Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas

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für meine Eltern

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1. General introduction

1.1. Natural Killer cells

1.1.1. Discovery of Natural Killer cells

The discovery of Natural Killer cells, which happened in the early seventies, was an unexpected finding. At that time inbred mouse strains were generated which allowed the immunization of animals with irradiated tumor cells, and subsequently the growth of grafted viable cells of the same tumor was followed. The results revealed a contribution of the immune system to tumor rejection, and this led to the establishment of a new research field referred to as tumor immunology. Immunological effector mechanisms were identified by *in vitro* assays such as ^{51}Cr release where mouse spleen cells collected from immunized mice killed lymphoma cells. When Rolf Kiessling, at that time a medical student at the Karolinska Institutet (Stockholm, Sweden), performed experiments using YAC cells derived from a Moloney virus induced tumor in the A/Sn strain as target, he noticed that spleen cells from non-immunized mice were also able to kill YAC tumor cells. The phenomenon of this "background" cytotoxicity was reported at the same time from researchers in the group of Ronald Herbermann (USA), and finally both groups could prove the existence of lymphocytes with spontaneous cytotoxicity. A phenotypic analysis of these effector cells revealed that they were certainly not B cells but not conventional cytotoxic T cells either, because they did not show MHC restriction. Hence, the cells were named after their natural cytotoxic activity Natural Killer (NK) cells by Eva Klein (Herberman *et al.*, 1975a; Herberman *et al.*, 1975b; Kiessling *et al.*, 1975a; Kiessling *et al.*, 1975b).

1.1.2. NK cell development and definition

NK cells are derived from hematopoietic stem cells. In experiments with thymectomized or splenectomized mice normal numbers of functional NK cells were observed, which led to the assumption that neither spleen nor thymus appears to be essential for the generation of NK cells. At present, it is believed that bone marrow is the main generation site for NK cells, however recent evidence suggests that they can also arise in lymph nodes. During

development, NK cells undergo a complex maturation process that leads to the acquisition of their effector functions. IL-15 was found to be the crucial factor for the development of human and murine NK cells (Cooper et al., 2002; Koka et al., 2003; Miller et al., 2005; Prlic et al., 2003). Due to changes in the expression of integrins and chemotactic receptors, NK cells acquire the ability to home to the blood, spleen, lung, and liver, where they could be localized using NKp46 as NK-specific marker in mice (Gregoire *et al.*, 2007).

NK cells represent a cell subset accounting for approximately 10-15% of blood lymphocytes and are thought to represent important effectors of the innate immune response. Human NK cells can be distinguished from other cell types by the expression of cell markers such as CD56 and CD16 and the absence of CD3. The CD56 antigen is an isoform of the human neural-cell adhesion molecule with unknown function in human NK cells (Lanier *et al.*, 1989). CD16 is the low-affinity FcRIIIa on the surface of NK cells that binds to the Fc part of antibodies (Lanier *et al.*, 1983; Perussia *et al.*, 1984). Binding of CD16 to antibody-coated targets results in activating NK cells that leads to the coated target cell killing in a process referred to as antibody-dependent cellular cytotoxicity (ADCC).

1.2. NK cell activation

Different from T or B lymphocytes, NK cells do not rearrange genes encoding antigen-specific receptors such as the antibody or the T cell receptor. In the early 80's, Klas Kärre started to investigate how NK cells recognize their targets in the course of his PhD thesis at the Karolinska Institutet. He suggested that NK cells rather sense the absence of 'self' than the presence thereof, leading to a hypothesis referred to as 'missing self'. In the course of experiments comparing the ability of NK cells to attack targets with diminished or lost MHC class I expression, Klas Kärre proved that NK cells recognize the absence of MHC class I molecules (Karre *et al.*, 1986). Subsequently, the discovery and the molecular characterization of MHC class I specific inhibitory receptors, such as Ly49 in mice (Karlhofer *et al.*, 1992), the killer immunoglobulin-like receptors (KIR) in humans (Moretta *et al.*, 1993), and CD94/NKG2A in both mice and humans (Lazetic *et al.*, 1996), supported these findings. The missing-self model was completed in the following years by the detection of activating receptors, such as the NKp46 (Sivori *et al.*, 1997) and other natural cytotoxicity receptors (NCRs) as well as NKG2D (Bauer *et al.*, 1999), which are sometimes able to com-

pletely override the inhibitory signal. Today, it is known that a delicate balance between activating and inhibiting signals determines the activation status of the NK cell.

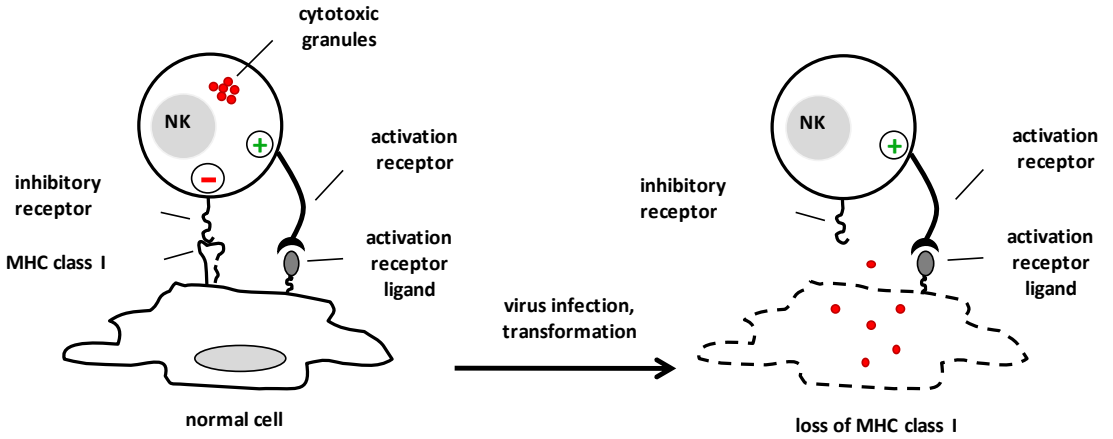


Figure 1. NK cell recognition of target cells. *NK cell activation is regulated by a delicate balance between signals initiated by activating and inhibitory receptors. Normal cells express ligands for activation receptors as well as MHC class I molecules, thus providing both activating and inhibitory signals to NK cells. Upon virus infection or transformation, MHC class I molecules can be reduced or completely lost, resulting in a shift of the signal balance towards activation. Subsequently, cytolytic and other effector cell functions are initiated leading to the killing of target cell.*

1.2.1. NK cell inhibitory receptors

There are two types of NK cell inhibitory receptors based on their ligand specificity: receptors recognizing MHC class I molecules and receptors recognizing non-MHC class I molecules. The cytoplasmic domains of all inhibitory receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM), and they recruit SH2 domain-containing protein phosphatase (SHP)-1, SHP-2 and SH2 domain-containing inositol 5'-phosphatase (SHIP) proteins upon binding to their ligands. These phosphatases inhibit the phosphorylation of proteins which are part of the signaling cascade involved in cell activation.

In mice, the C-type lectin family receptors Ly49 recognize MHC class I molecules and are encoded by highly polymorphic genes. The human equivalent to Ly49 receptors are the KIRs, which are encoded by roughly 12 polymorphic genes and recognize different allelic groups of HLA-A, HLA-B and HLA-C molecules. Importantly, each type of KIR is expressed only by a subset of NK cells. In humans and mice, the CD94/NKG2A heterodimer recognizes non-classical MHC class I molecules such as HLA-E, a molecule with li-

mited polymorphism, and its mouse homolog Qa-1. The non-MHC-binding inhibitory receptors, such as the killer cell lectin-like receptors KLRG1 and KLRB1 as well as the leukocyte-associated immunoglobulin-like receptor 1, LAIR-1, are a diverse group of proteins for which the ligands have not yet been identified.

1.2.2. Activating receptors

A number of receptors have been identified which mediate NK cell activation. In contrast to the inhibitory ones, stimulatory receptors are characterized by short cytoplasmic domains which are able to associate with adapter molecules such as CD3ζ and FcRγ as well as DAP-10/-12. These molecules initiate signal transduction leading to cell activation through either the immunoreceptor tyrosine-based activation motif (ITAM) or the YxxM motif. The natural cytotoxicity receptors (NCRs) NKp46, NKp30 and NKp44 are activating receptors unique for NK cells, for which the ligands have not yet been identified. The best characterized stimulatory receptor is NKG2D, which is not only expressed on NK cells but also on γδ T cells and can be upregulated on activated $CD8^+$ T cells. NKG2D ligands in humans are the stress-inducible MHC class I chain-related (MIC)A and MICB proteins as well as the widely expressed UL-16 binding proteins (ULBPs). In mice, the distant relatives to MHC class I molecules, Rae1 proteins and the H60 proteins, interact with the mouse NKG2D. Blocking of NKG2D results in its down-modulation and impairs effector cell activation (Groh *et al.*, 2002; Wiemann *et al.*, 2005). Moreover, some members of the receptors belonging to the inhibitory receptor families contain stimulatory isoforms such as e.g. KIR2DS1, which is the activating isoform of the inhibitory KIR2DL1.

1.2.3. NK cell licensing/education

It was widely believed that NK cells must express at least one inhibitory receptor for a self MHC class I molecule to achieve self-tolerance. Recently, several studies in humans and mice have demonstrated the existence of a NK population that does not express any inhibitory receptors, which is actually hyporesponisve (Anfossi *et al.*, 2006; Fernandez *et al.*, 2005; Kim *et al.*, 2005). The mechanisms behind this phenomenon might involve the lack of 'licensing' by the appropriate MHC class I ligands important for the acquisition of full effector functions by the NK cell (Yokoyama and Kim, 2006). Alternatively, these NK cells

might have been overstimulated and in turn became hyporesponsive (Gasser and Raulet, 2006).

1.3. NK cells in tumor therapy

Since the discovery of NK cells as cytotoxic lymphocytes which are able to destroy certain tumor cells *in vitro* without prior sensitization, they were further analyzed in order to evaluate the molecular mechanisms of tumor cell recognition by NK cells. A number of studies have demonstrated NK cell killing of many different mouse and human cell lines *in vitro*, e.g. the prototype NK cell targets K562 (human) and YAC (mouse). *In vivo*, NK cells are particularly efficient in eliminating metastases and small tumor grafts in mice (Kim *et al.*, 2000).

In humans, an alloreactive NK cell response was observed in acute myeloid leukemia (AML) patients when receiving mismatched haploidentical transplants resulting in an increase of survival and decrease in relapse (reviewed by (Ruggeri *et al.*, 2006)). The missing expression of a KIR ligand on mismatched allogeneic cells triggered NK cells to kill host target cells including the leukemic blasts, which was confirmed in a mouse model. Despite the high number of the transplanted alloreactive NK cells, there was no evidence of graft-versus-host-disease (GvHD) (Ruggeri *et al.*, 2002).

Several strategies were developed to enhance NK cell responses to tumors. In clinical trials as well as mouse models, the effect of IL-2 administration on activation and expansion of NK cells was investigated. IL-2 administration was shown to increase NK infiltration of both primary tumors and metastases (Basse *et al.*, 2001). Apart from cytokines, immunomodulatory drugs which are used in cancer treatment were shown to increase NK cell reactivity (Hayashi *et al.*, 2005). Monoclonal antibodies that specifically target inhibitory receptors on NK cells were used to tip the balance towards NK cell activation (Sheridan, 2006) with good preclinical evidence in mouse models (Koh *et al.*, 2001). Alternatively, the use of small interfering RNA (siRNA) in order to silence inhibitory receptor expression may be considered. Triggering the activation receptors by using monoclonal antibodies or by the genetic modification of the tumor were investigated by several groups (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001; Wilson *et al.*, 1999). Another therapeutic approach was the use of bispecific antibodies targeting CD30 on lymphoma cells as well a CD16 on NK cells showing a clinical response in patients with Hodgkin's lymphoma. Moreover, a humanized

monoclonal antibody targeting CD20 expressed on mature B cells (rituximab) was shown to deplete B cells *in vivo* in a pre-clinical trial (Reff *et al.*, 1994). Interestingly, CD16 on NK cells shows a gene dimorphism so that two CD16 receptors with different affinities to the Fc part of an antibody can be found. Patients bearing the genotype homozygous for CD16 with higher affinity were associated with higher clinical response to rituximab (Dall'Ozzo *et al.*, 2004). Aditionally, acute lymphoblastoid leukemia (ALL) cells were shown to be a good target for NK cells transduced with a CD19 specific receptor (Imai *et al.*, 2005). In future, similar to T cell mediated adoptive therapy (Rosenberg, 2004), it is likely that combinatory therapies will become more important for the development of immunotherapies involving NK cells. Currently the combination of IL-2 and rituximab to treat non-Hodgkin's lymphoma is being investigated in a Phase I clinical trial (Gluck *et al.*, 2004).

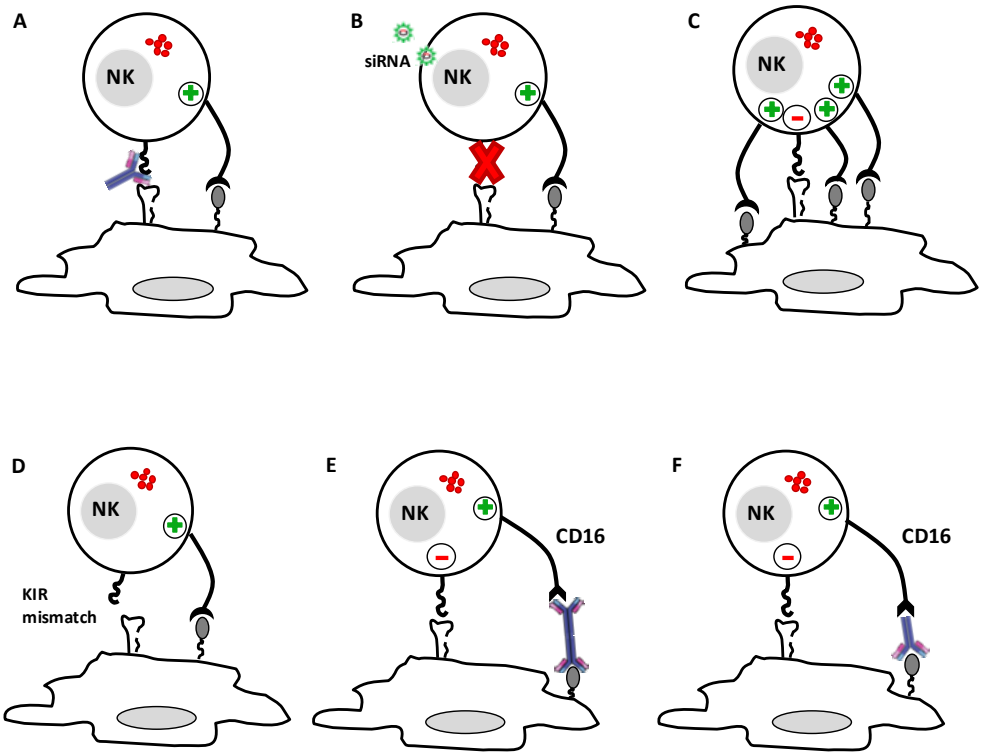


Figure 2. Strategies for the enhancement of tumor cell recognition by NK cells. *Several possibilities are being developed to improve NK cell mediated recognition of tumor cells. (A) Monoclonal antibody blocks signaling through inhibitory receptors. (B) siRNA mediated silencing of inhibitory receptors. (C) Overexpression of activating ligands on tumor cells or overexpression of activation receptors on NK cells in order to improve recognition. (D) KIR-ligand mismatch between donor and recipient. (E) Bispecific antibodies recognizing tumor antigens and stimulating CD16 on NK cells in order to initiate ADCC. (F) Monoclonal antibodies targeting a tumor antigen are being recognized through CD16 on NK cells mediating ADCC.*

1.4. HER-2

1.4.1. HER-2 mediated cell signaling

The human epidermal growth factor receptor-2 (HER-2) (also reffered to as *neu* or c-erbB-2) gene encodes a transmembrane tyrosine kinase receptor protein belonging to the HER protein family consisting of four members: EGFR (HER-1), HER-2, HER-3, and HER-4. Upon ligand binding, the extracellular domain of a particular HER protein changes conformation leading to receptor dimerization and phosphorylation of their c-terminal tails. The HER family receptors may homodimerize or it may be required to dimerize with a different member of the family (heterodimerization). Following dimerization, the phosphorylated tyrosine residues activate multiple intracellular signal transduction pathways initiating the transcription of various genes, which ultimately affects cell proliferation, survival, motility, and adhesion.

HER-2 is unique in the HER family because its extracellular domain does not have ligand-binding activity and constitutively exists in an activated conformation ready for dimerization (Fig. 3). HER-2 is the preferred dimerization partner of the other family members and its specific tyrosine residues that are phosphorylated, and hence the signaling pathways that are activated, depend on the dimerization partner.

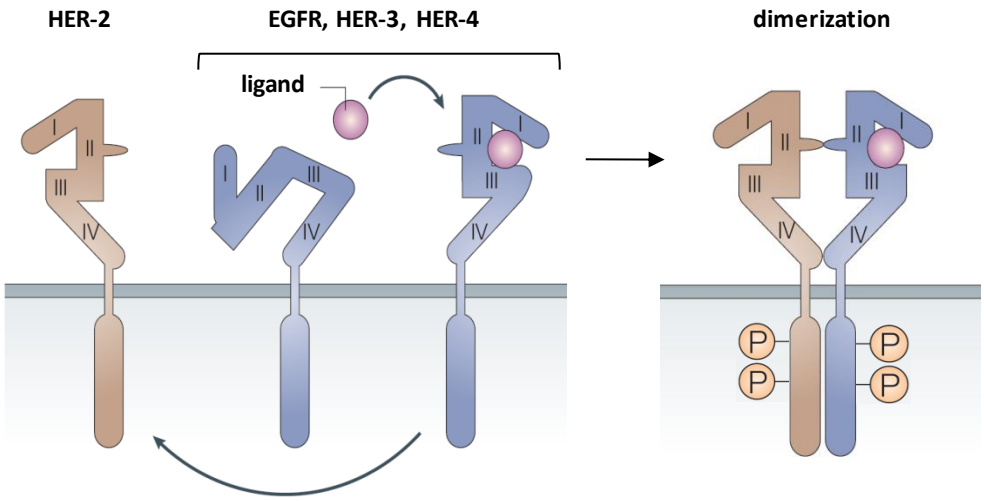


Figure 3. HER-2 heterodimerization with other members of the HER family. *Unlike other HER family members, the extracellular domain of HER2 does not have ligand-binding activity and constitutively exists in an activated conformation. All other HER proteins require ligand binding in order*

to reconfigure into an activated conformation ready for dimersiation. When HER-2 heterodimerizes with another HER protein, the intracellular kinase domain phosphorylates the c-terminal tails leading to activation of downstream pathways (adapted from (Hynes and Lane, 2005)).

1.4.2. Immunotherapy for HER-2 overexpressing tumors

HER-2 represents a valid target for immunotherapy. Overexpression of HER2 in cell culture models induces cell transformation and tumorigenesis (Hudziak *et al.*, 1987). In transgenic mouse models it was shown that the protein Neu, the rat homolog of HER-2, is linked with the development of breast tumors (reviewed by (Ursini-Siegel *et al.*, 2007)). HER-2 is amplified and overexpressed in 30- 80% of human breast, ovarian, pancreatic, colon, gastric, lung and prostate carcinomas and melanoma (Eshhar, 2008; Menard *et al.*, 2003; Meric-Bernstam and Hung, 2006). HER-2 overexpression on carcinomas of various origins correlates with a more aggressive progression of the disease (Slamon *et al.*, 1987). Furthermore, breast cancer lymph node metastatic lesions generally overexpress HER-2 to the same extent as the corresponding primary tumours (Carlsson *et al.*, 2004) and the HER-2 gene amplification status remains quite conserved in distant metastases and is not affected by chemotherapy (Gong *et al.*, 2005; Tapia *et al.*, 2007). As a consequence, all these observations have led to the evaluation of several therapies targeting HER-2.

A number of vaccine strategies were developed, and peptide vaccines targeting HER-2 have been studied extensively in phase I studies and have been found to be both safe and immunogenic (Disis *et al.*, 2004; Zaks and Rosenberg, 1998). However, there are disadvantages to the use of peptides as vaccine antigens such as HLA restrictions. Antibody (trastuzumab) based therapy that targets HER-2 received FDA-approval in 1998, and is clinically used for the treatment of breast cancer (Finn and Slamon, 2003). Trastuzumab is believed to manifest its effect through direct antibody binding, NK-mediated antibody-dependent cell cytotoxicity and by blocking angiogenesis through inhibition of VEGF expression (Menard *et al.*, 2003; Meric-Bernstam and Hung, 2006). Nonetheless, not all HER-2 positive tumors or cell lines are responsive to this antibody based therapy (Choudhury *et al.*, 2004; Finn and Slamon, 2003; Menard *et al.*, 2003; Meric-Bernstam and Hung, 2006; Robert *et al.*, 2006) or to this extent to siRNA mediated HER-2 targeting that was recently used (Choudhury *et al.*, 2004). In fact, only tumor cells that have gene amplified HER-2, accounting for one third of the HER-2 positive tumors, represent good targets for these antibody and siRNA treatments. Adoptive transfer of autologous HER2-specific T-

lymphocyte clones to a patient with metastatic HER2-overexpressing breast cancer was shown to eliminate disseminated HER-2 positive tumor cells in the bone marrow, but the transferred T cells were unable to penetrate into the solid metastases (Bernhard *et al.*, 2008). Further studies will thus be of importance in order to develop more precise and effective immunotherapies against HER-2 overexpressing cancer.

1.5. Chimeric receptors

1.5.1. Composition of a CR

In the late 80's, recombinant chimeric receptors have been developed by Eshhar *et al.* in order to combine the antigen-specific binding properties of a monoclonal antibody with the cytotoxic activity of lymphocytes in a single molecule. Thus, a CR is typically composed of an antibody fragment which recognizes the tumor associated antigen fused to an intracellular activation motif such as CD3ζ or FcεRIγ (reviewed by (Eshhar, 2008)). Often, a hinge IgG_1 region separates the recognition and activation domains, which was shown to stabilize the CR expression (Hombach *et al.*, 1998). In general, antigen recognition is not restricted by MHC class I molecules, but is rather directed to native cell surface structures. However, human TCR-like CRs obtained by *in vitro* selection of Fab phages on soluble peptide/MHC complexes were functionally expressed on human T cells (reviewed by (Engberg *et al.*, 1999)).

1.5.2. Advantages of a CR

In T cells, the introduction of a CR bypasses problems associated with the introduction of TCR genes, such as activation of the second TCR and pairing of introduced TCR chains with endogenous TCR chains (Gladow *et al.*, 2004; Sommermeyer *et al.*, 2006) leading to the formation of TCRs with unknown specificity which bears the risk of severe autoimmunity (Bendle, May 2008). Furthermore, a CR utilizes the antibody derived scFv for antigen recognition that can be isolated with a relative ease from phage libraries, as compared to TCR selection and cloning, which remains a cumbersome endeavor. Moreover, the TCRs are MHC restricted, so that several receptors or vectors must be made to cover common MHC

polymorphisms for every peptide, which needs to be identified, and these TCRs have each time to be matched to the patient's HLA. Additionally, some tumors evade T cell immune responses due to a down-regulation of their MHC expression or due to mutations in the antigen-processing mechanisms (reviewed by (Zou, 2005)). Thus, T cells expressing a certain TCR are of value for a restricted number of patients.

In NK cells, the introduction of a CR could improve tumor infiltration, cytokine release and cytotoxicity, because the CR is constitutively expressed on the cell surface, in contrast to monoclonal antibodies specific for the tumor-associated antigen, which show slow tumor penetration and short half-life. Furthermore, cells engineered with CRs can be used in many animal models and ultimately for any patient apart from MHC polymorphism, e.g. a CR targeting CD20 on B cells of patients with B cell leukemia. Additionally, lymphocytes grafted with chimeric receptors are not affected by down-regulation of MHC class I antigens or by defects in the antigen-processing machinery. Furthermore, a CR is modular, and therefore the antigen recognition domain can be fused to many signaling and costimulation domains leading to optimized activation.

1.5.3. CRs in immunotherapy

CR constructs can be introduced *ex vivo* into T cells and NK cells from peripheral lymphocytes of a given patient typically using retroviral vectors. Following infusion of the engineered lymphocytes back into the patient, they are assumed to traffic and reach their target site. There, they shall undergo activation upon interaction with their target cell, and perform their specific effector function. Until today, many CRs with different specificities were generated, leading to better insight into the mechanism of action and improving the protocol design. From work conducted in the lab of Hinrich Abken and others it is known that the intracellular signaling domain substantially affects CR expression and effector function in grafted T cells (Heuser *et al.*, 2003; Hombach *et al.*, 2001; Maher *et al.*, 2002) and that the receptor binding affinity has an impact on antigen recognition (Chmielewski *et al.*, 2004). Moreover, the fusion of costimulation domains to the CD3ζ signaling domain increases IL-2 secretion and *in vitro* T-cell expansion ((Hombach *et al.*, 2001) and reviewed by (Sadelain *et al.*, 2009)).

Some CRs are currently implied in clinical trials investigating safety and the proof of concept. Lamers *et al.* targeted carboxy-anhydrase-IX (CAIX) positive metastatic renal cell can-

cer (RCC) in 3 patients by adoptive transfer of autologous gene-modified T-cells expressing a CR specific for CAIX and reported preserved CR-mediated functions *in vivo*. Initially, infusions of the gene-modified T cells were well tolerated, but at a later stage liver cytotoxicity was observed due to expression of CAIX on hepatocytes (Lamers *et al.*, 2007). In a study targeting the α-folate receptor (FR) on ovarian cancer by CR-modified T cells, the infusions of a large number of T cells were well tolerated, but the transferred T cells did not persist long term (Kershaw *et al.*, 2006). Another report on the feasibility of isolating, and the safety of infusing, autologous $CD8^+$ cytolytic T cell clones co-expressing a CR specific for the L1-cell adhesion molecule (CD171) and the selection-suicide enzyme HyTK (fusion protein of hygromycin phosphotransferase with the herpes simplex virus type 1 thymidine kinase) in children with recurrent/refractory neuroblastoma demonstrated short T cell persistence (1-7 days) in patients with bulky disease, but significantly longer (42 days) in a patient with a limited disease burden and no autoimmunity (Park *et al.*, 2007). A further study utilized a CD20 specific CR for engineering T cells where previous data revealed that these modified T cells secrete IL-2 in an antigen-dependent manner (Jensen *et al.*, 1998), selectively kill $CD20^+$ target cells *in vitro* (Wang *et al.*, 2004), and eradicate human xenograft tumors in mice (Chen *et al.*, 2004). In this subsequent proof-of-concept study, *ex vivo*-expanded, genetically modified autologous CD20-specific T cells were used as adoptive therapy for patients with relapsed or refractory indolent B-cell non-Hodgkin lymphoma and mantle cell lymphoma (Till *et al.*, 2008). The authors show that these T cells can be reproducibly generated and expanded, persist *in vivo* for up to 9 weeks, and appear to be safe, well tolerated, and potentially capable of mediating *in vivo* antitumor activity. Clinical responses in this study were modest, potentially due to the inadequate CR surface expression leading to poor killing, and lack of costimulatory signaling from the CR construct. A recent study focused on engineered EBV-specific CTLs to express a chimeric receptor directed to the diasialoganglioside GD2, a nonviral tumor–associated antigen expressed by human neuroblastoma cells (Pule *et al.*, 2008). The results show that virus-specific CTLs expressing the GD2-specific CR persist in higher numbers and for a longer time (>6 weeks vs. 3 weeks) after administration to patients with neuroblastoma than do activated T cells expressing the same CR, but lacking viral specificity, however the number of infused cells was low. None of the 11 patients developed an immune response towards CR-CTLs, and there were no adverse events related to the CR-modified T cells followed for up to 2 years. Four of the 8 patients with evaluable tumors had evidence of tumor necrosis or regressions, including a sustained complete remission, yet this effect could not be directly as-

signed to the CR-modified T cells. As in the study described above, the chimeric receptor used in this study contained only the ζ-chain and no additional costimulatory domains such as CD28. Altogether, further investigation of the *in vivo* function of CRs is necessary to elucidate their therapeutic impact.

1.6. Aim and outline of this study

HER-2 is overexpressed by the primary and metastatic tumor cells compared with normal tissues. This overexpression allows a relatively selective cellular immunotherapy, while minimizing the risk of side effects.

Thus, the objective of this thesis was to genetically engineer primary human NK cells in order to provide them with antigen specificity towards HER-2 expressing carcinomas. The potential clinical application for such engineered NK cells needs to meet several criteria, such as the feasibility for large-scale expansion and genetic modification, a constitutive maintenance of specificity, and efficient effector function. To achieve these, a chimeric receptor composed of a HER-2 specific scFv antibody domain fused to the joined CD3ζ and CD28 signaling domains was transferred into primary human NK cells by retroviral transduction. The expression as well as its functionality and specificity of these engineered NK cells were confirmed *in vitro* and *in vivo* (Fig. 4).

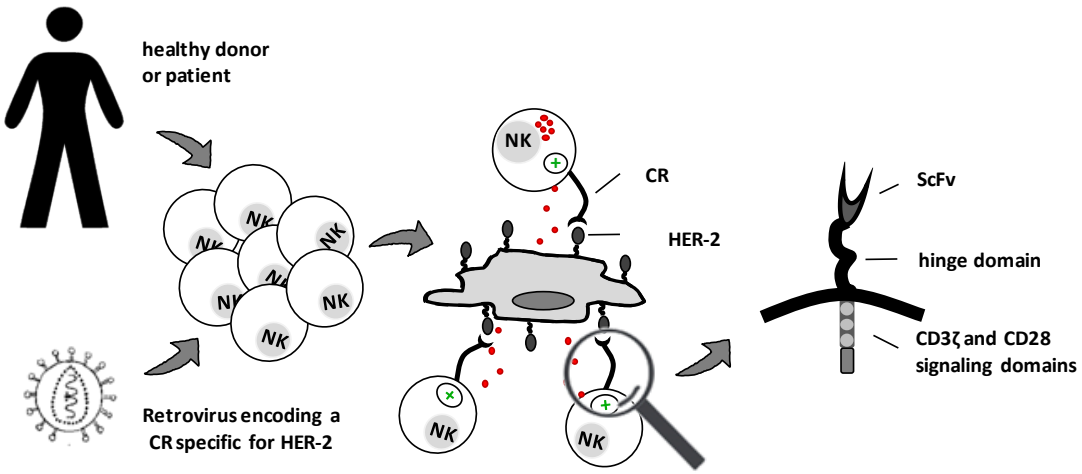


Figure 4. Outline of this study. *Primary human NK cells isolated from patients or healthy donors were genetically engineered using a retroviral vector to express a CR specific for HER-2, which is overexpressed on carcinomas. The CR is composed of a single chain variable fragment (ScFv) specific for HER-2, an extracellular hinge domain and CD3ζ as well as CD28 signaling domains.*

To be able to transfer the CR into NK cells, a protocol for the expansion of primary human NK cells from PBMCs had to be optimized and a subsequent retroviral transduction method had to be established. Following that, the chimeric receptor expression had to be evaluated on NK cells by flow cytometry and its functional properties had to be evaluated by several *in vitro* assays:

First, the recognition specificity of engineered CR-NK cells was evaluated by measuring IFN-γ and IL-2 cytokine production. Additionally, I investigated whether the amount of cytokines produced by the engineered NK cells correlated with varying HER-2 expression levels and/or varying MHC class I expression levels on the tumor cells. Next, a degranulation assay and a ^{51}Cr release assay were performed to see whether engineered NK cells were able to exert cytotoxicity. Using autologous tumor cells as targets, I tested whether CR mediated recognition could override inhibition to self. Moreover, I investigated whether freshly isolated tumor cells from cancer patients could be recognized not only by allogeneic but also by autologous CR-engineered NK cells.

Having proved the concept of specific HER-2 recognition by CR-transduced NK cells, I investigated whether blocking NKG2D, which was reported to result in reduced NK cell responsiveness, had any impact on CR mediated recognition of HER-2. Another interesting question was whether this new approach would compare favorably to the currently approved therapy of HER-2 overexpressing carcinomas using the humanized monoclenal antibody trastuzumab (Herceptin).

Finally, I established an *in vivo* mouse model using luciferase-expressing tumor cells to monitor tumor cell outgrowth or rejection by engineered NK cells by an *in vivo* imaging system.

2. Materials and Methods

2.1. Materials

2.1.1. Consumables and chemicals

Unless otherwise stated, the plastic material was purchased from Costar (Bodenheim, Germany), Eppendorf (Hamburg, Germany), Roth (Karlsruhe, Germany), BD Biosciences (Heidelberg, Germany), PerkinElmer, Jügesheim, Germany. The chemicals were purchased from Sigma-Aldrich (Munich, Germany), Invitrogen (Karlsruhe, Germany), Amersham (Freiburg, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Chiron/Novartis (Marburg, Germany), Calbiochem (EMD Biosciences, Darmstadt, Germany), Biosynth (Staat, Switzerland), TaKaRa (Apen, Germany) and New England Biolabs (Beverly, MA, USA).

2.1.2. Antibodies

Following antibodies conjugated to FITC, PE, APC, Cy-5, or PE-Cy5 were used:

Anti human Abs	Clone	Supplier
Anti CD3	UCHT1	BD Biosiences, Heidelberg, Germany
Anti CD56	NCAM16.2	BD Biosiences, Heidelberg, Germany
Anti HER-2	Neu 24.7	BD Biosiences, Heidelberg, Germany
Anti CD107a	H4A3	BD Biosiences, Heidelberg, Germany
Anti HLA-ABC	G46-2.6	BD Biosiences, Heidelberg, Germany
Anti CD16	B73.1	BD Biosiences, Heidelberg, Germany
Anti NKG2D	1D11	BD Biosiences, Heidelberg, Germany
Anti MICA/B	6D4	BD Biosiences, Heidelberg, Germany
Anti IFN-γ	4S.B3	BioLegend via Biozol, Eching, Germany

Anti ULBP-2	BUMO1	BAMOMAB GmbH, Munich, Germany
Anti human Ig	Goat polyclonal	SouthernBiotech via Biozol, Eching, Germany

Isotype controls	**Clone**	**Supplier**
mouse IgG1	MOPC-21	BD Biosiences, Heidelberg, Germany
mouse IgG2a	G155-178	BD Biosiences, Heidelberg, Germany
mouse IgG2b	MPC-11	BD Biosiences, Heidelberg, Germany
Mouse IgG3	J606	BD Biosciences, Heidelberg, Germany
Goat $F(ab)_2$ fragment	Goat polyclonal	SouthernBiotech via Biozol, Eching, Germany

Anti mouse antibodies	**Clone**	**Supplier**
Rat anti mouse IgG1	X56	BD Biosiences, Heidelberg, Germany
Rat anti mouse IgG2a	R19-15	BD Biosiences, Heidelberg, Germany
Rat anti mouse IgG2b	R12-3	BD Biosiences, Heidelberg, Germany

Humanized antibodies	**Supplier**
trastuzumab (anti HER-2)	Roche, Grenzach-Wyhlen, Germany
rituximab (anti CD20)	Roche, Grenzach-Wyhlen, Germany

2.1.3. Machines

Following machines were used:

FACSCalibur	BD Biosciences, Heidelberg, Germany
TopCount	Canberra Packard, Schwadorf, Austria

IVIS 200	Xenogen, Caliper Life Sciences GmbH, Rüsselsheim, Germany

2.1.4. Software

Following software was used:

Microsoft Office	Microsoft Deutschland GmbH, Munich, Germany
CellQuest Pro	BD Bioscience, Heidelberg, Germany
FlowJo	Treestar, San Carlos, USA
SigmaStat 3.5	Statcon, Witzenhausen, Germany
LivingImage 2.6.1	Xenogen, Caliper Life Sciences GmbH, Rüsselsheim, Germany
EndNote X.0.2	Thomson Reuters, Philadelphia, USA

2.1.5. Statistics

P values were calculated using the Wilcoxon-Mann-Whitney test. Correlation between cytokine production and HER-2 expression level or MHC class I expression level, respectively, was calculated using Microsoft Excel 2007.

2.2. Cell culture

2.2.1. Cell lines

The human embryonic kidney cell line 293T was kindly provided by W. Uckert (MDC, Berlin) and was used as a packaging cell line for retroviral vectors. It was grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS. C1R/A2, a LCL line, and its stable transfectant C1R/A2HER2, which expresses HER-2, were available in our lab. These cell lines were kept in RPMI 1640 medium with 10% (v/v) FBS and 200 U/ml Hygromycin B. Ovarian tumor cells were isolated from the ascites fluid of patients with advanced epithelial

ovarian cancer and the HER-2 status of the tumor cells was determined by flow cytometry in the lab of R. Kiessling (Karolinska Institutet, Stockholm, Sweden). These ovarian tumor cells together with matched PBMC samples from 2 patients, the erythromyeloid leukemia K562, the ovarian carcinoma SKOV3 and the breast carcinoma MCF-7 cell lines were kindly provided by R. Kiessling. The other breast carcinoma cell lines were kindly provided by the following investigators from MDC, Berlin, Germany: MDA MB 468 and MDA MB 453 (W. Uckert), HBL 100 and Cal 51 (W. Birchmeier, MDC, Berlin) and MDA-N, ZR-75-1 and MDA MB 435 (S. Scherneck, MDC, Berlin), all cultured in RPMI 1640 medium, 10% (v/v) FBS, except Cal 51, which was cultured in DMEM, 10% (v/v) FBS, and ZR-75-1 which was cultured in RPMI 1640 medium, 20% (v/v) FBS. Control or HER-2 extracellular domain expressing lymphoplastoid cell lines (LCL ctr and LCL HER-2, respectively) were produced by transforming B cells by control or HER-2 extracellular domain expressing mini-EBV constructs (Moosmann *et al.*, 2002). They were kindly provided along with matched PBMC samples by R. Kiessling and A. Moosmann (LMU Munich) and were cultured in RPMI 1640 medium, 10% (v/v) FBS. The p815 mouse lymphoblast-like mastocytoma cell line was available in our lab and was cultured in RPMI 1640 medium, 10% (v/v) FBS.

2.2.2. Isolation of human PBMCs

Buffy coats from the blood of anonymous healthy donors were obtained from the blood bank of the Deutsches Rotes Kreuz (Berlin, Germany) and were diluted 1:2 with PBS. Mononuclear cells were collected from the diluted samples by centrifugation using a Lymphoprep density step (Bicoll, Biochrom AG, Berlin, Germany; or PANcoll ready-to-use, PAN-Biotech, Aldenbach, Germany). The PBMCs were washed two to four times in PBS and were frozen to maintain standard conditions.

2.3. Retroviral vectors

The chimeric receptor construct specific for HER-2, C6.5-scFv-Fc-CD3ζ-CD28, was kindly provided by H. Abken. The CR was cloned into the modified retroviral vector pMIG kindly provided by L. v. Parijs replacing the GFP and IRES fragment. The resulting construct was designated pMSCV-CR. The pMIG vector encoding GFP, or the pMSCVred

vector encoding red fluorescent protein, which was generated by replacing the IRES and the GFP encoding region of pMIG by the RFP encoding region from the DSred-Express plasmid (Clontech, BD biosciences, Heidelberg, Germany), were used as controls. The CBG (click beetle green) luciferase encoding retroviral vector was constructed by cloning the luciferase cDNA obtained from the pCBG99 plasmid (Promega, Mannheim, Germany) and shuttled through the phRL-null plasmid into the pMIG vector. The resulting plasmid was designated pMIG-CBG. Cloning of the various constructs was confirmed by restriction mapping and partial sequencing. All plasmids were kindly provided by J. Charo.

2.4. Retrovirus production by 293T cells

The 10A1-pseudotyped retrovirus was generated by cotransfection of 293T cells with the above mentioned plasmids and the gag, pol and env encoding pCL-10A1 vector (Naviaux *et al.*, 1996) using Lipofectamine 2000. Briefly, $1x10^6$ 293T cells were seeded into a T 25 flask one day prior to transfection. The following day, medium was replaced with 4 ml fresh medium. Ten µl Lipofectamine 2000 and 3 µg DNA of each plasmid were used (6 µg total DNA) diluted in 1 ml Opti-MEM for transfection of one T 25 flask of 293T cells according to the manufacturers manual. The following day, medium was changed to 5 ml RPMI 1640 medium, 10% (v/v) FBS, and the cells were moved to 32°C, 5 % CO_2 humidified incubator. Virus supernatant was collected on three successive days and filtered through a 0.45 µm sterile filter prior to use.

2.5. Expansion of primary human NK cells

Thawed PBMCs were washed twice in RPMI 1640 medium. The cells were counted and $2x10^7$ cells were seeded into a T 125 tissue culture flask. The cells were incubated at 37°C for 30 min to allow adherent cells to attach to the flask surface. Non-adherent PBLs were carefully harvested and counted. Meanwhile, RPMI 8866 feeder cells were collected, irradiated at 30 Gy, washed twice and also counted. The cells were then cocultured in a 6 well plate at $1.5x10^6$ PBLs/well together with $3x10^5$ RPMI 8866 cells/well for the ratio 5:1, at $1.5x10^6$ PBLs/well together with $5x10^5$ RPMI 8866 cells/well for the ratio 3:1, at $5x10^5$ PBLs/well together with $1.5x10^6$ RPMI 8866 cells/well for the ratio 1:3 and at $3x10^5$ PBLs/well together with $1.5x10^6$ RPMI 8866 cells/well for the ratio 1:5.

2.6. Retroviral transduction

Transduction was performed on day 6 or 8 and was repeated on two subsequent days using either of the two following protocols:

2.6.1. RetroNectin assessed transduction protocol

Six-well tissue culture plates were coated with 50 μg per well RetroNectin as recommended by the manufacturer. One day later, 4 ml virus supernatant was added to each well and plates were incubated for 30 min at 32°C and then for additional 24 hours at 4°C. Virus supernatant was removed and replaced by cells ($1x10^6$ cells/ml) in RPMI 1640 medium, 10% (v/v) FBS, containing 200 IU/ml IL-2. Half a ml of fresh virus supernatant was added to each well and cells were incubated at 32°C for 24 hours. Transduction was repeated on two successive days. After the third transduction, cells were maintained in RPMI 1640 medium, 10% (v/v) FBS, 200 IU/ml IL-2 at 37°C.

2.6.2. Spinocculation transduction

$2x10^5$ cells/well were seeded into a 24-well tissue culture plate in a total volume of 2 ml virus supernatant diluted 1:1 in culture medium in the presence of 8 μg/ml polybrene (Sigma-Aldrich, Munich, Germany) and 200 IU/ml IL-2. Cells were centrifuged at 2000 rpm, 32°C for 90 min. Plates were placed afterwards in 32°C, 5 % CO_2 humidified incubator for 24 hours. Transduction was repeated on two successive days. After the third transduction, cells were maintained in RPMI 1640 medium, 10% (v/v) FBS, 200 IU/ml IL-2 at 37°C.

2.7. Antibody staining and FACS analysis

To analyze the phenotype of PBMCs or cell lines, approximately $1x10^6$ cells were stained with the respective antibody diluted according to the manufacturer's recommendation for 30 min on ice in the dark. The cells were washed twice and resuspended in 400 μl PBS for subsequent analysis by flow cytometry.

2.8. T cell depletion

T cells and NKT cells were depleted using the "Dynabead CD3 negative selection protocol" (Invitrogen). Briefly, the amount of $CD3^+$ cells in each sample which needs to be depleted was determined by flow cytometry. The magnetic dynabeads were washed 4 times in PBS/1% FBS in order to remove sodium azide. Cells were collected, washed twice in PBS, counted and resuspended in 1-2 ml PBS/1% FBS in sterile 4 ml FACS tubes. Cells and dynabeads were mixed in a ratio of 1:4 of $CD3^+$ cells to dynabeads. The mixture was slowly rotated for 45 min at 4°C to allow the dynabeads to bind to CD3 on the T cell and NKT cell surface. Next, $CD3^+$ cells were removed by a magnet and the procedure was repeated. CD3 depleted NK cells were analyzed by flow cytometry in order to determine purity.

2.9. Cytokine release assay (ELISA)

Target cells ($5x10^4$) were cocultured with an equal number of effector cells in 96-well flat bottom plates for 24 hours. The supernatant was assayed for IFN-γ and IL-2 by ELISA (Mabtech, Hamburg, Germany, or BD Biosciences) according to the manufacturer's protocol. The data represents mean values of triplicates derived from one representative experiment. Experiments were performed at least twice with similar results. The detection limit of the ELISA is 100 pg/ml IFN-γ or 20 pg/ml IL-2.

2.10. Pre-incubation with plate-bound recombinant MICA

For the pre-incubation of effector cells with plate-bound recombinant MICA protein, 96-well flat bottom plates were coated with 2 μg/ml of the recombinant MICA protein in a volume of 50 μl PBS and incubated at 37°C. Two hours later, the liquid was aspirated and $5x10^4$ effector cells (mock- or CR-transduced NK cells) were added in a volume of 200 μl per well. They were incubated overnight and harvested and counted the next day to be utilized in a subsequent IFN-γ ELISA.

2.11. Intracellular cytokine staining for IFN-γ

For qualitative detection of IFN-γ production on a single-cell level, $5x10^5$ mock transduced or CR-transduced NK cells were cultured either alone or with equal number of K562 or SKOV3 target cells overnight in the presence of Brefaldin A (BD GolgiPlug, 1 µl/ml). Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm Kit according to the manufacturer's protocol. In brief, cells were stained with an anti human Ig antibody, fixed and permeabilized and stained intracellularly with an anti IFN-γ Ab. NK cells were gated based on size and granularity and analyzed by flow cytometry.

2.12. IL-2 secretion assay

For detetion of IL-2 secretion on a single-cell level, $5x10^5$ mock transduced or CR-transduced NK cells were cultured either alone or with equal number of SKOV3 target cells for 5 h. IL-2 secretion assay was performed using the MACS IL-2 secretion Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cells were additionally stained for CD3 and CD56 and analyzed by flow cytometry.

2.13. CFSE labeling of NK cells

To be able to distinguish NK cells from fresh tumor cells from ascites fluid by flow cytometry in the following degranulation assay, effector cells had to be labeled with CFSE using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) prior to coculture with target cells. For this purpose, mock- and CR-transduced NK cells were washed with PBS, pelleted and resuspended in 1 ml PBS/0.1 % BSA. CFSE was added to a final concentration of 0.4 µM and cells were labeled for 10 min at RT in the dark. The staining was quenched by addition of 10 ml culture medium. Cells were washed one more time with culture medium.

2.14. CD107a degranulation assay

Target cells ($5x10^5$) were cocultured with an equal number of effector cells in 1 ml per well in a 48-well plate. Control wells contained either NK cells alone or NK cells stimulated

with PMA (2.5 μg/ml) and ionomycin (0.5 μg/ml). Anti-CD107a antibody (10 μl per well) conjugated to either PE or to PE-Cy5 was added and incubated for 1 hr at 37°C. Golgi Stop (BD Biosciences) was added to a final concentration of 6 μg/ml and cells were further incubated for 4 more hours. Cells were washed two times with PBS and analyzed on a FACSCalibur.

2.15. ^{51}Cr release assay

For direct measurement of NK cell mediated lysis, the SKOV3 cell line was labeled for 1 h with 50 μCi sodium ^{51}chromate (PerkinElmer), washed three times and adjusted to 5.000 cells per well in 100 μl volume of a 96 well V- bottom microtiter plate. Mock-transduced or CR-NK cells from three donors were added in 100 μl volume at various effector to target (E:T) ratios in triplicates, respectively. After four hours of incubation at 37°C, 50 μl of supernatant from each well were harvested and transferred to a solid scintillator coated LumaPlate (PerkinElmer), dried over night and measured in a TopCount counter. Maximal ^{51}Cr release was determined by mixing wells and transferring supernatant containing SKOV3 cells. Percentage of specific lysis was calculated using the standard formula: 100 x (cpm experimental release – cpm spontaneous release) / (cpm maximum release – cpm spontaneous release).

2.16. Tumor cell challenge and *in vivo* imaging

NOD/SCID mice were obtained from Charles River Laboratories, (Sulzfeld, Germany) and were bread in our animal facility. *RAG2* knockout mice were obtained from Jackson (via Charles River Laboratories) and bred in our animal facility. SKOV3 cells were virally transduced with pMIG-CBG to express the click beetle green luciferase (SKOV/CBG). The cells were sorted based on GFP expression to be 100% GFP positive on a FACSAria cell sorter (BD Biosiences). NK cells from one healthy donor were transduced using virus containing supernatants of either the pMSCVred or pMSCV-CR transfected 293T cells. 5x10^6 SKOV3/CBG cells were mixed in 200 μl PBS with either mock- or CR-NK cells in an effector to target ratio of 1:1 of SKOV/CBG cells to CR-NK cells, mock-NK cells were adjusted to the same cell number, for the injection into 6 NOD/SCID mice. The cell mixture was inoculated subcutaneously (s.c.) on the flank of 2 NOD/SCID mice per group.

For *in vivo* imaging, animals were injected with 200 µl of an aqueous solution of the substrate D-luciferin (150 µg/g, Biosynth, Staat, Switzerland) intraperitoneally and imaged using the IVIS 200 *in vivo* imaging system. The animals were imaged for the CBG-Luc signal initially several times a day and subsequently 3 times a week.

For the following experiment using a larger group of 23 *RAG2* knockout mice, NK cells from two donors were transduced using virus containing supernatants of either the pMSCVred or pMSCV-CR transfected 293T cells. $5x10^6$ SKOV3/CBG cells were mixed in 200 µl PBS with either mock- or CR-NK cells in an effector to target ratio of 1:1 of SKOV/CBG cells to CR^+-NK cells, mock-NK cells were adjusted to the same cell number. The cell mixture was inoculated subcutaneously (s.c.) on the flank of 3-4 *RAG2* knockout mice per group, respectively. In total, four mice received control tumor cells and 7-8 mice received either mock-NK or CR-NK cells, respectively, together with tumor cells. Twenty five percent of the mice in each group were imaged daily for the CBG-Luc signal from days 1-6. From day 7 to the end of the experiment all mice were imaged at least twice a week.

For both mice groups, a pseudocolor image representing light intensity was generated and the relative light intensity from each mouse was quantified using the Living-Image 2.6.1 software. Tumor measurements were performed at least twice a week using a caliper and mice were sacrificed once tumor reached 15 mm in any of the three perpendicular.

3. Results

3.1. Expansion and transduction of primary human NK cells

To efficiently transduce NK cells, the culture conditions that primarily favor the proliferation of NK cells based on coculture of PBLs with irradiated RPMI 8866 feeder cells were optimized. Figure 5 gives a schematic overview of the steps which were involved in isolation, expansion and transduction of NK cells.

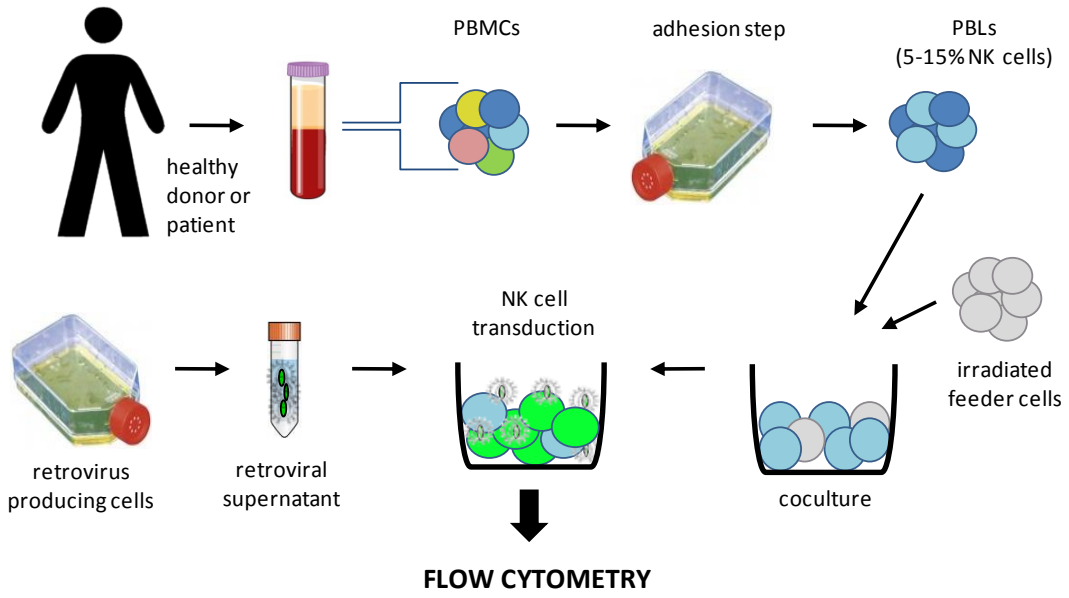


Figure 5. Schematic overview of NK cell expansion and transduction with a GFP encoding virus. *PBMCs were isolated from healthy donors by density separation and a subsequent adhesion step allowed the removal of adherent cells, leaving mainly PBLs which were cocultured with the irradiated feeder cell line RPMI 8866. Meanwhile, retrovirus was produced by virus packaging cells. Finally, NK cells were transduced using supernatant containing GFP encoding retroviral particles and analyzed by flow cytometry.*

3.1.1. Effect of PBL to feeder cell ratio on transduction efficacy

Efficient retroviral transduction requires a vigorous cell expansion, since the integration of the introduced gene by the virus involves the transition of the cell cycle. The *in vitro* expansion of primary NK cells is regulated not only by soluble factors such as interleukins, but

also by stimuli from accessory cells. In an early study, B lymphoblastoid cells were found to induce a preferential proliferation of NK cells upon coculture for at least 6 to 12 days (Perussia *et al.*, 1987). Thus, the B lymphoblastoid cell line RPMI 8866 was chosen as feeder cell line for this study, mainly because of its reported ability to secrete IL-12, a cytokine considered to be a strong stimulator of *NK* cells.

Initially, various ratios between PBLs and RPMI 8866 feeder cells were tested to determine the optimal NK cell culture conditions, that can lead to the best transduction efficacy (Fig. 6).

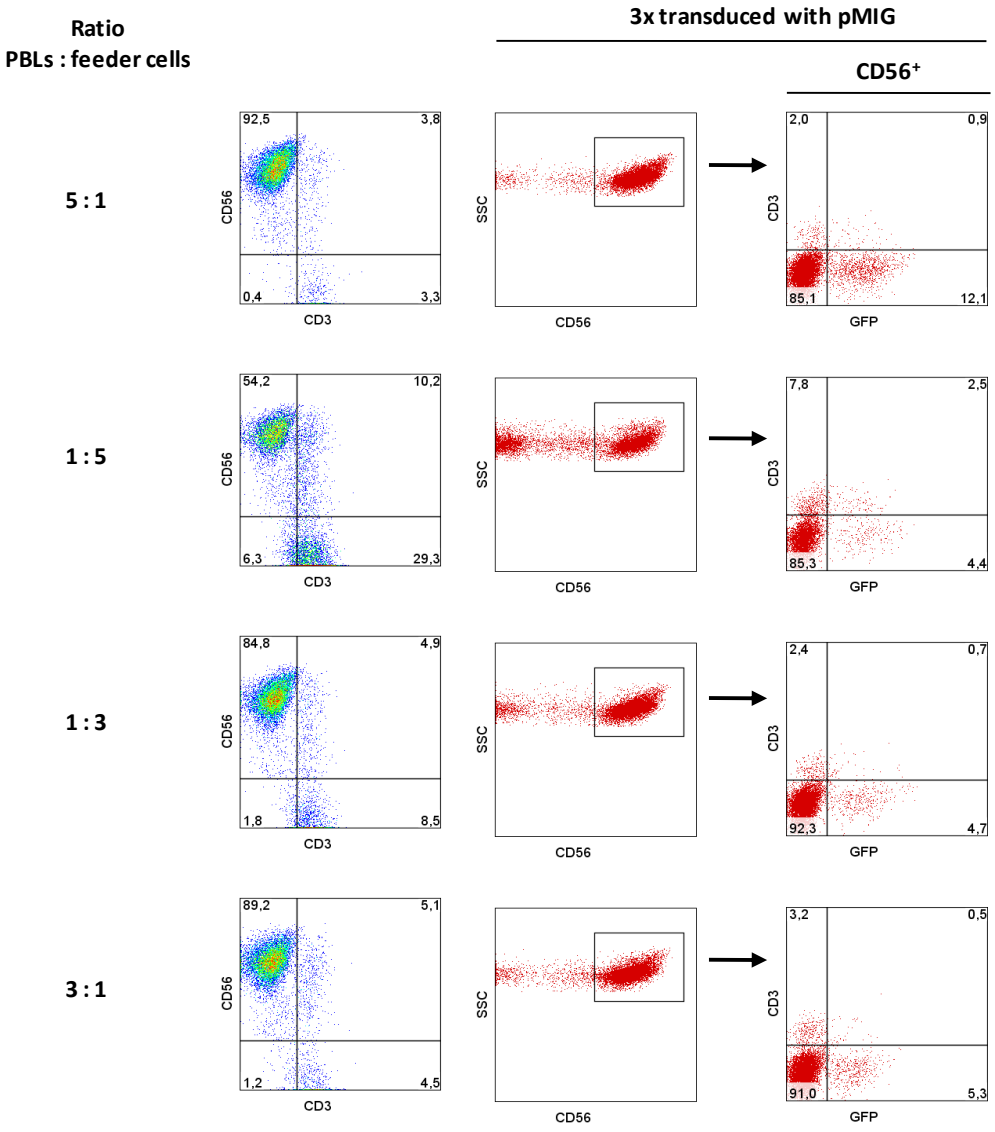


Figure 6. PBL and feeder cell ratio of 5:1 leads to highest NK expansion and transduction efficacy. *PBLs from one donor were cultured in different ratios with the irradiated feeder cell line RPMI 8866 and transduced with pMIG for 3 subsequent days using the RetroNectin protocol. The cells were then stained with a mouse anti-humanCD3 antibody conjugated to Cy-5 and a mouse anti-humanCD56 antibody conjugated to APC (left panel) to determine the percentage of $CD3^-CD56^+$ NK cells. Cells were then gated on the $CD56^+$ population (middle panel) and analyzed for GFP and CD3 expression (right panel) by flow cytometry.*

Using the pMIG vector that expresses GFP as a reporter, optimal NK cell expansion and best transduction efficacy were found based on the RetroNectin protocol culturing PBLs together with RPMI 8866 cells in a ratio of 5:1. This ratio resulted in a NK cell population of high purity (92.5 % $CD3^-CD56^+$ NK cells) and the best transduction efficacy of 12.1 % $GFP^+CD3^-CD56^+$ NK cells. Similar expansion achieved the coculture ratio of 3:1 (89.2 % $CD3^-CD56^+$ NK cells), but here the transduction efficacy was only 5.3 % GFP^+ $CD3^-CD56^+$ NK cells. The other 2 coculture ratios, 1:3 and 1:5, led to a less efficient NK expansion of 84.8 % and 54.2 % $CD3^-CD56^+$ NK cells, respectively, and a less efficient transduction of 4.7 % and 4.4 % $GFP^+CD3^-CD56^+$ NK cells, respectively.

3.1.2. Role of IL-2 in transduction efficacy

Next, the effect of IL-2 added during the transduction process on the transduction efficacy was analyzed. Figure 7 shows GFP expression on cells that were transduced based on the RetroNectin protocol either without IL-2 (upper panel) or with 200 IU/ml IL-2 (lower panel).

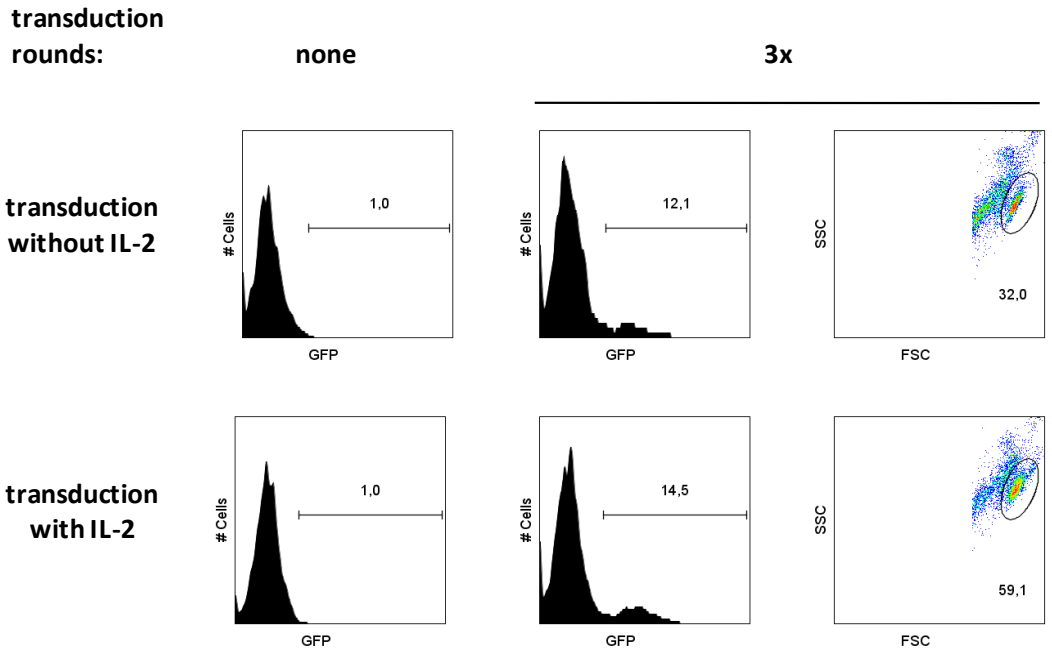


Figure 7. IL-2 addition during transduction increases cell viability. *PBLs from one donor were transduced with pMIG either without IL-2 (upper panel) or with 200 IU/ml IL-2 (lower panel) for three successive days. Cells were analyzed for GFP expression and viability by flow cytometry.*

While the presence of IL-2 during the transduction process slightly improved the transduction efficacy by 2.4 % (middle panels), the cell viability was about twice as high (32 % live cells versus 59.1 % live cells, respectively, right panels) when IL-2 was added during transduction.

3.1.3. Effect of timing and number of transductions on maximizing transduction efficacy

The optimal time point and frequency to perform retroviral transduction following NK cell activation upon coculture with the feeder cell line RPMI 8866 was investigated. Figure 8 shows GFP expression on cells that were transduced using the RetroNectin assisted transduction protocol either on day 6 (upper panel) or on day 8 (lower panel) after coculture initiation.

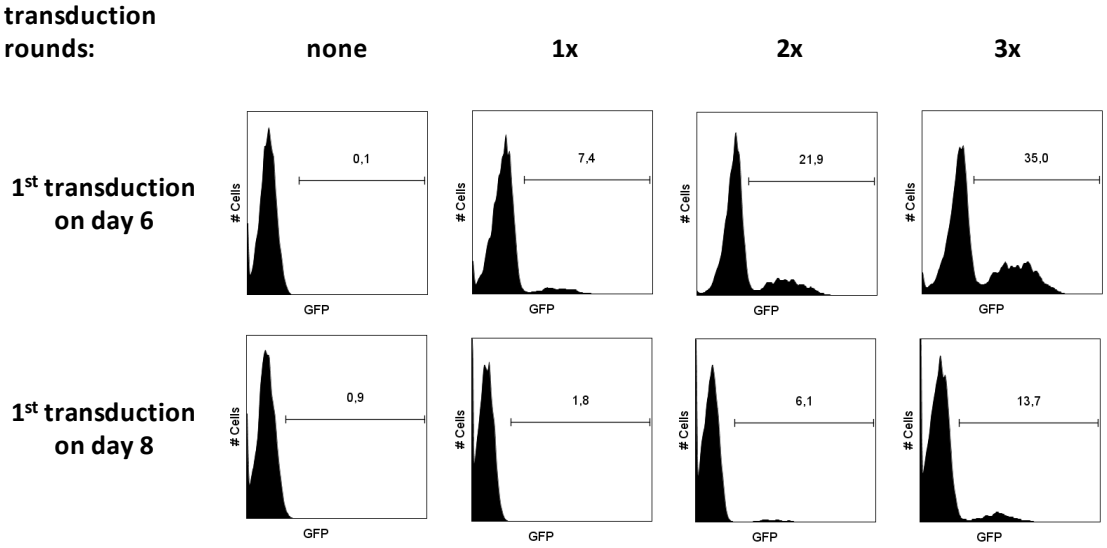


Figure 8. Improved transduction efficacy is achieved when transduction is performed on day 6 as compared to day 8 of coculture initiation. *PBLs from one donor were transduced with pMIG on day 6 (upper panel) or 8 (lower panel) for three successive days. On day 12, cells were analyzed for GFP expression by flow cytometry.*

A more efficient transduction was achieved when performed on day 6 after initiation of coculture, which led to a GFP expression of 7.4 % after the 1st transduction round, 21.9 % after the 2nd transduction round and 35 % after the 3rd transduction round (Fig. 8, upper panels). Transduction performed on day 8-10 after initiation of coculture was less efficient with 1.8 %, 6.1 % and 13.7 % after the 1st, 2nd and 3rd transduction round, respectively (Fig. 8, lower panels).

3.1.4. Impact of transduction method on transduction efficacy

Finally, two retroviral transduction protocols were compared; the first based on RetroNectin and the second based on spinocculation assisted transduction (Fig. 9).

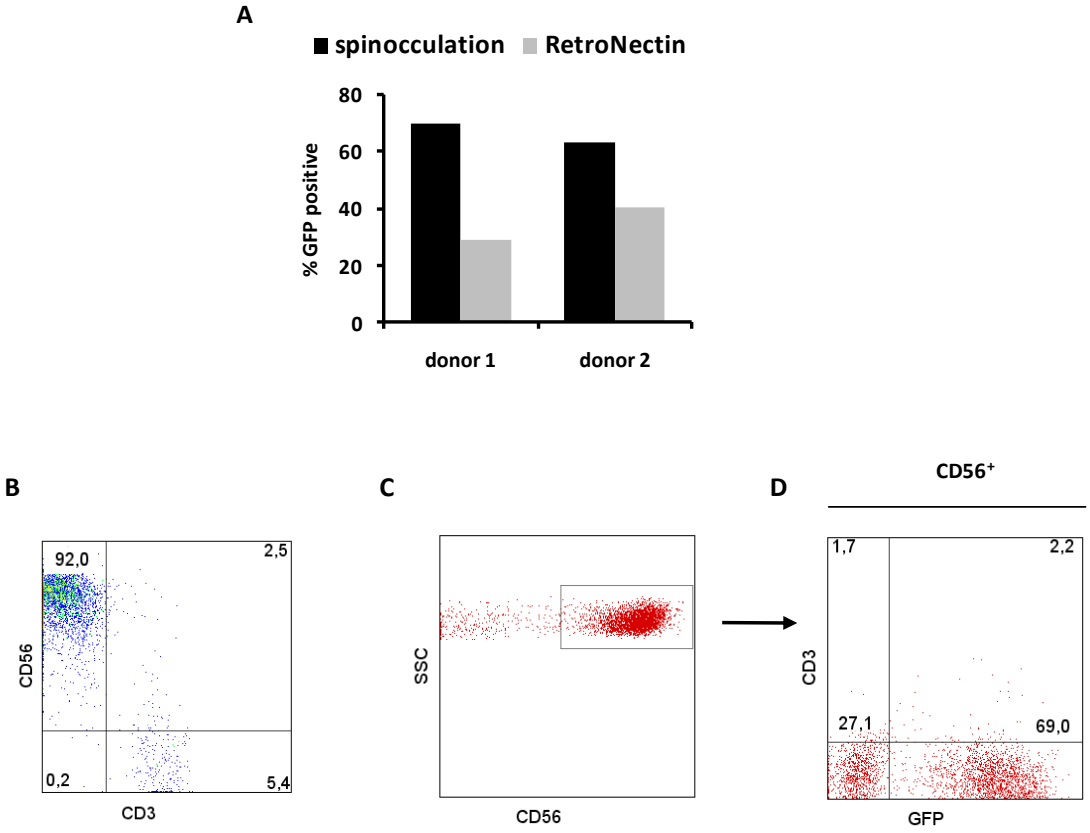


Figure 9. Highest level of NK transduction can be achieved by spinocculation. *(A) PBLs from 2 donors were transduced with pMIG using either the spinocculation protocol or RetroNectin coated plates and cells were then analyzed for GFP expression by flow cytometry. (B-D) Staining of transduced cells using the spinocculation protocol from one representative donor. Cells were stained with a mouse anti-human CD56 antibody conjugated to APC and a mouse anti-human CD3 antibody conjugated to Cy-5. (B) CD3 and CD56 expression on transduced PBLs. (C-D) FACS analysis of GFP expression on NK cells. Cells were gated on the CD56$^+$ population (C) and were then analyzed for CD3 and GFP expression (D).*

Higher transduction efficacies were achieved using the spinocculation protocol (Fig. 9A). PBLs transduced by RetroNectin showed an average GFP expression of 35 ± 8 %, whereas those transduced by spinocculation were up to 66 ± 4 % GFP positive (Fig. 9A). On day 6 from the first transduction, >90 % of the PBLs transduced via spinocculation were NK cells as judged by the CD3$^-$CD56$^+$ phenotype (Fig. 9B) and the percentage of transduced (GFP positive) NK cells was 69 % (Fig. 9D).

3.2. Genetically engineered NK cells express the transduced targeting receptor

To investigate the potential of engineering antigen-specific NK cells, a chimeric receptor construct consisting of a HER-2 specific binding domain fused to the joined CD3ζ and CD28 signaling domains was used. To obtain retroviral particles encoding the CR, the construct was cloned into the pMIG vector (Fig. 10).

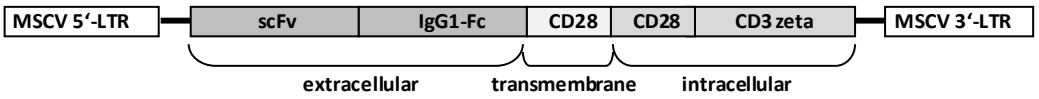
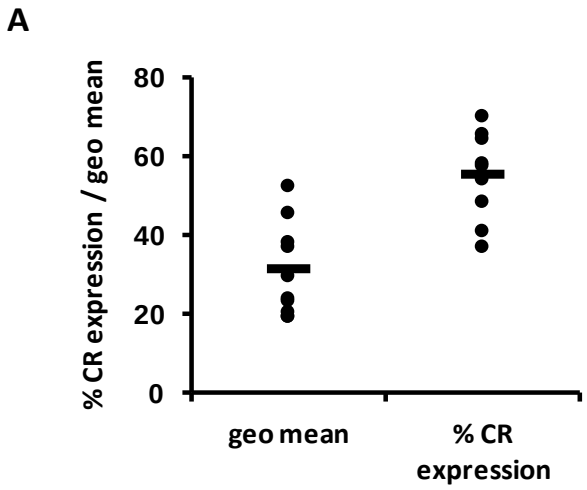


Figure 10. Schematic representation of the CR used in this study. *The chimeric receptor construct was cloned into pMIG replacing IRES and GFP. The resulting construct is designated pMSCV-CR. scFv, single-chain fragment variable; hIgG1-Fc, human IgG1 crystallizable fragment.*

293T cells were used as a packaging cell line for the transient production of virus particles, and the virus supernatant was harvested for the transduction of PBL-derived primary human NK cells of 10 different healthy donors via spinocculation (Fig. 11).



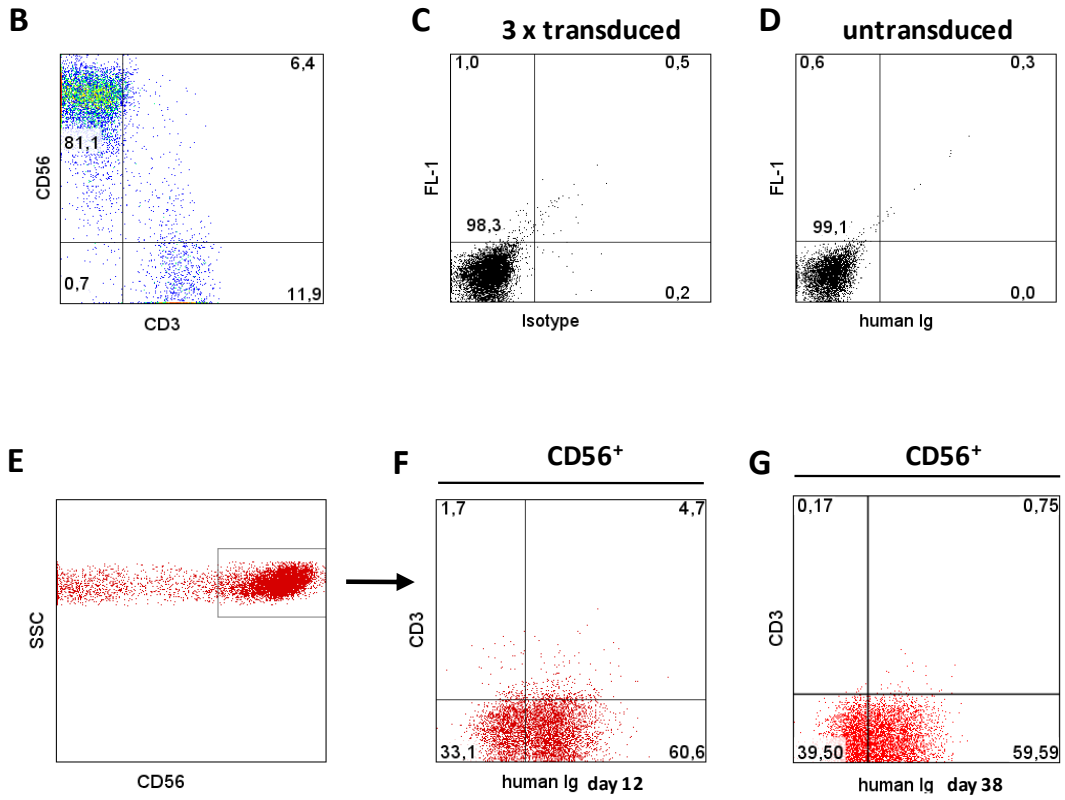


Figure 11. Her-2 specific CR is efficiently expressed on transduced NK cells. *PBLs from 10 different donors were transduced with the CR and were then analyzed for CR expression by flow cytometry. (A) Percent and geometric mean of the expression of the CR on primary NK cells from 10 different donors. (B-F) Staining of CR transduced primary human NK cells from one representative donor. Cells were stained with a mouse anti-human CD56 antibody conjugated to APC, a mouse anti-human CD3 antibody conjugated to Cy-5 and a goat anti-human Ig antibody conjugated to PE recognizing the CR. (B) CD3 and CD56 expression on CR transduced cells. (C) Isotype control staining and (D) untransduced cells stained for CR. Cells were gated on the CD56$^+$ population (E) and were then analyzed for the expression of CR and CD3 on day 12 (F) and day 38 (G) after coculture initiation.*

Flow cytometry analysis revealed that the average of transduction of the 10 different donors was 55 ± 11 %, and the geometric mean of the fluorescence signal was 31 ± 10 % (Fig. 11A). PBL-derived NK cells represented 81 % of the cell population of one representative donor (Fig. 11B), 60.6 % of which expressed the CR (Fig. 11E and F) as detected by antibodies specific for human Ig recognizing the extracellular domain of the CR. The CR expression on NK cells remained stable for more than one month from the transduction date (Fig. 11F and G).

3.3. Generation of pure NK cell cultures in order to investigate functionality

To assess the function and specificity of CR-NK cells, they had to be purified in order to remove residual T cells and NKT cells. Therefore, CR-transduced or mock-transduced PBLs were CD3 depleted using magnetic panT dynabeads (Fig. 12).

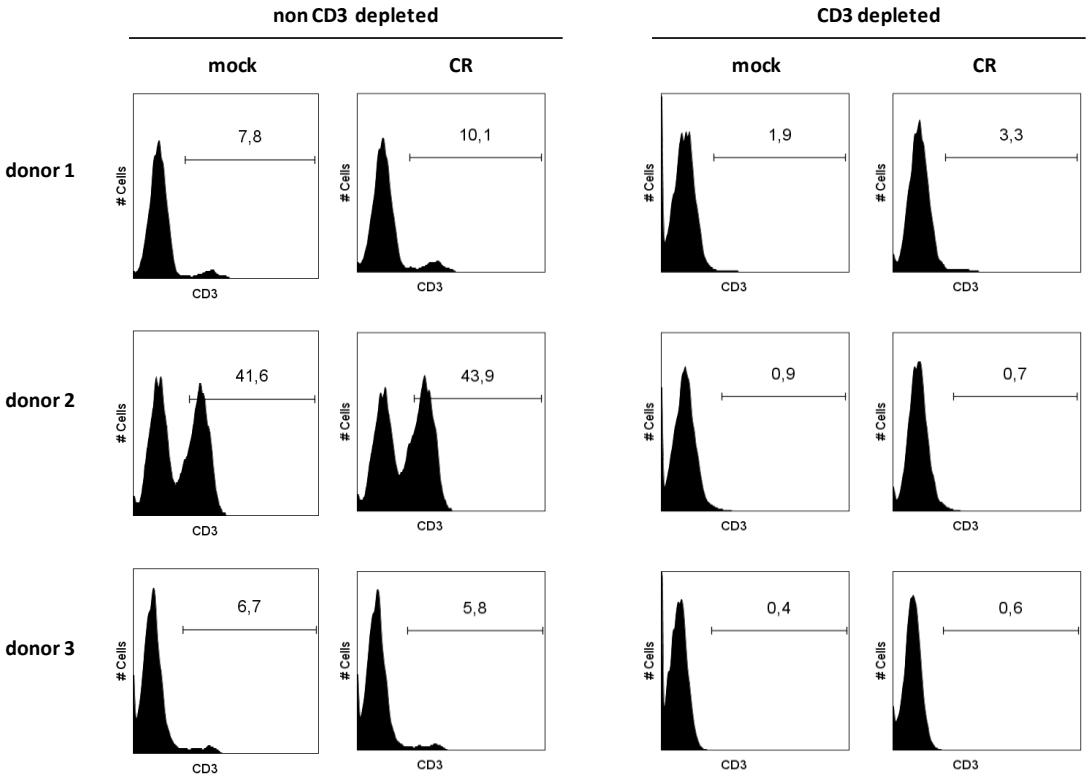


Figure 12. High purity of NK cell population after CD3 depletion. *PBLs were transduced with either the mock or the CR construct and were subsequently depleted using magnetic dynabeads conjugated to a mouse anti-human CD3 antibodies. Staining of 3 representative donors with a mouse anti-human CD3 antibody conjugated to Cy-5 which were not depleted (left panels) and on day 6 after depletion (right panels) is depicted.*

CD3 depletion by magnetic dynabeads resulted in high purity with approximately 95-99 % of the cells displaying the $CD3^{-}CD56^{+}$ NK cell phenotype (Fig. 12), even if the T cell and NKT cell amount was originally high as depicted for donor 2 (Fig. 12, middle panels).

3.4. HER-2 expressing targets specifically activate genetically engineered NK cells

The ability of genetically engineered cells to recognize HER-2 expressing target cells was gradually analyzed by various assays investigating NK cell activation and cytotoxicity.

3.4.1. HER-2 expressing transfectant specifically stimulates CR-NK cells

CD3 depleted CR- or mock-engineered NK cells were cocultured with either the HER-2 negative tumor cell line C1R/A2 or the HER-2 expressing transfectant of this cell line, C1R/A2HER2. K562 cells were included as control target cells. Figure 13 summaries the results obtained from NK cells of 10 healthy donors included in this assay.

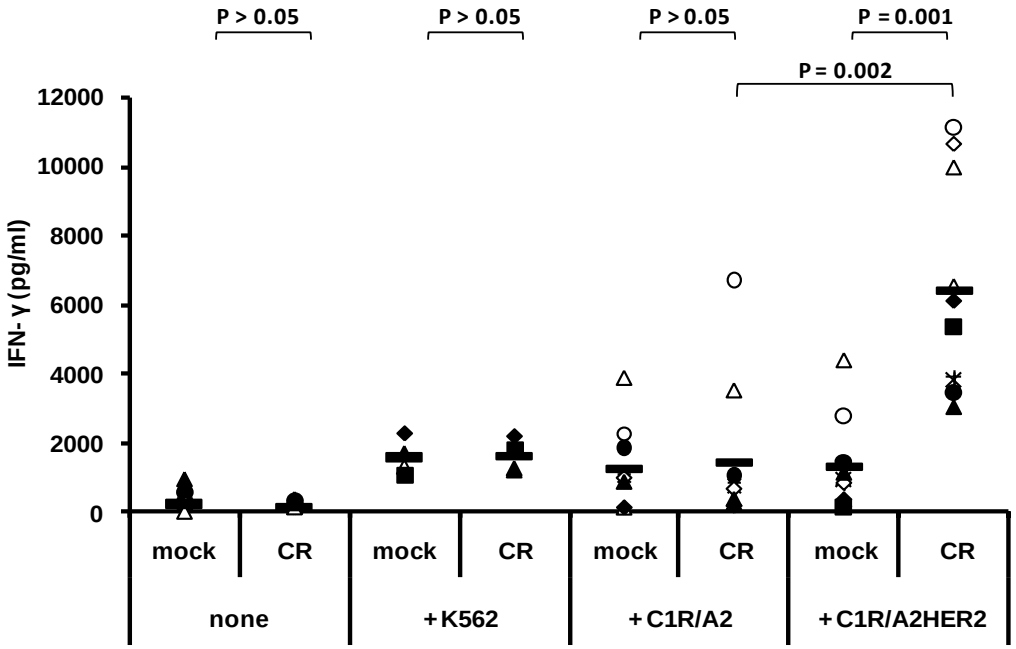


Figure 13. CR-NK cells are specifically stimulated by HER-2 expressing cells. *IFN-γ was measured in the supernatant of stimulated NK cells from 10 different donors. Values shown represent mean values of triplicates obtained from IFN-γ-specific ELISA. Effector cells ($5x10^4$) were mock transduced or CR transduced NK cells. They were either cultured without target cells (none) or cocultured with equal number of K562 cells (4 donors), C1R/A2 cells or C1R/A2HER2 cells (10 donors). P values were calculated using the Wilcoxon-Mann-Whitney test and indicate the difference between the groups.*

CR-NK as well as mock-NK cells were stimulated by C1R/A2 cells to produce on average 1300 pg/ml of IFN-γ (Fig. 13). Significantly higher levels of IFN-γ were produced by CR-NK cells, but not by mock-NK cells, in response to stimulation by C1R/A2HER2 cells with an average of 6400 pg/ml (Fig. 13). As expected, when cultured with K562 cells, all engineered NK cells produced significant levels of IFN-γ (1600 pg/ml) (Fig. 13). Neither CR- nor mock-NK cells produced IFN-γ spontaneously (Fig. 13).

3.4.2. Classification of endogenously expressed HER-2 on carcinoma lines

To determine the ability of CR-NK cells to specifically recognize the endogenously expressed HER-2 on cancer cells, a panel of breast- and ovarian-carcinoma cell lines was tested for the potential expression of HER-2 by flow cytometry. The majority of these carcinoma lines expressed HER-2 (7 of 8) and the cell lines were classified into 4 groups based on the MFI designated as negative, low, intermediate and high HER-2 expressing cells (Fig. 14).

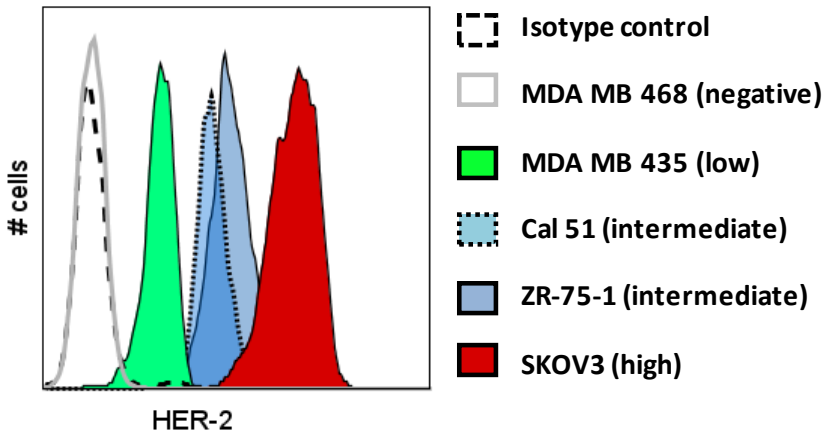
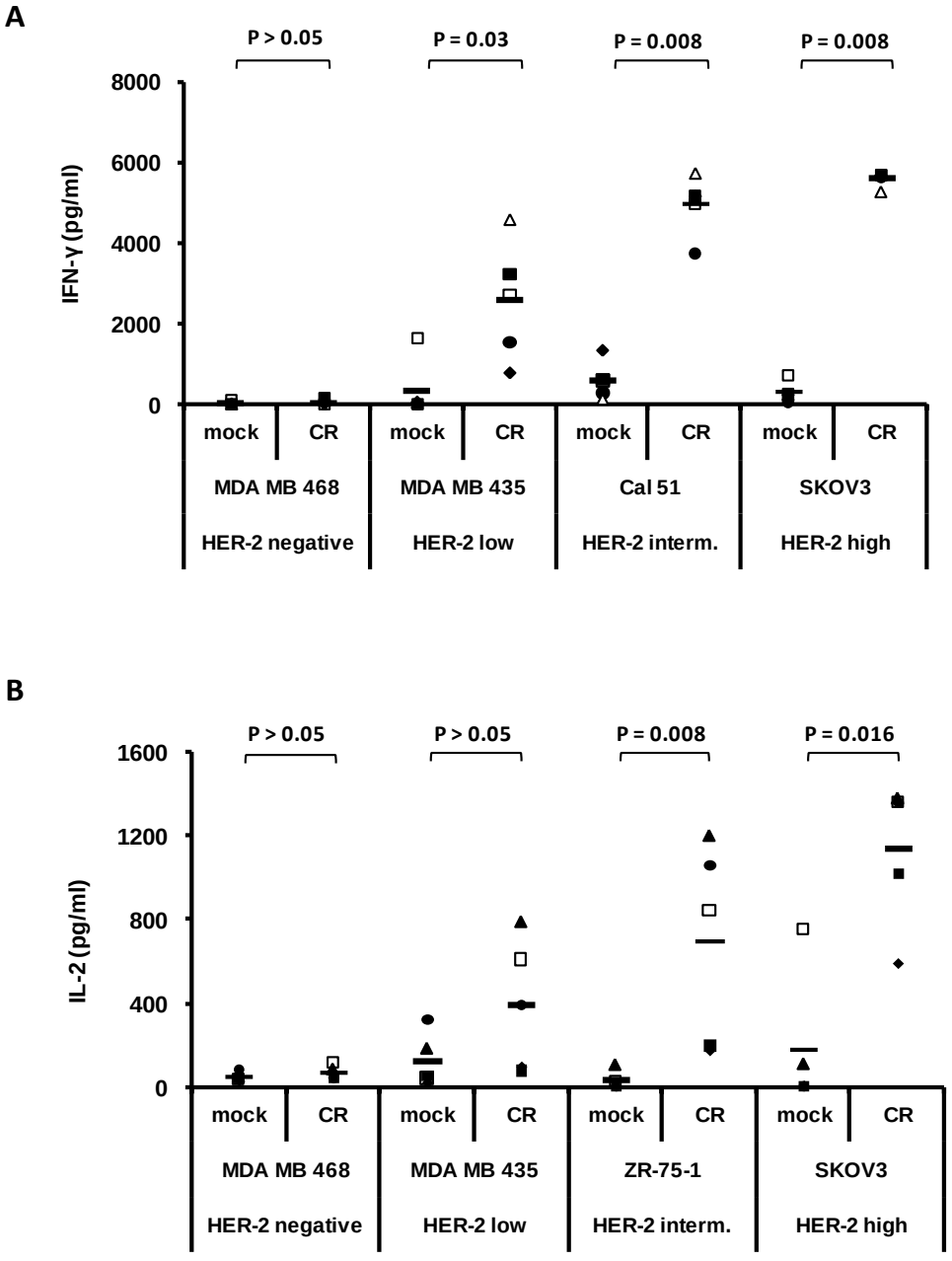


Figure 14. Different HER-2 expression levels on carcinoma cells. *Staining of carcinoma cell lines with a mouse anti-human HER-2 antibody conjugated to PE. Five different breast and ovarian cancer cell lines selected based on the increasing level of HER-2 expression on their surface are depicted. Definition of HER-2 expression (negative, low, intermediate, high) based on MFI is indicated in parentheses.*

3.4.3. CR-NK cells produce cytokines upon HER-2 recognition

Next, these 5 selected lines were tested for their ability to induce cytokine production by CR-NK cells (Fig. 15).



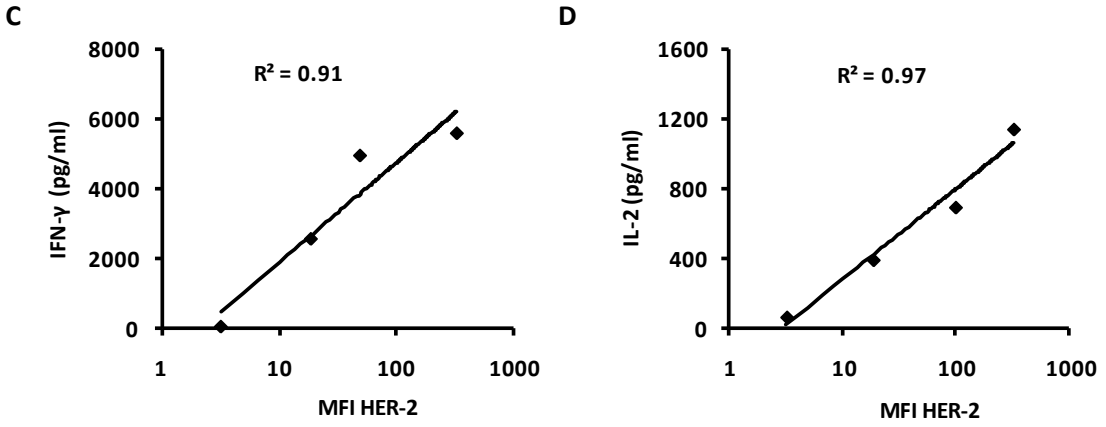


Figure 15. CR-NK cells recognize HER-2 positive but not HER-2 negative carcinomas. *Effector cells ($5x10^4$) were pMIG (mock) transduced or CR transduced NK cells. They were cocultured with equal number of the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ or IL-2 using specific ELISA. IFN-γ (A) and IL-2 (B) production by CR-NK cells derived from 5 different donors in response to stimulation. Correlation between HER-2 expression levels on the different carcinoma lines and IFN-γ (C) or IL-2 (D) production levels by CR-NK cells in response to stimulation by these lines. P values were calculated using the Wilcoxon-Mann-Whitney test and indicate the difference between the groups.*

CR-NK cells from 5 different donors were able to recognize all HER-2 positive cell lines and produced significantly higher levels of IFN-γ and IL-2 than mock-NK cells (Fig. 15A and B). There was a good correlation between the level of IFN-γ or IL-2 production by the CR-NK cells and HER-2 expression on the cancer cells (Fig. 15C and D). While the recognition of cancer cell lines expressing higher levels of HER-2 was better than that of cells with lower HER-2 expression level, those cells that did not express HER-2 were not recognized, confirming the specificity of the recognition pattern of CR-NK cells.

3.4.4. All tested HER-2 positive carcinoma lines are specifically recognized by CR-NK cells

Furthermore, additional HER-2 positive breast carcinoma cell lines were tested for the ability of inducing IFN-γ or IL-2 production by CR-NK cells derived from different donors and found to induce high levels of IFN-γ and IL-2 production by CR- but not mock-NK cells (Fig. 16).

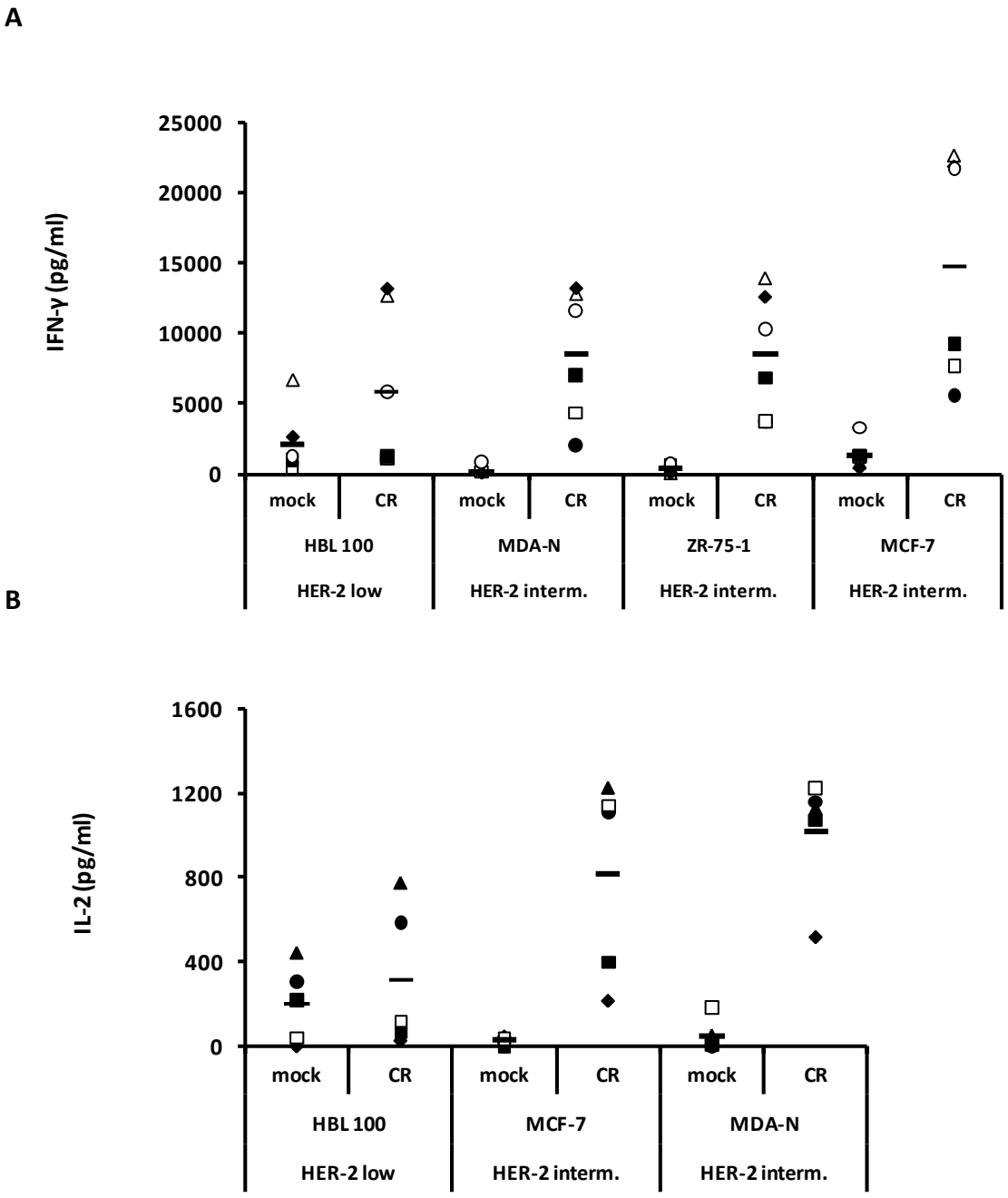


Figure 16. CR-NK cells recognize all HER-2 positive carcinomas. *Effector cells ($5x10^4$) were pMIG (mock) transduced or CR transduced NK cells. They were cocultured with equal number of the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ or IL-2 using specific ELISA. (A) IFN-γ production by NK cells derived from 6 different donors in response to stimulation by 4 HER-2 positive carcinoma cell lines. (B) IL-2 production by NK cells derived from 5 different donors in response to stimulation by 3 HER-2 positive carcinoma cell lines.*

3.4.5. MHC class I expression on tumor cells does not impact on tumor recognition

To investigate if varying expression of MHC class I molecules on tumor cells influences recognition by CR-NK cells, MHC class I expression on the different tumor cell lines was determined and IFN-γ expression and IL-2 expression of CR-NK cells was correlated with the MHC class I expression on tumor cells (Fig. 17).

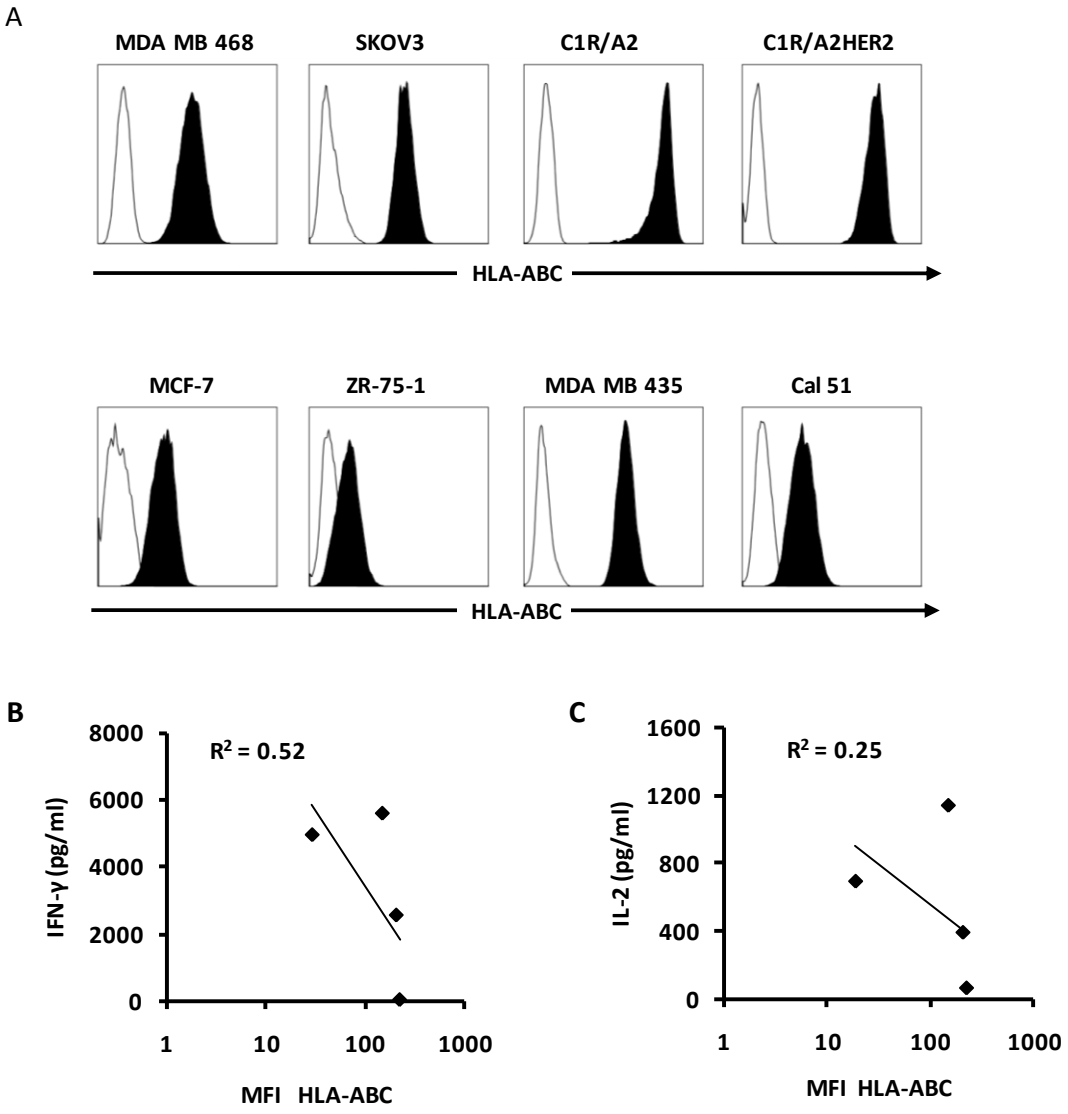


Figure 17. No correlation of MHC Class I expression on tumor cell lines and cytokine production. *(A) Various tumor cell lines were stained with a mouse anti-human HLA-ABC antibody conjugated to FITC and analyzed for MHC Class I expression by flow cytometry. Open histograms represent the isotype control staining, filled histograms represent HLA-ABC expression in the respective overlay of each tumor cell line. (B-C) Correlation between HLA-ABC expression levels on the*

different carcinoma cell lines and IFN-γ (B) or IL-2 (C) production levels by CR-NK cells in response to stimulation by the same tumor cell lines as depicted in Fig. 15.

Most carcinoma cell lines tested expressed high level of MHC class I (Fig. 17A). However, cytokine production which was measured by ELISA depicted in Fig. 15 did not correlate with the expression of MHC class I molecules (Fig. 17B and C).

3.4.6. Analysis of IFN- γ production by CR-NK cells at single cell level

To confirm that NK cells are producing IFN-γ upon activation, an intracellular staining for this cytokine was performed together with a surface staining for the CR (Fig. 18).

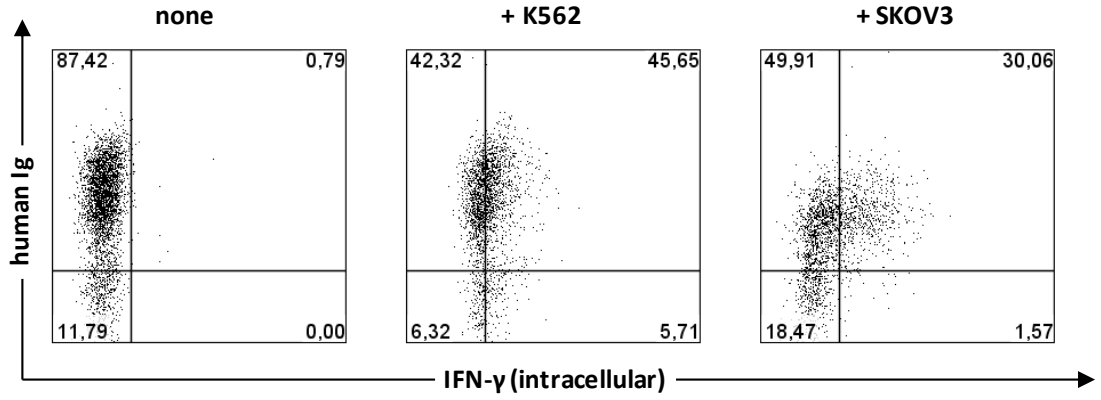


Figure 18. NK cells produce IFN-γ upon specific stimulation. *$5x10^5$ CR-transduced NK cells were cultured over night either alone (none) or cocultured with equal number of K562 or SKOV3 cells in the presence of Brefaldin A. Cells were stained with a mouse anti-human Ig antibody conjugated to PE, fixed, permeabilized and stained intracellularly with a mouse anti-human IFN-γ antibody conjugated to APC. NK cells were gated based on granularity and size and analyzed by flow cytometry.*

NK cells cultured alone produced no detectable IFN-γ (Fig. 18, left histogram). When co-cultured with K562, a fraction of approximately 50 % of both the CR negative and the CR positive population produced IFN-γ (Fig. 18, middle histogram). In contrast, when cocultured with SKOV3, no significant IFN-γ was detected in CR negative NK cells (less than 1 %), whereas 30 % of the NK cells representing 38 % of CR positive NK cells was positive for IFN-γ (Fig. 18, right histogram).

3.4.7. IL-2 is specifically produced by CR-NK cells

To confirm that CR-NK cells can produce IL-2, an IL-2 secretion assay was performed which enables the determination of IL-2 expression at the single cell level (Fig. 19).

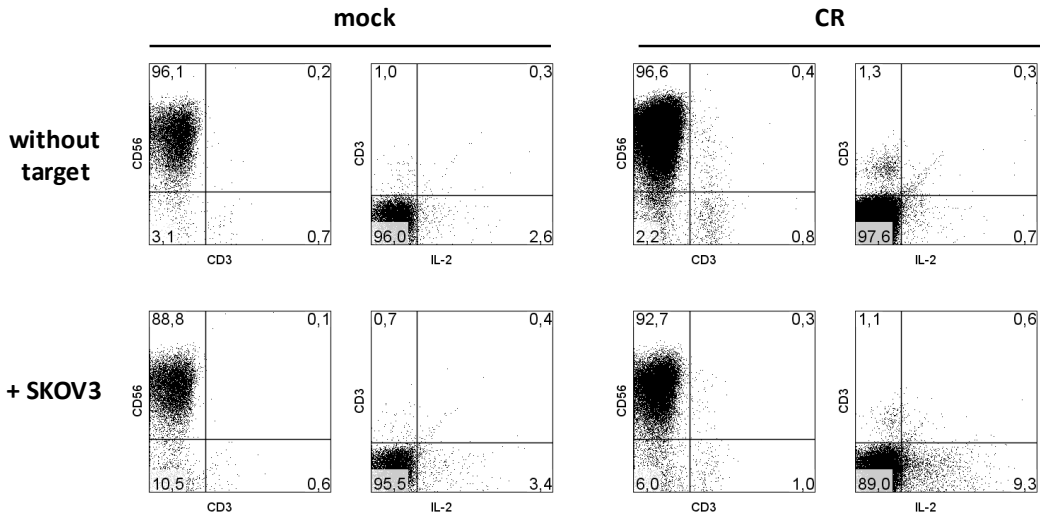


Figure 19. A subpopulation of CR-NK cells is the main source of IL-2. *$5x10^5$ mock-transduced or CR-transduced NK cells were either cultured alone or cocultured with equal number of the carcinoma cell line SKOV3 for 5 hours. Subsequently, an IL-2 secretion assay was performed and cells were additionally stained for CD3 and CD56 and analyzed by flow cytometry. One exemplary staining out of 4 is depicted.*

The CD3 and CD56 patterns confirmed the purity of NK cells (Fig. 19). A small, but clearly distinguishable subset of CR-engineered NK cells secreted IL-2. This IL-2 producing CR-NK cell subset represented 15 % of the transduced NK population taking into account that transduction efficacy for this donor was about 60 % (Fig. 19).

3.5. CR-NK cell cytotoxicity response towards HER-2 expressing targets

In addition to cytokine release, HER-2-specific NK-mediated cytotoxicity was induced by HER-2 positive target cells as indicated by the degranulation assay (Fig. 20).

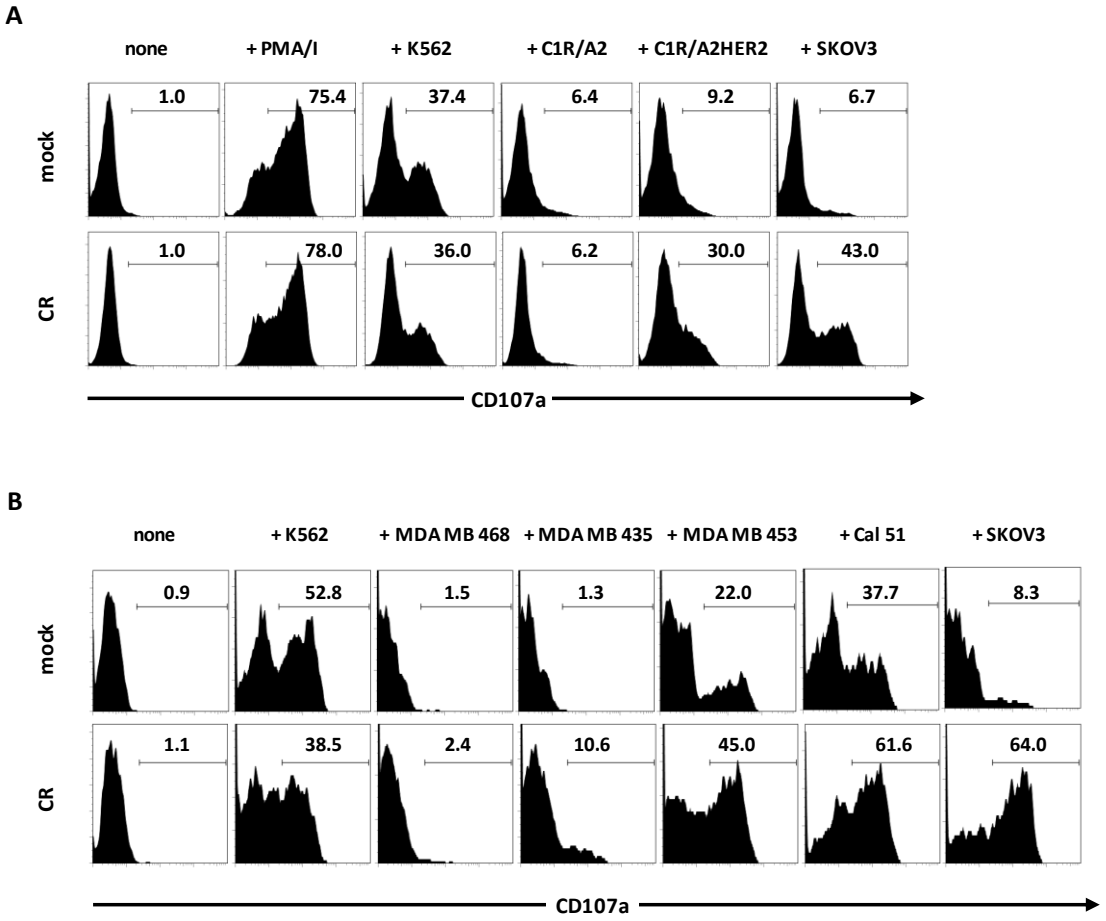


Figure 20. CR-NK cells degranulate in response to HER-2 specific stimulation. *Effector cells were pMIG (mock) transduced or CR transduced NK cells. They were either cultured without target cells (none), or with PMA and ionomycin (+PMA/I), or cocultured with the indicated cell line for 5h while being stained for CD107a conjugated to PE or PE-Cy5. Two representative experiments out of 5 performed are depicted.*

In this assay, mock-NK cells significantly degranulated in response to mitogenic (75 %) and K562 cell (37-53 %) stimulation and to the carcinoma lines MDA MB 453 and Cal 51 (22 % and 38 %, respectively) (Fig. 20A and B). CR-engineered NK cells, while degranulated in response to mitogenic (78 %) and K562 (36-39 %) cell stimulation, also responded well to all HER-2 expressing targets to a much higher extent (up to 64 %) than mock-NK cells. Notably, CR-NK cells did not degranulate in response to the HER-2 negative targets C1R/A2 or MDA MB 468 (Fig. 20A and B). The level of degranulation by CR-NK cells correlated with the level of HER-2 expression on the target carcinoma cell lines, which was in accordance with the cytokine release data.

In addition, the ability of CR-engineered NK cells to lyse HER-2 positive tumor cells was analyzed. A ^{51}Cr release assay was performed using SKOV3 cell line as target (Fig. 21).

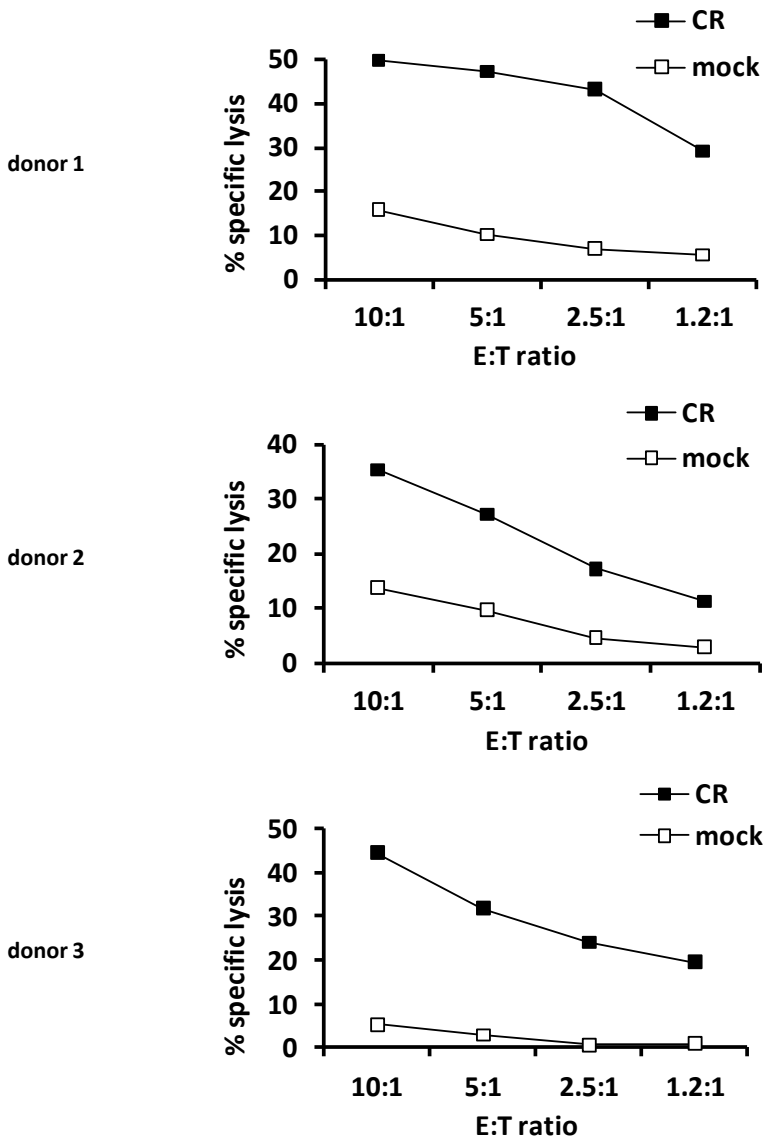


Figure 21. CR-NK cells specifically lyse HER-2 positive target cells. *Effector cells were pMIG (mock) transduced or CR transduced NK cells from 3 donors. They were cocultured with ^{51}Cr labeled SKOV3 cells in different E:T ratios for 4h and supernatants were analyzed for ^{51}Cr release.*

During 4 h, CR-engineered NK cells from all 3 donors specifically lysed up to 50 % of SKOV3 cells in an effector to target ratio of 10:1 as measured by ^{51}Cr release (Fig. 21, filled squares). In contrast, mock transduced NK cells from the same donors did not specifically lyse SKOV3 tumor cells as no significant ^{51}Cr release above background was detected (Fig. 21, open squares).

3.6. CR-NK activation by autologous cells expressing HER-2

CR-NK cell activation by autologous cells requires the overcoming of all kinds of inhibition provided by these cells. Therefore, it was tested if engineered NK cells bearing the HER-2 specific receptor will be able to overcome inhibition to 'self'. For this purpose, paired autologous LCLs, which were immortalized using a mini-EBV construct (LCL ctr) or a HER-2 encoding mini-EBV-HER-2 construct (LCL HER-2), were obtained (Fig. 22).

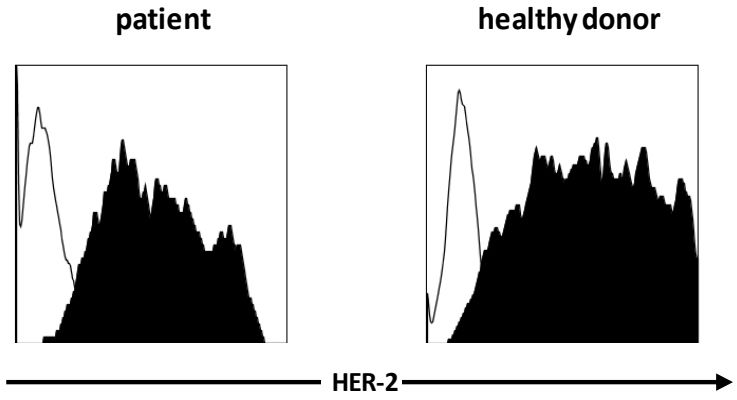


Figure 22. HER-2 expression on mini-EBV transformed LCL lines. *Paired LCL lines from a patient and a healthy donor were stained with a mouse anti-human HER-2 antibody conjugated to PE and analyzed by flow cytometry. Open histograms represent control mini-EBV transformed LCLs, filled histograms represent mini-EBV-HER-2 transformed LCLs in the respective overlay.*

HER-2 expression was broadly distributed on both the patient's and the healthy donor's LCL HER-2 line (Fig. 22, filled histograms), whereas their relevant LCL ctr cells did not stain positive for HER-2 (Fig. 22, open histograms). PBMCs from the same patient and the healthy donor from whom these LCLs were prepared were obtained and were used for the expansion and transduction of NK cells, which were then cocultured with the transformed autologous LCLs. Specific stimulation of CR-engineered NK cells by autologous HER-2 expressing LCLs was evaluated in an IFN-γ ELISA (Fig. 23).

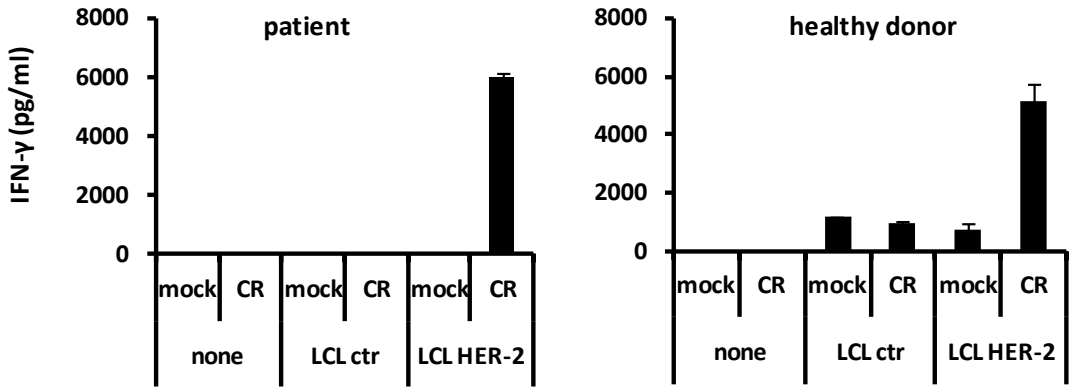


Figure 23. CR-NK cells recognize specifically autologous target cells. *$5x10^4$ paired control or HER-2 LCLs from a patient or healthy donor were cocultured with equal number of autologous mock or CR transduced NK cells. Supernatants were harvested and measured for IFN-γ using specific ELISA.*

Remarkably, CR-NK cells from the patient recognized autologous LCLs only when these LCLs expressed HER-2 (Fig. 23, left panel) and produced 6000 pg/ml IFN-γ, indicating that the activation provided by the CR signaling was sufficient to override the collective inhibitory signals provided by the corresponding inhibitory ligands on the autologous targets. Mock- and CR-NK cells from the healthy donor recognized autologous LCLs to a certain extent producing on average 900 pg/ml IFN-γ. However, the IFN-γ production by CR-NK cells significantly increased to 5200 pg/ml when these LCLs expressed HER-2 (Fig. 23, right panel).

3.7. Fresh tumor cells are recognized by both allogeneic and autologous CR-NK cells

Next, the potential of allogeneic and autologous NK cells to recognize fresh tumor cells isolated from the ascitic fluid of patients with advanced epithelial ovarian cancer was investigated. Five tumor cell samples were obtained which were positive for HER-2 at different expression levels. From two patients, matched PBMCs were also obtained, which were used for the expansion and transduction of NK cells. These autologous NK cells as well as NK cells from two additional allogeneic healthy donors were used for a coculture with fresh tumor cells. Specific stimulation of CR-NK cells was measured in an IFN-γ ELISA (Fig. 24).

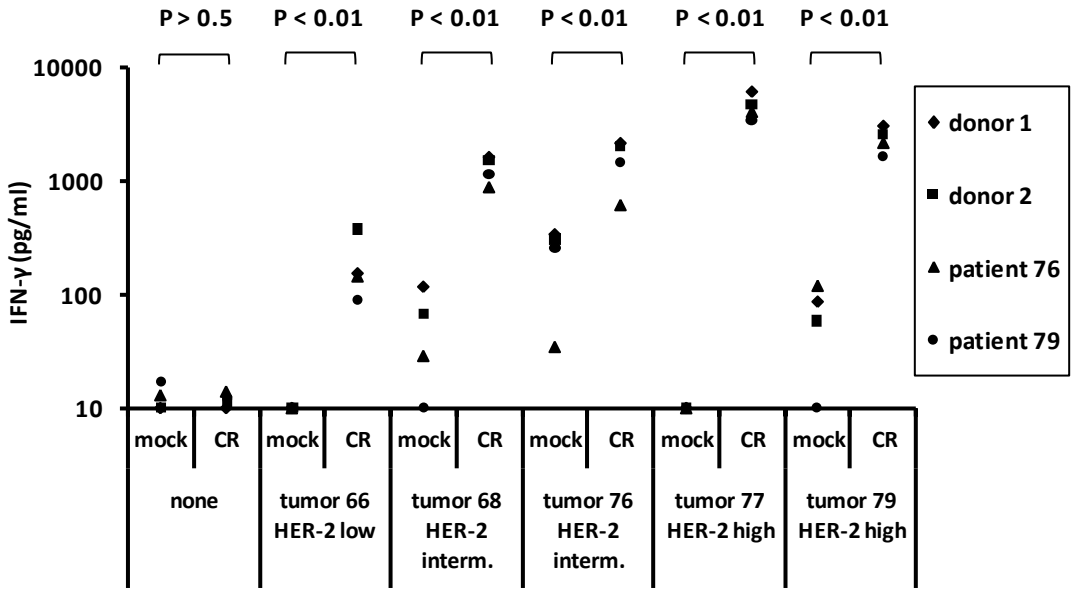


Figure 24. Autologous and allogeneic CR-NK cells specifically recognize HER-2 positive ovarian tumor cells ex vivo. *$5x10^4$ freshly isolated ovarian tumor cells were cocultured with equal number of autologous or allogeneic mock- or CR-transduced NK cells. Supernatants were harvested and measured for IFN-γ using specific ELISA. P values were calculated using the Wilcoxon-Mann-Whitney test and indicate the difference between the groups.*

Allogeneic CR-NK cells from healthy donors as well as autologous CR-NK cells obtained from ovarian carcinoma patients, but not their mock-transduced counterparts, were able to recognize fresh ovarian tumor cells *ex vivo* (Fig. 24). Tumor cells from patient 66, which featured low HER-2 expression, induced no IFN-γ production by mock-NK cells and induced an average of 300 pg/ml IFN-γ by CR-NK cells (Fig. 24). Tumor cells from patients 68 and 76, which featured intermediate HER-2 expression, induced no IFN-γ produced above detection limit by mock-NK cells. However, significantly higher amounts of 1000 pg/ml IFN-γ was induced by these tumor cells to be produced by CR-NK cells (Fig. 24). The highest IFN-γ production by CR-NK cells of 6000 – 8000 pg/ml on average was induced when the CR-NK cells were cocultured with tumor cells with high HER-2 expression from patients 77 and 79, whereas mock-NK cells produced no IFN-γ above detection limit when cocultured with tumor cells from these two patients (Fig. 24). Notably, tumor cells from patient 76 and 79 were similarly well recognized by the matched autologous CR-NK cells (triangle and circle, respectively) as by the allogeneic CR-NK cells (Fig. 24).

Additionally, a degranulation assay was performed to determine the ability of CR-NK cells to degranulate upon specific HER-2 stimulation by fresh ovarian cancer cells. In contrast to well established tumor cell lines, fresh tumor cells are heterogeneous in term of size and granularity. Therefore, NK cells were labeled with CSFE prior to coculture in order to be able to clearly distinguish them from the tumor cells (Fig. 25).

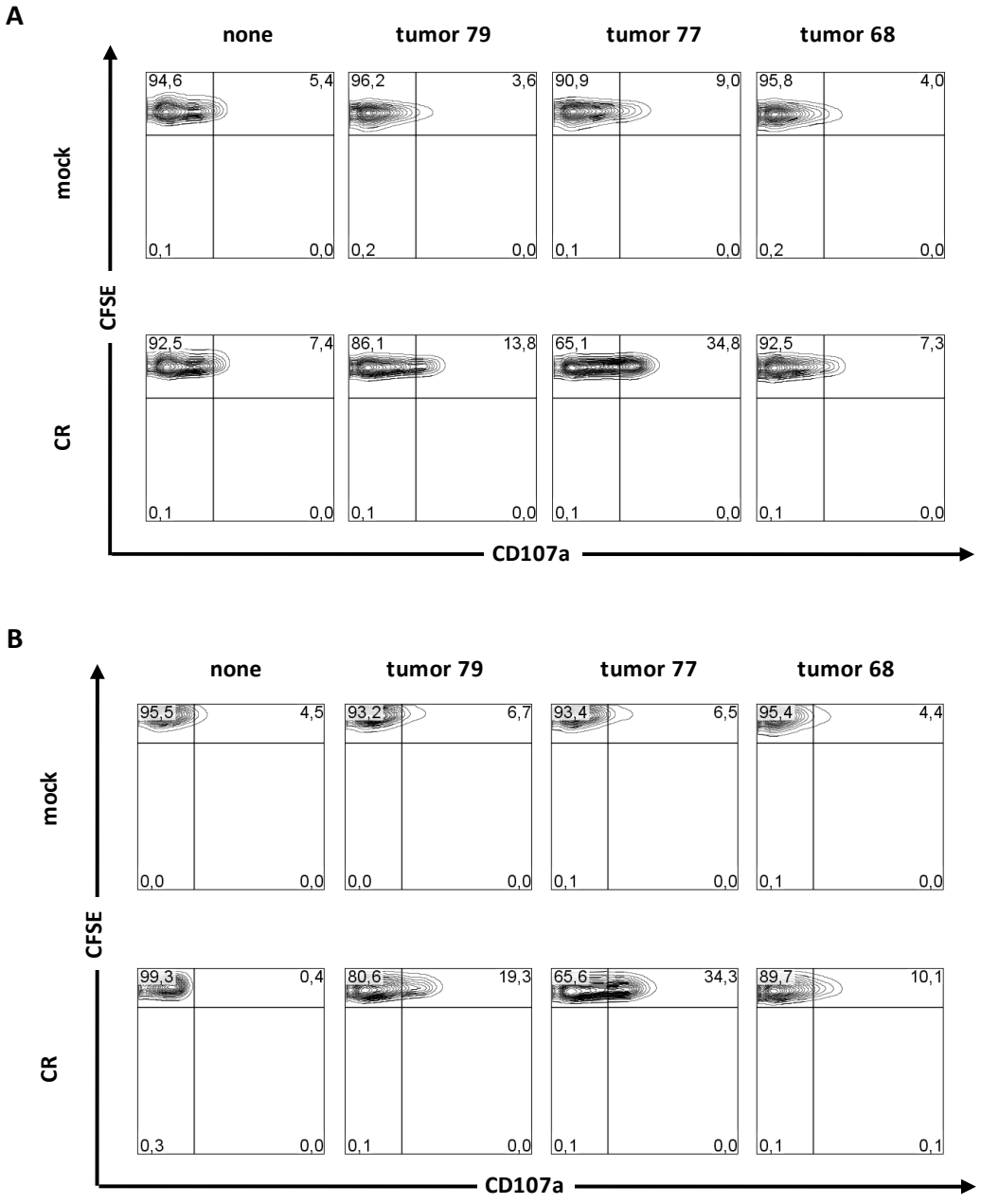


Figure 25. CR-NK cells degranulate in response to HER-2 positive fresh tumor cells. *Effector cells gated on CFSE were pMIG (mock) transduced or CR transduced NK cells from patient 79 (A) or a healthy donor (B). They were either cultured without target cells (none), or cocultured*

with the indicated tumor cells from ascites fluid for 5h while being stained for CD107a conjugated to PE-Cy5.

In line with the cytokine production, autologous CR-NK cells obtained from an ovarian carcinoma patient as well as allogeneic CR-NK cells from a healthy donor specifically degranulated in response to HER-2 stimulation by fresh ovarian tumor cells, whereas mock-NK cells did not significantly degranulate (Fig. 25A and B).

3.8. NKG2D has no impact on specific HER-2 recognition

Many tumors of epithelial origin such as carcinomas shed MICA and other soluble ligands for the activating NK cell receptor NKG2D. Since blocking of NKG2D with 100 ng/ml recombinant soluble MICA was shown to down-modulate this receptor and prevent effector cell activation (Groh *et al.*, 2002), the effect of soluble NKG2D ligands on HER-2 specific CR-NK cell activation was investigated. This question was addressed in following experiments:

3.8.1. Binding of MICA to NKG2D on NK cells

First, binding of a commercially available recombinant human MICA protein (R&D Systems, Wiesbaden, Germany) to NKG2D was confirmed by flow cytometry (Fig. 26).

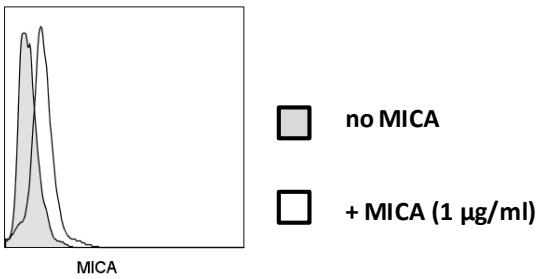


Figure 26. MICA binds to NKG2D on NK cells. *NK cells from one donor were cultured in the presence or absence of 1µg/ml of the recombinant protein MICA for 30 min and the cells were subsequently stained for MICA using a mouse anti-human MICA/B antibody conjugated to PE. Filled histogram represents MICA staining of cells which were not exposed to recombinant MICA, open histogram represents staining of cells incubated with 1 µg/ml recombinant MICA in the overlay.*

3.8.2. No down-modulation of NKG2D on NK cells after overnight exposure to soluble recombinant MICA

An overnight culture of NK cells in the presence or absence of 2 μg/ml of the recombinant MICA protein was performed to investigate a potential down-modulation of NKG2D by this protein (Fig. 27).

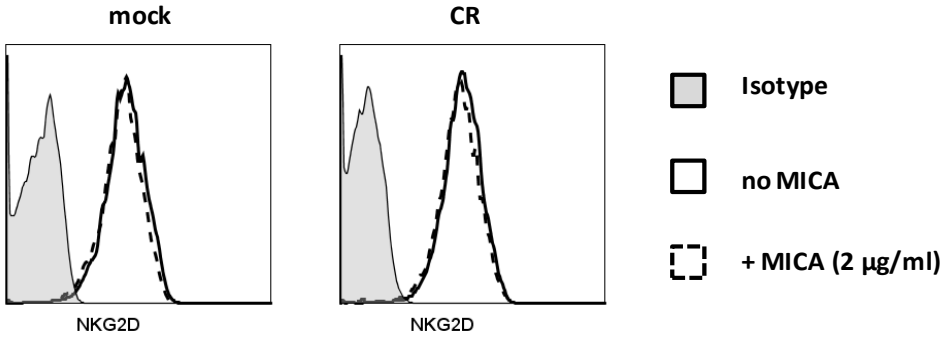


Figure 27. No NKG2D down-modulation after overnight MICA exposure. *Mock- and CR-NK cells form one donor were incubated over night either with or without 2 μg/ml of the recombinant protein MICA and were subsequently stained for NKG2D using a mouse anti-human NKG2D antibody conjugated to PE. Filled histograms represent isotype control staining, open histograms represent staining with a mouse anti-human NKG2D antibody depicted in the respective overlays. Open histograms solid line: no MICA was added to the overnight culture; open histograms dotted line: 2 μg/ml of the recombinant MICA was added to the overnight culture.*

Even if highly concentrated (2 μg/ml) recombinant MICA protein was added to the overnight NK cell cultures, no effect could be detected regarding NKG2D down-modulation (Fig. 27). This experiment was confirmed using another recombinant human MICA protein form a different company (IBT systems, Reutlingen, Germany) with similar results.

3.8.3. Selection of tumor cell lines based on NKG2D ligand expression

Three breast- and ovarian cancer cell lines were chosen based on their expression of NKG2D ligands such as MICA/B and ULBP-2 (Fig. 28).

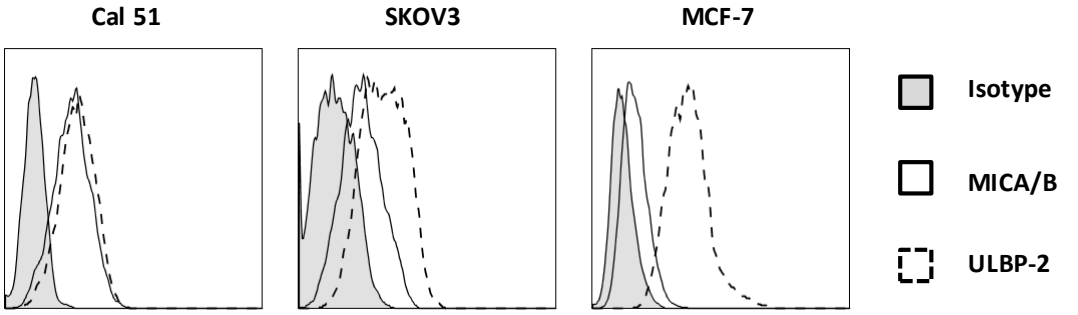


Figure 28. NKG2D ligand expression on selected tumor cell lines. *Staining of carcinoma cell lines with a mouse anti-human MICA/B antibody conjugated to PE as well as a mouse anti-human ULBP-2 antibody and a secondary anti-mouse IgG1 antibody conjugated to PE. Filled histograms represent isotype control staining, open histograms solid line represent staining with an anti MICA/B antibody, open histograms dotted line represent staining with an anti ULBP-2 antibody for each cell line depicted in the respective overlay.*

The breast cancer cell line Cal 51 expressed both NKG2D types of ligands, MICA/B and ULBP-2 (Fig. 28, left histogram). The ovarian cancer cell line SKOV3 expressed the ligand ULBP-2 and to some extent the ligand MICA/B (Fig. 28, middle histogram). The breast cancer cell line MCF-7 expressed well the ligand ULBP-2 and at a low level the ligand MICA/B (Fig. 28, right histogram).

3.8.4. No negative impact of NKG2D blockade on HER-2 specific recognition by CR-engineered NK cells

Due to expression of NKG2D ligands, recognition of these cell lines by CR-engineered NK cells likely involved the interaction of MICA/B and ULBP-2 with NKG2D in addition to CR-mediated recognition of HER-2. Therefore, I wanted to investigate whether blocking NKG2D interaction with its ligands could prevent the HER-2 specific NK cell activation. For that purpose, a coculture of these 3 cell lines together with mock- or CR-transduced NK cells was initiated, while 2 μg/ml of recombinant human MICA was added to one part of the coculture. Furthermore, I stimulated part of the effector cells overnight with 2 μg/ml plate-bound recombinant human MICA before they were further cocultured with the 3 target cell lines in the continuous presence of the recombinant protein. Subsequently, an IFN-γ ELISA was performed in order to examine the effect of NKG2D blockage by soluble MICA (Fig. 29).

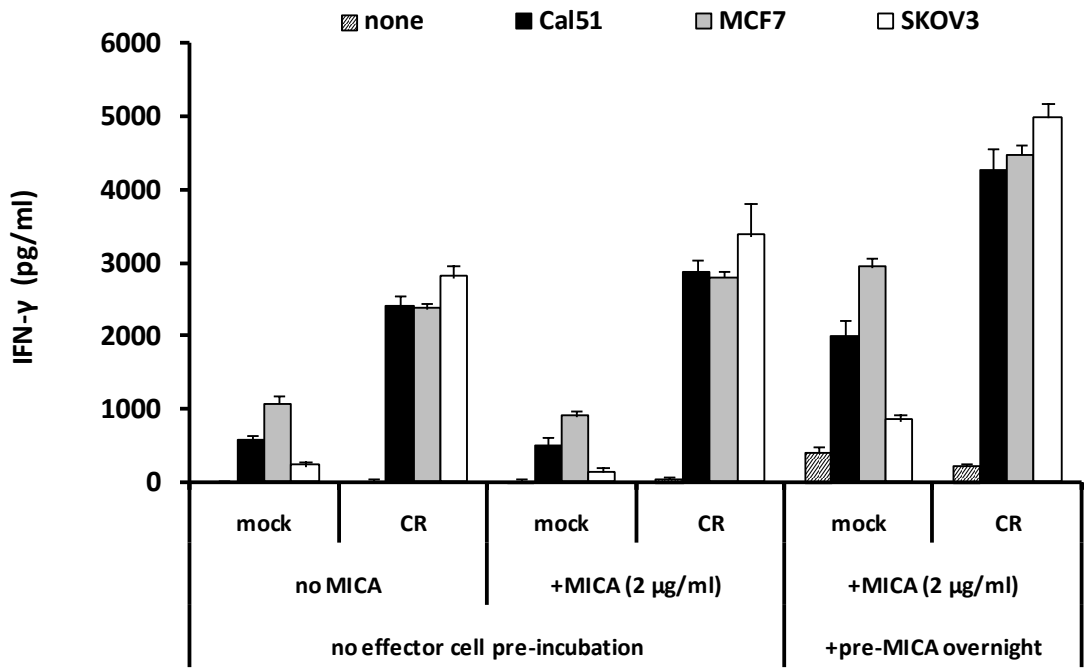


Figure 29. Blocking of NKG2D does not impair HER-2 specific recognition. *Effector cells ($5x10^4$) were mock-transduced or CR-transduced NK cells from one donor. Part of the effector cells was previously cultured overnight with 2 µg/ml plate-bound recombinant MICA protein (+ pre-MICA overnight). The effector cells were then cultured alone (none) or cocultured with equal number of the indicated carcinoma cell line in the presence or absence of 2 µg/ml of recombinant MICA protein. Supernatants were harvested and measured for IFN-γ using specific ELISA.*

As expected, in absence of recombinant human MICA significantly higher levels of IFN-γ were produced by CR-NK cells, as compared to mock-NK cells, in response to stimulation by all HER2 positive cell lines with an average of 3000 pg/ml (Fig. 29, left columns). Mock-NK cells produced some IFN-γ when cocultured with Cal 51 and MCF-7 cells (500 – 1200 pg/ml), but no IFN-γ when cocultured with SKOV3 cells. Interestingly, no considerable difference in IFN-γ levels were observed when MICA was present in these cocultures (Fig. 29, middle columns), but higher amounts of IFN-γ were produced by both mock- and CR-NK cells that were previously cultured overnight in the presence of plate-bound recombinant MICA (Fig. 29, right columns). In this setting, mock-NK cells and CR-NK cells produced already little amounts of IFN-γ when cultured alone (410 pg/ml and 220 pg/ml, respectively), but when cocultured with the 3 target cell lines, mock-NK cells

produced more IFN-γ (860 – 3000 pg/ml), however an even higher level of IFN-γ was produced by CR-engineered NK cells (4300 – 5000 pg/ml).

3.9. CR-mediated recognition compares favorably with trastuzumab mediated NK cell activation

NK cells express Fc receptors, which have been shown to be critical for an anti-HER-2 monoclonal antibody-mediated therapy such as trastuzumab (Herceptin) therapy. However, trastuzumab therapy is limited to patients with amplified HER-2, which is not a criterion for CR-mediated recognition of HER-2 positive tumors. Therefore, I was interested in comparing the two recognition systems (monoclonal antibody-based and CR-based) for their ability to trigger the NK cell response.

Initially, NK cells from 5 different donors were tested for the expression of CD16, the Fc receptor involved in ADCC (Fig. 30).

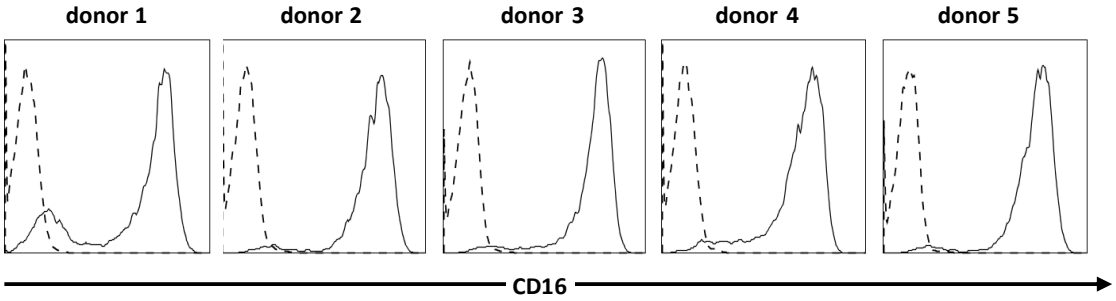


Figure 30. High levels of CD16 are expressed on NK cells. *Mock-NK cells from 5 donors were stained for CD16 and analyzed by flow cytometry. Open histograms dotted line represent isotype control staining, open histograms solid line represent stained cells using a mouse anti-human CD16 antibody conjugated to PE in the respective overlay.*

All tested NK cell lines from 5 donors expressed high levels of CD16 (Fig. 30). This fact was a prerequisite to test these cells for their ability to become activated via CD16. Additionally, allelic variation in CD16 was reported to affect its function. Hence, an overnight coculture of mock- and CR-transduced NK cells with p815 cells expressing the murine Fc receptor was initiated to confirm functional expression of CD16. 10 µg/ml of mouse anti-

human CD16 antibody was added to one part of the coculture. The Fc part of the antibody binds to the Fc receptors on the p815 cells and stimulates NK cells via CD16 leading to NK cell activation which can be measured in an IFN-γ ELISA (Fig. 31).

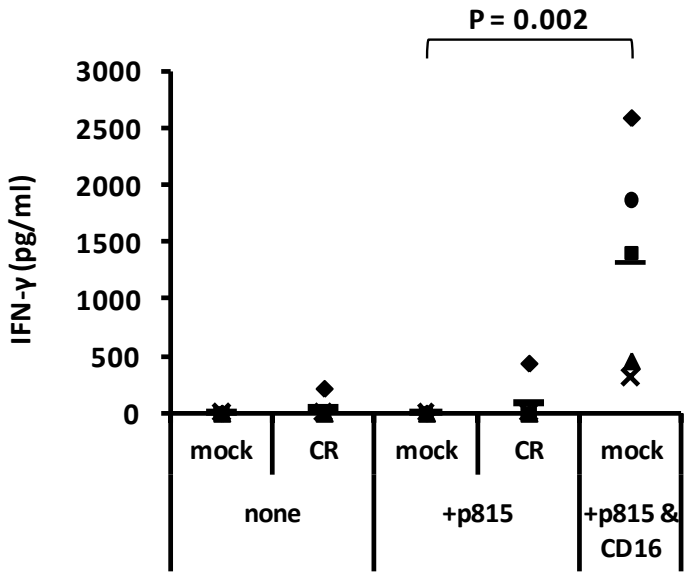


Figure 31. NK cells cann be stimulated via via CD16 to produce IFN-γ. *Effector cells ($5x10^4$) were mock transduced or CR transduced NK cells from five CD16 positive donors. They were either cultured alone or cocultured with the same number of p815 cells in the presence or absence of 10 μg/ml of a mouse anti human CD16 antibody. Supernatants were harvested and measured for IFN-γ using specific ELISA. P values were calculated using the Wilcoxon-Mann-Whitney test and indicate the difference between the groups.*

Neither mock- nor CR-transduced NK cells produce significant IFN-γ levels when cultured alone or cocultured with p815 cells (Fig. 31). However, mock-NK cells cocultured with p815 cells in the presence of 10 μg/ml of mouse anti human CD16 antibody become activated and produce significant levels of IFN-γ (on average 1500 pg/ml) (Fig. 31).

Next, I wanted to confirm that trastuzumab, a monoclonal antibody specific for HER-2, will be able to activate mock-NK cells via CD16 to recognize the high HER-2 expressing SKOV3 cell line. Therefore, an overnight coculture of mock-NK cells from two donors with SKOV3 cells in the absence or presence of 10 μg/ml trastuzumab was performed. Mock-NK cells cocultured with SKOV3 cells in the absence or presence of 10 μg/ml of the rituximab antibody specific for CD20 were used as control. IFN-γ production was measured in supernatants using a specific ELISA (Fig. 32).

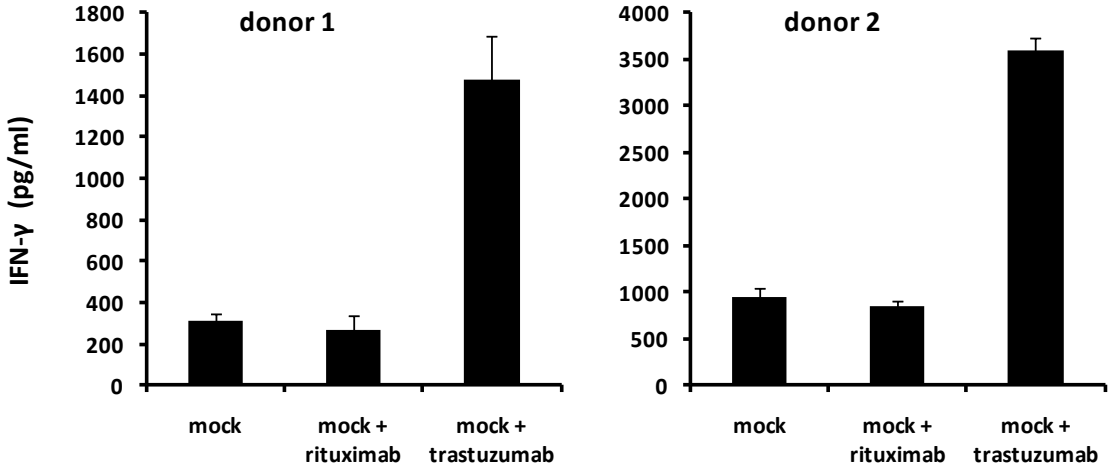


Figure 32. Trastuzumab activates mock-NK cells via CD16. *Effector cells ($5x10^4$) were mock transduced NK cells from two CD16 positive donors. They were cocultured with the same number of SKOV3 cells, which express high levels of HER-2, in the presence or absence of 10 μg/ml of either rituximab or trastuzumab. Supernatants were harvested and measured for IFN-γ using specific ELISA.*

Mock-NK cells produced little IFN-γ (309 pg/ml and 941 pg/ml, respectively) when cocultured with SKOV3 cells (Fig. 32). In the presence of 10 μg/ml rituximab, similar amounts of IFN-γ (267 pg/ml IFN-γ and 846 pg/ml, respectively) were produced. However, if mock-NK cells were cocultured with SKOV3 cells in the presence of 10 μg/ml trastuzumab, they were activated and produced high levels of IFN-γ (1474 pg/ml and 3594 pg/ml, respectively) (Fig. 32).

Finally, I performed a side-by-side comparison of the trastuzumab-based and CR-based approach for their ability to trigger the NK cell response towards an intermediate HER-2 expressing cell line. Therefore, CD16 positive NK cells from 5 different donors were cocultured over night with MCF-7 cells. Mock-NK cells cocoultured with MCF-7 cells in the absence or presence of 10 μg/ml of the rituximab antibody specific for CD20 were used as controls. Additionally, MCF-7 cells were cocultured together with mock-NK cells in the presence of 10 μg/ml of the trastuzumab antibody specific for HER-2, or with CR-NK cells. IFN-γ production was measured in supernatants using a specific ELISA (Fig. 33).

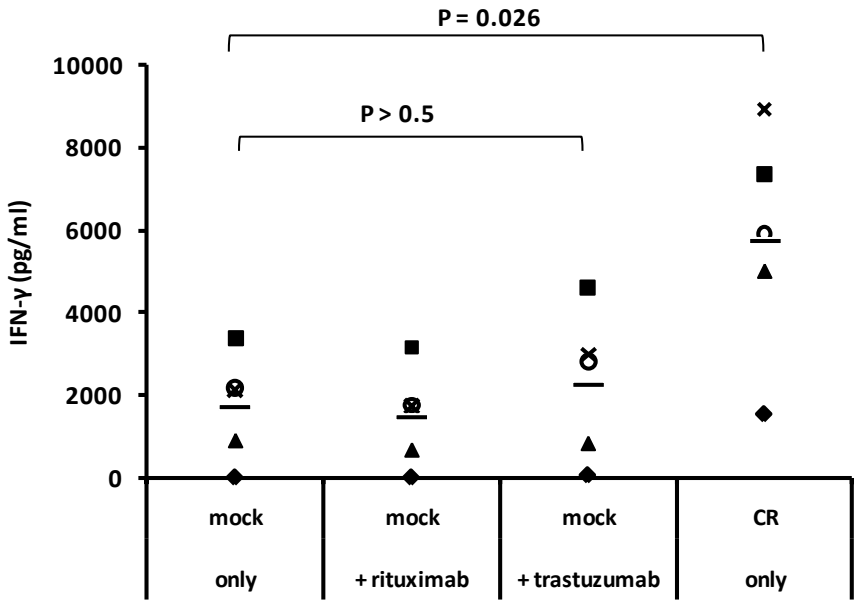


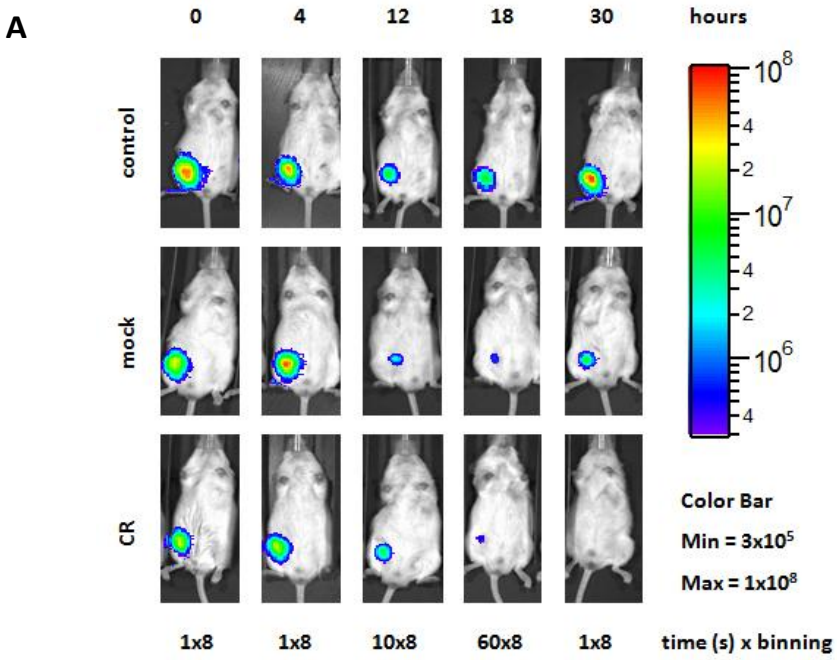
Figure 33. CR-mediated recognition performs superior to trastuzumab mediated recognition. *Effector cells ($5x10^4$) were mock transduced or CR transduced NK cells from five CD16 positive donors. They were cocultured with equal number of MCF-7 cells in the presence or absence of 10 μg/ml of either trastuzumab or rituximab. Supernatants were harvested and measured for IFN-γ using specific ELISA. P values were calculated using the Wilcoxon-Mann-Whitney test and indicate the difference between the groups.*

In the control cocultures of MCF-7 cells together with mock-NK cells in the absence or presence of 10 μg/ml rituximab antibody, average levels of 1700 pg/ml IFN-γ were detected (Fig. 33). Remarkably, no significant difference in IFN-γ production could be measured if the MCF-7 cells were cocultured with mock-NK cells in the presence of 10 μg/ml trastuzumab antibody. Here, a comparable IFN-γ level of 2200 pg/ml on average could be detected (Fig. 33). However, a significantly higher level of IFN-γ production (5700 pg/ml) was produced, when MCF-7 cells were cocultured with CR-transduced NK cells, indicating that the HER-2 specific, CR-mediated recognition is more efficient in activating NK cells in comparison to trastuzumab mediated activation.

3.10. Tumor challenge model and *in vivo* imaging

To evaluate the *in vivo* potential of these antigen specific primary human NK cells, the SKOV3 cell line was chosen due to its ability to grow in immune deficient mice such as the NOD/SCID or *RAG2* knockout mice (Fig. 33D and 34C). Subsequently, the SKOV3 cell line was transduced with a CBG construct to express the luciferase gene in order to follow its survival and outgrowth (SKOV3/CBG).

In the first experiment, 6 NOD/SCID mice were inoculated s.c. with either SKOV/CBG cells alone (control) or together with mock- or CR-NK cells from one donor and the animals were imaged for bioluminescence. The advantage of this bioluminescence imaging model is the ability to monitor tumor cell fate during the first few hours and days after injection, at the time that tumor formation cannot be detected by palpation. Figure 33 shows one representative mouse form each group measured for bioluminescence during the first 30 hours (Fig. 32A) and the following days (Fig. 32B).



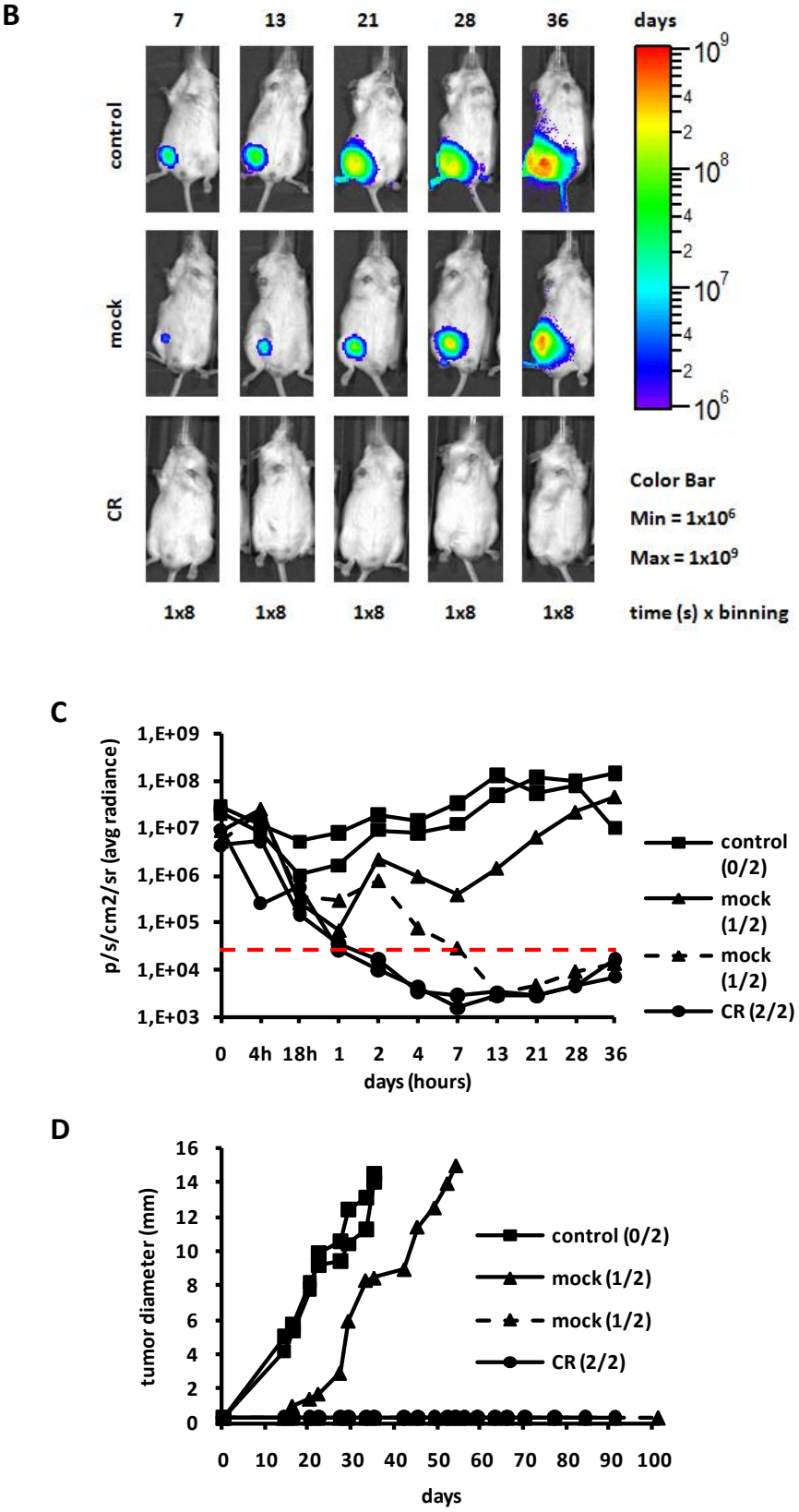


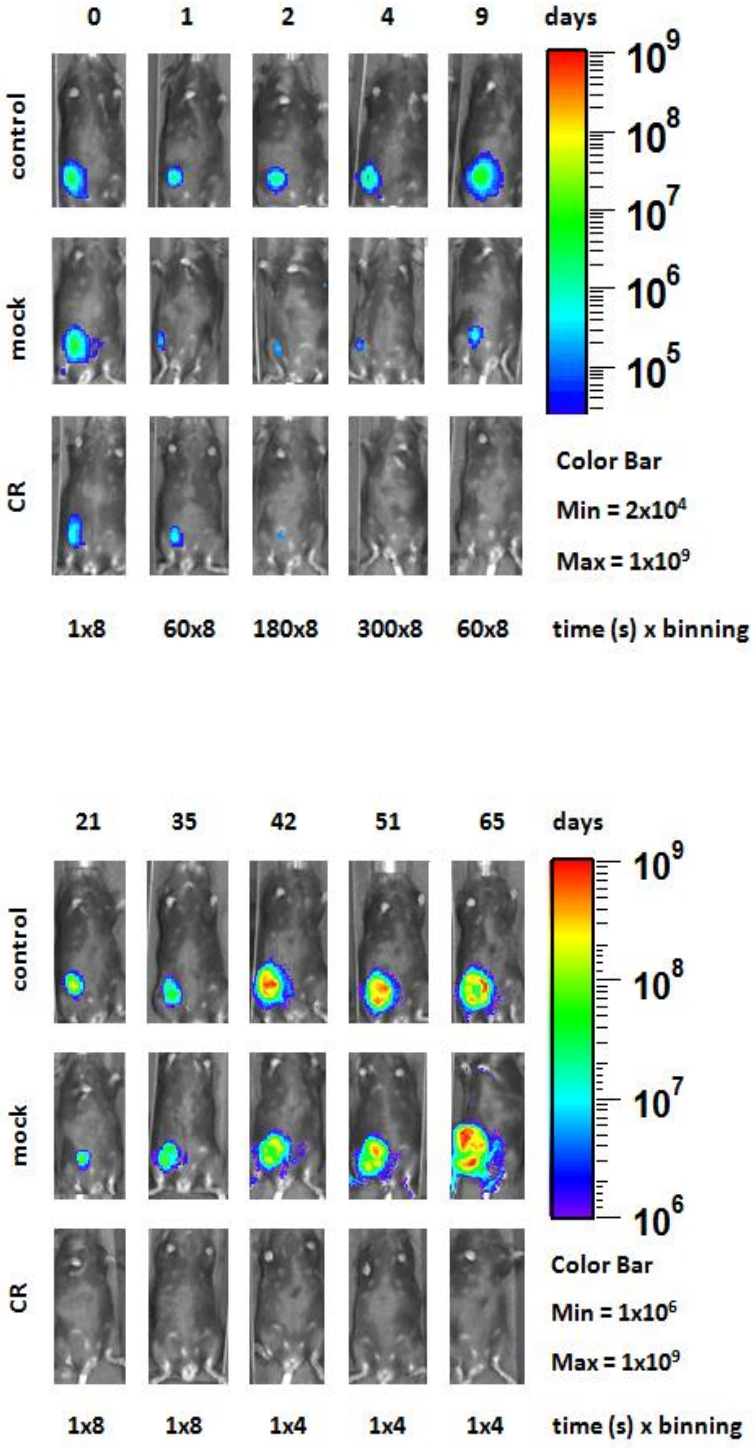
Figure 32. CR-NK cells efficiently eradicate HER-2 positive carcinoma in a NOD/SCID mouse model. *NOD/SCID mice were inoculated s.c. with 5x10^6 SKOV/CBG cells alone or together with the same number of CR-NK cells or mock-NK cells from one donor. In vivo*

imaging of tumor cell outgrowth or death in one mouse from each group during the first 30 hours (A) and the following days (B) is depicted. Due to increase in signal intensity correlating with tumor outgrowth, acquisition time and binning were changed as indicated and the pseudocolor scale bar was adjusted accordingly. (C) Quantification of signal intensity kinetics from tumor region. Background signal as measured for the imaging platform is indicated by the dashed line. (D) Mean tumor diameter. Tumors were measured 1-2 times per week using a digital caliper (C-D). Symbol description: filled squares represent 2 control mice, filled triangles represent 2 mice which received mock-NK cells, tumor grew in one of these mice (continuous line) but not in the other mouse (dashed line), filled circles represent 2 mice which received CR-NK cells. Numbers of live mice in each group are described in parentheses.

During the first 4 hours after injection, light signal didn't change significantly in all groups (Fig. 32A and C). If SKOV/CBG cells were injected alone, light signal decreased after 12 hours but started to recover after 18 hours to reach the original signal intensity after 30 hours (Fig. 32A and C, squares). The signal intensity increased during the following days as a result of increased tumor size (Fig. 32B and C, squares), and mice were sacrificed due to large tumor burden when mean tumor diameter reached 15 mm (Fig. 32D, squares). Similar signal kinetics was seen in one of the mice that were coinjected with SKOV/CBG cells together with mock-NK cells. In this mouse, light signal decreased more dramatically but nonetheless started to recover after 30 hours and increased over time (Fig. 32A and B, middle panels, and Fig. 32C, triangle solid line), which correlated with the tumor outgrowth in this mouse (Fig. 32D, triangle solid line). The other mouse that received similar coinjection with mock-NK cells, however, showed a slow but continuous signal decrease and survived the tumor challenge (Fig. 32C and D, triangle dotted line). When SKOV/CBG cells were injected together with CR-NK cells, light signal started to decrease rapidly after 12 hours and was hardly detectable after 30 hours (Fig. 32A and B, lower panels and Fig. 32C, circles). By day 2, no signal above background (Fig. 32C, circle) was detectable from the SKOV/CBG cells in this group despite prolonged imaging and all mice survived the tumor challenge (Fig. 32D).

The experiment was repeated using a larger group of animals on a *RAG2* knockout background. After inoculating 23 *RAG2* knockout mice subcutaneously with either SKOV/CBG cells alone (control) or together with mock- or CR-NK cells from two different donors, the animals were imaged for bioluminescence (Fig. 33).

A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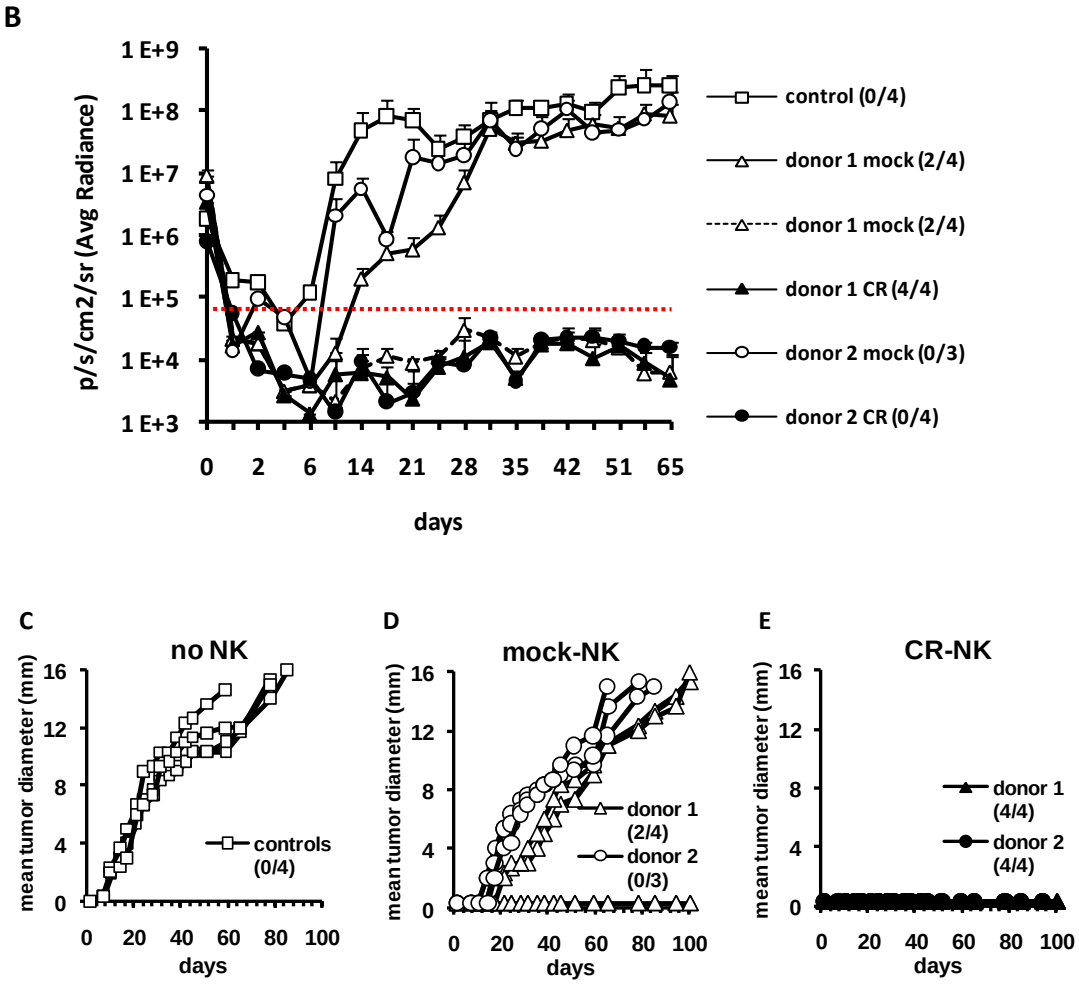

Figure 33. CR-NK cells efficiently eradicate HER-2 positive carcinoma in a RAG2$^{-/-}$ mouse model. *RAG2 $^{-/-}$ mice were inoculated s.c. with 5x10^6 SKOV/CBG cells alone or together with the same number of CR$^+$-NK cells or mock-NK cells from two different donors. (A) In vivo imaging of tumor cell outgrowth or death in one representative mouse from each group. Due to increase in signal intensity correlating with tumor outgrowth, acquisition time and binning were changed as indicated, and two pseudocolor scales corresponding to signal intensity are depicted with a minimum of 2x10^4 for days 1-9 and 1x10^6 for days 21-65. (B) Exact quantification of signal intensity kinetics from tumor region. Background signal as measured for the imaging platform is indicated by the dashed line. Open squares represent 4 control mice, open triangles represent 4 mice which received mock-NK cells from donor 1, tumor grew in two of these mice (continuous line) but not in the other 2 mice (dashed line), filled triangles represent 4 mice which received CR-NK cells from donor 1, open circles represent 3 mice which received mock-NK cells from donor 2, filled circles represent 4 mice that received CR-NK cells from donor 2. Error bars represent standard deviations. (C-E) Mean tumor diameter. Tumors were measured 1-2 times per week using a digital caliper. Survival comparison was performed using the Wilcoxon-Mann-Whitney test. The P values for the difference between the groups are P<0.001, P= 0.02 for differences between C and E and between D and E, respectively. The difference between C and D is not statistically significant. Numbers of live mice in each group are described in parentheses.*

In Fig. 33A one representative mouse of each group is shown. When SKOV/CBG cells were injected alone, light signal decreased during the first 6 days but recovered on day 9 and increased over time, which was followed by increase in mean tumor diameter (Fig. 33A, upper panels, 33B and C). A similar signal kinetic was seen in most of the mice that were coinjected with SKOV/CBG cells together with mock-NK cells. In these mice, light signal decreased more dramatically but nonetheless started to recover on day 9 and increased over time followed by an increase in the mean tumor diameter (Fig. 33A, middle panels, 33B and D). All mice that have received coinjection with mock-NK cells from donor 2 succumbed to tumor outgrowth. Two of 4 mice that received similar coinjection but with mock-NK cells from donor 1 also succumbed, while the other 2 mice survived the tumor challenge (Fig. 33B and D). When SKOV/CBG cells were injected together with CR-NK cells, the light signal decreased initially to the same extent as the group with mock-NK coinjection but by day 4 no signal above background (Fig. 33B) was detectable from the SKOV/CBG cells despite prolonged imaging and no tumor could be palpated in these mice (Fig 33A, lower panels and 33E). All mice that have received the coinjection of SKOV/CBG cells together with CR-NK cells survived and remained tumor free.

4. Discussion

NK cells were discovered more than 30 years ago based on their ability to directly lyse certain tumor cells *in vitro* without prior sensitization. Since that time, much has been elucidated about how NK cell recognition of tumor cells occurs. Today it is known that NK cells developed a recognition mechanism that depends on sensing the expression of activation versus inhibitory ligands on their target cells (Gasser and Raulet, 2006; Johansson and Hoglund, 2006; Lanier, 2005; Lanier, 2006; Parham, 2006; Trinchieri, 1989). While loss or downregulation of ligands for inhibitory receptors is often associated with NK recognition, some tumors that lack MHC class I molecules are nonetheless resistant to NK killing, probably due to the decrease or absence of a recognizable activation ligand. Therefore, various approaches were evaluated to overcome NK cell tolerance to tumor cells. These included the use of allogeneic NK cells (Ruggeri *et al.*, 2006), blocking the inhibitory signal (Koh *et al.*, 2001), or triggering the activation receptors by using monoclonal antibodies or by the genetic modification of tumor cells (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001; Wilson *et al.*, 1999).

Unlike transformed cells from hematopoietic origin which express several activation ligands (Bryceson *et al.*, 2006; Wilson *et al.*, 1999), cells derived from solid tumors are rather resistant to NK recognition. Hence, the objective of this study was to exploit the potential for primary human NK cells to be genetically manipulated in order to specifically target carcinomas. For this purpose, an approach to enhance specific tumor recognition by NK cells based on the recognition of the tumor-associated antigen HER-2 via a CR (Abken *et al.*, 2002) was tested. The expansion and genetic engineering of primary human NK cells generated up to $1x10^9$ tumor-specific effector cells within 2 weeks from the PBL isolation day. These engineered NK cells recognized and lysed tumor cells *in vitro* and *in vivo*. Notably, freshly isolated tumor cells from cancer patients were targeted with similar efficiency by allogeneic as well as autologous engineered NK cells.

4.1. Optimization of a transduction protocol in order to engineer primary human NK cells

Proliferation is a prerequisite for an efficient retroviral transduction, since the retroviral DNA can only enter the nucleus and subsequently integrate into the host genome upon breakdown of the nuclear membrane, which happens shortly before cell division. Protocols for retroviral transduction of T cells include an effective T cell activation leading to proliferation prior to the addition of the retroviral particles (Charo *et al.*, 2005). Thus, various factors were tested in order to identify optimal conditions for the expansion and proliferation of primary human NK cells isolated from PBMCs. As a basis, a protocol for NK cell expansion form Perussia *et al.* (Perussia *et al.*, 1987) was used which utilized irradiated LCL lines as feeder cells. Among these, the B lymphoblastoid cell line RPMI 8866 was considered for this study because it was reported to secrete IL-12. This cytokine, also called NK cell stimulatory factor (NKSF), was reported to induce IFN-γ production and enhance NK cell cytotoxicity (D'Andrea *et al.*, 1992). Based on this study, NK cells which were cocultured with irradiated RPMI 8866 cells at a ratio of 5:1 showed the highest expansion and best transduction efficacy using GFP as a reporter. Apparently, this cell ratio allowed preferential NK cell activation. IL-2, which was administered during the transduction process, did not increase transduction efficacy, but increased cell viability. This might be due to the fact that IL-2 was shown to not only induce NK cell proliferation, but also to protect the NK cells from death by apoptosis (Armant *et al.*, 1995). Optimal transduction efficacy was achieved when the first transduction was performed on day 6 after the initiation of coculture, and was repeated two more times on the subsequent two days. This might be because between day 6 and 8 after coculture initiation NK cells have their highest proliferation. Furthermore, improved results were obtained when performing transduction by spinoculation in comparison to the RetroNectin assessed protocol. Taken together, the optimized protocol for transduction of primary human NK cells included coculture of PBLs with irradiated RPMI 8866 cells at a ratio of 5:1, performing the first transduction step by spinocculation on day 6 after coculture initiation, and repeating transduction on two subsequent days in the continuous presence of IL-2.

4.2. Expression and functionality of the CR *in vitro*

The CR composed of a scFv domain specific for HER-2 fused via a linker to the joined CD3ζ and CD28 signaling domains was cloned into the pMIG vector in order to be transiently packaged into retroviral particles in 293T cells. After transduction using the optimized protocol, the expression of the CR was confirmed by flow cytometry and remained stable for more than one month, confirming the integration of the retroviral construct into the NK cell genome. In order to investigate the functionality of CR-engineered NK cells, it was important to obtain a pure NK cell culture, which was achieved by the depletion of residual T cells using magnetic anti CD3 dynabeads.

4.2.1. HER-2 specific recognition

To evaluate the possibility of targeting NK cells towards tumor cells by genetic engineering, I tested the ability of HER-2 specific CR-NK cells to recognize tumor cells in a HER-2 dependent fashion. I found that 10 out of 10 CR-engineered NK cell lines specifically recognized the HER-2 expressing C1R/A2 cell line to a much higher extent than the control C1R/A2 cell line, while the mock-engineered NK cell lines did not show any difference in recognition of both, C1R/A2 and C1R/A2HER2 cell lines. C1R/A2 is an EBV transformed cell line, which expresses high levels of various activation ligands that might have contributed to the recognition (Bryceson *et al.*, 2006; Lanier, 2005; Wilson *et al.*, 1999), which can explain why mock-NK cells recognized the control C1R/A2 cells to some extent. Notably, both CR- and mock-NK cells recognized well their prototype target, the cell line K562, indicating that the genetic modification did not hinder the intrinsic NK cell capability to recognize MHC class I negative or low cells.

4.2.2. Adjusted NK cell activation level based on HER-2 expression level

It was important to investigate whether carcinomas, which were not reported to express higher levels of activation ligands, and which expressed HER-2 constitutively, are specifically recognized by CR-NK cells. This was indeed the case, as all tested HER-2 expressing breast- and ovarian cancer cells were recognized by CR-NK cells, but not by their mock-transduced counterpart. Interestingly, the extent of recognition correlated with the level of

HER-2 expression on the tumor cells, pointing out the finely-tuned NK cell recognition system. Thus, the more activation provided by the CR, the higher the activation level measured by cytokine production. Remarkably, the HER-2 negative cell line MDA MB 468 did neither stimulate CR-NK cells nor mock-NK cells to produce cytokines such as IFN-γ and IL-2. Contrary to a generally perceived view that NK cells target all tumor cells effectively, at least *in vitro*, most of the carcinoma lines that I have analyzed were not good targets for NK cells, regardless of the donor from which the NK cells were derived. This resistance to NK killing is probably natural since many of these carcinoma lines express normal levels of MHC class I on their surface. Notably, the MHC class I expression did not correlate with cytokine production by CR-NK cells.

4.2.3. IFN-γ and IL-2 are specifically produced by NK cells

NK cell cultures used for functional assays were of a very high purity, in most cases containing not more than 1-2 % residual T cells. NK cell cultures that showed a T cell contamination higher than 5 % were re-depleted from these T cells. To determine the cytokine production by the NK cells on a single cell level, CR-NK cells were cocultured with the HER-2 positive carcinoma SKOV3 or the prototype NK target K562, following an intracellular cytokine staining for IFN-γ and a simultaneous surface staining for the CR. As expected, approximately 50 % of NK cells stained positive for intracellular IFN-γ when cocultured with K562, regardless of the CR expression. In contrast, when cocultured with SKOV3 cells, only CR positive NK cells stained positive for intracellular IFN-γ, confirming the HER-2 specificity. IL-2 production, which is more difficult to determine because it is usually produced at a lower level than IFN-γ, was measured using the IL-2 secretion assay with SKOV3 cells as stimulator. The cytokine secretion assay was designed for highly sensitive cytokine detection. As expected, a small, but clearly distinguishable subset of CR-NK cells secreted IL-2. This IL-2 producing CR-NK cell subset represented 15 % of the transduced NK population, taking into account that this donor´s transduction efficacy was about 60 %.

4.2.4. CR-NK cells exert cytotoxicity towards HER-2 positive targets

Cytotoxic lymphocytes contain secretory lysosomes with a dense core, including various proteins that are involved in cytotoxic function like perforin and granzymes. The core is surrounded by a lipid bilayer that contains lysosomal-associated membrane glycoproteins (LAMPs) (Bossi and Griffiths, 1999; Peters *et al.*, 1991). Degranulation by cytotoxic lymphocytes results in CD107a (LAMP-1) appearance at the cell surface which can be detected by flow cytometry (Alter *et al.*, 2004; Betts *et al.*, 2003). Therefore, I investigated if CR-NK cells are able to degranulate in response to HER-2 stimulation. This was indeed the case, as all HER-2 positive tumor cell lines induced degranulation by CR-NK cells based on CD107a expression. In contrast, mock-transduced NK cells showed little or no degranulation. Notably, the cell lines MDA MB 453 and Cal 51 stimulated mock-NK cells to some extent, most likely because the NK cells from this particular donor were less inhibited and thus exerted alloreactivity. However, CR-NK cells from the same donor showed twice as much CD107a positive cells in response to stimulation by these cell lines confirming the HER-2 specificity. Remarkably, the stimulation with the mitogen phorbol myristate acetate (PMA) and ionomycin or with the prototype NK target K562 induced similar degranulation levels in CR-NK cells as compared to mock-NK cells. As degranulation of NK cells is an indirect measurement of target cell lysis, additionally a ^{51}Cr release assay was performed to confirm the degranulation results. In this assay, the SKOV3 target cells were labeled with radioactive ^{51}Cr that was released upon cell lysis. This assay confirmed the HER-2 specific lysis by CR-NK cells.

Taken together, these data indicate that CR-NK cells have the capacity to respond specifically to HER-2 expressing targets by IFN-γ and IL-2 production, the two cytokines essential for tumor rejection and NK cell survival, as well as by degranulation and specific lysis of HER-2 positive target cells while maintaining their classical NK specificity as indicated by the response to K562 cells, hence using two independent mechanisms, which are non-MHC class I restricted, to recognize their target cell.

4.3. CR mediated activation overrides inhibition to 'self'

NK cells use an array of activating and inhibitory receptors upon interaction with autologous cells and allogeneic cells. The balance between signals from these receptors decides whether the NK cell is going to be activated or not. It is thus of particular importance that the NK cell is appropriately tuned to prevent autoreactivity. There are several mechanisms by which the interaction between inhibitory receptors and host MHC class I molecules during NK cell development could result in a functional, yet self-tolerant, NK cell population. These mechanisms are based on licensing, arming or adaptation. In each of these suggested mechanisms, qualitative and/or quantitative encounter, or its absence therein, between the NK cell and a cell expressing MHC class I molecules can lead to either a positive outcome that produce a functional NK cell, or to a negative outcome that leads to the development of an unresponsive NK cell (Gasser and Raulet, 2006; Johansson and Hoglund, 2006; Parham, 2006; Yokoyama and Kim, 2006).

The functional assays described so far were performed in an allogeneic setting, since the breast- and ovarian cancer cell lines were not HLA-matched with the NK cells. Therefore, the 'missing self' response might have positively contributed to the HER-2 specific activation. Nonetheless, most of the tested cancer cell lines expressed high MHC class I levels and thus the natural 'missing self' response mediated by mock-engineered NK cells was rather low or absent. Yet, the question whether CR-engineered NK cells would be able to overcome self-tolerance, a prerequisite for successful immunotherapy, needed to be formally addressed in an autologous system, where NK cells and HER-2 expressing tumor cells are derived from the same donor or patient and thus are fully matched for KIR and KIR ligand. The model system which I have used consisted of LCLs from a patient and a healthy donor, which were immortalized with mini-EBV constructs including or excluding the extracellualar HER-2 domain, and the matching NK cells that were generated from the same patient and healthy donor, respectively. Hence, this system allowed monitoring the natural response to EBV-transformed 'self' LCLs as well as the HER-2 specific response. Remarkably, CR-NK cells from a patient recognized autologous LCLs only when these LCLs expressed HER-2. Interestingly, NK cells from this patient did not at all respond to the HER-2 negative autologous LCLs, which was not expected considering the generally perceived observation that NK cells do target autologous cells upon transformation (Cerwenka and Lanier, 2001; Moretta and Moretta, 2004). This targeting of transformed cells could yet be observed with NK cells from the healthy donor, which responded to some

extent to the transformed LCL line, however a much higher response was observed by CR-NK cells specifically targeting the HER-2 expressing autologous LCL line. In summary, these results indicate that the activation provided by the CR signaling was sufficient to override the collective inhibitory signals provided by the corresponding inhibitory ligands on the autologous targets.

4.4. Freshly isolated tumor cells are similarly targeted by allogenic and autologous NK cells

Eventually, it was important to investigate whether freshly isolated carcinomas expressing HER-2 constitutively would be recognized by CR-engineered NK cells. For that reason, HER-2 positive ovarian carcinoma cells collected from 5 different patients were obtained, which expressed HER-2 at various levels. From two patients, matched PBMCs were obtained, which were used for the expansion and transduction of NK cells. These fresh ovarian tumor cells were well recognized by allogeneic CR-NK cells from healthy donors as well as autologous CR-NK cells obtained from matching ovarian carcinoma patients, but not by the mock-transduced counterparts, respectively. Remarkably, the magnitude of this *ex vivo* recognition was similar for allogeneic and autologous CR-NK cells.

In general, this universal recognition of HER-2 positive target cells may be explained by the level of activation provided by the CR used in this study that may have overridden the sum of inhibitory signals provided by any of the tested cell lines. Apart from the qualitative difference in signaling, the affinity of the binding domain (KD = 10^{-8} M) of this CR is much higher than that of the reported interactions between known inhibitory NK-receptors and their ligands (Vales-Gomez *et al.*, 2000).

4.5. NKG2D blockade does not impair HER-2 recognition

In certain tumor models, the role of NKG2D, an activation receptor expressed on NK cells, γδ+ T cells, NKT cells and activated CD8+ T cells, was investigated by several groups (Cerwenka *et al.*, 2001; Gasser and Raulet, 2006). Constitutive, induced expression of NKG2D ligands strongly increased the sensitivity of tumor cells to NK cell recognition *in vitro*, and the ectopic expression of NKG2D ligands Rae-1 and H60 by tumor cell lines resulted in their rejection (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001; Oppenheim *et al.*,

2005), suggesting that tipping the balance of signaling towards activation can overcome NK and T cell tolerance to tumor cells. Moreover, Guerra *et al.* have shown that in spontaneous tumor models of NKG2D-defficient mice, there is an early incidence of primary prostate adenocarcinomas and B lymphomas which expressed higher amounts of NKG2D ligands (Guerra *et al.*, 2008). On the other hand, few studies have pointed out that many tumors of epithelial origin shed soluble NKG2D ligands such as MICA, which can be detected in sera of tumor patients at significant levels. Soluble MICA was shown to down-modulate NKG2D expression on $CD8^+$ T-cells and NK cells (Groh *et al.*, 2002; Wu *et al.*, 2004), which might render the NK cells less responsive. However, soluble NKG2D ligands are not expected to affect target recognition by CR-NK cells, since the effect of these soluble ligands can be overcome when cells are cultured in the presence of IL-2 (Wu *et al.*, 2004). To confirm these data, I used two different commercially available recombinant MICA preparations to test their effect on the recognition of HER-2 carcinomas by CR-NK cells. Although MICA binding to NKG2D on NK cells was confirmed by flow cytometry, this binding did not down-modulate the receptor even when high concentrations (2μg/ml) of the soluble protein were used. Interestingly, some studies reported similar findings when using soluble MICA and MICB, where no change in NKG2D expression could be observed (Salih *et al.*, 2006). A further study confirmed these results using another NKG2D ligand, soluble ULBP-2, which did not down-modulate NKG2D on NKL cells nor did it affect the cell's cytotoxic activity (Waldhauer and Steinle, 2006). In line with these findings, I did not observe any blocking of the CR-mediated recognition of the HER-2 positive carcinoma cell lines in the presence of soluble recombinant MICA in my study, even if these carcinoma cell lines expressed high levels of NKG2D ligands on their surface. On the contrary, I could observe a slightly better recognition of the tumor cell lines by mock- and CR-engineered NK cells, when these were pre-incubated with plate-bound recombinant MICA over night. Thus, in my studies the exposure to plate-bound MICA had a rather pre-activating effect, because both mock-NK cells and CR-modified NK cells produced some IFN-γ even without any target, and upon coculture with the 3 target cell lines, the IFN-γ levels were higher than those observed in the two conditions without prior pre-incubation of the effector cells. Therefore, the NK cells used in my study were not susceptible to the negative effect of the soluble NKG2D ligand MICA.

4.6. Advantage of the CR-NK approach over trastuzumab-mediated therapy

Distinguishing the CR-NK approach from the use of trastuzumab or other Ab-based strategies targeting HER-2 is that not all HER-2 positive tumors or cell lines are responsive to this Ab based therapy. Only tumor cells that have gene amplified HER-2, accounting for one third of the HER-2 positive tumors, represent good targets for these Ab and siRNA treatments. Neve *et al.* have recently reported that breast cancer cell lines have the molecular features that mirror primary breast tumors, which permits prediction of response to targeted therapy by trastuzumab (Neve *et al.*, 2006). Thus, the majority of breast carcinoma lines that I have tested would not be responsive to trastuzumab. This is in contrast to the CR-NK cells, which were able to recognize all tested HER-2 positive carcinomas regardless of their gene amplification status.

Data from genetically disrupted mouse models describe the role of NK and other innate immune cells expressing Fc receptors as a critical component for anti-HER-2 mAb-mediated therapy. However, these models have also shown a very significant contribution of the inhibitory Fc receptor. Clynes *et al.* have shown that genetic deletion of FcγRIIb, an inhibitory Fc receptor expressed on monocytes, significantly improves the antitumor response to Rituximab (Clynes *et al.*, 2000). Furthermore, several studies have shown that polymorphisms in Fc receptors have a very significant impact on the ADCC and can predict clinical outcome of mAb therapy. The FcγRIIIa (CD16), which is responsible for ADCC mediated by NK cells, has two allelic single-nucleotide polymorphic (SNP) variants, valine (V) or phenylalanine (F), at amino acid position 158. The FcγRIIIa bearing a (V) at this position has a higher affinity for both monomeric and immune complexed IgG1, IgG3, and IgG4 than an FcγRIIIa bearing an (F) (Hatjiharissi *et al.*, 2007). Thus, the 158 V/V genotype is associated with higher response rate to rituximab treatment (Cartron *et al.*, 2002; Varchetta *et al.*, 2007; Weng and Levy, 2003). Similar results were observed in a retrospective study with trastuzumab in patients with metastatic HER-2 positive breast cancer. ADCC analysis revealed that the 158 V/V genotype showed a significantly higher trastuzumab-mediated cytotoxicity than other genotypes. Furthermore, patients with a 158 V/V genotype had a significantly higher response to trastuzumab therapy than the so called "F-carriers", referring to patients with either a 158 F/V or 158 F/F genotype (Musolino *et al.*, 2008). Since only 15 - 20 % of the population have the homozygous 158 V/V genotype (Musolino *et al.*, 2008; Treon *et al.*, 2005; Weng and Levy, 2003), these studies

indicate that most of the treated patients, which have either a mixed genotype or are homozygous for the less favorable genotype, will only moderately benefit from a trastuzumab therapy.

Additionally, all these studies have focused on investigating the impact of ADCC on tumor cells expressing high level of HER-2. However, from my current study, it becomes obvious that not only this group of HER-2 positive tumors, but also those with relatively low HER-2 expression level such as MCF-7 can be efficiently targeted by CR-NK cells. MCF-7 is resistant to HER-2 mAb mediated ADCC (Stein *et al.*, 2006; Yamaguchi *et al.*, 2005), unless transfected to overexpress HER-2 (Carson *et al.*, 2001). To confirm these data, the MCF-7 cell line was used for a side-by-side comparison of trastuzumab- and CR-mediated activation of NK cells. I fist confirmed that NK cells can be activated via CD16 to produce IFN-γ and that trastuzumab can mediate recognition of a high HER-2 expressing cell line SKOV3 by mock-NK cells. Next, I confirmed published observations that MCF-7, a cell line that expresses relatively low HER-2 levels, did not activate mock-NK cells in the presence of trastuzumab, but was recognized by CR-NK cells. These data therefore suggest that CR-modified NK cells can be very effective in targeting all HER-2 positive carcinomas irrespective of their HER-2 expression levels.

4.7. Tumor challenge and *in vivo* imaging

A mouse model is a valuable tool to investigate the potential of CR-engineered NK cells to reject tumor cells *in vivo*. Few reports have described the transduction of an NK-like lymphoma line (NK-92) and targeted these cells to HER-2 expressing tumor cells. These attempts demonstrated that tumor outgrowth was transiently delayed in mice coinjected with a HER-2 positive tumor and targeted NK-92 cells, but mice protection could not be achieved (Suck, 2006; Uherek *et al.*, 2002). Moreover, these NK-92 cells are transformed and therefore are not ideal for immunotherapy and do not reflect the biology of primary NK cells expressing a CR. A recent study reported the adoptive transfer of transiently (expression up to 72 hours) CR-modified mouse NK cells into *RAG* mice bearing RMA-HER-2 lymphoma using the Amaxa gene transfer technology. This approach achieved moderate survival differences and no mice protection (Pegram *et al.*, 2008).

In the initial *in vivo* study, I've chosen two xenograft models that offer a rapid tumor formation and its synchronized progression, which makes these models highly reproducible.

However, the mouse is not the natural host for human NK cells and its microenvironment will not support proper homing and engraftment of adoptively transferred NK cells. Therefore, the NK cells and tumor cells had to be coinjected at the same time.

The mouse models revealed that CR engineered NK cells from different donors can overcome NK cell intrinsic inhibition, which limited their function in most of the animals that received mock transduced NK cells. Thus, these CR-engineered NK cells did prevent tumor outgrowth in all mice that have received these cells.

In order to further the study, a suitable mouse model should be developed, ideally using a syngeneic tumor and CR-engineered mouse NK cells combined with *in vivo* imaging, which could improve analyzing the trafficking potential and homing capacity of CR-modified NK cells to the tumor site.

4.8. Improvement of safety and efficacy

In order to translate this study into a clinical setting, it will be very important to obtain the approval to use the irradiated feeder cell line RPMI 8866 and to be able to use it under good manufacturing practice (GMP) conditions in order to expand the primary human NK cells. Such an approval would require several studies proving that the irradiated feeder cell line could in no way induce leukemia upon being transferred into a patient. However, a similar cell line was already approved for studies with adoptive T cell therapy (Walter *et al.*, 1995).

A further complication for the translation of this approach into a clinical setting is the question of side effects such as cardiotoxicity associated with HER-2 targeting. In clinical trials using trastuzumab, cardiotoxicity was reported in patients, especially in combination with chemotherapy (Slamon *et al.*, 2001). Early clinical trials provided little or no indication of an increased risk of cardiotoxicity in patients treated with trastuzumab. It only become evident in Phase III clinical trials and therefore a retrospective analysis of cardiac events in seven Phase II and III trastuzumab clinical trials by an independent, blinded Cardiac Review and Evaluation Committee was prompted. The incidence of cardiotoxicity was greatest in patients receiving concomitant trastuzumab and anthracycline plus cyclophosphamide (27%). The risk was substantially lower in patients receiving paclitaxel and trastuzumab (13%) or trastuzumab alone (3% to 7%) (Seidman *et al.*, 2002). However, the majority

of reported cardiac effects were mild to moderate and medically manageable, and cardiac-related deaths in patients treated with trastuzumab monotherapy or combination therapy were uncommon (1%) (reviewed by (Keefe, 2002)). The mechanisms of trastuzumab-associated cardiotoxicity are currently under investigation. Preclinical animal and small clinical studies are being conducted in order to obtain more detailed information regarding the nature of this adverse event. In my study, all tested cell lines which were efficiently targeted by CR-modified NK cells overexpress HER-2. In contrast, normal tissue cells express, if any, very low levels of HER-2 similar to the level of expression detected on the cell line MDA MB 468, which does not overexpress HER-2 (but express very low levels of HER-2 that can only be detected at the mRNA level (Faltus *et al.*, 2004) but not by flow cytometry) and therefore did not stimulate the CR-engineered NK cells to produce cytokines or exert cytotoxicity. However, in contrast to trastuzumab that has a short half-life, CR-engineered NK cells might persist long-term in the treated patient. Kieback *et al.* recently suggested the introduction of a myc-tag into the TCRα-chain of a therapeutic TCR to specifically eliminate such genetically modified T cells if these become autoreactive (Kieback *et al.*, 2008). Such a safety modality could also be introduced into the CR to eliminate CR-engineered NK cells *in vivo* if they become potentially autoreactive or show other adverse effects which might occur upon genetic modification (Hacein-Bey-Abina *et al.*, 2003). Altogether, the risk of HER-2 specific autoimmunity needs to be further addressed in a suitable experimental model.

It was reported that soluble antigens might interfere with the function of CR-modified T cells, however a CR that selectively targets a membrane-bound carcinoembryonic antigen (CEA) was capable of stimulating CR-modified T cells in the presence of soluble CEA (Hombach *et al.*, 1999; Treon *et al.*, 2005). Furthermore, it has been shown that recognition of tumor cells alone is not sufficient to reject large, established tumors, but it is also necessary to target the tumor stroma (Kammertoens *et al.*, 2005; Spiotto *et al.*, 2004; Zhang *et al.*, 2007). The eradication of large, long-term established tumors by immunotherapy has rarely been reported. Most preclinical models in a therapeutic setting utilized tumors that have been implanted for less than 2 weeks before initiation of therapy (Yu *et al.*, 2006). Recent work conducted in the lab of Carl June however showed that adoptive transfer of engineered T cells expressing a chimeric receptor with high affinity for human mesothelin fused to CDζ and CD28 or 4-1BB costimulatory domains into NOD/scid/IL2rγ$^{-/-}$ mice

engrafted with very large, established tumors (500 to 1000 mm^3) has led to reduction of the tumor load, and in some cases to complete eradication of tumors at low effector-to-target ratios (Carpenito *et al.*, 2009). Since the CR used in this study is not able to directly target the stroma, these data suggest that CR-mediated cytotoxicity might indirectly induce manipulation of the stromal microenvironment. However, if targeting of tumor stroma that cross-presents the tumor antigen is essential, one might consider to use a TCR-like chimeric receptor, which is MHC class I restricted (Lev *et al.*, 2002). Such an MHC-restricted CR might also improve the typically low intrinsic affinity of a TCR by combining the TCR-like specificity with the high-affinity binding characteristics of antibodies. Therefore, it would be interesting to investigate the potential of combining a conventional CR with an MHC-restricted CR for immunotherapy.

4.9. Prospective studies

Haploidentical hematopoietic stem cell transplantation (HSCT) from related donors for the treatment of leukemia was reported to exploit alloreactive NK cells to increase the probability of survival of high risk acute myeloid leukemia (Ruggeri *et al.*, 2002). However, some controversy exist about whether transplants from MHC class I partially matched related or unrelated donors can also contribute to the curative potential of allogeneic HSCT, if a KIR ligand incompatibility in graft-versus-host direction is present (Beelen *et al.*, 2005; Bornhauser *et al.*, 2004; Farag *et al.*, 2006; Giebel *et al.*, 2003; Leung *et al.*, 2004). These contradictory findings can partly be attributed to differences between the investigated patient populations and to varying transplantation procedures. In fact, T cell depletion of the donor stem cell transplant was performed in some studies using an anti CD6 antibody (Schetelig *et al.*, 2008). CD6 is also widely expressed on NK cells (Alonso-Ramirez R., 2009; Braun, 2009), and therefore such a graft manipulation will have an impact on NK cell effector function. Thus, the beneficial effect of alloreactive NK cells in HSCT from unrelated donors needs to be further elucidated in studies with comparable settings in terms of patient populations, underlying diseases, preparative regimens, graft manipulation techniques, and posttransplantation immunosuppressive regimens. Yet, the superior antileukemic efficacy of KIR mismatched HSCT from related donors has been convincingly demonstrated in several following studies (Hsu *et al.*, 2005; Miller *et al.*, 2005; Ruggeri *et al.*, 2006), supporting a positive precedent for adoptive NK cell therapy. Additionally, acute lymphoblastic

leukemia cells were shown to be a good target for NK cells transduced with CD19 specific receptor (Imai *et al.*, 2005).

Adoptively transferred primary NK cells have the potential of long-term persistence and proliferation in the recipient and do compete well for the utilization of homeostasis growth factors (Miller *et al.*, 2005). Interestingly, *in vivo* NK-cell expansion occurs after preparation with a high dose but not lower doses of immunosuppression, suggesting that lymphopenia is required to change the competitive balance between transferred NK cells and endogenous lymphocytes. These findings are consistent with data from a murine model, where Prlic *et al.* showed that mature NK cells proliferated only in an NK-cell-deficient host, where the endogenous NK cell pool was absent (Prlic *et al.*, 2003). The maintenance of mature NK cell *in vivo* depends on IL-15 in mice and human (Cooper *et al.*, 2002), and NK cell adoptive therapy is associated with a striking rise in endogenous IL-15 levels, most probably due to the intensive lymphocyte-depleting preparative regimen (Miller *et al.*, 2005). The fact that adoptively transferred human NK cells can persist and expand *in vivo* is a prerequisite for successful adoptive immunotherapy (Charo *et al.*, 2005; Rosenberg *et al.*, 2008). The prospective of using NK cells for the immunotherapy of cancer has thus become an attractive approach. This study demonstrated that genetic engineering of primary human NK cells rendered them reactive towards previously spared HER-2 positive carcinomas *in vitro* and *in vivo*, and that the CR-mediated signaling could override inhibition to 'self'.

The approach of engineering primary human NK cells to become antigen-specific is not limited to HER-2 as tumor antigen. Indeed, several CRs with different specificities are available which could be transferred into NK cells, conferring various specificities to these engineered NK cells. The advantage of this approach is the targeting of other types of cancer as well as the possible simultaneous targeting of several tumor antigens on the same tumor cell surface. Concerning HER-2, additional CRs are available (through collaboration with Hinrich Abken, Department I of Internal Medicine, Tumor Genetics, and Center for Molecular Medicine Cologne (CMMC), University of Cologne), which have different affinities ranging from K_d 10^{-7} to 10^{-11}. Therefore, it would be attractive to investigate whether NK cells engineered with CRs with lower or higher affinity would also be able to efficiently target HER-2 positive tumor cells.

A further exciting aspect of a genetic modification of primary human NK cells with activating receptors is to further our understanding of activating and inhibitory signaling in NK cells. Hence, it would be interesting to investigate whether NK cells engineered with an activating receptor with other signaling modalities than ζ and CD28 would be able to overcome tolerance to 'self' in a similar fashion. Additionally, inhibitory receptors such as KIRs could be introduced in order to study the impact of augmented inhibition on CR mediated recognition.

Taken together, the ability to genetically engineer primary NK cells, apart from providing an opportunity to further the analysis of NK cell biology, can represent an effective alternative or a complement to the currently used approaches in cancer immunotherapy.

Summary

NK cells are promising effectors for tumor adoptive immunotherapy, particularly when considering the targeting of MHC class I low or negative tumors. Yet, many tumor cells are resistant to NK killing, which is mainly the case for non-hematopoietic tumors such as carcinomas or melanoma, even when these cells lose MHC class I expression.

In this study, primary human NK cells were engineered by gene transfer of an activating chimeric receptor (CR) specific for HER-2, which is frequently overexpressed on carcinomas. To tip the signal balance towards activation, the antigen-binding domain in this CR was fused to the joined CD3ζ and CD28 signaling domains.

I found that these targeted NK cells were specifically activated upon recognition of any HER-2 positive tumor cell tested as indicated by high levels of cytokine secretion as well as degranulation and tumor cell lysis. The magnitude of this specific response correlated with the level of HER-2 expression on the tumor cells and was independent of their MHC class I expression level. Remarkably, CR-mediated activation was shown to override inhibition to 'self' in an autologous setting. Also, freshly isolated HER-2 positive tumor cells from ovarian cancer patients were efficiently targeted by allogeneic and autologous CR-engineered NK cells. Importantly, blocking of NKG2D, which is known to render NK cells less responsive, did not have any impact on this CR-meidated HER-2 specific recognition. Moreover, a side-by-side comparison revealed a superior recognition of the CR over trastuzumab-mediated NK cell activation. Finally, these CR transduced NK cells, but not their mock transduced counterpart, eliminated coinjected tumor cells in NOD/SCID and *RAG2* knockout mice as visualized by *in vivo* imaging.

The major advantage of this approach is the direct coupling of the specificity of the chimeric receptor to the function of NK cells that makes it superior to the currently widely used approach based on monoclonal antibodies, which have a short half-life. Moreover, the antibody therapy strongly depends on the patient's given FcγR composition. Finally, only 30 % of HER-2 positive tumors are responsive to trastuzumab therapy.

Taken together, these results indicate that the expression of this activating chimeric receptor overrides inhibitory signals in primary human NK cells and directs them specifically towards all tested HER-2 expressing tumor cells both *in vitro* and *in vivo*.

Zusammenfassung

NK-Zellen sind vielversprechende Effektoren im Hinblick auf adoptive Immuntherapie von Tumoren, vor allem wenn man auf Tumore mit wenig oder keiner MHC-Klasse-I-Expression abzielt. Trotzdem sind viele Tumore resistent gegen den Angriff durch NK-Zellen, was besonders für Tumore des nicht-hämatopoietischen Ursprungs wie Karzinome und Melanome zutrifft.

In dieser Studie wurden primäre humane NK-Zellen durch Gentransfer mit einem aktivierenden, chimären Rezeptor (CR) spezifisch für HER-2 versehen, welches häufig auf Karzinomen überexprimiert ist. Durch Fusion der Antigen-Bindungsdomäne mit der gekoppelten CD3ζ- und CD28-Signaldomäne wurde die Signal-Balance zugunsten von Aktivierung verschoben. Die CR-modifizierten NK-Zellen wurden aufgrund der Erkennung jeglicher getesteter HER-2 positiver Tumorzellen spezifisch aktiviert, was durch einen hohen Grad von Zytokin-Produktion, Degranulation sowie Tumorzell-Lyse belegt wurde. Das Ausmaß der spezifischen Antwort korrelierte mit dem HER-2-Expressionsniveau und war unabhängig von dem MHC-Klasse-I Expressionsniveau. Die CR-vermittelte Aktivierung hat überdies die Selbstinhibition, welche in einem autologen Milieu das Angreifen eigener Zellen verhindert, aufgehoben, und auch frisch isolierte, HER-2-positive Tumorzellen von Ovarialkarzinom-Patienten wurden effizient durch allogene und autologe CR-modifizierte NK-Zellen erkannt. Des Weiteren hatte die Blockierung von NKG2D keinen Einfluss auf die CR-vermittelte, HER-2-spezifische Erkennung. Im direkten Vergleich war der CR-Ansatz der trastuzumab-vermittelten Erkennung überlegen. Schließlich haben die CR-transduzierten NK-Zellen effizient Tumorzellen in NOD/SCID- sowie RAG2-knockout-Mäusen beseitigt.

Der Vorteil dieser Studie liegt in der Koppelung der Spezifität des CR an die Funktion der NK-Zellen, wodurch dieser Ansatz den zur Zeit eingesetzten monoklonalen Antikörpern überlegen ist, da diese eine kurze Lebensdauer haben. Außerdem hängt die Antikörpertherapie stark von der gegebenen FcγR-Komposition des Patienten ab. Überdies sprechen lediglich 30 % der HER-2-positiven Tumore auf die trastuzumab-Therapie an. Zusammengefasst zeigen diese Ergebnisse, dass die Expression eines aktivierenden, chimären Rezeptors die inhibierenden Signale in primären humanen NK-Zellen aufhebt, und dass diese NK-Zellen gegen alle getesteten HER-2 positiven Tumorzellen sowohl *in vitro* als auch *in vivo* spezifisch vorgehen.

Abbreviations

- ADCC antibody dependent cell-mediated cytotoxicity
- ALL acute lymphoblastic leukaemia
- ALV antigen loss variants
- allo-SCT allogeneic stem-cell transplantation
- AML acute myeloid leukemia
- APC allophycocyanin
- CBG click beetle green
- CD cluster of differentiation
- CEA carcinoembryonic antigen
- cDNA complementary deoxyribonucleic acid
- CFSE carboxyfluorescein diacetate succinimidyl ester
- CML chronic myelogenous leukaemia
- cpm counts per minute
- CR chimeric receptor
- ^{51}Cr chromium isotope 51
- CTL cytolytic T lymphocyte
- DAP-10 DNAX-activation protein 10
- DAP-12 DNAX-activation protein 12
- DMEM Dulbeco's modified Eagle's medium
- DNA deoxyribonucleic acid
- EBV Epstein-Barr virus
- ELISA enzyme-linked immunosorbent assay
- erbB-2 erythroblastic leukemia viral oncogene homolog 2
- (F) phenylalanine
- FACS fluorescence-activated cell sorting
- Fc crystallizable fragment
- FcR Fc receptor
- FCS fetal calf serum

- Fig figure
- FITC fluorescein isothiocyanate
- FR α-folate receptor
- GFP green fluorescent protein
- GMP good manufacturing practice
- GvHD graft-versus-host-disease
- H60 histocompatibility 60
- HER human epidermal growth factor receptor
- HER-2 human epidermal growth factor receptor-2
- HLA human leukocyte antigen
- HSCT hematopoietic stem cell transplantation
- IFN-γ interferon-γ
- Ig immunoglobulin
- IL interleukin
- IRES internal ribosomal entry site
- ITAM immunoreceptor tyrosine-based activation motif
- ITIM immunoreceptor tyrosine-based inhibitory motif
- K_d dissociation constant
- KIR killer immunoglobulin-like receptor
- KLRA1 killer cell lectin-like receptor subfamily A, member 1
- KLRB1 killer cell lectin-like receptor subfamily B, member 1
- KLRC1 killer cell lectin-like receptor subfamily C, member 1
- KLRG1 killer cell lectin-like receptor subfamily G, member 1
- KLRK1 killer cell lectin-like receptor subfamily K, member 1
- LAIR-1 leukocyte-associated immunoglobulin-like receptor 1
- LCL B-lymphoblastoid cell line
- LTR long terminal repeat
- Ly49 see KLRA1
- mAb monoclonal antibody

- MFI mean fluorescence intensity
- MHC major histocompatibility complex
- MICA/B MHC class I chain-related protein A/B
- mRNA messenger ribonucleic acid
- MSCV mouse stem cell virus
- NCR natural cytotoxicity receptor
- NCR1 natural cytotoxicity triggering receptor 1
- NK natural killer
- NKG2A see KLRC1
- NKG2D see KLRK1
- NKp46 see NCR1
- NKSF NK cell stimulatory factor
- NOD non-obese diabetic
- PMA phorbol myristate acetate
- PBL peripheral blood lymphocyte
- PBMC peripheral blood mononuclear cell
- PBS phosphate-buffered saline
- PCR polymerase chain reaction
- PE phycoerythrin
- Rae1 RNA export 1 homolog
- RAG2 recombination-activating gene 2
- RFP red fluorescent protein
- RNA ribonucleic acid
- rpm rounds per minute
- RT-PCR reverse transcriptase-polymerase chain reaction
- scFv single-chain variable fragment
- siRNA small interfering RNA
- SCID severe combined immunedeficieny
- SHP H2 domain-containing protein phosphatase

- SHIP SH2 domain-containing inositol 5'-phosphatase
- SNP single nucleotide polymorphism
- TCR T cell receptor
- ULBP UL-16 binding protein
- (V) valine

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Publication

Anna Kruschinski, Andreas Moosmann, Isabel Poschke, Håkan Norell, Markus Chmielewski, Barbara Seliger, Rolf Kiessling, Thomas Blankenstein, Hinrich Abken, and Jehad Charo.

‚Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas'.

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Presentations at Conferences

Anna Kruschinski, Markus Chmielewski, Thomas Blankenstein, Hinrich Abken, and Jehad Charo. Poster: *‚Introduction of a chimeric receptor antibody specific for Her-2/neu into Natural Killer cells'* .
European Congress of Immunology in Paris, France 2006

Anna Kruschinski, Markus Chmielewski, Thomas Blankenstein, Hinrich Abken, and Jehad Charo. Poster: *‚Breaking NK cell tolerance to HER-2 expressing carcinomas'.*
10th Meeting of the Society for Natural Immunity in Cambridge, UK 2007

Anna Kruschinski, Markus Chmielewski, Thomas Blankenstein, Hinrich Abken, and Jehad Charo. Poster: *'Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas'*
International Symposium 'Adoptive T Cell Therapy' in Berlin, Germany 2008

Anna Kruschinski, Markus Chmielewski, Thomas Blankenstein, Hinrich Abken, and Jehad Charo. Poster: *'The prototype inhibitory receptor KIR2DL1 is overridden by engineering primary human Natural Killer cells with an activating receptor targeting HER-2 expressing carcinomas'*
3. Mildred Scheel Cancer Conference of the DKH in Königswinter, Germany 2008

Curriculum Vitae

For data privacy protection, the Curriculum Vitae will not appear in the online version.

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Ich bin mir bewusst, dass eine falsche Erklärung rechtliche Folgen haben wird.

Ort und Datum

Unterschrift