oxide (NO) production, and inhibition of NO

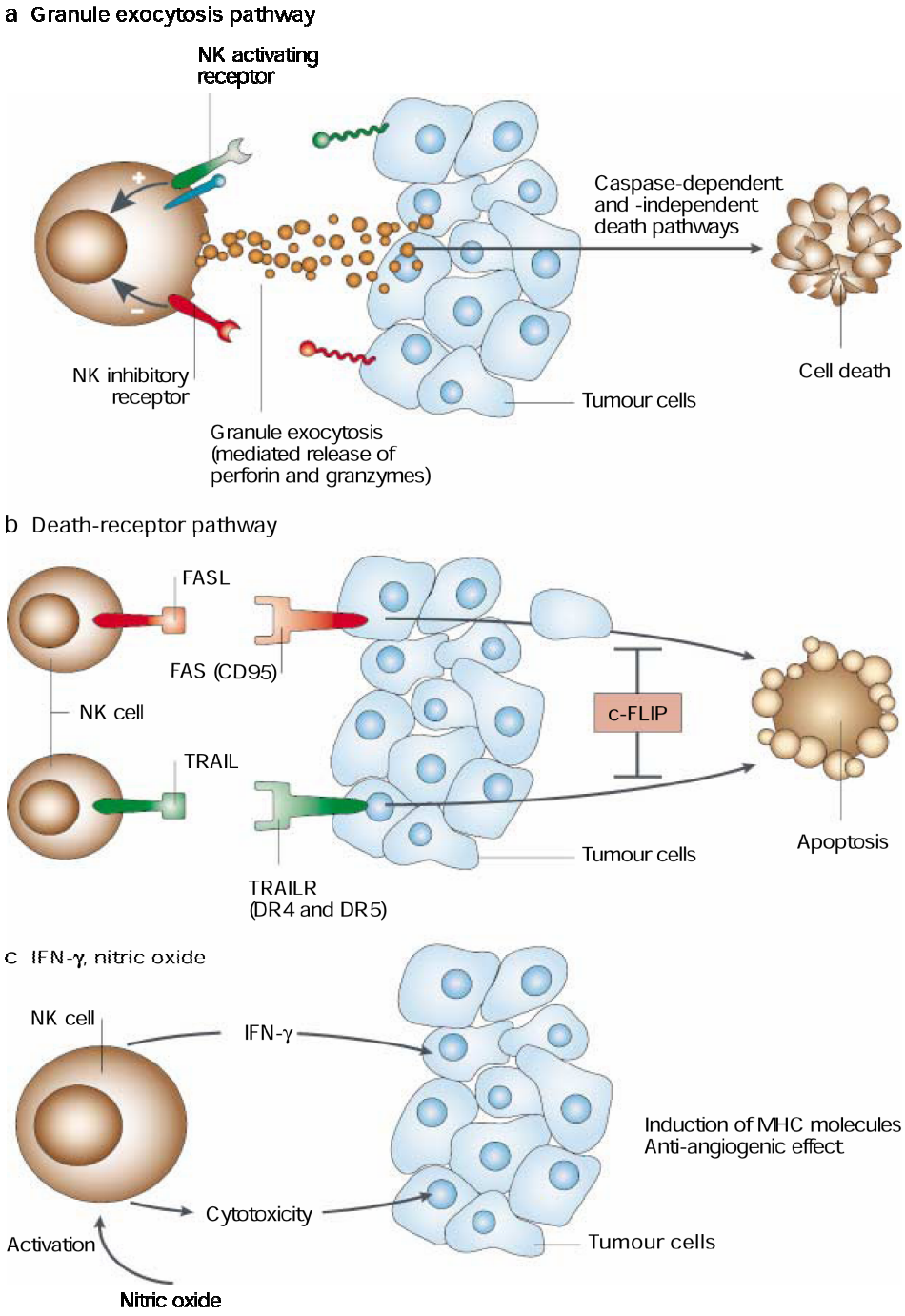

Figure 4. Schematic illustration of effector functions of NK cells that control tumour cells. NK cells can use the perforin/granzyme-containing granule exocytosis pathway (**a**), the death-receptor-ligand pathway (**b**) or the nitric oxide pathway **(c)** to kill tumour cells (Reproduced with permission from Nature Reviews Cancer (Smyth MJ et al., Nat Rev Cancer 2002, Nov;2 (1):850-861) copyright (2002) Macmillan Magazines Ltd.).

synthesis impairs NK cell activation, IFN-γ production and NK cell-mediated killing (Cifone et al., 1999; Furuke et al., 1999).

NK cells also mediate antibody-dependent cellular cytotoxicity (ADCC) of targets through FcγRIII (CD16), a receptor that binds the Fc portion of antibody (Lotzova et al., 1987; Oshimi et al., 1986; Landay et al., 1987). The alpha subunit of CD16, which binds to the Fc portion of IgG molecules, associates noncovalently with the signal transducing molecules CD3ζ and FcεRI-γ (Leibson et al., 1997). It is thought that antigen density and structure, as well as the isotype specificity of Fc binding, all contribute to the induction of ADCC (Hooijiberg et al., 1996). Addition of Trastuzumab (Herceptin), a humanized antibody against HER2/neu (Lewis et al., 1993) to normal resting NK cells without IL-2 activation significantly enhanced killing of all breast cancer targets (Cooley et al., 1999).

Supporting this therapy, a recent study has shown ADCC to be the mechanism of tumour lysis in a breast cancer model *in vivo* (Clynes et al., 2000). The use of monoclonal antibodies in combination with NK cells may therefore increase their specificity other than by mediating ADCC.

4.7 NK cells in cancer

NK cells were originally defined as fresh-isolated white blood cells that are capable of lysing certain tumour targets (Kiessling et al., 1975). Activation of NK cells with IL-2 or IFN-αβ further enhances their cytolytic function, resulting in killing of a broad array of other tumour targets that are not lysed by resting NK cells (Henney et al., 1981). IL-2 activated lymphocytes with the ability to kill tumour cells were initially designated "lymphokine-activated killer" (LAK) cells (Grimm et al., 1982). However, it was subsequently demonstrated that almost all of this activity was mediated by activated NK cells (Phillips et al., 1986). Numerous studies have also shown that NK cells preferentially kill MHC class I–deficient tumour cells *in vitro* and can reject class I–deficient tumours *in vivo* (Ljunggren et al., 1985). This may be relevant to human cancer, in that human tumours apparently frequently show loss of expression of MHC class I antigens on the cell surface (Algarra et al., 2000).

NK cells are frequently able to prevent outgrowth of small numbers of tumours, but this can be overwhelmed by using large tumour challenges. Depending on the tumour, administration of factors that recruit or activate NK cells may show therapeutic benefit. NK cells do not have the

capacity for "memory" and, therefore, on a secondary exposure to a tumour that has been eliminated by NK cells, there is no accelerated response or a larger pool of effector cells than on the first encounter. There is an emerging concept that although NK cells alone may be insufficient to totally eliminate established tumours, they may kill some tumour cells, providing apoptotic tumour bodies to dendritic cells for priming of a subsequent T cell response. In addition, IFN-γ or other cytokines produced by NK cells in response to tumours may activate local macrophages and dendritic cells, which reciprocally stimulate NK cells through cell–cell contact or soluble factors (Ferlazzo et al., 2002; Fernandez et al., 1999; Gerosa et al., 2002; Pawlowska et al., 2001; Peron et al., 1998; Piccioli et al., 2002).

Although IL-2 activated NK cells have the ability to kill many types of tumours *in vitro*, the more relevant question is if this happens *in vivo*? A role for NK cells in protection against tumour cells *in vivo* in experimental animal models has often relied on experiments in which participation by B and T cells has been excluded by use of SCID mice or RAG1−/− or RAG2−/− immunodeficient mice and through the depletion of NK cells by using anti-NK1.1 mAb or an antisera against asialoGM-1 (reviewed in Smyth et al., 2002). In humans, activated human NK cells posses the capacity to secrete cytokines and kill tumour cells, including autologous tumours. Most of the clinical trials that have been performed with adoptively transferred activated NK cells have been performed in terminal patients with extensive tumour burdens. Therapeutic manipulation of NK cells for cancer treatment may be therefore more realistic in an adjuvant setting with minimal residual disease.

Numerous studies have been conducted, and are still ongoing to improve the antitumour effect of NK cells. These include endogenous activation of the patient's own NK cells through administration of cytokines (Maraninchi et al., 1998; Chang et al., 2001; Mulatero et al., 2001) or through adoptive use of *ex vivo* expanded autologous (Benyunes et al., 1993; Hayes et al., 1995) or donor-derived (Rosenberg et al., 1993), lymphokine-activated killer (LAK) cells. Some success has been demonstrated for patients with renal cell carcinoma and malignant melanoma (Rosenberg et al., 1993, Margolin et al., 2000) as well as in patients with lung and hepatic cancers (Semino et al., 1999). The mechanism of antitumour efficacy of IL-2 is closely related to its ability to expand and activate NK and T cells that express IL-2 receptors. Affinity columns and new culture conditions have been developed to obtain more highly purified *ex vivo* populations of NK cells, and a recent study describes the use of enriched NK cells to treat patients with breast cancer (Burns et al., 2000). Unfortunately, the efficacy of IL-2 has been offset by accompanying toxicities that are mediated by cytokines and other small molecules that

are secreted by IL-2-activated effector cells. Attempts to reduce IL-2-mediated toxicity have been disappointing. Adoptive immunotherapy of cancer with regional or systemic administration of autologous NK cells is still in an experimental phase, as the cells are difficult to expand and gene-modify and localize poorly to tumour sites after culture.

4.7.1 Role of NK cells in autologous transplantation

Although high-dose chemotherapy with or without total body irradiation and supported by autologous stem cell transplantation may be able to induce long-term survival in a certain percentage of patients, relapse continues to be a major problem in autologous transplantation settings. The immunotherapeutic approaches for decreasing the potential for post-transplantation relapse after both autologous in specific areas of cytokine-based immunotherapy, donor lymphocyte infusions and novel cellular therapies with T- and NK cells therefore needs to be further investigated (Table 2).

Cytokine-Based
Interferon
IL-2
Interferon plus IL-2
IL-2 *ex vivo* purging with IL-2 posttransplantation
Autologous GVHD with cyclosporine
Cellular-Based
IL-2 induced LAK cells
Autologous *ex vivo* activated/expanded NK cells
Specific T cells
Autologous NKT cells

Table 2. Immunotherapy after autologous stem cell transplantation

In earlier studies, IL-2 with or without LAK cells represented a potentially non–cross-resistant treatment modality for preventing or delaying relapses if used as consolidative immunotherapy after autologous SCT and at a time of minimal residual disease. The feasibility of generating IL-2–induced LAK cells early after autologous bone marrow transplantation treatment for AML with promising results has been reported (Fefer et al., 1993). This therapeutic approach was

difficult for patients to tolerate because the administration of IL-2 soon after transplantation had side-effects and the repeated leukaphereses could induce severe thrombocytopenia (Benyunus et al., 1993). Other investigators have generated IL-2–activated blood mononuclear cells pre-transplantation and then infused them with the transplant, followed by injections of IL-2 to support the activity of the infused cells (Klingemann et al., 2000; Lister et al., 1995). Some other investigators have also explored the administration of IL-2 with IL-2-activated NK cells or LAK cells in the post-autologous setting (Benyunes et al., 1993 and 1995; Lister et al., 1995, Mazumder et al., 1997). However, many patients who achieve a complete remission following autologous transplantation will relapse and die of their disease. Most patients relapse at sites of previous bulk disease, suggesting that one reason for treatment failure is inability to eradicate chemotherapy-resistant clonogenic tumour. An inadequately functioning immune system may also contribute to early relapse. Specific, and better cell based approaches are therefore needed post-autologous transplantation. In addition, the feasibility and possibility of using different killer cell populations that use different recognition and killing mechanisms remain to be investigated.

4.7.2 Role of NK cells in allogeneic transplantation

Allogeneic stem cell transplantation is essentially a form of adoptive immunotherapy. The recognition of major or minor histocompatibility differences by donor lymphocytes can induce an alloimmune response which is predominantly mediated by T cells. The immune response leads to a release of cytokines that can further support the clonal expansion of T cells but that can also stimulate secondary effector cells such as NK cells, monocytes, and eosinophils, all of which can have antitumour effects. NK cells have not been given much attention until recently. Clinical studies using pure long-term expanded NK cells have not been performed to date, and most studies have used LAK cell preparations which only contain a small fraction of NK cells. However, newer separation and expansion technologies may make it possible to provide activated NK cells to patients with tumour. The immunotherapeutic approaches for decreasing the potential of post-transplantation relapse after allogeneic transplants in specific areas of cytokine-based immunotherapy, donor lymphocyte infusions, and novel cellular therapies with T- and NK cells also need to be further investigated (Table 3).

Cytokine-Based
Interferon
IL-2
IL-2 after donor lymphocyte infusion
Cellular-Based
Donor lymphocyte infusion
Selective CD8-depleted donor lymphocyte infusion
Allogeneic NK cells

Table 3. Immunotherapy after allogeneic stem cell transplantation

In human HSCT, several studies of HLA matching have suggested a role for NK cells in mediating complications of this procedure. The predominance of NK cells in the peripheral blood of HSCT recipients in the early stages of immune reconstitution, coupled with their immediate functional capacity, makes this particular lymphocyte subset a highly relevant factor in the successful outcome of HSCT. *In vivo* preclinical data indicated that infusion of allogeneic *in vitro* IL-2–activated NK cells provided a strong antileukemia effect in a syngeneic mouse leukemia model (Zeis et al., 1997). Mice that received allogeneic NK cell infusions 2 h after an allogeneic BMT to treat an established syngeneic leukemia displayed a survival rate of over 90% without graft-versus-host disease (GVHD). Syngeneic NK cells had an inferior antileukemia effect with about 50% of mice surviving, whereas 85% of mice given an allogeneic BMT without the addition of NK cells died from severe GVHD. This positive effect of NK cells in decreasing GVHD was confirmed in another study (Asai et al., 1998), in which GVHD was additionally confirmed histologically and determined to be dependent on TGF because anti-TGF antibodies eliminated the NK protective effect.

Several clues can be drawn from clinical studies that did not specifically look at NK cells but their setting allows for useful comparisons regarding the role of NK cells. A recent nonrandomized study examined the effect of using narrow specificity antibodies for T cell depletion of bone marrow grafts compared with broad specificity ones and with undepleted grafts (Champlin et al., 2000). The study included unrelated or mismatched related transplant recipients with CML or acute leukemias. Transplants depleted with narrow specificity antibodies produced a similar leukemia relapse rate and treatment failure rate after 5 years compared with non-T cell depleted transplants. In contrast, broad specificity antibody depletion

resulted in higher relapse and treatment failure rates. Acute GVHD was decreased through both methods of depletion whereas chronic GVHD was unaffected by broad specificity depletion but was marginally increased when depletion with narrow specificity antibodies was performed. Although this was a retrospective study, when NK cells are removed with the use of broad specificity antibodies, relapse rates are higher, giving evidence for the importance of NK cells in the GVL effect.

A second line of evidence comes from studies comparing related and unrelated allogeneic BMT. In this case NK cells in grafts from unrelated donors would be expected more often to display inhibitory receptors for the HLA-C molecules that are not ligated by the recipient's HLA-C alleles than grafts from related donors. An earlier report compared 78 patients with CML transplanted from an HLA identical sibling or an unrelated donor (Hessner et al., 1995). A statistically significant lower relapse rate of 8% was noted in patients with chronic phase disease who received unrelated grafts compared with sibling grafts in whom the relapse rate was 47%. As NK cells are expected to be preserved in the graft due to the depletion technique used, the GVL effect observed was most probably due to them. In contrast, as no KIR mismatch is present in HLA-identical siblings, no NK against leukaemia would be present.

4.7.2.1 GVHD and NK cells

The occurrence of GVHD after allogeneic transplantation is a significant cause of morbidity. The pathophysiology of acute GVHD has recently been summarized (Ferrara et al., 2005) as a three-step process that involves: activation of host antigen-presenting-cells (APC) and translocation of bacterial lipopolysaccharide (LPS) as a result of conditioning regimens; donor T cell activation with attainment of IFN-γ secretion capacity through “direct” interaction with alloantigen expressed on host APC; induction of a pro-inflammatory “cytokine storm” and further T cell effector function, including cytotoxicity through perforin- and fas-mediated pathways. Donor T cells present in the graft mediate GVT responses but are also able to initiate GVHD after recognizing the host as foreign (through both major and minor histocompatibility differences), and then they attack the host. Because NK cells do not directly induce solid tissue destruction, these cells may be suitable mediators of GVT without exacerbating GVHD.

The contribution of NK cells in the control of tumour growth without causing GVHD is well established in mice. Zeis *et al* were able to induce antileukemia effects after BMT without inducing GVHD by infusing allogeneic donor-derived IL-2-activated NK cells in syngeneic

mice bearing leukaemia (Zeis et al., 1994). In addition, recent studies have generated a tremendous amount of interest in the role of NK cells in GVHD (Farag et al., 2002). Since NK cells are negatively regulated by MHC class I-specific inhibitory receptors, HLA mismatched transplants may trigger donor NK-mediated alloreactivity (Murphy et al., 2001). In murine models of BMT, infusion of donor NK cells can reduce GVHD, probably through the elimination of host APCs (Ruggeri et al., 2002), or through the secretion of TGF-β (Asai et al., 1998). The timing of NK cell administration also seems to be critical for the control of GVHD in murine models. Preclinical studies indicate that NK cells must be given early after BMT before the amplification of GVHD occurs. If the administration of NK cells is delayed after the initiation of GVHD by the donor T cells, the incidence and severity of GVHD will increase. This is consistent with the detection of NK cells in GVHD lesions and with the finding that administration of cytokines such as IL-2 and IL-12 can prevent GVHD when given early but augment it when given late after BMT. Once GVHD is active and ongoing, NK cells can intensify GVHD and participate in GVHD pathology, possibly through the release of inflammatory cytokines (Sykes et al., 1995). IL-2-induced GVHD protection is not inhibited by cyclosporine and is maximal when IL-2 is given during a 25 h period beginning on the day following bone marrow transplantation (Murphy et al., 1992).

4.7.3 Role of NK cells in haploidentical transplantation

In haploidentical transplantation, alloreactivity is mediated by NK cells through mismatches between KIRs on donor NK cells and HLA class I molecules on recipient cells. This is known as donor-vs-recipient NK cell alloreactivity.

As KIRs are distributed clonally, the NK cell population in each individual constitutes a repertoire of NK cells; each bears a different receptor (or different receptor combinations) and they therefore have different allospecificities. Some NK cells in the repertoire will mediate alloreactions when the mismatched allogeneic target cells do not express a class I allele group that blocks them (Figure 5).

In HLA-haplotype-mismatched haematopoietic transplantation with a potential for GVH NK-mediated reactions, the engrafted stem cells give rise to an NK cell wave of donor origin that regenerates the same repertoire as the donors, and so includes high frequencies of donor-vs-recipient alloreactive NK cells (Ruggeri et al., 1999). Donor-vs-recipient NK cell alloreactivity dramatically reduced the risk of leukaemia relapse in 57 acute myeloid leukaemia (AML)

patients at high risk of relapse, while improving engraftment and protecting against GVHD (Ruggeri et al., 2002; Velardi et al., 2002). Given such a powerful donor-vs-recipient immune reaction, why do NK cells not mediate severe GVHD? Experimental evidence has suggested that NK cells predominantly attack the haematopoietic cells of the host but not other tissues which are common targets for T cell-mediated GVH disease. Murine alloreactive NK cells, even when infused in large numbers, do not cause GVHD in the mouse (Ruggeri et al., 2002). In contrast, in the same model allogeneic T cells kill all mice within 2 weeks. Interestingly, alloreactive NK cells kill host type dendritic cells (DCs) (Ruggeri et al., 2002), thus preventing presentation of host antigens to graft T cells, i.e. the crucial step that initiates GVH reactions (Shlomchik et al., 1999). In the mouse model, alloreactive NK cells therefore not only fail to cause GVHD but also block T cell-mediated GVHD. Indeed, alloreactive NK cells administered to recipient mice during pre-transplant conditioning protected from GVHD to such an extent as to allow a safe infusion of otherwise lethal doses of allogeneic T cells (Ruggeri et al., 2002).

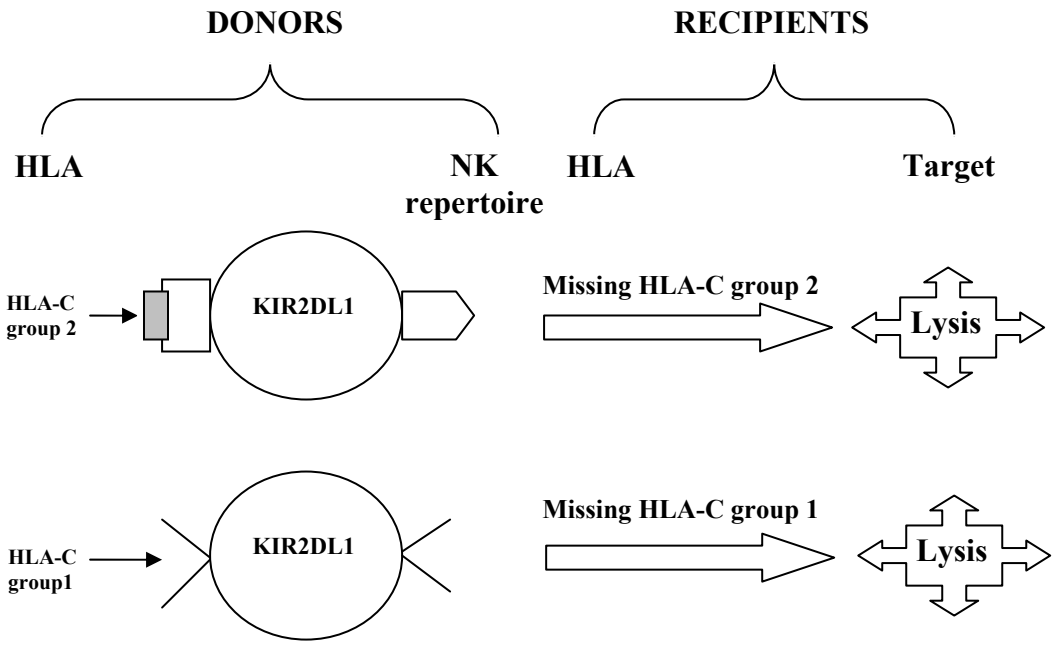

Figure 5. Donor-vs-recipient NK cell alloreactivity

Thus NK alloreactivity combines all the features that make it uniquely suited for transplantation. As depicted in Figure 6, alloreactive NK cells eradicate leukaemia, favour engraftment by killing host lympho-haematopoietic cells, and reduce GVHD by eliminating host-type DCs. One consequence of these studies is the need to exploit these results rapidly by revising current criteria for haploidentical donor selection. Donor selection for AML now

involves a search for the donor who is able to mount donor-vs-recipient NK cell alloreactivity (Karre et al., 2002).

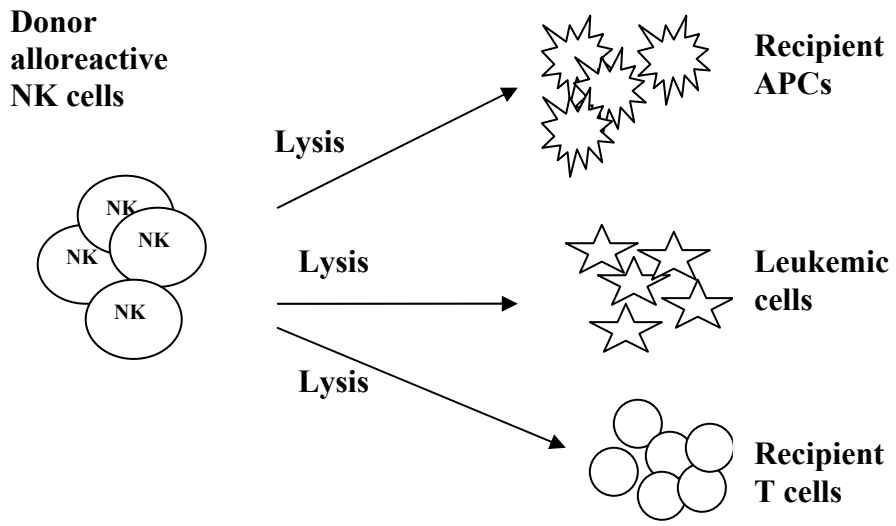

Figure 6. Summary of data from preclinical murine models and clinical transplantation.

4.7.4 Role of NK cells in solid tumour transplantation

The relevance of NK cell repertoires in the transplantation of allogeneic vascularized organ grafts has not been established but has not yet been investigated in depth (Bishara et al., 2001; Oertel et al., 2001). Although NK cells are traditionally thought not to be involved in solid organ graft rejection, results from experimental animal models have suggested a contributory role for NK cells in organ rejection in the absence of T cell responses (Maier et al., 2001). A novel and intriguing proposal is that NK cells may be directly involved in the processes of chronic vascular rejection (Russell et al., 2001). NK cells are known to activate vascular endothelial cells, and therefore the potential exists for genetic NK repertoire differences to influence this pathological process in recipients of solid organ transplants (Ayalon et al., 1998). The highly polymorphic MIC antigens are expressed in donor organs (Hankey et al., 2002), presumably as a result of inflammation associated with the transplant process, and can stimulate specific antibody production in mismatched recipients. These MIC-specific antibodies are associated with early graft rejection (Sumitran-Holgersson et al., 2002). The role of cellular NKG2D recognition in graft rejection has not been addressed as yet but must be regarded as a

potential contributory factor in view of the level of molecular diversity of both MIC and other NKG2D ligands.

4.7.5 Role of NK cells in xenotransplantation

Although the use of nonhuman donor organs remains a topic of interest in the transplant community, it has become increasingly apparent that abrogation of hyperacute rejection mechanisms by genetically modifying donor animals will not be sufficient to ensure long-term survival of such organs in transplant recipients. NK cells contribute to mechanisms of delayed xenograft rejection through direct recognition of xenogeneic cells (Xu et al., 2002), antibody-dependent cytotoxicity and procoagulant activity mediated through interaction with xenogeneic endothelial cells (Dawson et al., 2000). Whether these obstacles can be overcome through further genetic modulation of potential donor species with human histocompatibility molecules remains an area of relevant scientific interest (Forte et al., 2000).

4.8 NK cells in viral infections

The importance of NK cells in immunity against viruses is well documented in humans and experimental animals. NK cells have been implicated directly in host resistance to mouse cytomegalovirus (MCMV). Antibody depletion of NK cells resulted in elevated levels of viral titers in susceptible mouse strains, whereas adoptive transfer of NK cells provided protection against MCMV infection (Bukowski et al., 1985). Similar to certain tumours, some viruses (including CMV) downregulate MHC class I molecules on infected cells, leading to impairment of $CD8^+$ CTL effector function (Tortorella et al., 2000). In principle, the virus-infected cells that downregulated expression of MHC class I may be more easily killed by NK cells because the inhibitory MHC receptors on NK cells were no longer engaged (Cerwenka et al., 2001). Another activating NK receptor, NKp46, which was initially discovered because of its role in NK cell killing of human tumours (Pessino et al., 1998), has been claimed to recognize and kill influenza virus–infected cells (Mandelboim et al., 2001). Some other experiments have also implicated NK cells in Epstein-Barr virus (EBV) infection. Another activating NK receptor, CD244/2B4 signals by associating with a cytoplasmic adapter molecule (Tangye et al., 1999) called the signaling lymphocyte activation molecule-associated protein (SAP) (Sayos et al., 1998). Patients who have X-linked lymphoproliferative syndrome were found to have a defective SAP (Sayos et al., 1998), and the NK cells from these patients could not be activated through CD244 (Nakajima et al., 2000; Parolini et al., 2000). Intriguingly, these patients are highly susceptible to EBV infection. They often develop fulminating and fatal infectious

mononucleosis or B lymphomas, indicating that NK cells may play a role in controlling EBV infection. NK cells have also been implicated in immunity against another Herpesvirus—human herpesvirus 8 (HHV8)—the virus causing Kaposi's sarcoma. HHV8 encodes viral proteins that block expression of MHC class I, but that also downregulate other cell surface proteins such as CD54 (ICAM-1), that potentiate NK cell recognition and activation (Coscoy et al., 2000 and 2001; Ishido et al., 2000). A recent study has reported that NK cell function in HIV-infected patients inversely correlates with HHV8 viremia and that response to highly active antiretroviral therapy resulted in enhanced NK cell activity and control of HHV8 (Sirianni et al., 2002). Collectively, these studies indicate that NK cells may actively participate in immunity against viruses that cause cancer, such as EBV and HHV8, and that the NK cell immune strategies and receptors that have evolved to primarily deal with virus infections are applicable to the control of transformed cells.

CMV and EBV infections are particularly prevalent in the immediate post-transplant period, especially in recipients of T cell-depleted HSCT. At the initiation of the innate immune response to viral infection, monocyte-derived cytokines (IL-1, IL-12, IL-15 and IL-18) activate $CD56^{bright}$ NK cells which in turn secrete high levels of IFN-γ, IL-10, IL-13, macrophage colony-stimulating factor (G-CSF) and TNF-α. The relative concentrations of the NK-derived cytokines will influence the local T helper cell response and thus the nature of the acquired immune response (Cooper et al., 2001b). This close interrelationship between NK cells and T cells in the generation of anti-viral immunity appears to be an absolute requirement for the generation of a primary immune response, as mice depleted of NK cells *in vivo* have been shown to be incapable of generating CD8 T cell immunity to influenza virus (Kos et al., 1996). While IFN-γ has direct anti-viral activity, it also activates lytic NK cells which may target virally infected cells. The importance of NK cell lysis in CMV infection is demonstrated by the fact that some isolates of CMV have evolved mechanisms to downregulate LFA-3 expression on infected cells to render them non-susceptible to NK cell-mediated lysis (Fletcher et al., 1998).

4.9 *Ex vivo* NK cell expansion

4.9.1 NK cell expansion from bone marrow stem cells

The common B/T/NK lymphoid progenitor has been identified in human fetal liver, cord blood and bone marrow in both $CD34^+CD38^+$ and $CD34^+CD38^-$ cell populations. Differentiation of these early $CD34^+$ progenitors into functional NK cells was initially reported to require both the presence of human stromal cells and IL-2 (Lotzova et al., 1993; Miller et al., 1992). Since then, a stromal cell derived cytokine has been characterized as IL-15 (Tagaya et al., 1996). IL-15 has emerged as a key regulator of both NK cell differentiation and activation (Puzanov et al., 1996), displaying a similar effect to IL-2 on the different properties of NK cells. Availability of this cytokine has greatly facilitated the generation of NK cells *in vitro,* as recently demonstrated for thymic and fetal liver precursors (Mingari et al., 1997; Jaleco et al., 1997). Miller *et al* demonstrated that culture of $CD34^+DR^-$ HPCs in the presence of an allogeneic irradiated BM stromal cell layer and IL-2 led to 147-fold expansion of human $CD3^-CD56^+$ NK cells (Miller et al., 1992). Silva *et al* also established a cell culture system without stromal cells that allowed the $CD34^+$ HPCs to differentiate into NK cells in the presence of IL-1α, IL-2, and c-kit ligand (Silva et al., 1994). Most recently, Shibuya *et al* showed that cultures of HPCs with KL (c-kit ligand) alone or K L and IL-3 for 7 days followed by the addition of IL-2 for 21 days resulted in generation of $CD3^-CD56^+$ NK cells (Shibuya et al., 1995). It has also been suggested that cultivation of subsets of the human $CD34^+$ population *in vitro* or transplantation into SCID mice can result in the production of human T and B lymphocytes (Baum et al., 1992). Several other investigators have cultured BM populations with or without the depletion of mature NK to obtain various activated NK populations (Hakoda et al., 1989; Denning et al., 1991). Although NK cell differentiation and expansion is feasible from bone marrow cells, this is time-consuming, expensive, and more labour-intensive way of generating NK cells for the treatment of tumours in clinical settings.

4.9.2 NK cell expansion from cord blood cells

Human umbilical cord blood (HUC-blood) or the blood remaining in the placenta after delivery of full-term neonates, known as placental blood, contains considerable amounts of hemopoietic stem and progenitor cells. Study of the NK cell biology of developmentally early blood sources such as cord blood (CB) may provide more accurate insights into the intermediate stages of NK cell development and their proliferation capacity. CB leukocytes, including NK cells, seem to be different from their adult blood source counterparts in frequency, function and phenotype

(Seki et al., 1985; Lowdell et al., 2001; Kundig et al., 1993; Noguchi et al., 1993; Nguyen et al., 1998). It has previously been shown that: (a) the majority of CB samples have less NK lytic activity than adult PB samples (Cavazzana-Calvo et al., 1996; Lodolce et al., 2001; Kennedy et al., 2000; Veiby et al., 1996; Moore et al., 1997; Broxmeyer et al., 1995; Mckenna et al., 2000); (b) IL-2 or IL-12 can increase the NK activity and induce LAK activity in CB mononuclear cells (MNC) and increase the numbers of NK cells (Cavazzana-Calvo et al., 1996; Broxmeyer et al., 1995); (c) a novel subset of $CD16^{+}56^{-}$ NK cells is present in CB (Cavazzana-Calvo et al., 1996; Lodolce et al., 2001; Yu, 1998). The $CD34^{+}$ $CD38^{-}$ subset defines a primitive cell population and interestingly this subset is fourfold higher in UCB than in bone marrow, which correlates with functional assays indicating a higher proportion of primitive haemopoietic cells in cord blood collections (Hows et al., 1992; Sutherland et al., 1989; Verfaillie et al., 1990). Because the number of UCB cells obtained from the placental umbilical cord is limited, several methods to expand and/or activate UCB-derived NK cells using various cytokines have been attempted (Condiotti et al., 2001; Lin et al., 2000). Lin *et al* first reported that the combination of IL-15 and Flt3L could synergistically expand and activate NK cells derived from UCB mononuclear cells (MNCs) (Lin et al., 2000).

4.9.3 NK cell expansion from peripheral blood cells

Due to its easy accessibility, peripheral blood cells are the first-line of cells used for NK cell expansion. NK cells can be expanded from PBMCs upon activation by cytokines, by co-culturing with cell lines (such as K562, HFWT, RPMI 8866, LCLs) or by using irradiated autologous or allogeneic PBMCs. NK cells could be expanded with different protocols by directly culturing PBMCs or by separating pure NK cells for expansion. Reports from Perussia *et al* and Silva *et al* suggested that human B lymphoblastoid cell lines and leukapheresed peripheral blood stem cell grafts were also useful for human NK cell expansion (Perussia et al., 1987; Silva et al., 1995). Sekine *et al* developed an alternative method for lymphocyte expansion from peripheral blood by cultivating cells with IL-2 and immobilized anti-CD3 monoclonal antibodies (Sekine et al., 1993). Igarashi *et al* also reported NK cell expansion from PBMCs by isolating NK cells by magnetic depletion and culturing with or without feeder cells (allogeneic EBV-LCL). After 14 days of expansion, NK cells were expanded 100 times without feeder cells, whereas 1000-fold expansion was obtained using feeder cells (Igarashi et al., 2004).

After PBMC of healthy donors on the other hand was directly cultured on irradiated Wilms tumour cell line, HFWT for 10-21 days, the lymphocytes expanded 58- to 401-fold. This NK cell expansion required direct contact of PBMC with live, but not fixed, HFWT cells. The PBMC from an end-stage brain tumour patient also expanded 156-fold, whereas those cultured with irradiated NK-sensitive K562 grew only 30.5-fold. In a recent study, $CD16^{+}CD56^{+}$ NK cells accounted for more than 70% of the population expanded on HFWT cells (Harada et al., 2002). Using the same feeder cell line, PBMCs from hepatocellular carcinoma (HCC) were cultured for 10-21 days, $CD56^{+}CD16^{+}$ cells comprising more than 50% of the cell population, and more than 80% of fresh HFWT cells were killed at an effector/target ratio of 2 during 24 h (Peng et al., 2004).

Not only the Wilms tumour cell line, but also the K562 cell line has been used for NK cells expansion where a median of 2.5-fold cell expansion was obtained. However, when the K562 cell line transfected with 4-1BBL and used as a feeder cell line, a median of 10000-fold NK cell expansion was obtained after 14 days of culturing (Imai et al., 2005). NK cell expansion was also obtained from the blood of patients with acute lymphocytic leukemia (ALL) when co-cultured with either irradiated allogeneic PBMC or the RPMI 8866 cell line. After 12 days of expansion NK cells represented 62-95% of bulk culture. NK cell expansion was about 40-fold (Torelli et al., 2002).

In addition, Perez *et al* reported NK cell expansion from healthy donor PBMCs cultured in a medium supplemented with hydrocortisone, IL-2 and IL-15. Under such conditions 100-fold expansion was obtained (Perez et al., 2005). Although the feasibility of NK cells expansion has been demonstrated with different protocols, the usage of feeder cell lines or irradiation of cells during co-culturing makes such protocols unuseful for NK cell immunotherapy in the clinic. Thus a protocol that will give selective expansion advantage to NK cells without using tumour-derived feeder cells may be useful for immunotherapy of such cells.

4.10 Clinical trials with NK cells

There have not been clinical trials with NK cells until recently, mainly due to the lack of a large-scale NK cell expansion protocol with GMP quality products. First attempts were performed with short-term IL-2 activated cells with or without external IL-2 infusions. LAK cells exhibit an unrestricted pattern of lysis against a wide spectrum of malignant cells (Fox et al., 1989; Yron et al., 1980). It has been shown that LAK cells with IL-2 could result in a better clinical response than IL-2 alone (Rosenberg et al., 1985, and 1993). For instance, out of 106 evaluable patients receiving LAK cells plus IL-2, 8 had complete responses, 15 had partial responses, and 10 had minor responses. Of 46 evaluable patients treated with high-dose IL-2 alone, 1 had a complete response, 5 had partial responses and 1 had a minor response (Rosenberg et al., 1987). Despite these positive results, the effect of LAK cells seems to be limited to some certain tumour types, since the same positive effect could not be demonstrated for other tumour types (Weiss et al., 1992; Dutcher et al., 1989; Negrier et al., 1991).

Due to limited anti-tumour activity of LAK cells and increasing knowledge of NK cell activities, scientists began to study NK cells alone. Frohn *et al* performed a clinical trial with purified NK cells without additional cytokines in patients with renal cell carcinoma. Although the approach was safe and no severe side-effects were observed, no clinical response was apparent except in 1/11 patients enrolled (Frohn et al., 2000). In a separate clinical trial with purified heat shock protein 70 (HSP-70)-activated NK cells, in 11 patients with lung cancer, only one patient had stable disease, although similar, no side effects were again experienced in the same study (Krause et al., 2004). A safe and partial effective NK treatment was shown for patients with malignant glioma and post-tranplant period for lymphoma (Ishikawa et al., 2004; Lister et al., 1995). Furthermore, after promising results with haploidentical NK cells, such cells have also been used in clinical trials. In the study of Miller *et al* IL-15 dependent *in vivo* expansion of NK cells occured in 5 out of 19 cases of AML, and complete remission was evident (Miller et al., 2005; Koehl et al., 2004). However, in none of these studies were long-term IL-2 activated NK cells infused. Thus immunotherapy with long-term cytokine activated NK cells could still be of great value in different pre- or post-transplantation settings.

4.11 Gene transfer vectors

In general, gene transfer involves the delivery of therapeutic genes into target cells. The administration of genes as therapy requires transportation vehicles, such as viral vectors, which encapsulate the gene and carry it into the target cell. Once the vector binds to the target cell membrane, internalization takes place. The administered genome is then transported into the cell nucleus, where it is integrated into the genome of the cell (retrovirus, lentivirus ext.) or remains

outside the genome as an episome in the nucleus (adenovirus), depending on the type of vector used. Every step in this process and the ultimate expression of the gene constitute potential limitations. Some characteristics of the most commonly investigated vectors are presented in Table 4.

Vector group	**Genome**	**Capacity**	**Persistence**	**Titre $\log_{10}$ IU/ml**	**Immuno-genecity**
AAV	ssDNA	4-5 kb	Integrated tandem	11	low
AdV	dsDNA	8 kb (E3) 28-32 kb (gutless)	Transient episomal	13	high
Retrovirus	+RNA	8 kb	Integrated	6-7	low
Lentivirus	+RNA	8 kb	Integrated	10	low
Liposome	-	Unlimited	Transient	-	low
Molecular conjugate	-	10 kb	Transient	-	low

Table 4. Characteristics of the most commonly used gene transfer vectors.

4.12 Gene transfer into NK cells

A long-standing goal of cancer research has been to influence the immune system to attack and eliminate tumour cells. Attempts to involve the immune system in the therapy of cancer are based on either active or passive approaches. The passive or adoptive cellular immunotherapies involve the transfer of immune competent cells into the patients. The *ex vivo* manipulations of immune effector cells (such as NK cells) has involved the transfer of genes encoding immune stimulatory or antigen-specific targeting molecules to immune competent cells.

4.12.1 Gene transfer into NK cell lines

There are several gene delivery systems available, many of which, however, are not appropriate for gene transfer of human lymphocytes. Nonviral gene transfer methods, including calcium phosphate coprecipitation, electroporation, and liposomal transfection, are not very efficient in transducing both primary T and NK cells (Fregeau et al., 1991; Ebert et al., 1997; Van Tendeloo et al., 2000). On the other hand, NK cell lines appear to be more permissive to gene transfer. Two recent reports described instances of stable gene transfer into NK cell lines, one of the CD18 gene into mutant YT-1 cells by electroporation (Liu et al., 1994), and the other of the chimeric zeta-chain gene into NK 3.3 cells using a retroviral vector (Tran et al., 1995). Varying transfection efficiencies have been achieved for different NK cell lines. Moreover, NK-92 cells were also transfected with 5–15% efficiency using particle-mediated gene transfer (Tam et al., 1999), and with 10–20% efficiency using retroviral transduction (Nagashima et al., 1998, Konstantinidis et al., 2005). Nucleofection using the Amaxa system™ of NK3.3 and NKL cell lines has been reported (Trompeter et al., 2003). For the NK3.3 cell line, only 9% efficiency was achieved. Nucleofection of NKL cells only resulted in about 8% transfection efficiency of eYFP expression.

4.12.2 Gene transfer into primary NK cells

A variety of tumour cell targets, normal tissue cells or progenitor cells have been successfully transduced with cytokine genes (Foa et al., 1994; Patel et al., 1994). However, primary NK cells appear to be resistant to retroviral transduction, as well as to infection by other viral vectors. For reasons that are not understood, NK cells do not survive during the infection and selection process. While considerable success has been obtained with vaccinia vectors, this technique is limited to short-term experiments, given the transient nature of poxviral infections (Jiang et al.,

2000). More recently, chimeric adenoviral vectors have been described for transient transduction of primary NK cells (Schroers et al., 2004). In addition, the refinement of plasmid electroporation of NK cells has offered an alternative to viral vectors (Maasho et al., 2004; Trompeter et al., 2003). Retroviral vectors offer a potential complementary strategy to these existing methods, with respect to their ability to integrate into the host genome and mediate long-term, stable gene expression (Palu et al., 2000). In addition, hybrid EBV/retroviral vectors transduce viral packaging lines with high efficiency, leading to high viral titers, and this is an efficient method for retroviral infection of human primary NK cells using the PINCO hybrid EBV/retroviral transfer vector (Grignani et al., 1998; Becknell et al., 2005).

4.12.3 Safety issues of viral gene transfer

Clinical utility of viral transduction vectors may be hampered by biosafety concerns, such as the occurrence of replication-competent pathogenic viruses and insertional mutagenesis due to vector integration into the host genome, which has recently been observed following transduction of hematopoietic progenitor cells (Hacein-Bey-Abina et al., 2003; Fischer et al., 2000; Baum et al., 2003). In addition, expression levels are often low and insertion site–dependent silencing of the transgene expression is a frequent phenomenon (Lu et al., 1996). Therefore, due to safety concerns, both vectors and transductions protocols need to be further optimized in detail for different cell types and expansion protocols and application areas.

4.13 B-CLL and NK cells

4.13.1 B-CLL

B-CLL is the most common adult B-cell malignancy of the western world and is characterized by the expansion and accumulation of malignant monoclonal B lymphocytes in blood, bone marrow and secondary lymphoid organs (Dighiero et al., 1991). Neoplastic cells with the appearance of small mature lymphocytes exhibit a characteristic phenotype and particularly express CD5, CD19 and CD23 molecules in combination with an absence or low expression of the CD20 molecule (Marten et al., 2003; Ternynck et al., 1974; Zomas et al., 1996). Although current treatment modalities have improved secondary outcome measures (response rate and time to progression), relapse inevitably occurs and B-CLL remains a relatively indolent yet incurable disease (Dighiero et al., 2000). A variety of combination chemotherapy studies with the purine analogs have yielded mixed results. Preclinical data (Hamblin et al., 2002), suggest

synergistic interaction between DNA damaging agents and the purine analogs. Such synergy likely occurs as a consequence of alkylator-induced DNA damage and subsequent inhibition of DNA repair by the purine analog. Combination studies with alkylating agents and each of the purine analogs have been performed (Cheson et al., 1996; Tobin et al., 2002).

Campath-1H is a humanized anti-CD52 antibody whose antigen is expressed on greater than 95% of mature B and T lymphocytes. Studies with Campath-1H in untreated and heavily pre-treated CLL have yielded promising results (Flynn et al., 2000). Across all of the studies performed in CLL it is clearly demonstrated that Campath-1H preferentially eliminates CLL tumour cells from the blood, bone marrow, and spleen. Common toxicities observed with Campath-1H include self-limited cytokine release syndrome, immunosuppression and neutropenia. This immunosuppression can be quite severe when Campath-1H is administered at high doses or for a prolonged period, resulting in a high frequency of opportunistic infections. In addition, a good response to fludarabine has been reported, although, without significant prolongation of survival time (Montserrat et al., 1997).

Several Phase II studies have demonstrated the feasibility of autologous stem cell transplantation in CLL patients (Dreger et al., 2002). These studies have generally noted improved results when patients were transplanted during complete remission or with minimal residual clinically detectable disease and could be converted to a PCR negative status (Schultze et al., 1999). The role of standard allogeneic stem cell transplant in CLL is much less defined, except for the very young patient with refractory disease, due to the high treatment-related morbidity. Less toxic preparative regimens (i.e mini transplants) that induce significant immunosuppression to allow engraftment but do not produce significant regimen-related morbidity have broadened the use of allogeneic stem cell transplant in CLL (Khouri et al., 1998).

4.13.2 Role of NK cells in B-CLL

While the primary event in B-CLL is the malignant transformation of the CLL B cell, it is likely that progression is linked to an abnormal immune system in the host. Progression of this disease could be related to dysfunctional components of the host immune cellular arm. Early work showed that while absolute T cell numbers are elevated in untreated B-CLL, there are a large number of immune cell defects (Bartik et al., 1998). These defects affect all cellular components of the immune system, including quantitative and qualitative aspects of the normal B cell pool,

T cell subsets, NK cells, and dendritic cells. NK cells are deficient in function, as are dendritic cells (Orsini et al., 2003). These aspects are likely to have important roles in disease progression, since NK cells are the first line of defense in dealing with malignancy, and dendritic cells are crucial in the induction of an effective cytotoxic T cell response to tumour cells. In addition, since CLL patients have problems with an increased incidence of infection (Morra et al., 1999), autoimmune disorders (Stahl et al., 2001), and increased risk of skin and solid tumours, these cells are likely playing a role in the well-being of CLL patients.

The extensive cellular immune deficiency of B-CLL patients along with the clinical complications apparent in CLL comprising an increased rate of bacterial infections and certain malignancies (Manusow et al., 1975) makes immune effector cells such as NK and NKT cells relevant for B-CLL treatment. While there are significant deficiencies of the cellular component of the immune system in B-CLL patients (Bartik et al., 1998), the most constant defect appears to be that of the NK cell (Ziegler et al., 1981). Although it has been shown that the function of NK cells can be restored by IL-2 (Kay et al., 1987), there has been little or no systematic activity directed at methods that could enlarge the resident NK population in CLL patients, given the known deficiency of NK cells and the need for more effective therapies for B-CLL. Even though progress has been achieved in CLL management and prolonged remissions can be obtained, relapse inevitably occurs and the disease is presently considered as being incurable. Novel therapeutic strategies aimed at reducing minimal residual disease are therefore very much needed to decrease the relapse rate. The long natural course of the disease provides sufficient time for immune intervention of tumour cells; most patients with CLL are advanced in age and are therefore not suitable for aggressive chemotherapeutic regimens.

4.14 Natural killer-like T (NKT) cells

Natural killer-like T (NKT) cells constitute a lymphocyte subpopulation that coexpresses the CD56 and CD3–T-cell receptor (TCR) complex (Bendelac et al., 1997). NKT cells usually express a heavily biased TCR (Lantz et al., 1994; Cui et al., 1997; Kawano et al., 1997; Burdin et al., 1998). NKT cells possess activation or memory phenotype characterized by the expression of CD44, CD69, and CD122, and are present in most tissues where T cells are found, in particular the liver, bone marrow, spleen, and thymus (Bendelac et al., 1994; Ohteki, 1994). NKT cells are potentially capable of very rapid secretion of large amounts of T_H1 and T_H2 cytokines (Zlotnik et al., 1992; Arase et al., 1993), but also express perforin and surface molecules such as Fas ligand (Dao et al., 1998; Arase et al., 1994). Activated NKT cells can

mediate lysis mainly through the TCRs or CD16, and kill classical NK-sensitive targets such as K562 *in vitro* (Koyasu et al., 1992, and 1994). CD1d-restricted NKT cells have been reported to regulate a range of disease conditions, including type 1 diabetes mellitus and tumour rejection or promotion, mainly through the secretion of T_H2 or T_H1 cytokines, respectively (Wilson et al., 2002).

The fact that NKT cells have the potential to secrete these types of cytokines and thereby of being capable to regulate opposing immune responses, led to the conclusion that these may consist of functionally distinct subsets. To this end, Lee *et al* recently reported that $CD4^+$ and $CD4^-CD8^-$ NKT cell subsets represent functionally distinct lineages with significant differences in their profile of cytokine secretion and pattern of expression of chemokine receptors, integrins, and NK receptors (Lee et al., 2002). $CD4^+$ NKT cells represent the exclusive producers of the T_H2 cytokines IL-4 and IL-13, whereas the $CD4^+CD8^+$ NKT cell subset has a strict T_H1 profile producing TNF-α and IFN-γ. NKT cells rapidly produce a series of cytokines including IL-4, IL-10, IL-13, IFN-gamma, and TNF-alpha upon TCR engagement (Bendelac et al., 1997; Godfrey et al., 2000). NKT cells activated in this manner possess cytotoxic activities. However, the capacity of NKT cells to secrete large amounts of cytokines suggests that these cells have a great impact in influencing adoptive immune responses.

Because NKT cells secrete both IFN-gamma and IL-4, these cells can promote T_H1 and T_H2 responses (Joyce et al., 2001). The role of NKT cells in antitumour immunity has been demonstrated in models of natural tumour immunosurveillance and in models where antitumour immunity is induced by IL-12 or αGalCer (Smyth et al., 2000a, and 2001b). In these models, the antitumour capacity of NKT cells was associated with their capacity to secrete large amounts of IFN-γ. In contrast, two other studies suggested that NKT cells might also suppress T-cell–mediated antitumour responses by mechanisms involving production of IL-13 (Terabe et al., 2000; Moodycliffe et al., 2000). Most recently scientists have demonstrated the capacity of NKT cells activated *in vitro* by a combination of IL-12 plus IL-18 and adoptively transferred into syngeneic animals to induce host NK-cell activation, resulting in syngeneic tumour rejection by a mechanism involving IL-2 and IFN-γ production (Baxevanis et al., 2003). Thus, NKT cells can influence tumour immunity both positively and negatively depending primarily on the stimulus used for their activation. Finally, a significant amount of evidence indicates that NKT cells are critical in the regulation of autoimmune responses. Defects in NKT cell development and function were observed in mice susceptible to induction of experimental autoimmune encephalomyelitis and in lupus-prone mice (Godfrey et al., 2000; Joyce et al.,

2001). Although the mechanisms through which NKT cells regulate autoimmune phenomena are not precisely known, most studies have provided evidence for the crucial role of IL-4 and IL-10.

Immunotherapy using activating NKT cell ligands has so far been successful with αGalCer. This glycolipid ligand has significant efficacy in the treatment of a variety of experimental tumours (Smyth et al., 2000a). Although NKT cells activated with γGalCer can kill a wide range of tumour cell targets *in vitro*, accumulating evidence indicates that other cell types are the predominant effectors *in vivo*. The finding that CD1d expression on tumour cells is not required for their *in vivo* clearance by αGalCer (Kawano et al., 1998) also implies the existence of an alternative effector population. αGalCer-activated NKT cells produce IFN-γ and induce IL-12 production by DCs. Both of these cytokines are potent activators of NK cells, enhancing IFN-γ production and cytotoxicity. There is also evidence that IFN-γ induced by αGalCer potentiates the generation of cytotoxic T cells (Nishimura et al., 2000) which might also contribute to the antitumour activities of αGalCer. However, NKT cells may also be induced to engage in antitumour activities *in vivo* in a TCR-independent manner. For instance, IL-12 has been demonstrated to activate *in vivo* NKT cells and to promote an effective collaboration with syngeneic NK cells for chemically induced sarcomas (Smyth et al., 2000b). A recent report demonstrated synergism between IL-12 and IL-18 in activating NKT cells to produce high levels of IFN-γ and IL-2 transactivating NK cells to exhibit strong cytotoxicity *in vitro* and *in vivo* against syngeneic ALC lymphoma and MC57X fibrosarcoma cell lines (Baxevanis et al., 2003).

5 AIMS OF THE PRESENT STUDY

The overall aim of this study was to investigate the therapeutical potential of NK cells for the immunotherapy of cancers in order to study the biology of NK cells *in vivo* and their precise role in tumour cell combat.

In addition, the more specific aims were:

I. To study the development of a new GMP compatible NK cell expansion protocol from healthy donors (Paper I)
II. To study the feasibility of NK cell expansion from patients with B-cell chronic lymphocytic leukemia (B-CLL) (Paper II)
III. To study optimization of conditions for gene transfer into NK cells (Paper III)
IV. To study immunotherapy of tumours established in SCID-beige mice with *ex vivo* expanded NK cells (Paper IV)
V. To study feasibility and safety of *ex vivo* expanded NK and NKT cells in a Phase I/II clinical trial (Paper V)

6 METHODOLOGY

6.1 NK cell expansion

In papers I-V, buffy-coat cells or heparinized whole blood were obtained from either healthy blood-bank donors (Paper I, III, and IV) or from patients (Paper II), if not from HSC donors (Paper V) on the day before starting the cultures. On day 0, PBMCs were isolated by gradient centrifugation using Lymphoprep. After washing twice with phosphate-buffered saline, cell viability was assessed by trypan blue dye exclusion and the cells were plated into six-well dishes at 0.2 to 1.0 x 10^6 cells/ml. CellGro SCGM serum-free medium was used in all the cultures with the addition of 5% human serum and 500 Ũml IL-2. However, in Paper I, in order to optimize the protocol, not only CellGro medium, but other media such as RPMI 1640, In Vivo 10, AIM-V were used with different doses and amounts of IL-2, HS, and OKT-3. For the first 5 days, the medium was further supplemented with anti-CD3 antibody, OKT-3, to a final concentration of 10 ng/ml. Total cell numbers were assessed by staining cells with trypan blue dye on days 0, 5, 10, 15, and 21. Absolute cell counts were calculated by multiplying the total number of cells in each subset with the percentage of these subsets as determined by flow cytometry. On days 5, the OKT-3-containing medium was washed out, and fresh medium with IL-2 (500 U/ml) but without OKT-3 was added. The cultures were replenished with fresh medium every 2-3 days throughout the culture period. To prevent contact inhibition of cell growth, the cells were transferred to T25 flasks on days 10-11. *Ex vivo* NK cell expansion is illustrated in Figure 7.

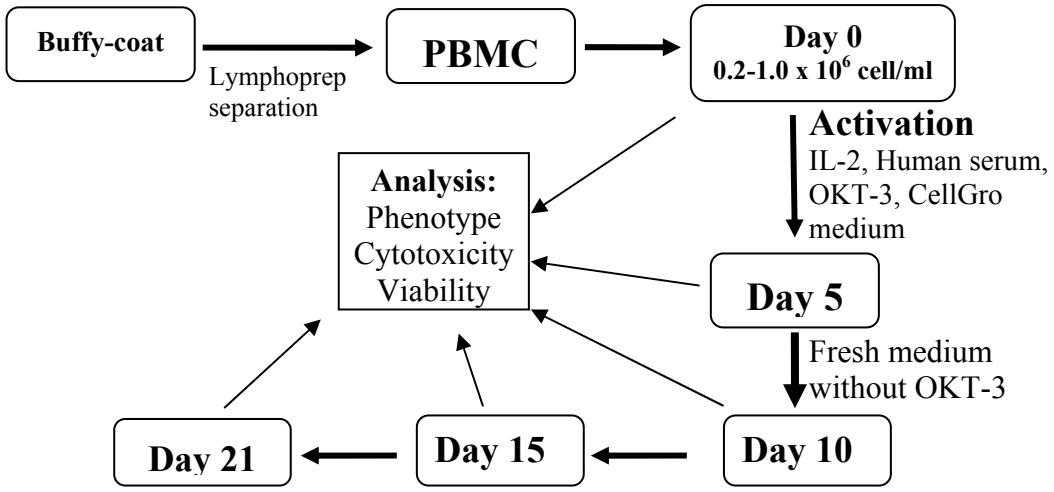

Figure 7. Schematic illustration of NK cell expansion.

6.2 Flow cytometric analysis

In all papers (I-V), the phenotype of fresh and expanded cells was analyzed by flow cytometry on days 0, 5, 10, 15, and 21. Three (Paper I), four (Paper II, III, IV) or eight-colour (Paper V) fluorescence was used according to standard procedures. Generally, cells were mixed with appropriate concentrations of fluorochrome-conjugated Mabs to CD2, CD3, CD4, CD5, CD7, CD8, TCRγδ, CD16, CD19, CD25, CD45, CD56, CD62L, and CD69, and to further characterize expanded cells also against CD95L, HLA-A, -B, -C, NKB1 (KIR3DL1), CD161, CD158a (KIR2DL1), CD158b (KIR2DL2/3), Vα24, Vβ11, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, 2B4, and DNAM-1 receptors whenever applicable. After the addition of primary antibody and incubation for 30 min at room temperature, cells were washed with PBS. Propidium iodide (PI) staining was used for dead cell exclusion. For data acquisition and analysis a minimum of 10000-100000 cells were acquired.

6.3 B cell depletion

In paper II, $CD19^+$ B cells were depleted from PBMC using MACS colloidal super-paramagnetic microbeads conjugated to monoclonal CD19 antibody. PBMCs were washed with PBS and spun down at 1500 rpm for 5 min. After removal of supernatant, cells were resuspended in 80 μl of the separation buffer per 10^7 total cells. A volume of 20 μl of MACS anti-CD19 microbeads per 10^7 total cells was added, mixed, and incubated for 15 min at 4°C. After incubation cells were washed with separation buffer in a 10-20 x labeling volume and spun down, after which supernatants were removed completely. Cell suspensions were then applied to an LS+ column, which was prewashed with the separation buffer and placed in the magnetic field of an appropriate MACS separator. The column was rinsed with 4 x 3 ml separation buffer. Negative (B-cell-depleted) and positive (B-cell-enriched) selected cells were collected. After separation, cells were washed with PBS and viability was assessed with trypan blue dye exclusion.

6.4 Cell-mediated cytotoxicity

In papers I-V, to assess the cytotoxic capacity of cells obtained after *in vitro* expansion, cells from two patients were analyzed on day 21 of culture. The NK-sensitive K562 cell line was used as a target. The cytotoxic function of cultured cells was measured in a standard 4 h ^{51}Cr-release assay in triplicates at various effector:target ratios. Target cells were labeled with 100 Ci of ^{51}Cr for 2 h at 37°C, washed three times with PBS and resuspended in 10 ml of RPMI 1640 medium. A total of 3 x 10^4 target cells in 100 µl RPMI 1640 medium were placed in triplicates into U-bottomed 96-well plates and incubated for 4 h with 100 µl of effector cells at appropriate concentrations to obtain effector:target ratios from 0.3:1 to 10:1. Cells were then collected by centrifugation and aliquots of supernatants were counted in a counter. The percentage specific ^{51}Cr release was calculated according to the formula: percent specific release= ((experimental release-spontaneous release) / (maximum release-spontaneous release)) x 100.

6.5 Transduction

In papers III, in order to study the optimization of retroviral gene transfer into NK cells, the stably transduced retrovirus producing cell line, SF11GFP, was grown until they become subconfluent, then new fresh medium was added for 24 hours. Finally the supernatant was collected, filtered through a 0.45µm filter and frozen at -70 °C. The supernatant collected and harvested had a titer of $0.5x10^6$ virus particles per ml measured on HeLa cells. Viral particles produced from this producer cell line contain a GALV (Gibbon Ape Leukemia Virus) envelope. PBMCs were subsequently transduced with the SF11GFP vector. All transductions were carried out by replacing the media with the retrovirus-containing supernatant at a multiplicity of infection (MOI) of 3 in the presence of 8 µg/ml polybrene and 500 U/ml IL-2 by centrifugation at 1000 *g* at room temperature for two hours on days 3, 4, 5, 6, 10, 15 or 21, respectively. In some cultures cells were transduced on day 5 as well as on day 6 of culture (double transduction). After centrifugation, the supernatant was replaced with fresh medium and cells were expanded until day 21.

6.6 Cryopreservation and thawing of cells

In papers I-V, PBMCs obtained by lymphoprep separation and cells after expansion were cryopreserved using the same procedure. Briefly, cells obtained after lymphoprep or expansion were washed 2 times with PBS and then resuspended in 0.5 ml human serum albumin (HSA) and left on ice for 20 min. After 20 min on ice the cells in HSA were gently mixed with 0.5 ml PBS containing 12 % DMSO and the stored at -80°C for short term or at -140°C for future use. When needed, cells were thawed at 37°C for a couple of minutes, they were then gently mixed with cold CellGro with 20% HS, and spun down for 5 min and then resuspended for second wash CellGro with 5% HS. The cell pellet was then washed once with PBS and the cells were counted in trypan blue and put into culture with fresh medium.

6.7 Human K562 leukemia/SCID-beige model and *in vivo* anti-tumour activity of *ex vivo* expanded NK cells

In paper IV, the mouse model of human K562 leukemia was established by titration of the amount of K562 cells that induced leukemia in irradiated SCID-beige mice. Leukemia was induced by the combination of sub-lethal total body irradiation (2 Gy) followed by intravenous (i.v.) injection of $1x10^6$ K562 cells into the tail of the mice. In order to evaluate the *in vivo* antitumour activity of *ex vivo* expanded human NK cells, mice (n=10) were infused i.v. with 20 x 10^6 cells obtained on day 21 of human PBMC culture, one day after K562 cell injection. Thereafter mice were, observed for signs of morbidity. Other groups included mice injected i.v. with tumour cells (n=15, $1x10^6$ K562 cells), expanded NK cell preparation (n=8, $20x10^6$ cells) or PBS (n=8, 200 µl) alone. Sick mice were sacrificed when presenting with more than 40 % of body weight loss and/or worsening of symptoms. Presence of tumour cells infiltrating tissues of sick mice was evaluated by flow cytometry analysis of single-cell suspensions from the bone marrow, spleen, blood, lung, and liver. Tumour cells were detected using the human-specific antibody for CD235a.

6.8 Injection and detection of human effector cells in SCID-beige mice

To track the enriched NK cell preparation after i.v. injection into mice, human cells pre-stained with fluorescent dye carboxyfluorescein-diacetate-succinimydil-ester (CFSE) were detected in single cell suspensions of mouse tissues by FACS analysis. Human cells obtained on day 21 of culture were labeled with CFSE dye (10 μM in PBS) for 15 min at 37˚C, washed once in serum containing media and resuspended in PBS to a final concentration of 1 x 10^7 cells/ml. 20 x 10^6 of the labeled cells were injected i.v. into the tail of irradiated (2 Gy) SCID-beige mice. On 2, 4 or 9 days after injection, single cell suspensions of the bone marrow, spleen, blood, lung and liver were prepared and incubated with appropriated concentrations of human-specific, direct-labeled antibodies for CD56 or HLA-ABC molecules. After incubation for 30 min, cells were washed and resuspended with PBS for flow cytometry analysis.

6.9 HLA and KIR typing

In paper V, HLA-A, -B and -C types were determined using polymerase chain reaction sequence-specific primers (PCR-SSP) and a regularly updated version of the HLA genonotype method. KIR genotyping was determined using the PCR-SSP method.

6.10 Patients

In paper V, the patients included in this study protocol had undergone allogeneic HSCT for renal cell cancer (RCC), colorectal cancer (CRC), hepatocellular cancer (HCC) or chronic lymphocytic leukemia (CLL). In patients with solid cancer, the allogeneic HSCT had been performed a median of 15.5 (range 13-38) months prior to the first NK/NKT cell infusion. The patient with HCC had undergone orthotopic necro liver grafting two months prior to allogeneic HSCT. Pre-treatment using RIC had been applied in cancer patients. The patient with CLL had received a booster stem cell infusion due to relapse in CLL 15.5 months prior to the first NK/NKT cell infusion. Two patients with HCC and CLL, respectively, were on immunosuppressive therapy during the study period. All patients with solid cancer presented with progressive disease as evaluated by computed tomography (CT) with increasing number and/or size of metastases. The CLL patient presented with increasing number of lymphoblasts and increased size of lymph nodes. All patients but the CLL case had received a minimum of three DLI without development of acute GVHD a median of 11 (range 2-26) months prior to the

first infusion of NK/NKT cells. The booster stem cell infusion to the CLL patient was considered as a comparable cell infusion as DLI of the other patients.

6.11 Cell infusions

In paper V, according to the study protocol, three patients received three infusions with one log increase of the cell count per infusion of NK/NKT cells with one month interval in the absence of acute GVHD after the previous dose. The infusions were given via a central vein catheter (CVC) without premedication. Since none of the first three patients (NKP1-3) presented with acute GVHD, the protocol required the next patients to be administered increasing monthly doses of NK/NKT cell infusions with IL-2 of 6 x 10^6 IU / m(2) / day (s.c.) for three consecutive days. The first IL-2 dose was given on the day of NK/NKT cell infusion using prior oral premedication with antihistamine, clemastine 2 mg. Patients NKP1 and NKP3 were also included into this second group of patients receiving cell infusions with IL-2 s.c. in addition to their initial participation when NK/NKT cells were infused without IL-2. The NK/NKT infusions without IL-2 injections were administrated at the outpatient clinic followed by 3-4 hours of observation for possible adverse reactions. During the cell infusions with IL-2 injections the patients were at the ward for 3-4 days. Vital signs and symptoms were monitored daily.

6.12 Follow-up of patients

In paper V, peripheral blood samples were obtained 1 hour prior, and 1 hour as well as 1 week after, and monthly during 3 months after each cell infusion. The patients' peripheral blood samples were analysed for soluble serum cytokines (sIL-2R, IL-6, TNF-α, IL-8 and IL-10) using ELISA and for lymphocyte markers ($CD19^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $CD16^+$, $CD5^+$, $CD52^+$, $CD25^+$, $CD69^+$) using flow cytometry. In addition, blood chemistry of haemoglobin (Hb), white blood cell count (WBC), neutrophils (ANC), platelets (TRC), serum reactive protein (CRP), liver, kidney and pancreas function tests as well as alpha-feto protein (AFP) and hepatitis C virus (HCV) load using quantitative PCR for the patient with HCC were analysed. Chimerism was studied in peripheral blood by PCR based analysis of sorted $CD3^+$, $CD19^+$ and $CD33^+$ cell populations using magnetic beads before, and monthly until full donor chimera (DC) was reached after the cell infusions. The tumour load was evaluated using CT images performed within one-month prior to and at three months after the last cell infusion. The follow-up of patients for this study was three months after the last NK/NKT cell infusion.

6.13 Statistics

In papers I, and II, nonparametric Mann-Whitney U-test was used to compare the results. In paper III, nonparametric Kruskal Wallis test was used for comparison of absolute cell counts of all cultures and non-parametrical unpaired *t*-test for comparison of transduction rates in percentage. In paper IV, Logrank test was performed to analyze statistical significance of the survival curves. In paper V, nonparametric Fisher exact test (two-tailed) was used when comparing not normally distributed measurements. P-value > 0.05 was considered to give a statistical difference between groups in all tests performed.

6.14 Ethical considerations

All the experiments performed in this study have been done after obtaining ethical permissions from both institutional and national ethical committees.

7 RESULTS AND DISCUSSION

7.1 NK cell expansion (Papers I-V)

In our expansion protocol, PBMCs originating from seven human donors expanded from the starting number to a median of 193-fold (range 21–277) after 21 days of culture (Figure 8). Cells from seven donors, expanded in IL-2, OKT3 and HS, resulted in a median NK cell proportion of 55% (range 7–92) CD3-CD56+ cells. Among the CD3+ cells (45%) the median value for co-expression of CD56 was 22% (range 2–68); thus, these cells were clearly CIK cells (Figure 9).

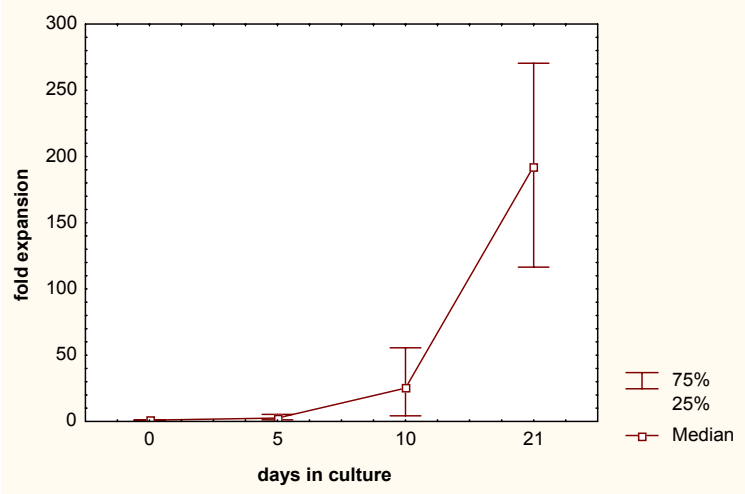

Figure 8. Median cell expansion for seven donors. Values are presented as median and inter-quartile range.

To our knowledge, no previous reports have yet dealt with the practical issues of establishing a protocol that could potentially be used for large scale expansion of cells under GMP conditions for clinical use. In our expansion protocol, expanding cells beyond 21 days seems to be feasible; however, a prolonged culturing time (>21 days) does not seem to consistently increase the final number of $CD3^{-}CD56^{+}$ cells (data not included). Both IL-2 and OKT-3 proved to be essential for the cell expansion procedure. The fact that, to our knowledge, no clinical trials have been performed in which long-term activated NK cells are adoptively transferred as immunotherapy is most likely due to the traditional difficulty of culturing and enriching human

CD3-CD56+ cells in large amounts. Our findings indicate that large numbers of activated NK cells can now be produced and used in the setting of adoptive immunotherapy. With refractory relapses

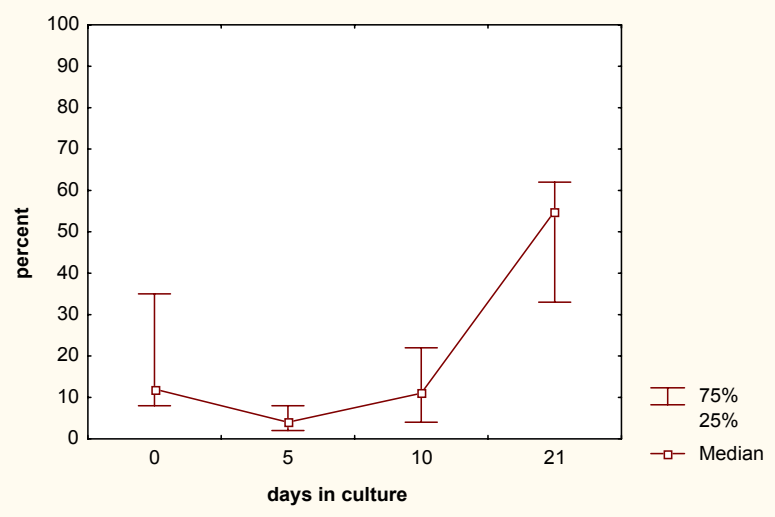

Figure 9. Percent CD3-CD56+ cells for seven donors. Values are presented as median and inter-quartile range.

still being a major complication after HSCT, especially for acute leukemias, alternative DLI regimens are clearly needed. So called non-myeloablative preparative regimens, given before HSCT, are increasingly used, widening the applicability of HSCT for patients with such conditions as solid tumours and autoimmune diseases. DLI is the most likely potential means of converting these patients from a state of mixed haematopoietic chimerism to full donor chimerism, which would, in turn, facilitate remission of the underlying disease. The novelty in our method lies in the use of a clinically defined serum-free medium that in a simple way promotes a selective enrichment of large numbers of activated NK cells.

7.2 Effect of tumour cells, disease progress and sampling time on NK cell expansion (Paper II)

All PBMC samples from the patients with indolent disease showed a marked CD3-CD56+ NK cell expansion. In contrast, under the same conditions, cultured PBMCs from patients with progressive disease ($LB1^{Before}$ and $RL1^{Before}$) had far less NK cell expansion after 21 days. Moreover, in patient LB1 the NK expansion capacity was restored after fludarabine therapy, at

which time the patient had achieved a complete remission. B cells remained in the cultures but were not expanded. B cells were more markedly reduced in cultures from patients with indolent disease (AD1, KO2, LS1, and YO2) compared samples obtained from patients with progressive untreated disease. In contrast, in samples obtained from patients after treatment, the number of B cells was markedly reduced during culture.

To assess the reproducibility of our culture conditions, PBMCs obtained from the same patients at different time points were cultured under identical conditions. The time interval between the first and second samples was not longer than 1 month, except in patient KO2, for whom it was 4 months, and for LB with 3 months between the sample before therapy and the sample after therapy. As indicated in Figure 10, the absolute NK cell counts were quite similar in repeated samples, both in those obtained from patients with indolent (KO and YO) and progressive disease (LB), indicating a high degree of reproducibility in NK cell expansion capacity from the same patients at different time points.

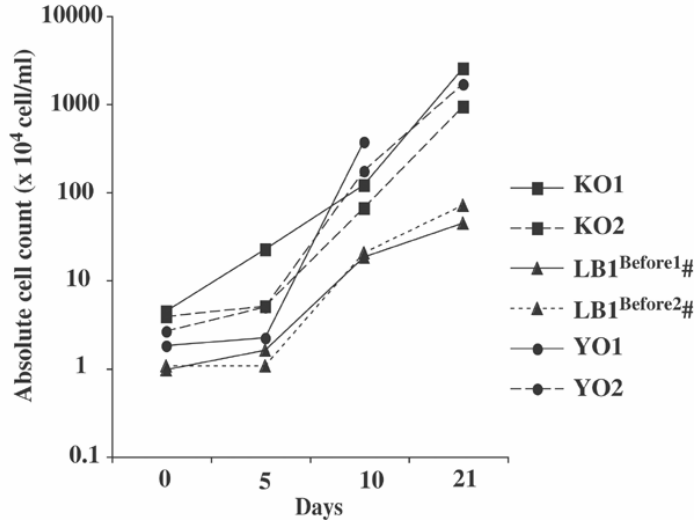

Figure 10. $CD3^-CD56^+$ NK cell expansion rates in repeated samples obtained from three individuals and cultured under identical conditions. Two patients had indolent (KO and YO) and one patient had progressive (LB) disease. Samples from the patient with progressive disease were labeled with the symbol '# '. 'Before' after patients' initials refers to samples obtained before fludarabine treatment. Numbers after patients' initials refer to sample numbers. Time interval between first and second samples of the same patients was 1 (LB, YO) to 4 (KO) months.

Our finding that cells with the NK as well as the NKT phenotype can be expanded in culture indicates that multiple subsets of killer lymphocytes utilizing partly different killer mechanisms are generated simultaneously, which may be therapeutically beneficial.

In conclusion, our results clearly demonstrate that several potentially cytolytic subtypes of lymphocytes from B-CLL patients can be expanded *in vitro*. These cells express CD16 and Granzyme B and have cytolytic capacity, indicating that their use alone or in combination with Mab may be a feasible clinical strategy for controlling B-CLL.

7.3 Gene transfer into NK cells (Paper III)

For detailed studies of the efficiency of retroviral transduction, two transduction strategies were employed (Figure 11). In single transduction experiments, cells were transduced on day 5. In double transduction experiments, cells were transduced on days 5 and 6. In both settings transduced cells were readily detectable 24 h after exposure to retrovirus (Figure 11b and c; days 6 and 7, respectively). In the following experiments, transduction efficiency was always evaluated 24 h after exposure to retroviral vectors unless otherwise noted. In single transduction experiments the median transduction efficiency of NK cells was 27.2% (range 16.3-41.5%) and in double transduction experiments 47.1% (range 35.0-64.7%) (Figure 11b and d; days 6 and 7, respectively). Due to the selective expansion of NK cells during the total culture period, median percentages of transduced NK cells were 51.9% (range 31.7-72.3%) in single transduced cultures and 75.4% (33.5-76.7%) in double transduced cultures after 21 days of culturing (Figure 11c and e; day 21).

Several factors may contribute to the efficient expansion of NK cells in the present protocol, including the specific tissue culture medium used. Although the reason for this outcome is not entirely clear, the OKT-3 mediated stimulation of T cells in the cell culture may have contributed to the secretion of cytokines and/or other growth factors necessary for optimal NK cell growth.

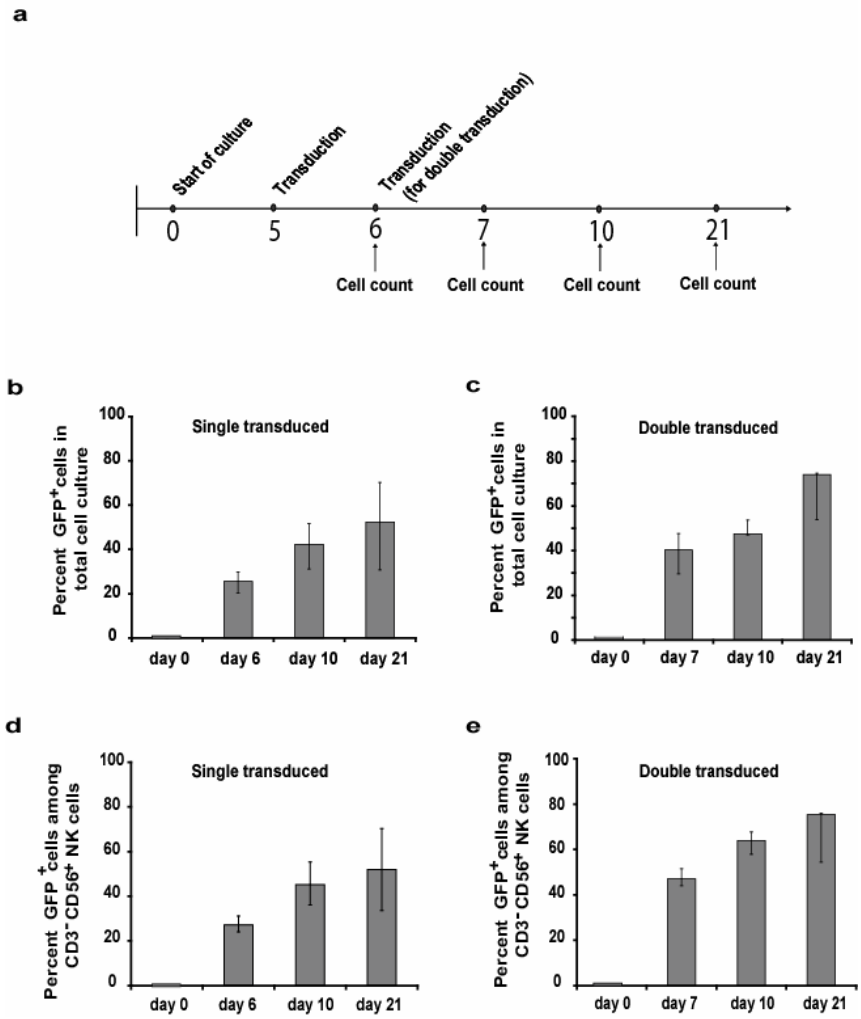

Figure 11. Percent of total cells as well as NK cells transduced by retroviral transduction after single and double transduction. (a) Experimental set-up and transduction rate (%) of total cells in (b) single and (c) double transduced cell cultures, as well as transduction rate (%) of $CD3^-$ $CD56^+$ NK cells in (d) single and (e) double transduced cultures at time points indicated. Results are presented as median ± percentile (0.25) of three individual experiments. *P-value* > 0.05 for all analyzed data groups.

Our study indicates that a single round of transduction yields a high percentage of transduced NK cells. Efficient transduction with single transduction and a low MOI may therefore prevent possible problems with gene transfer such as insertional mutagenesis. Importantly, we found neither any significant inhibition of NK cell proliferation and/or NK cell death among transduced cells, nor any loss of cytotoxic activity after gene transfer into NK cells when compared to parental nontransduced cells.

With respect to the possible implications of the present protocol, gene transfer of cytokines such as IL-2, IL-12, IL-15, or IL-21 might create NK cells that are less dependent on such cytokines for growth and effector mechanisms. Apart from cytokines, chemokine receptors such as CXCR3 and CXCR4 have been shown to be important for NK cell homing to different organs in mice *in vivo*. Therefore, introduction of different chemokines into NK cells may enable NK cells to migrate *in vivo* to more specific target areas of interest. Moreover, retargeting NK cells to some specific molecules on tumour cells such as CD19 or Erb/neu may make NK cells more powerful tools in immunotherapy regimens against tumour cells such as B-cell chronic lymphocytic leukemia or breast cancer. Furthermore, over-expression of activation receptors, e.g., for stress or transformation-associated target cell molecules, or the silencing of inhibitory receptors on NK cells for target cell MHC class I molecules may serve as attractive possibilities for gene therapy approaches using NK cells.

7.4 Toxicity, distribution and survival of human ex vivo expanded NK cells in SCID-beige mice (Paper IV)

An enriched NK cell population (74 %) obtained after *ex vivo* expansion was labelled with CFSE fluorescent dye prior injection into the tail vein of mice. Dot plots obtained from FACS analysis of single cell suspensions of mouse tissues are presented in Figure 12. At day 2, CFSE labeled human cells were found at high numbers in the lungs and liver of mice. At day 4, lung, liver, bone marrow and blood presented a reduction in the percentage of CSFE labeled cells compared with day 2, and they were not detected in the mouse spleen. After that time, human cells were no longer detected in mouse tissues. Taken together, CFSE labeled cells detected in the spleen, lungs and liver of the mice represented 86 % of the total of human cells detected by FACS, whereas the other 14 % was found in the bone marrow plus blood.

In our animal model of K562 leukemia the number of tumour cells detected infiltrating mouse tissues were similar to those found in residual disease. Within the first 24 hours following i.v. injection low numbers of tumour cells were found infiltrating mostly the blood and liver of mice, though bone marrow, spleen and lungs were also infiltrated to a lesser extent. Despite the low number of tumour cells present in mouse tissues, total body pre-irradiation favoured leukemia development, since leukemia was not observed when non-irradiated SCID-beige mice were injected with the same amount of tumour cells. FACS analysis showed that the number of human cells in the mouse tissues decreased gradually with time, supporting that the life-span of

human NK cells in immunodeficient mice might not be longer than 4 days when cytokines such as IL-2 or IL-15 are not provided.

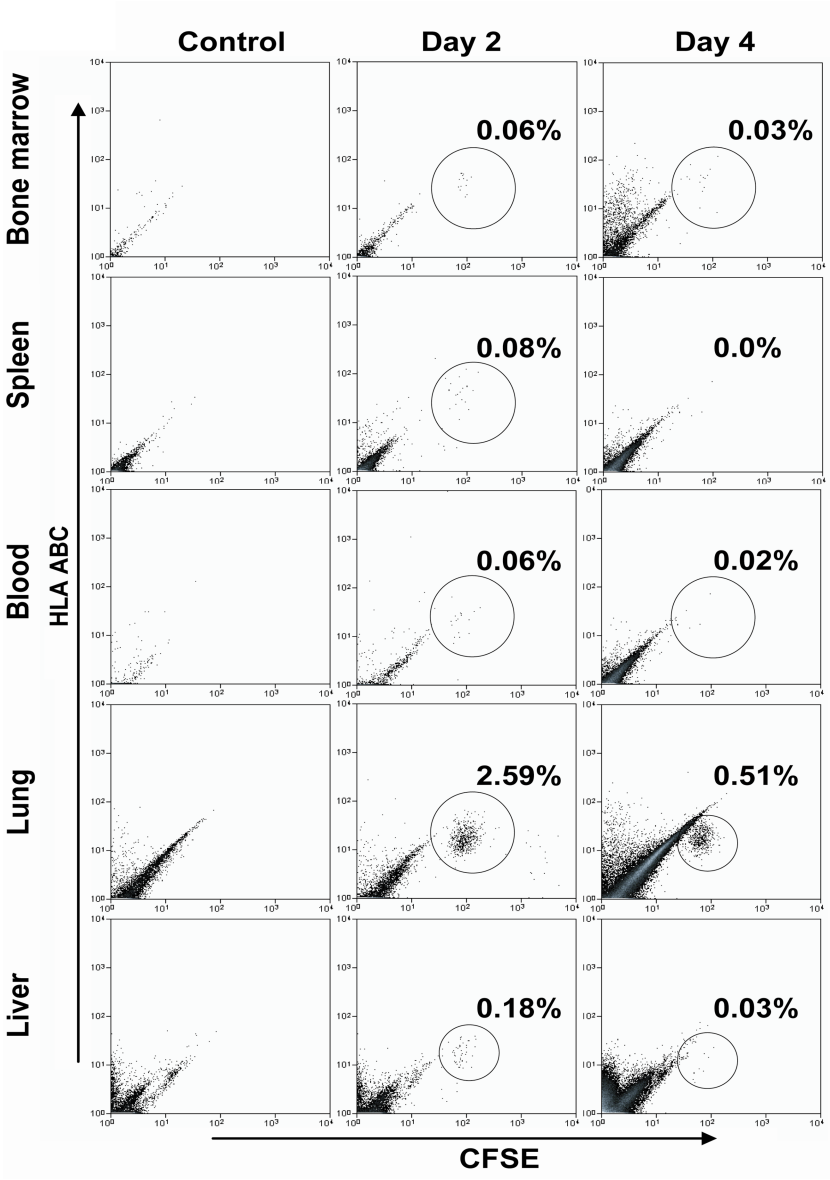

Figure 12. Flow cytometric analysis of organ distribution of the NK enriched effector cell preparation in SCID-beige mice on days 2 and 4 after i.v. injection of CFSE-labelled effector cells. Single-cell suspensions were prepared from different mouse tissues were stained with anti-human HLA-ABC mAb and analyzed by FACS. In each sample a minimum of 100,000 cells were acquired.

7.5 Anti-tumour activity of expanded cells *in vivo* (Paper IV)

Effector cells were injected i.v. into mice one day after tumour cell injection. Figure 13 depicts the *in vivo* therapeutic effects of the effector cell preparation against K562 tumour cells as measured by the survival of tumour-bearing treated *versus* tumour-bearing untreated animals.

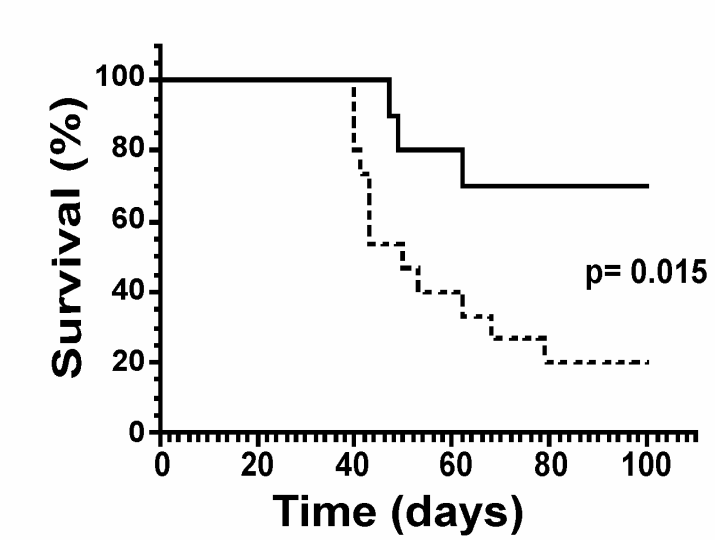

Figure 13. *In vivo* therapeutic effects of the NK enriched effector cell preparation obtained after 21 days of culture from human PBMC. Leukemia was induced in irradiated mice by i.v. injection of K562 tumour cells. 24 h after tumour cell injection mice were treated with the effector cell preparation. Treatment with the effector cell preparation resulted in a significant increase in survival (solid line), as compared to untreated tumour-bearing mice (dashed line). (p=0.015 Log rank test).

As indicated, the enriched NK cell preparation was effective in preventing leukemia development, resulting at the end of the observation period in a higher number (p=0.015) of surviving mice in the treated group than in the untreated group. In the untreated group, 80 % of the mice were sacrificed within 80 days after tumour cell injection due to leukemia development. In contrast, 70 % of the mice treated with the enriched NK cell preparation remained healthy until 100 days after tumour cell injection. Presence of tumour cells was confirmed in the blood of untreated mice during the sickness period, whereas in the treated survivor mice tumour cells were not found (data not included).

Our data confirmed what has been shown for *in vitro* IL-2 activated lymphocyte trafficking, i.e. a tendency of these cells to migrate towards the liver and inflammatory areas. However, we also confirmed the presence of CD56+ cells in the blood and bone marrow of the mice.

However, the fact that cells with the NK as well NKT phenotype could be expanded in culture indicates that multiple subsets of killer lymphocytes utilizing partly different killer mechanisms were generated simultaneously, which may be therapeutically beneficial. As an advantage of our system, human effector cells were expanded in compliance with GMP conditions and the adoptive treatment of mice was performed systemically in an attempt to mimic the therapy applied to human patients. In conclusion, the data presented validates our method of NK cells expansion as a source for cellular immunotherapy.

7.6 Safety and possible anti-tumour activity of NK/NKT cells in a Phase I-II clinical trial (Paper-V)

Infusions of the NK/NKT cells, either fresh (n=2) or thawed (n=7), without IL-2 injections did not give any immediate allergic signs or symptoms. None of the three patients developed any infectious complications. Cell infusions with the IL-2 s.c. injections were associated in all patients with fever for up to one week. A spontaneous regression was seen within 24 hours. Both patients were known to have progression of lung metastases. A decrease of AFP from 117 to 13 ng/ml within 11 weeks as well as regress of pleural fluid after the third NK/NKT cell infusion was recorded in the patient with known increasing AFP coexisting with findings of lung metastases of hepatitis C virus (HCV) associated HCC and progress of right-sided pleural effusion (data not included). The level of HCV RNA in serum was not influenced in connection to NK/NKT cell infusions and the period of progressive metastatic disease. In all remaining patients, the malignancy progressed during the follow-up of three months after the last cell infusions. All patients were alive at the end of the follow-up time.

Although the cell infusion consisted of mixed cell populations including $CD3^+$ cells, only one patient was considered to develop limited and short duration of skin rash similar to acute GVHD (Table 5).

The immunological setting with sibling donors and without the required NK cell receptor/ligand mismatches for NK cell killing demonstrated that adjuvant cell infusions with this type of mixed cell populations of $CD3^-CD56^+$ and $CD3^+CD56^+$ were safe. Purposeful selection of unrelated donors with mismatch of inhibitory NK cell receptors that ligate HLA molecules of

patients with haematopoietic or solid malignancy might be argued to benefit from anti-leukemic and anti-tumour effects of allogeneic NK cells. In conclusion, infusions of *ex vivo* long-term expanded NK/NKT cells was proven to be safe with no risk of GVHD or other severe side-effects either with or without IL-2 infusion. Separation and infusion of *ex vivo* expanded pure NK cells could be an alternative approach in the future of cancer immunotherapy.

Pat. ID	**RFA prior to NK/NKT infusion (months)**	**Irradiation prior to NK/NKT infusion (months)**	**Surgery prior to NK/NKT infusion (months)**	**Tumor status after NK/NKT cell infusions**	**Patient outcome**
NKP1/ 02	3.5 / liver met.	0	0	P lung+liver met.	Alive
NKP2/ 02	0	0	0	R pleura+lung met.	Alive
				a-FP 117-->13 after NK/NKT#3	Stable HCV-RNA in serum
NKP3/ 02	16 / liver met.	16 / lung met.	0	SD lung+liver met.	Alive
NK/NKT cell infusion + IL-2 s.c					
NKP1/ 02,G2	11 / liver met.	1 / lung met.	0	P lung met.	Alive
NKP5/ 03	0	0	0	P lgll met.	Alive
NKP3/ 02,G2	26 / liver met.	26 / lung met.	0	P pleura, lung, bone met.	Alive
NKP6/ 03	0	0	2 / lung met.	P lgll, lung met.	Alive

Table 5. Additional tumor reducing therapy, tumor and patient status 3 months after the last NK/NKT cell infusions. RFA indicates radio frequency ablation; P progression; SD stable disease; R regression; lgll lymph node

8 CONCLUDING REMARKS

There has been increasing knowledge in the area of NK cell biology and of their clinical and therapeutical potential in tumour as well as infection settings. Allogeneic T-lymphocytes possess a powerful anti-tumour activity. However, they also play a key role in the induction of GVHD. Severe GVHD after hematopoietic stem cell transplantation is associated with a high morbidity and mortality. The role of new cytokines in enhancing the anti-neoplastic activity of NK cells and the infusion of expanded NK cells instead of DLI for GVL without severe GVHD need to be investigated. Achieving more detailed data regarding the anti-leukemic effect of the NK cells will be crucial in order to develop a new therapeutic modality for treatment of minimal residual disease after allogeneic hematopoietic stem cell transplantation. Patients undergoing autologous hematopoietic stem cell transplantation for diseases such as myeloma, and lymphoma have in general a poor prognosis with high incidence of post transplant progressive disease. We believe that immunotherapy with ex vivo expanded autologous NK cells, or gene modified NK cells could potentially help these patients in raising an immune response against their tumour and increase the chances of a remission. In addition, their vital role was also shown in haploidentical transplantation and in adoptive immunotherapy in animal models. However, there is still much more to learn about NK cells in terms of NK cell development, differentiation, receptor acquisition, role of different subsets, *ex vivo* expansion kinetics, biodistribution kinetics after expansion and gene modification. One of the important drawbacks in the studies of NK cell biology and immunotherapy has been the lack of a large scale NK cell expansion method. Being able to expand such cells *ex vivo* provides us with an opportunity to study their exact role, especially in human cancers. In the future, being able to proliferate NK cells or a specific subset without decreasing their killing capacity and changing the receptor profile may be instrumental for their clinical usage. NK cells in cancer patients need to be further studied, in order to understand why and how they become unresponsive to tumour cells and how they can be reactivated. Since NK cells do not rearrange their receptors as do T cells, it seems the gene modification of NK cells is essential in order to make them a better killers in the complex tumour environment. Gene transfer of different cytokines, chemokines, activatory receptors and even specific T cell receptors may induce specific NK cell killing and make their responses more targeted. One of the other areas that needs to be studied more is the crosstalk of NK cells with other elements of the immune system such as T cells and dendritic cells. Even the role of NK-NK cell contact in the initiation of other effector functions could be studied in order to better understand the communication between NK cells in tumour combat. The large scale

expansion of very specific NK cell subsets will provide cancer patients with specific and targeted NK cell lysis. In addition, their vital role was also shown in haploidentical transplantation and in adoptive immunotherapy in animal models. However, there is still much more to learn about NK cells in terms of NK cell development, differentiation, receptor acquisition, role of different subsets, *ex vivo* expansion kinetics, biodistribution kinetics after expansion and gene modification. One of the important drawbacks in the studies of NK cell biology and immunotherapy has been the lack of a large scale NK cell expansion method. Being able to expand such cells *ex vivo* provides us with an opportunity to study their exact role, especially in human cancers. In the future, being able to proliferate NK cells or a specific subset without decreasing their killing capacity and changing the receptor profile may be instrumental for their clinical usage. NK cells in cancer patients need to be further studied, in order to understand why and how they become unresponsive to tumour cells and how they can be reactivated. Since NK cells do not rearrange their receptors as do T cells, it seems the gene modification of NK cells is essential in order to make them better killers in the complex tumour environment. Gene transfer of different cytokines, chemokines, activatory receptors and even specific T cell receptors may induce specific NK cell killing and make their responses more targeted. One of the other areas that needs to be studied more is the crosstalk of NK cells with other elements of the immune system such as T cells and dendritic cells. Even the role of NK-NK cell contact in the initiation of other effector functions could be studied in order to better understand the communication between NK cells in tumour combat. The large scale expansion of very specific NK cell subsets may provide cancer patients with specific and targeted NK cell lysis.

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10 REFERENCES

Akashi K., Traver D., Miyamoto T. and Weissman I. L. (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404, 193-7.

Algarra I., Cabrera T. and Garrido F. (2000) The HLA crossroad in tumor immunology. Hum Immunol 61, 65-73.

Arase H., Arase N., Kobayashi Y., Nishimura Y., Yonehara S. and Onoe K. (1994) Cytotoxicity of fresh NK1.1+ T cell receptor alpha/beta+ thymocytes against a CD4+8+ thymocyte population associated with intact Fas antigen expression on the target. J Exp Med 180, 423-32.

Arase H., Arase N., Nakagawa K., Good R. A. and Onoe K. (1993) NK1.1+ CD4+ CD8- thymocytes with specific lymphokine secretion. Eur J Immunol 23, 307-10.

Asai O., Longo D. L., Tian Z. G., Hornung R. L., Taub D. D., Ruscetti F. W. and Murphy W. J. (1998) Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. J Clin Invest 101, 1835-42.

Ayalon O., Hughes E. A., Cresswell P., Lee J., O'Donnell L., Pardi R. and Bender J. R. (1998) Induction of transporter associated with antigen processing by interferon gamma confers endothelial cell cytoprotection against natural killer-mediated lysis. Proc Natl Acad Sci U S A 95, 2435-40.

Bahram S. (2000) MIC genes: from genetics to biology. Adv Immunol 76, 1-60.

Bartik M. M., Welker D. and Kay N. E. (1998) Impairments in immune cell function in B cell chronic lymphocytic leukemia. Semin Oncol 25, 27-33.

Bauer S., Groh V., Wu J., Steinle A., Phillips J. H., Lanier L. L. and Spies T. (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285, 727-9.

Baum C., Dullmann J., Li Z., Fehse B., Meyer J., Williams D. A. and von Kalle C. (2003) Side effects of retroviral gene transfer into hematopoietic stem cells. Blood 101, 2099-114.

Baum C. M., Weissman I. L., Tsukamoto A. S., Buckle A. M. and Peault B. (1992) Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A 89, 2804-8.

Baxevanis C. N., Gritzapis A. D. and Papamichail M. (2003) In vivo antitumor activity of NKT cells activated by the combination of IL-12 and IL-18. J Immunol 171, 2953-9.

Becknell B., Trotta R., Yu J., Ding W., Mao H. C., Hughes T., Marburger T. and Caligiuri M. A. (2005) Efficient infection of human natural killer cells with an EBV/retroviral hybrid vector. J Immunol Methods 296, 115-23.

Bendelac A., Killeen N., Littman D. R. and Schwartz R. H. (1994) A subset of CD4+ thymocytes selected by MHC class I molecules. Science 263, 1774-8.

Bendelac A., Rivera M. N., Park S. H. and Roark J. H. (1997) Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 15, 535-62.

Bennett I. M., Zatsepina O., Zamai L., Azzoni L., Mikheeva T. and Perussia B. (1996) Definition of a natural killer NKR-P1A+/CD56-/CD16- functionally immature human NK cell subset that differentiates in vitro in the presence of interleukin 12. J Exp Med 184, 1845-56.

Benyunes M. C., Higuchi C., York A., Lindgren C., Thompson J. A., Buckner C. D. and Fefer A. (1995) Immunotherapy with interleukin 2 with or without lymphokine-activated killer cells after autologous

bone marrow transplantation for malignant lymphoma: a feasibility trial. Bone Marrow Transplant 16, 283-8.

Benyunes M. C., Massumoto C., York A., Higuchi C. M., Buckner C. D., Thompson J. A., Petersen F. B. and Fefer A. (1993) Interleukin-2 with or without lymphokine-activated killer cells as consolidative immunotherapy after autologous bone marrow transplantation for acute myelogenous leukemia. Bone Marrow Transplant 12, 159-63.

Biassoni R., Cantoni C., Pende D., Sivori S., Parolini S., Vitale M., Bottino C. and Moretta A. (2001) Human natural killer cell receptors and co-receptors. Immunol Rev 181, 203-14.

Biewenga J. E., Destree O. H. and Schrama L. H. (1997) Plasmid-mediated gene transfer in neurons using the biolistics technique. J Neurosci Methods 71, 67-75.

Biron C. A. (1997) Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol 9, 24-34.

Biron C. A., Nguyen K. B., Pien G. C., Cousens L. P. and Salazar-Mather T. P. (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 17, 189-220.

Bishara A., Brautbar C., Eid A., Sherman L. and Safadi R. (2001) Killer inhibitory receptor mismatching and liver transplantation outcome. Transplant Proc 33, 2908.

Borrego F., Ulbrecht M., Weiss E. H., Coligan J. E. and Brooks A. G. (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med 187, 813-8.

Bradley M., Zeytun A., Rafi-Janajreh A., Nagarkatti P. S. and Nagarkatti M. (1998) Role of spontaneous and interleukin-2-induced natural killer cell activity in the cytotoxicity and rejection of Fas+ and Fas- tumor cells. Blood 92, 4248-55.

Braud V. M., Allan D. S., O'Callaghan C. A., Soderstrom K., D'Andrea A., Ogg G. S., Lazetic S., Young N. T., Bell J. I., Phillips J. H., Lanier L. L. and McMichael A. J. (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391, 795-9.

Brooks A. G., Posch P. E., Scorzelli C. J., Borrego F. and Coligan J. E. (1997) NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med 185, 795-800.

Broxmeyer HE, Lu L, Cooper S, Ruggieri L, Li ZH, Lyman SD (1995) Flt3 ligand stimulates/costimulates the growth of myeloid stem/progenitor cells. Exp Hematol 23, 1121-29.

Bukowski J. F., Warner J. F., Dennert G. and Welsh R. M. (1985) Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J Exp Med 161, 40-52.

Burdin N., Brossay L., Koezuka Y., Smiley S. T., Grusby M. J., Gui M., Taniguchi M., Hayakawa K. and Kronenberg M. (1998) Selective ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14+ NK T lymphocytes. J Immunol 161, 3271-81.

Burns L. J., Weisdorf D. J., DeFor T. E., Repka T. L., Ogle K. M., Hummer C. and Miller J. S. (2000) Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer. Exp Hematol 28, 96-103.

Cantoni C., Bottino C., Vitale M., Pessino A., Augugliaro R., Malaspina A., Parolini S., Moretta L., Moretta A. and Biassoni R. (1999) NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J Exp Med 189, 787-96.

Carretero M., Cantoni C., Bellon T., Bottino C., Biassoni R., Rodriguez A., Perez-Villar J. J., Moretta L., Moretta A. and Lopez-Botet M. (1997) The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. Eur J Immunol 27, 563-7.

Cavazzana-Calvo M., Hacein-Bey S., de Saint Basile G., De Coene C., Selz F., Le Deist F. and Fischer A. (1996) Role of interleukin-2 (IL-2), IL-7, and IL-15 in natural killer cell differentiation from cord blood hematopoietic progenitor cells and from gamma c transduced severe combined immunodeficiency X1 bone marrow cells. Blood 88, 3901-9.

Cerwenka A. and Lanier L. L. (2001) Natural killer cells, viruses and cancer. Nat Rev Immunol 1, 41-9.

Champlin R. E., Passweg J. R., Zhang M. J., Rowlings P. A., Pelz C. J., Atkinson K. A., Barrett A. J., Cahn J. Y., Drobyski W. R., Gale R. P., Goldman J. M., Gratwohl A., Gordon-Smith E. C., Henslee-Downey P. J., Herzig R. H., Klein J. P., Marmont A. M., O'Reilly R. J., Ringden O., Slavin S., Sobocinski K. A., Speck B., Weiner R. S. and Horowitz M. M. (2000) T-cell depletion of bone marrow transplants for leukemia from donors other than HLA-identical siblings: advantage of T-cell antibodies with narrow specificities. Blood 95, 3996-4003.

Chang C., Rodriguez A., Carretero M., Lopez-Botet M., Phillips J. H. and Lanier L. L. (1995) Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. Eur J Immunol 25, 2433-7.

Chang E. and Rosenberg S. A. (2001) Patients with melanoma metastases at cutaneous and subcutaneous sites are highly susceptible to interleukin-2-based therapy. J Immunother 24, 88-90.

Cheson B. D., Bennett J. M., Grever M., Kay N., Keating M. J., O'Brien S. and Rai K. R. (1996) National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 87, 4990-7.

Cifone M. G., D'Alo S., Parroni R., Millimaggi D., Biordi L., Martinotti S. and Santoni A. (1999) Interleukin-2-activated rat natural killer cells express inducible nitric oxide synthase that contributes to cytotoxic function and interferon-gamma production. Blood 93, 3876-84.

Clynes RA, TL Towers, LG Presta and JV Ravetch. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nature Med 6:443–446.

Colucci F., Caligiuri M. A. and Di Santo J. P. (2003) What does it take to make a natural killer? Nat Rev Immunol 3, 413-25.

Condiotti R., Zakai Y. B., Barak V. and Nagler A. (2001) Ex vivo expansion of CD56+ cytotoxic cells from human umbilical cord blood. Exp Hematol 29, 104-13.

Cooley S, Burns LJ, Repka T, Miller JS. (1999) Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. Exp Hematol 27(10),1533-41.

Cooper M. A., Fehniger T. A. and Caligiuri M. A. (2001a) The biology of human natural killer-cell subsets. Trends Immunol 22, 633-40.

Cooper M. A., Fehniger T. A., Turner S. C., Chen K. S., Ghaheri B. A., Ghayur T., Carson W. E. and Caligiuri M. A. (2001b) Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 97, 3146-51.

Coscoy L. and Ganem D. (2000) Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc Natl Acad Sci U S A 97, 8051-6.

Coscoy L. and Ganem D. (2001) A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. J Clin Invest 107, 1599-606.

Cui J., Shin T., Kawano T., Sato H., Kondo E., Toura I., Kaneko Y., Koseki H., Kanno M. and Taniguchi M. (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278, 1623-6.

Dao T., Mehal W. Z. and Crispe I. N. (1998) IL-18 augments perforin-dependent cytotoxicity of liver NK-T cells. J Immunol 161, 2217-22.

Dawson J. R., Vidal A. C. and Malyguine A. M. (2000) Natural killer cell-endothelial cell interactions in xenotransplantation. Immunol Res 22, 165-76.

Denning S. M., Jones D. M., Ware R. E., Weinhold K. J., Brenner M. B. and Haynes B. F. (1991) Analysis of clones derived from human CD7+CD4-CD8-CD3- thymocytes. Int Immunol 3, 1015-24.

Dighiero G. and Binet J. L. (2000) When and how to treat chronic lymphocytic leukemia. N Engl J Med 343, 1799-801.

Dighiero G., Travade P., Chevret S., Fenaux P., Chastang C. and Binet J. L. (1991) B-cell chronic lymphocytic leukemia: present status and future directions. French Cooperative Group on CLL. Blood 78, 1901-14.

Dreger P. and Montserrat E. (2002) Autologous and allogeneic stem cell transplantation for chronic lymphocytic leukemia. Leukemia 16, 985-92.

Dutcher J. P., Creekmore S., Weiss G. R., Margolin K., Markowitz A. B., Roper M., Parkinson D., Ciobanu N., Fisher R. I., Boldt D. H. and et al. (1989) A phase II study of interleukin-2 and lymphokine-activated killer cells in patients with metastatic malignant melanoma. J Clin Oncol 7, 477-85.

Ebert O., Finke S., Salahi A., Herrmann M., Trojaneck B., Lefterova P., Wagner E., Kircheis R., Huhn D., Schriever F. and Schmidt-Wolf I. G. (1997) Lymphocyte apoptosis: induction by gene transfer techniques. Gene Ther 4, 296-302.

Farag S. S., Fehniger T. A., Ruggeri L., Velardi A. and Caligiuri M. A. (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. Blood 100, 1935-47.

Farag SS, VanDeusen JB, Fehniger TA, Caligiuri MA. (2003) Biology and clinical impact of human natural killer cells. Int J Hematol 78, 7-17.

Fefer A., Benyunes M., Higuchi C., York A., Massumoto C., Lindgren C., Buckner C. D. and Thompson J. A. (1993) Interleukin-2 +/- lymphocytes as consolidative immunotherapy after autologous bone marrow transplantation for hematologic malignancies. Acta Haematol 89 Suppl 1, 2-7.

Ferlazzo G., Tsang M. L., Moretta L., Melioli G., Steinman R. M. and Munz C. (2002) Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med 195, 343-51.

Fernandez N. C., Lozier A., Flament C., Ricciardi-Castagnoli P., Bellet D., Suter M., Perricaudet M., Tursz T., Maraskovsky E. and Zitvogel L. (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5, 405-11.

Ferrara JL, Yanik G. (2005) Acute graft versus host disease: pathophysiology, risk factors, and prevention strategies. Clin Adv Hematol Oncol 3, 415-9.

Fischer A. (2000) Cautious advance. Gene therapy is more complex than anticipated. EMBO Rep 1, 294-6.

Fletcher J. M., Prentice H. G. and Grundy J. E. (1998) Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA. J Immunol 161, 2365-74.

Flynn J. M. and Byrd J. C. (2000) Campath-1H monoclonal antibody therapy. Curr Opin Oncol 12, 574-81.

Foa R., Guarini A., Cignetti A., Cronin K., Rosenthal F. and Gansbacher B. (1994) Cytokine gene therapy: a new strategy for the management of cancer patients. Nat Immun 13, 65-75.

Fogler W. E., Volker K., McCormick K. L., Watanabe M., Ortaldo J. R. and Wiltrout R. H. (1996) NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction. J Immunol 156, 4707-14.

Forte P., Matter-Reissmann U. B., Strasser M., Schneider M. K. and Seebach J. D. (2000) Porcine aortic endothelial cells transfected with HLA-G are partially protected from xenogeneic human NK cytotoxicity. Hum Immunol 61, 1066-73.

Fox B. A. and Rosenberg S. A. (1989) Heterogeneous lymphokine-activated killer cell precursor populations. Development of a monoclonal antibody that separates two populations of precursors with distinct culture requirements and separate target-recognition repertoires. Cancer Immunol Immunother 29, 155-66.

Fregeau C. J. and Bleackley R. C. (1991) Factors influencing transient expression in cytotoxic T cells following DEAE dextran-mediated gene transfer. Somat Cell Mol Genet 17, 239-57.

Frohn C., Doehn C., Durek C., Bohle A., Schlenke P., Jocham D. and Kirchner H. (2000) Feasibility of the adoptive transfusion of allogenic human leukocyte antigen-matched natural killer cells in patients with renal cell carcinoma. J Immunother 23, 499-504.

Furuke K., Burd P. R., Horvath-Arcidiacono J. A., Hori K., Mostowski H. and Bloom E. T. (1999) Human NK cells express endothelial nitric oxide synthase, and nitric oxide protects them from activation-induced cell death by regulating expression of TNF-alpha. J Immunol 163, 1473-80.

Gerosa F., Baldani-Guerra B., Nisii C., Marchesini V., Carra G. and Trinchieri G. (2002) Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 195, 327-33.

Glas R., Franksson L., Une C., Eloranta M. L., Ohlen C., Orn A. and Karre K. (2000) 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 191, 129-38.

Godfrey D, Hammond KJL, Poulton LD, Smyth MJ, Baxter AG (2000) NKT cells: facts, functions and fallacies. Immunol Today, 21, 573–83.

Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Grignani F, Lanfrancone L, Peschle C, Nolan GP, Pelicci PG. (1998) High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res 58, 14-9.

Grimm E. A., Mazumder A., Zhang H. Z. and Rosenberg S. A. (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 155, 1823-41.

Hacein-Bey-Abina S., von Kalle C., Schmidt M., Le Deist F., Wulffraat N., McIntyre E., Radford I., Villeval J. L., Fraser C. C., Cavazzana-Calvo M. and Fischer A. (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348, 255-6.

Hakoda M., Hirai Y., Shimba H., Kusunoki Y., Kyoizumi S., Kodama Y. and Akiyama M. (1989) Cloning of phenotypically different human lymphocytes originating from a single stem cell. J Exp Med 169, 1265-76.

Hamblin T. J., Orchard J. A., Ibbotson R. E., Davis Z., Thomas P. W., Stevenson F. K. and Oscier D. G. (2002) CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood 99, 1023-9.

Hankey K. G., Drachenberg C. B., Papadimitriou J. C., Klassen D. K., Philosophe B., Bartlett S. T., Groh V., Spies T. and Mann D. L. (2002) MIC expression in renal and pancreatic allografts. Transplantation 73, 304-6.

Harada H., Saijo K., Watanabe S., Tsuboi K., Nose T., Ishiwata I. and Ohno T. (2002) Selective expansion of human natural killer cells from peripheral blood mononuclear cells by the cell line, HFWT. Jpn J Cancer Res 93, 313-9.

Hayes R. L., Koslow M., Hiesiger E. M., Hymes K. B., Hochster H. S., Moore E. J., Pierz D. M., Chen D. K., Budzilovich G. N. and Ransohoff J. (1995) Improved long term survival after intracavitary interleukin-2 and lymphokine-activated killer cells for adults with recurrent malignant glioma. Cancer 76, 840-52.

Henney C. S., Kuribayashi K., Kern D. E. and Gillis S. (1981) Interleukin-2 augments natural killer cell activity. Nature 291, 335-8.

Hessner M. J., Endean D. J., Casper J. T., Horowitz M. M., Keever-Taylor C. A., Roth M., Flomenberg N. and Drobyski W. R. (1995) Use of unrelated marrow grafts compensates for reduced graft-versus-leukemia reactivity after T-cell-depleted allogeneic marrow transplantation for chronic myelogenous leukemia. Blood 86, 3987-96.

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

Hows J. M., Bradley B. A., Marsh J. C., Luft T., Coutinho L., Testa N. G. and Dexter T. M. (1992) Growth of human umbilical-cord blood in longterm haemopoietic cultures. Lancet 340, 73-6.

Hwu P. and Rosenberg S. A. (1994) The genetic modification of T cells for cancer therapy: an overview of laboratory and clinical trials. Cancer Detect Prev 18, 43-50.

Igarashi H., Gregory S. C., Yokota T., Sakaguchi N. and Kincade P. W. (2002) Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity 17, 117-30.

Igarashi T., Wynberg J., Srinivasan R., Becknell B., McCoy J. P., Jr., Takahashi Y., Suffredini D. A., Linehan W. M., Caligiuri M. A. and Childs R. W. (2004) Enhanced cytotoxicity of allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility against melanoma and renal cell carcinoma cells. Blood 104, 170-7.

Imai C., Iwamoto S. and Campana D. (2005) Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 106, 376-83.

Ishido S., Choi J. K., Lee B. S., Wang C., DeMaria M., Johnson R. P., Cohen G. B. and Jung J. U. (2000) Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. Immunity 13, 365-74.

Ishikawa E., Tsuboi K., Saijo K., Harada H., Takano S., Nose T. and Ohno T. (2004) Autologous natural killer cell therapy for human recurrent malignant glioma. Anticancer Res 24, 1861-71.

Jaleco A. C., Blom B., Res P., Weijer K., Lanier L. L., Phillips J. H. and Spits H. (1997) Fetal liver contains committed NK progenitors, but is not a site for development of CD34+ cells into T cells. J Immunol 159, 694-702.

Joyce S (2001) CD1d and natural T cells: how their properties jump-start the immune system. Cell Mol Life Sci, 58, 442–69.

Jiang K., Zhong B., Gilvary D. L., Corliss B. C., Hong-Geller E., Wei S. and Djeu J. Y. (2000) Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat Immunol 1, 419-25.

Kagi D., Ledermann B., Burki K., Seiler P., Odermatt B., Olsen K. J., Podack E. R., Zinkernagel R. M. and Hengartner H. (1994) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369, 31-7.

Karre K. (2002) Immunology. A perfect mismatch. Science 295, 2029-31.

Karre K., Ljunggren H. G., Piontek G. and Kiessling R. (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 319, 675-8.

Kawano T., Cui J., Koezuka Y., Toura I., Kaneko Y., Motoki K., Ueno H., Nakagawa R., Sato H., Kondo E., Koseki H. and Taniguchi M. (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 278, 1626-9.

Kawano T., Cui J., Koezuka Y., Toura I., Kaneko Y., Sato H., Kondo E., Harada M., Koseki H., Nakayama T., Tanaka Y. and Taniguchi M. (1998) Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. Proc Natl Acad Sci U S A 95, 5690-3.

Kay NE, Zarling J. (1987) Restoration of impaired natural killer cell activity of B-chronic lymphocytic leukemia patients by recombinant interleukin-2. Am J Hematol, 24, 161167.

Kayagaki N., Yamaguchi N., Nakayama M., Takeda K., Akiba H., Tsutsui H., Okamura H., Nakanishi K., Okumura K. and Yagita H. (1999) Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. J Immunol 163, 1906-13.

Kennedy M. K., Glaccum M., Brown S. N., Butz E. A., Viney J. L., Embers M., Matsuki N., Charrier K., Sedger L., Willis C. R., Brasel K., Morrissey P. J., Stocking K., Schuh J. C., Joyce S. and Peschon J. J. (2000) Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 191, 771-80.

Khouri I. F., Keating M., Korbling M., Przepiorka D., Anderlini P., O'Brien S., Giralt S., Ippoliti C., von Wolff B., Gajewski J., Donato M., Claxton D., Ueno N., Andersson B., Gee A. and Champlin R. (1998) Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. J Clin Oncol 16, 2817-24.

Kiessling R., Klein E. and Wigzell H. (1975) "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 5, 112-7.

Klingemann H. G. (2000) Relevance and potential of natural killer cells in stem cell transplantation. Biol Blood Marrow Transplant 6, 90-9.

Koehl U., Sorensen J., Esser R., Zimmermann S., Gruttner H. P., Tonn T., Seidl C., Seifried E., Klingebiel T. and Schwabe D. (2004) IL-2 activated NK cell immunotherapy of three children after haploidentical stem cell transplantation. Blood Cells Mol Dis 33, 261-6.

Kondo M., Weissman I. L. and Akashi K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91, 661-72.

Konstantinidis K. V., Alici E., Aints A., Christensson B., Ljunggren H. G. and Dilber M. S. (2005) Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells. Exp Hematol 33, 159-64.

Kos F. J. and Engleman E. G. (1996) Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. Cell Immunol 173, 1-6.

Koyasu S. (1994) CD3+CD16+NK1.1+B220+ large granular lymphocytes arise from both alpha-beta TCR+CD4-CD8- and gamma-delta TCR+CD4-CD8- cells. J Exp Med 179, 1957-72.

Koyasu S., D'Adamio L., Arulanandam A. R., Abraham S., Clayton L. K. and Reinherz E. L. (1992) T cell receptor complexes containing Fc epsilon RI gamma homodimers in lieu of CD3 zeta and CD3 eta components: a novel isoform expressed on large granular lymphocytes. J Exp Med 175, 203-9.

Krause S. W., Gastpar R., Andreesen R., Gross C., Ullrich H., Thonigs G., Pfister K. and Multhoff G. (2004) 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 10, 3699-707.

Kundig T. M., Schorle H., Bachmann M. F., Hengartner H., Zinkernagel R. M. and Horak I. (1993) Immune responses in interleukin-2-deficient mice. Science 262, 1059-61.

Landay A. L., Zarcone D., Grossi C. E. and Bauer K. (1987) Relationship between target cell cycle and susceptibility to natural killer lysis. Cancer Res 47, 2767-70.

Lanier L. L., Le A. M., Civin C. I., Loken M. R. and Phillips J. H. (1986) 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 136, 4480-6.

Lanier L. L., Testi R., Bindl J. and Phillips J. H. (1989) Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J Exp Med 169, 2233-8.

Lantz O. and Bendelac A. (1994) An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. J Exp Med 180, 1097-106.

Lazetic S., Chang C., Houchins J. P., Lanier L. L. and Phillips J. H. (1996) Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol 157, 4741-5.

Lee P. T., Benlagha K., Teyton L. and Bendelac A. (2002) Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med 195, 637-41.

Leibson PJ. (1997). Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity 6:655–661.

Lewis GD, I Figari, B Fendly, WL Wong, P Carter, C Gorman and HM Shepard. (1993). Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol Immunother 37:255–263.

Lin S. J., Yang M. H., Chao H. C., Kuo M. L. and Huang J. L. (2000) Effect of interleukin-15 and Flt3-ligand on natural killer cell expansion and activation: umbilical cord vs. adult peripheral blood mononuclear cells. Pediatr Allergy Immunol 11, 168-74.

Lister J., Rybka W. B., Donnenberg A. D., deMagalhaes-Silverman M., Pincus S. M., Bloom E. J., Elder E. M., Ball E. D. and Whiteside T. L. (1995) Autologous peripheral blood stem cell transplantation and

adoptive immunotherapy with activated natural killer cells in the immediate posttransplant period. Clin Cancer Res 1, 607-14.

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

Ljunggren H. G. and Karre K. (1985) Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med 162, 1745-59.

Ljunggren H. G. and Karre K. (1990) In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 11, 237-44.

Lodolce J. P., Burkett P. R., Boone D. L., Chien M. and Ma A. (2001) T cell-independent interleukin 15Ralpha signals are required for bystander proliferation. J Exp Med 194, 1187-94.

Lotzova E., Savary C. A. and Champlin R. E. (1993) Genesis of human oncolytic natural killer cells from primitive CD34+CD33- bone marrow progenitors. J Immunol 150, 5263-9.

Lotzova E., Savary C. A. and Herberman R. B. (1987) Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. J Immunol 138, 2718-27.

Lowdell M. W., Lamb L., Hoyle C., Velardi A. and Prentice H. G. (2001) Non-MHC-restricted cytotoxic cells: their roles in the control and treatment of leukaemias. Br J Haematol 114, 11-24.

Lu M., Zhang N., Maruyama M., Hawley R. G. and Ho A. D. (1996) Retrovirus-mediated gene expression in hematopoietic cells correlates inversely with growth factor stimulation. Hum Gene Ther 7, 2263-71.

Maasho K., Marusina A., Reynolds N. M., Coligan J. E. and Borrego F. (2004) Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system. J Immunol Methods 284, 133-40.

Maier S., Tertilt C., Chambron N., Gerauer K., Huser N., Heidecke C. D. and Pfeffer K. (2001) Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. Nat Med 7, 557-62.

Mandelboim O., Lieberman N., Lev M., Paul L., Arnon T. I., Bushkin Y., Davis D. M., Strominger J. L., Yewdell J. W. and Porgador A. (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409, 1055-60.

Manusow D. and Weinerman B. H. (1975) Subsequent neoplasia in chronic lymphocytic leukemia. Jama 232, 267-9.

Maraninchi D., Vey N., Viens P., Stoppa A. M., Archimbaud E., Attal M., Baume D., Bouabdallah R., Demeoq F., Fleury J., Michallet M., Olive D., Reiffers J., Sainty D., Tabilio A., Tiberghien P., Brandely M., Hercend T. and Blaise D. (1998) A phase II study of interleukin-2 in 49 patients with relapsed or refractory acute leukemia. Leuk Lymphoma 31, 343-9.

Margolin K. A. (2000) Interleukin-2 in the treatment of renal cancer. Semin Oncol 27, 194-203.

Marten A., Renoth S., Heinicke T., Albers P., Pauli A., Mey U., Caspari R., Flieger D., Hanfland P., Von Ruecker A., Eis-Hubinger A. M., Muller S., Schwaner I., Lohmann U., Heylmann G., Sauerbruch T. and Schmidt-Wolf I. G. (2003) Allogeneic dendritic cells fused with tumor cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma. Hum Gene Ther 14, 483-94.

Mazumder A. (1997) Experimental evidence of interleukin-2 activity in bone marrow transplantation. Cancer J Sci Am 3 Suppl 1, S37-42.

McKenna H. J., Stocking K. L., Miller R. E., Brasel K., De Smedt T., Maraskovsky E., Maliszewski C. R., Lynch D. H., Smith J., Pulendran B., Roux E. R., Teepe M., Lyman S. D. and Peschon J. J. (2000) Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood 95, 3489-97.

Miller J. S., Alley K. A. and McGlave P. (1994) Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. Blood 83, 2594-601.

Miller, J., Verfaillie, C. and McGlave, P. (1992). The generation of human natural killer cells from CD34+/DR− primitive progenitors in long-term bone marrow culture. Blood 1992. 80: 2182–2187.

Miller J. S., Soignier Y., Panoskaltsis-Mortari A., McNearney S. A., Yun G. H., Fautsch S. K., McKenna D., Le C., Defor T. E., Burns L. J., Orchard P. J., Blazar B. R., Wagner J. E., Slungaard A., Weisdorf D. J., Okazaki I. J. and McGlave P. B. (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105, 3051-7.

Mingari M. C., Vitale C., Cantoni C., Bellomo R., Ponte M., Schiavetti F., Bertone S., Moretta A. and Moretta L. (1997) Interleukin-15-induced maturation of human natural killer cells from early thymic precursors: selective expression of CD94/NKG2-A as the only HLA class I-specific inhibitory receptor. Eur J Immunol 27, 1374-80.

Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE (2000) Immune suppression and skin cancer development: regulation by NKT cells. Nat Immunol, 1, 521–25.

Moore T. A. and Zlotnik A. (1997) Differential effects of Flk-2/Flt-3 ligand and stem cell factor on murine thymic progenitor cells. J Immunol 158, 4187-92.

Montserrat E, Bosch F, Rozman C (1997). Treatment of B-cell chronic lymphocytic leukemia: current status and future perspectives. J Intern Med Suppl ; 740: 6367

Moretta A., Bottino C., Vitale M., Pende D., Cantoni C., Mingari M. C., Biassoni R. and Moretta L. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19, 197-223.

Morra E., Nosari A. and Montillo M. (1999) Infectious complications in chronic lymphocytic leukaemia. Hematol Cell Ther 41, 145-51.

Moullier P., Bohl D., Heard J. M. and Danos O. (1993) Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts. Nat Genet 4, 154-9.

Mrozek E., Anderson P. and Caligiuri M. A. (1996) Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood 87, 2632-40.

Mulatero C. W., Penson R. T., Papamichael D., Gower N. H., Evans M. and Rudd R. M. (2001) A phase II study of combined intravenous and subcutaneous interleukin-2 in malignant pleural mesothelioma. Lung Cancer 31, 67-72.

Murphy W. J., Bennett M., Kumar V. and Longo D. L. (1992) Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogeneic bone marrow transplantation. J Immunol 148, 2953-60.

Murphy W. J., Koh C. Y., Raziuddin A., Bennett M. and Longo D. L. (2001) Immunobiology of natural killer cells and bone marrow transplantation: merging of basic and preclinical studies. Immunol Rev 181, 279-89.

Nagashima S., Mailliard R., Kashii Y., Reichert T. E., Herberman R. B., Robbins P. and Whiteside T. L. (1998) 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 91, 3850-61.

Nakajima H., Cella M., Bouchon A., Grierson H. L., Lewis J., Duckett C. S., Cohen J. I. and Colonna M. (2000) Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. Eur J Immunol 30, 3309-18.

Negrier S., Michon J., Floret D., Bouffet E., Gentet J. C., Philip I., Cochat P., Stamm D., Costil J., Gaspard M. and et al. (1991) Interleukin-2 and lymphokine-activated killer cells in 15 children with advanced metastatic neuroblastoma. J Clin Oncol 9, 1363-70.

Nguyen Q. H., Roberts R. L., Ank B. J., Lin S. J., Thomas E. K. and Stiehm E. R. (1998) Interleukin (IL)-15 enhances antibody-dependent cellular cytotoxicity and natural killer activity in neonatal cells. Cell Immunol 185, 83-92.

Nishimura T., Kitamura H., Iwakabe K., Yahata T., Ohta A., Sato M., Takeda K., Okumura K., Van Kaer L., Kawano T., Taniguchi M., Nakui M., Sekimoto M. and Koda T. (2000) The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int Immunol 12, 987-94.

Nitta T., Yagita H., Sato K. and Okumura K. (1989) Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction. J Exp Med 170, 1757-61.

Noguchi M., Nakamura Y., Russell S. M., Ziegler S. F., Tsang M., Cao X. and Leonard W. J. (1993) Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science 262, 1877-80.

Oertel M., Kohlhaw K., Diepolder H. M., Schroder S., Schwarz R., Tannapfel A., Mossner J., Hauss J. and Berr F. (2001) Alloreactivity of natural killer cells in allogeneic liver transplantation. Transplantation 72, 116-22.

Ohteki T. and MacDonald H. R. (1994) Major histocompatibility complex class I related molecules control the development of CD4+8- and CD4-8- subsets of natural killer 1.1+ T cell receptor-alpha/beta+ cells in the liver of mice. J Exp Med 180, 699-704.

Orsini E, Guarini A, Chiaretti S, Mauro FR, Foa R. (2003) The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. Cancer Res 63, 4497-506.

Oshimi K., Oshimi Y., Akutsu M., Takei Y., Saito H., Okada M. and Mizoguchi H. (1986) Cytotoxicity of interleukin 2-activated lymphocytes for leukemia and lymphoma cells. Blood 68, 938-48.

Palu G., Parolin C., Takeuchi Y. and Pizzato M. (2000) Progress with retroviral gene vectors. Rev Med Virol 10, 185-202.

Parish C. R. and O'Neill E. R. (1997) Dependence of the adaptive immune response on innate immunity: some questions answered but new paradoxes emerge. Immunol Cell Biol 75, 523-7.

Parolini S., Bottino C., Falco M., Augugliaro R., Giliani S., Franceschini R., Ochs H. D., Wolf H., Bonnefoy J. Y., Biassoni R., Moretta L., Notarangelo L. D. and Moretta A. (2000) X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. J Exp Med 192, 337-46.

Patel P. M., Flemming C. L., Fisher C., Porter C. D., Thomas J. M., Gore M. E. and Collins M. K. (1994) Generation of interleukin-2-secreting melanoma cell populations from resected metastatic tumors. Hum Gene Ther 5, 577-84.

Pawlowska A. B., Hashino S., McKenna H., Weigel B. J., Taylor P. A. and Blazar B. R. (2001) In vitro tumor-pulsed or in vivo Flt3 ligand-generated dendritic cells provide protection against acute myelogenous leukemia in nontransplanted or syngeneic bone marrow-transplanted mice. Blood 97, 1474-82.

Pende D., Cantoni C., Rivera P., Vitale M., Castriconi R., Marcenaro S., Nanni M., Biassoni R., Bottino C., Moretta A. and Moretta L. (2001) Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol 31, 1076-86.

Pende D., Parolini S., Pessino A., Sivori S., Augugliaro R., Morelli L., Marcenaro E., Accame L., Malaspina A., Biassoni R., Bottino C., Moretta L. and Moretta A. (1999) Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med 190, 1505-16.

Peng BG, Liang LJ, He Q, Huang JF, Lu MD. (2004) Expansion and activation of natural killer cells from PBMC for immunotherapy of hepatocellular carcinoma. World J Gastroenterol 10, 2119-23.

Perez S. A., Mahaira L. G., Demirtzoglou F. J., Sotiropoulou P. A., Ioannidis P., Iliopoulou E. G., Gritzapis A. D., Sotiriadou N. N., Baxevanis C. N. and Papamichail M. (2005) A potential role for hydrocortisone in the positive regulation of IL-15-activated NK-cell proliferation and survival. Blood 106, 158-66.

Peron J. M., Esche C., Subbotin V. M., Maliszewski C., Lotze M. T. and Shurin M. R. (1998) FLT3-ligand administration inhibits liver metastases: role of NK cells. J Immunol 161, 6164-70.

Perussia B., Ramoni C., Anegon I., Cuturi M. C., Faust J. and Trinchieri G. (1987) Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat Immun Cell Growth Regul 6, 171-88.

Pessino A., Sivori S., Bottino C., Malaspina A., Morelli L., Moretta L., Biassoni R. and Moretta A. (1998) Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med 188, 953-60.

Phillips J. H. and Lanier L. L. (1986) Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J Exp Med 164, 814-25.

Piccioli D., Sbrana S., Melandri E. and Valiante N. M. (2002) Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med 195, 335-41.

Puzanov I. J., Bennett M. and Kumar V. (1996) IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. J Immunol 157, 4282-5.

Rosenberg S. A., Anderson W. F., Blaese M., Hwu P., Yannelli J. R., Yang J. C., Topalian S. L., Schwartzentruber D. J., Weber J. S., Ettinghausen S. E. and et al. (1993) The development of gene therapy for the treatment of cancer. Ann Surg 218, 455-63; discussion 463-4.

Rosenberg S. A., Lotze M. T., Muul L. M., Chang A. E., Avis F. P., Leitman S., Linehan W. M., Robertson C. N., Lee R. E., Rubin J. T. and et al. (1987) 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 316, 889-97.

Rosenberg S. A., Lotze M. T., Muul L. M., Leitman S., Chang A. E., Ettinghausen S. E., Matory Y. L., Skibber J. M., Shiloni E., Vetto J. T. and et al. (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 313, 1485-92.

Rosenberg S. A., Lotze M. T., Yang J. C., Topalian S. L., Chang A. E., Schwartzentruber D. J., Aebersold P., Leitman S., Linehan W. M., Seipp C. A. and et al. (1993b) Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst 85, 622-32.

Rosenberg S. A., Packard B. S., Aebersold P. M., Solomon D., Topalian S. L., Toy S. T., Simon P., Lotze M. T., Yang J. C., Seipp C. A. and et al. (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 319, 1676-80.

Rosmaraki E. E., Douagi I., Roth C., Colucci F., Cumano A. and Di Santo J. P. (2001) Identification of committed NK cell progenitors in adult murine bone marrow. Eur J Immunol 31, 1900-9.

Ruggeri L., Capanni M., Casucci M., Volpi I., Tosti A., Perruccio K., Urbani E., Negrin R. S., Martelli M. F. and Velardi A. (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood 94, 333-9.

Ruggeri L., Capanni M., Urbani E., Perruccio K., Shlomchik W. D., Tosti A., Posati S., Rogaia D., Frassoni F., Aversa F., Martelli M. F. and Velardi A. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295, 2097-100.

Russell P. S., Chase C. M., Sykes M., Ito H., Shaffer J. and Colvin R. B. (2001) Tolerance, mixed chimerism, and chronic transplant arteriopathy. J Immunol 167, 5731-40.

Sayos J., Wu C., Morra M., Wang N., Zhang X., Allen D., van Schaik S., Notarangelo L., Geha R., Roncarolo M. G., Oettgen H., De Vries J. E., Aversa G. and Terhorst C. (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature 395, 462-9.

Schroers R., Hildebrandt Y., Hasenkamp J., Glass B., Lieber A., Wulf G. and Piesche M. (2004) Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35 chimeric adenoviral vectors. Exp Hematol 32, 536-46.

Schultze J. L., Donovan J. W. and Gribben J. G. (1999) Minimal residual disease detection after myeloablative chemotherapy in chronic lymphatic leukemia. J Mol Med 77, 259-65.

Screpanti V., Wallin R. P., Ljunggren H. G. and Grandien A. (2001) A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. J Immunol 167, 2068-73.

Seki H., Ueno Y., Taga K., Matsuda A., Miyawaki T. and Taniguchi N. (1985) Mode of in vitro augmentation of natural killer cell activity by recombinant human interleukin 2: a comparative study of Leu-11+ and Leu-11- cell populations in cord blood and adult peripheral blood. J Immunol 135, 2351-6.

Sekine T., Shiraiwa H., Yamazaki T., Tobisu K. and Kakizoe T. (1993) A feasible method for expansion of peripheral blood lymphocytes by culture with immobilized anti-CD3 monoclonal antibody and interleukin-2 for use in adoptive immunotherapy of cancer patients. Biomed Pharmacother 47, 73-8.

Semino C., Martini L., Queirolo P., Cangemi G., Costa R., Alloisio A., Ferlazzo G., Sertoli M. R., Reali U. M., Ratto G. B. and Melioli G. (1999) Adoptive immunotherapy of advanced solid tumors: an eight year clinical experience. Anticancer Res 19, 5645-9.

Shibuya A., Nagayoshi K., Nakamura K. and Nakauchi H. (1995) Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. Blood 85, 3538-46.

Shlomchik W. D., Couzens M. S., Tang C. B., McNiff J., Robert M. E., Liu J., Shlomchik M. J. and Emerson S. G. (1999) Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science 285, 412-5.

Silva M. R., Hoffman R., Srour E. F. and Ascensao J. L. (1994) Generation of human natural killer cells from immature progenitors does not require marrow stromal cells. Blood 84, 841-6.

Silva M. R., Parreira A. and Ascensao J. L. (1995) Natural killer cell numbers and activity in mobilized peripheral blood stem cell grafts: conditions for in vitro expansion. Exp Hematol 23, 1676-81.

Sirianni M. C., Vincenzi L., Topino S., Giovannetti A., Mazzetta F., Libi F., Scaramuzzi D., Andreoni M., Pinter E., Baccarini S., Rezza G., Monini P. and Ensoli B. (2002) NK cell activity controls human herpesvirus 8 latent infection and is restored upon highly active antiretroviral therapy in AIDS patients with regressing Kaposi's sarcoma. Eur J Immunol 32, 2711-20.

Sivori S., Vitale M., Morelli L., Sanseverino L., Augugliaro R., Bottino C., Moretta L. and Moretta A. (1997) p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med 186, 1129-36.

Smyth, M. J., Thia, K. Y., Street, S. E., Cretney, E., Trapani, J. A., Taniguchi, M., et al. (2000a). Differential tumour surveillance by natural killer (NK) and NKT cells. J Exp Med, 191(4), 661-668.

Smyth, M. J., Taniguchi, M., & Street, S. E. (2000b). The anti-tumour activity of il-12: Mechanisms of innate immunity that are model and dose dependent. J Immunol, 165(5), 2665-2670.

Smyth, M. J., Cretney, E., Takeda, K., Wiltrout, R. H., Sedger, L. M., Kayagaki, N., et al. (2001a). Tumour necrosis factor-related apoptosis-inducing ligand (trail) contributes to interferon gamma-dependent natural killer cell protection from tumour metastasis. J Exp Med, 193(6), 661-670.

Smyth, M. J., Crowe, N. Y., & Godfrey, D. I. (2001b). NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol, 13(4), 459-463.

Smyth, M. J., Crowe, N. Y., Hayakawa, Y., Takeda, K., Yagita, H., & Godfrey, D. I. (2002). NKT cells - conductors of tumour immunity? Curr Opin Immunol, 14(2), 165-171.

Snyder R. O., Miao C. H., Patijn G. A., Spratt S. K., Danos O., Nagy D., Gown A. M., Winther B., Meuse L., Cohen L. K., Thompson A. R. and Kay M. A. (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 16, 270-6.

Stahl D., Lacroix-Desmazes S., Sibrowski W., Kazatchkine M. D. and Kaveri S. V. (2001) Broad alterations of self-reactive antibody-repertoires of plasma IgM and IgG in B-cell chronic lymphocytic leukemia (B-CLL) and B-CLL related target-restricted autoimmunity. Leuk Lymphoma 42, 163-76.

Street S. E., Cretney E. and Smyth M. J. (2001) Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood 97, 192-7.

Street S. E., Trapani J. A., MacGregor D. and Smyth M. J. (2002) Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med 196, 129-34.

Sumitran-Holgersson S., Wilczek H. E., Holgersson J. and Soderstrom K. (2002) Identification of the nonclassical HLA molecules, mica, as targets for humoral immunity associated with irreversible rejection of kidney allografts. Transplantation 74, 268-77.

Sutherland H. J., Eaves C. J., Eaves A. C., Dragowska W. and Lansdorp P. M. (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74, 1563-70.

Suzuki N., Suzuki T. and Engleman E. G. (1991) Evidence for the involvement of CD56 molecules in alloantigen-specific recognition by human natural killer cells. J Exp Med 173, 1451-61.

Sykes M., Pearson D. A. and Szot G. L. (1995) IL-2-induced GVHD protection is not inhibited by cyclosporine and is maximal when IL-2 is given over a 25 h period beginning on the day following bone marrow transplantation. Bone Marrow Transplant 15, 395-9.

Tagaya Y, Bamford RN, DeFilippis AP, Waldmann TA. (1996) IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. Immunity 4, 329-36.

Takeda K., Hayakawa Y., Smyth M. J., Kayagaki N., Yamaguchi N., Kakuta S., Iwakura Y., Yagita H. and Okumura K. (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 7, 94-100.

Tam Y. K., Maki G., Miyagawa B., Hennemann B., Tonn T. and Klingemann H. G. (1999) Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. Hum Gene Ther 10, 1359-73.

Tangye S. G., Lazetic S., Woollatt E., Sutherland G. R., Lanier L. L. and Phillips J. H. (1999) Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. J Immunol 162, 6981-5.

Tay C. H., Szomolanyi-Tsuda E. and Welsh R. M. (1998) Control of infections by NK cells. Curr Top Microbiol Immunol

Terabe M, Matsui O, Noben-Trauth N, Chen H, Watson C, Donaldson DD, Carbone DP, Paul WE, Berzofsky J (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol, 1, 515, 193-20.

Ternynck T., Dighiero G., Follezou J. and Binet J. L. (1974) Comparison of normal and CLL lymphocyte surface Ig determinants using peroxidase-labeled antibodies. I. Detection and quantitation of light chain determinants. Blood 43, 789-95.

TNF/TIL human gene therapy clinical protocol. (1990). Hum Gene Ther, 1(4), 441-480.

Tobin G., Thunberg U., Johnson A., Thorn I., Soderberg O., Hultdin M., Botling J., Enblad G., Sallstrom J., Sundstrom C., Roos G. and Rosenquist R. (2002) Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood 99, 2262-4.

Topalian S. L., Solomon D., Avis F. P., Chang A. E., Freerksen D. L., Linehan W. M., Lotze M. T., Robertson C. N., Seipp C. A., Simon P. and et al. (1988) Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. J Clin Oncol 6, 839-53.

Torelli G. F., Guarini A., Palmieri G., Breccia M., Vitale A., Santoni A. and Foa R. (2002) Expansion of cytotoxic effectors with lytic activity against autologous blasts from acute myeloid leukaemia patients in complete haematological remission. Br J Haematol 116, 299-307.

Tortorella D., Gewurz B., Schust D., Furman M. and Ploegh H. (2000) Down-regulation of MHC class I antigen presentation by HCMV; lessons for tumor immunology. Immunol Invest 29, 97-100.

Tran A. C., Zhang D., Byrn R. and Roberts M. R. (1995) Chimeric zeta-receptors direct human natural killer (NK) effector function to permit killing of NK-resistant tumor cells and HIV-infected T lymphocytes. J Immunol 155, 1000-9.

Trapani J. A., Davis J., Sutton V. R. and Smyth M. J. (2000) Proapoptotic functions of cytotoxic lymphocyte granule constituents in vitro and in vivo. Curr Opin Immunol 12, 323-9.

Trompeter H. I., Weinhold S., Thiel C., Wernet P. and Uhrberg M. (2003) Rapid and highly efficient gene transfer into natural killer cells by nucleofection. J Immunol Methods 274, 245-56.

Uhrberg M., Valiante N. M., Shum B. P., Shilling H. G., Lienert-Weidenbach K., Corliss B., Tyan D., Lanier L. L. and Parham P. (1997) Human diversity in killer cell inhibitory receptor genes. Immunity 7, 753-63.

Vales-Gomez M., Reyburn H. T., Mandelboim M. and Strominger J. L. (1998) Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. Immunity 9, 337-44.

Valiante N. M., Uhrberg M., Shilling H. G., Lienert-Weidenbach K., Arnett K. L., D'Andrea A., Phillips J. H., Lanier L. L. and Parham P. (1997) Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity 7, 739-51.

Van Tendeloo V. F., Willems R., Ponsaerts P., Lenjou M., Nijs G., Vanhove M., Muylaert P., Van Cauwelaert P., Van Broeckhoven C., Van Bockstaele D. R. and Berneman Z. N. (2000) High-level transgene expression in primary human T lymphocytes and adult bone marrow CD34+ cells via electroporation-mediated gene delivery. Gene Ther 7, 1431-7.

Veiby O. P., Lyman S. D. and Jacobsen S. E. (1996) Combined signaling through interleukin-7 receptors and flt3 but not c-kit potently and selectively promotes B-cell commitment and differentiation from uncommitted murine bone marrow progenitor cells. Blood 88, 1256-65.

Velardi A., Ruggeri L., Alessandro, Moretta and Moretta L. (2002) NK cells: a lesson from mismatched hematopoietic transplantation. Trends Immunol 23, 438-44.

Verfaillie C., Blakolmer K. and McGlave P. (1990) Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated bone marrow stroma. J Exp Med 172, 509-2.

Vitale M., Bottino C., Sivori S., Sanseverino L., Castriconi R., Marcenaro E., Augugliaro R., Moretta L. and Moretta A. (1998) 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 187, 2065-72.

Weiss G. R., Margolin K. A., Aronson F. R., Sznol M., Atkins M. B., Dutcher J. P., Gaynor E. R., Boldt D. H., Doroshow J. H., Bar M. H. and et al. (1992) A randomized phase II trial of continuous infusion interleukin-2 or bolus injection interleukin-2 plus lymphokine-activated killer cells for advanced renal cell carcinoma. J Clin Oncol 10, 275-81.

Williams N. S., Moore T. A., Schatzle J. D., Puzanov I. J., Sivakumar P. V., Zlotnik A., Bennett M. and Kumar V. (1997) Generation of lytic natural killer 1.1+, Ly-49- cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. J Exp Med 186, 1609-14.

Wilson M. T., Singh A. K. and Van Kaer L. (2002) Immunotherapy with ligands of natural killer T cells. Trends Mol Med 8, 225-31.

Wiltrout R. H., Mathieson B. J., Talmadge J. E., Reynolds C. W., Zhang S. R., Herberman R. B. and Ortaldo J. R. (1984) Augmentation of organ-associated natural killer activity by biological response modifiers. Isolation and characterization of large granular lymphocytes from the liver. J Exp Med 160, 1431-49.

Xu X. C., Goodman J., Sasaki H., Lowell J. and Mohanakumar T. (2002) Activation of natural killer cells and macrophages by porcine endothelial cells augments specific T-cell xenoresponse. Am J Transplant 2, 314-22.

Yron I., Wood T. A., Jr., Spiess P. J. and Rosenberg S. A. (1980) In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. J Immunol 125, 238-45.

Yu H., Fehniger T. A., Fuchshuber P., Thiel K. S., Vivier E., Carson W. E. and Caligiuri M. A. (1998) Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. Blood 92, 3647-57.

Yu Y. Y., Kumar V. and Bennett M. (1992) Murine natural killer cells and marrow graft rejection. Annu Rev Immunol 10, 189-213.

Zeis M., Uharek L., Glass B., Gaska T., Gassmann W. and Mueller-Ruchholtz W. (1994) Induction of graft-versus-leukemia (GVL) activity in murine leukemia models after IL-2 pretreatment of syngeneic and allogeneic bone marrow grafts. Bone Marrow Transplant 14, 711-5.

Zeis M., Uharek L., Glass B., Steinmann J., Dreger P., Gassmann W. and Schmitz N. (1997) Allogeneic MHC-mismatched activated natural killer cells administered after bone marrow transplantation provide a strong graft-versus-leukaemia effect in mice. Br J Haematol 96, 757-61.

Ziegler H. W., Kay N. E. and Zarling J. M. (1981) Deficiency of natural killer cell activity in patients with chronic lymphocytic leukemia. Int J Cancer 27, 321-7.

Zlotnik A., Godfrey D. I., Fischer M. and Suda T. (1992) Cytokine production by mature and immature CD4-CD8- T cells. Alpha beta-T cell receptor+ CD4-CD8- T cells produce IL-4. J Immunol 149, 1211-5.

Zomas A. P., Matutes E., Morilla R., Owusu-Ankomah K., Seon B. K. and Catovsky D. (1996) Expression of the immunoglobulin-associated protein B29 in B cell disorders with the monoclonal antibody SN8 (CD79b). Leukemia 10, 1966-70.

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