Chimeric antigen receptor-modified NK cells target breast carcinomas resistant to soluble antibody treatment

Dissertation zur Erlangung des akademischenGrades des

Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie

der Freien Universität Berlin

vorgelegt von

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Dezember 2010

Diese Arbeit wurde zwischen dem 01. Oktober 2008 und dem 14. Dezember 2010 erstellt. Die zugrundeliegenden Experimente wurden unter der Leitung von Prof. Dr. Thomas Blankenstein am Max-Delbrück-Centrum für Molekulare Medizin Berlin-Buch durchgeführt.

1. Gutachter: Prof. Dr. Markus Wahl

2. Gutachter: Prof. Dr. Tina Romeis

Disputation am: 02. März 2011

Acknowledgements

The last three years were not an easy journey for me, that‘s why I would like to thank all those people who supported and encouraged me to held my head up straight and get it done. Even if I do not name all, I do acknowledge all of you.

I would like to thank Prof. Dr. Abram Ulrich (Institute for Chemistry and Biochemistry, Free University), for his great support, and for giving me the opportunity to finish my PhD work.

Also I would like to thank Prof. Dr. Markus Wahl (Institute for Chemistry and Biochemistry, Free University) and Prof. Dr. Tina Romeis (Institute for Biology, Free University), for their generousness to review my thesis, and to give me the opportunity to finish my PhD work.

Probably the most, I would like to thank Dr. Ibrahim Kocman, not only for his superb supervision, but also for his patience with me. I have not met many people like him, who are always friendly and calm to everyone, regardless of any problems or personal distress or whatsoever. Thanks for answering the tons of questions I have asked you and for always having time to help me.

Dr. Anna Kruschinski, for her great help in establishing the project together. Hearing her laughing somewhere in the lab always cheered me up. Thank you, Anna, for being always optimistic, and for your whole friendly nature.

Dr. Tobias Jursch, for interesting discussion and for his great help during the preparation of this thesis and his comments on it. I am really lucky, dear Tobias, to have a friend like you here in Germany.

Dr. Oluwatosm Adaramoye, for sharing his knowledge about science and life, and also for being always available to discuss my project. I really appreciate your help during the preparation of this thesis and your critics on it.

Stephanie Kupsch, for her help (especially in the beginning and whenever I needed) in the lab, and also for her technical assistance and for being so uncomplicated, friendly and helpful.

My friends Ana Jukica, Abdullah Abdulkader, Abdulazim Alturkawi, Ibrahim Zaror, for their love and understanding about the limited time I was able to spend with them during the last years. Thank you for calling and lightening my evenings up. Thank you for helping me thinking of something else then work and cheering me up just with your presence. Thank you for making me realizing that having such good friends is a great gift from God.

My uncle Michiel, Pierina, Atilio, and Maya, whom I can never thank enough in my life, for being the best relatives on earth! You have not only initiated and influenced my interest in science but you always believed in me and helped me going through the deepest downs during the last three years. Thanks for your love and for teaching me to be patient with myself and to believe that God keeps me and takes good care of me.

My sister Yasmin, you were the most important person during the last years for me. Thank you for being my sister. Thank you so much not only for taking care of me here in Berlin all the time, the endless talks and the unlimited support I have needed so desperately, but also for your unconditional love, your help to believe in myself again.

My nieces Angel and Marilyn were the main source of true happiness for me during these last three years. Seeing them and playing with them was giving me a great push, to look forward and to face all work difficulties.

My Fiancée, Yasmin, my biggest love, for being with me all the time, for your patience and for tolerating and supporting all the decisions I have made, for being critical and loving at the same time and for making me laugh in times, when work had stressed and upset me. You are the one that gives me my strength and inspiration. You have brought “good music” into my life.

My uncle Eissa and aunt Sahar, for the great love and support. Thank you for keeping in touch, and for warm words to encourage me. Thanks God for having such a wonderful second parents like you. Thanks God for having wonderful sisters in law (Nadijda, Angela, Mai).

And last but not least, my family: my father Mufid, my mother Faizeh, my brother Wasim, for their unconditional love and support during all my life, for teaching me the virtue of hard working and patience, for believing in me. I owe you for everything I achieved in my life. You are the most valuable gift I have ever had. Thanks God for giving me such a lovely family.

My Lord, please help me to be a good son, a good brother, a good husband, a good friend, and a good member of the human society.

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1 Introduction

1.1 The immune system

The immune system of mammals has evolved a variety of mechanisms and effector arms to defend the individual against microbes. It recognizes and eliminates pathogenic viruses and bacteria and transformed cells. Highly specialized cell types derived from hematopoietic stem cells in the bone marrow have evolved to perform this function. These are antigen presenting cells, B cells, natural killer (NK) cells and T cells. In the following, I will briefly describe the divisions of the immune system, immune system cells and their functions, and also some of the soluble factors involved in the immune response [1-2].

1.1.1 The innate immune system

The innate immunity mediates the initial protection against infections. It is also called natural or native immunity, and named the body's first arm of defense. It is comprised of cellular components (macrophages (MØs), neutrophils, mast cells, dendritic cells (DCs) and NK cells) and soluble complements (e.g. complement proteins, acute phase proteins). Components of this arm have numerous ways to combat pathogens. Opsonization, for instance, is the binding of complement proteins to the surface of bacteria and thereby make them visible for other components of the immune system such as phagocytes (neutrophils and MØs), that either phagocytose these opsonized bacteria or secrete substances that lyse these pathogens (NK cells, eosinophils). The mechanisms of activating the innate immune system are more ancient and less variable than those of the adaptive immune system. For example, MØs and DCs recognize foreign organisms with their pattern recognition receptors such as toll-like receptors (TLRs) that bind to pathogen specific surface molecules. The innate immune system has a major role in alerting and helping the adaptive immune system to develop an efficient immune response. This is mediated by the secretion

of cytokines and chemokines and by the presentation of foreign antigens on their cell surface to the cells of adaptive immune system [3].

1.1.2 The adaptive immune system

The adaptive immunity develops more slowly and mediates the later, even more effective, defense against infections. It is also called specific or acquired immunity. This type of host defense is stimulated by microbes that invade tissues, thus it adapts to the presence of microbial invaders. Adaptive immunity consists of humoral immunity, in which antibodies neutralize and eradicate extracellular microbes and toxins, and cell-mediated immunity, in which T lymphocytes eradicate intracellular microbes. Adaptive immune responses consist of sequential phases: antigen recognition by lymphocytes, activation of the lymphocytes to proliferate and to differentiate into effector and memory cells, elimination of the microbes, decline of the immune response, and long-lived memory [4].

1.1.3 Antigen presenting cells

This term refers to the cell subsets associated with the induction of a primary immune response. Antigen presenting cells (APCs) are highly specialized cells that process antigens and present the resultant antigen fragments bound to antigen presenting molecules (APMs) to T cells. APCs include DCs, MØs and B cells. The APMs on the APC surface are membrane glycoproteins called the major histocompatibility complex (MHC) molecules class I and class II .The interaction between MHC molecules and their ligands on the surface of a lymphocyte provides the first signal for lymphocyte activation. Additional interaction, or co-stimulation, between accessory molecules present on the APC and the accessory molecules receptors on lymphocytes provide the second signal. Both signals are necessary for triggering the T cell-dependent or mediated immune response [5].

1.1.3.1 MHC molecules and antigen presentation

Three functionally and genetically different classes of APMs are specialized in presenting epitopes to T cells. The MHC is the most polymorphic gene cluster in the human genome, having a large number of alleles. APMs sample the content of the extra and the intracellular compartments and display them on the cell surface. The pathways for antigen processing and presentation by these molecules are presented below. In human, MHC is called the human leukocyte antigen (HLA) and in mice, is called H-2.

1.1.3.2 The MHC class I presentation pathway

MHC class I is expressed on all nucleated cells in mammals. It is specialized in presenting intracellular peptides and is composed of a membrane-spanning heavy α chain that is associated with the free light β2 microglobulin chain (Figure 1). Intracellular cytosolic proteins are digested by a large protease complex, the proteasome. The resulting short peptides are then transported into the endoplasmic reticulum (ER) lumen by the transporter associated with antigen presentation (TAP) molecules. In the ER, MHC class I, which is incompletely folded associates with at least three chaperone proteins (calreticulin, Erp57 and tapasin) and TAP, then binds the TAP-transported peptides and finishes folding. Completely folded MHC class I is released from the chaperone-TAP complex and is then exported to the cell surface [6-7], (Figure 2). This peptide-MHC class I complex can then be recognized by the $CD8^+$ αβ subset of T cells.

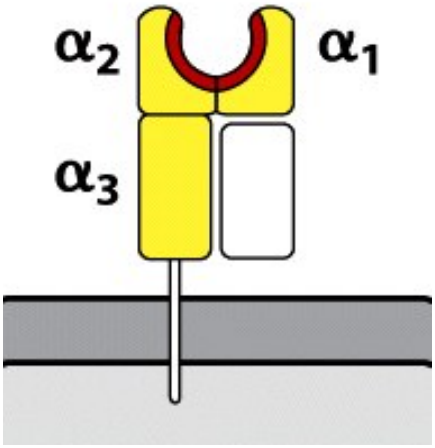

Figure 1: Structure of the MHC class I molecules. The MHC-encoded polypeptide is shown in yellow, the ß2-microglobulin in white and the peptide binding is shown in red. *Adapted from reference* [5].

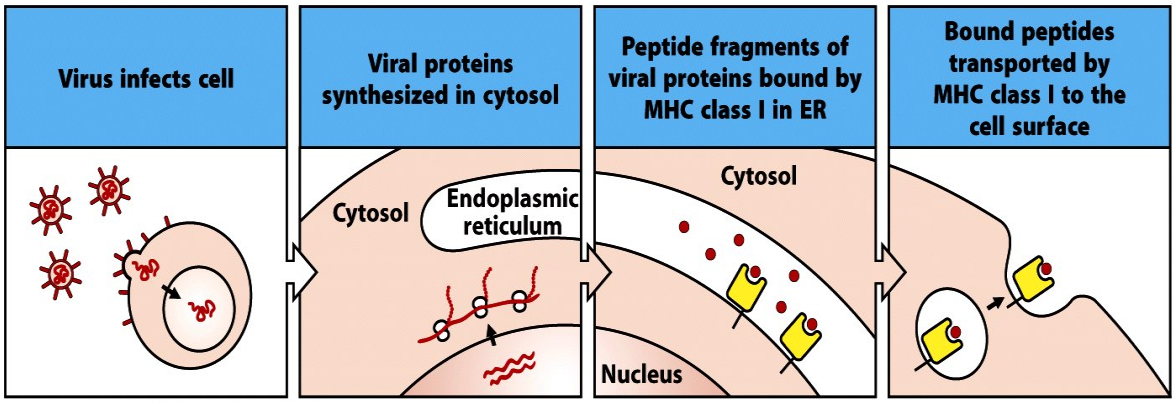

Figure 2: Antigen presentation by MHC class I molecules. In cells infected with viruses, viral proteins are synthesized in the cytosol. Peptide fragments of viral proteins are transported into the ER, where they are bound by MHC class I molecules, which then deliver the peptides to the cell surface. *Adapted from reference* [5].

1.1.3.3 The MHC class II presentation pathway

MHC class II is constitutively expressed on APCs and is specialized in presenting exogenous peptides. It is composed of two membrane-spanning glycoproteins, the α and the β chains (Figure 3). APCs internalize extracellular antigens into endosomes, which contain proteases active at low pH that degrade the antigen into peptide fragments. The endosome then fuses with vesicles containing a trimer complex composed of the MHC class II α/β heterodimer and the invariant chain (Ii), which blocks the MHC class II peptide-binding site prior to this fusion. After the fusion, Ii is cleaved, leaving a class II-associated invariant chain (CLIP) still bound to MHC class II. The class II-like molecule (DM in human and M in mice) then catalyzes the release of CLIP, thus allowing the antigenic peptides to bind. This MHC class II/peptide complex is then transported to the cell surface where it will be recognized by $CD4^+$ αβ T cells [4-5].

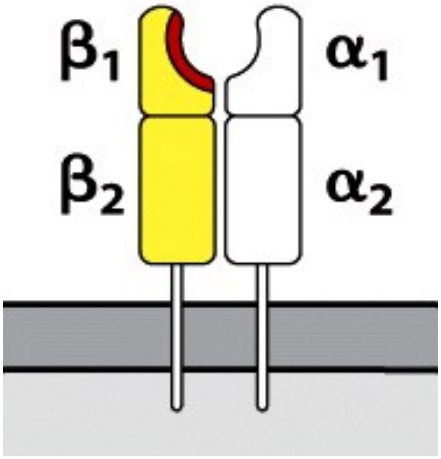

Figure 3: **Structure of the MHC class II molecules**. The MHC-encoded polypeptides are shown in yellow and white, while the peptide antigen-binding is shown in red. Adapted from reference [5].

1.1.3.4 Non-MHC class I and II molecules APMs

Several molecules unrelated to the "classical" MHC molecules are implicated in antigen presentation. Although some of these molecules are well defined, others are only implicated by their functions. These molecules, contrary to MHC class I and II molecules, mainly present non-peptidic antigens. CD1 molecules, which non-covalently associate with β2 micoglobulin, present lipid antigens to $CD8^+$ or double negative (DN) CD4-CD8-αβ T cells. Another APM that has not yet been identified presents non-proteinaceous phospholigands to γδ T cells, which also recognize the lipid antigen [8-9].

1.1.4 B cells

B cells acquired their name in mammals from the site where they are generated and mature within, the bone marrow (originally named after the bursa of Fabricius in birds). Mature B cells are defined by the expression of surface immunoglobulins (Ig), which are antigen-binding molecules. Ig functions as the B cell-specific antigen receptor. Antigen recognition by a mature B cells leads to the differentiation of this B cell into a plasma cell, which produces the soluble form of Ig, the antibody. In addition to their role as APCs, B cells exert protective immunity based on the production of neutralizing or opsonizing antibodies, which are capable of targeting viruses, bacteria and tumor cells [5].

1.1.5 T cells

T cells, which, like B cells, originate in the bone marrow, are named after the thymus, the site where they develop and mature. Mature T cells express a specialized antigen receptor, the T cell receptor (TCR). The TCR is a heterodimer which consists of α and β or γ and δ glycoprotein chains. TCRs on the surface of T cells are associated with other transmembrane proteins (Figure 4), like the signal transduction complex CD3 (which composed of two ε chains, one γ chain, one δ chain and two cytoplasmic ζ chains) and

either CD4 or CD8 co-receptor molecules, which bind the MHC molecule and thereby stabilize the TCR-peptide-MHC interaction and increase the sensitivity of the TCR recognition [3-4]. This interaction represents the first signal necessary for T cell activation. The second signal is provided by co-stimulatory or accessory receptors expressed on the surface of T cells. If a T cell receives only the first signal it becomes anergic, which means that the cells are unable to produce IL-2 or proliferate anymore upon encountering their specific peptides later. These accessory receptors on T cells belong to the CD28 family and bind to receptors of the B7 family expressed on APC. Upon TCR and CD28 ligation, T cells secrete IL-2, an autocrine growth factor, and also express the IL-2 receptor α, which enables them to proliferate rapidly and differentiate. T cells can be divided into four subsets based on their TCR and co-receptor expression [5].

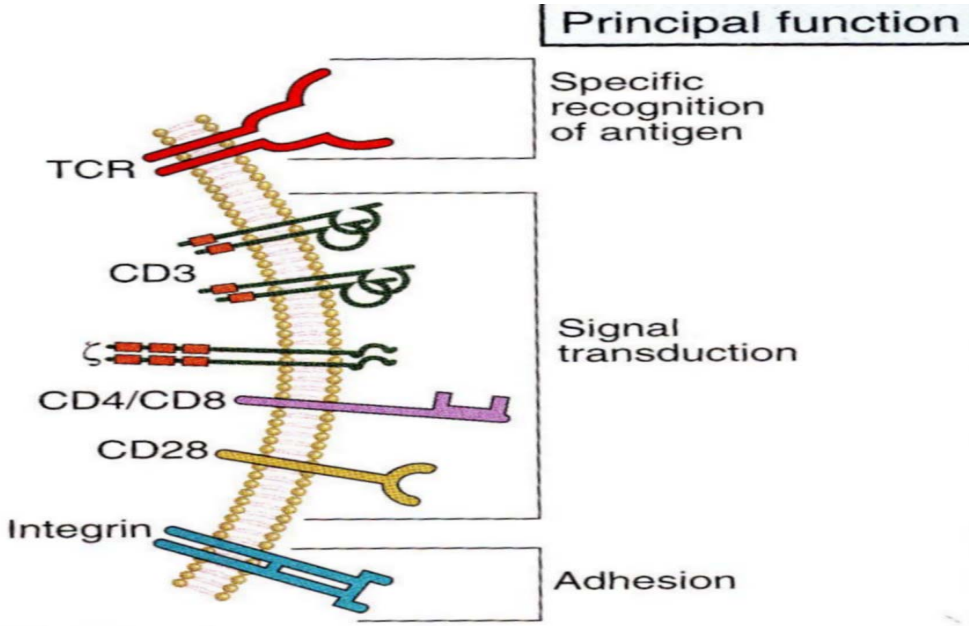

Figure 4: TCR and accessory molecules. The principal proteins involved in antigen recognition and in response to antigens are shown. The function of these proteins fall into three groups: antigen recognition, signal transduction, and adhesion. *Adapted from reference* [3].

1.1.5.1 CD8⁺ αβ TCR⁺ T cells

This T cell subset is often referred to as the cytotoxic T lymphocyte (CTL), and is mainly responsible for defending the body against viruses, intracellular bacteria and malignancies. Through its TCR (Figure 5), it recognizes MHC class I-presented non-self peptide epitopes on the target cell and responds by killing it or by producing cytokines. CTLs are the major players in protective immunity against infection by intracellular pathogens and against cancer. A certain population of CTLs, which recognizes non-classical MHC class I presented epitopes such as N-Formyl peptides, is important in mediating protection against and killing of intracellular bacteria [10].

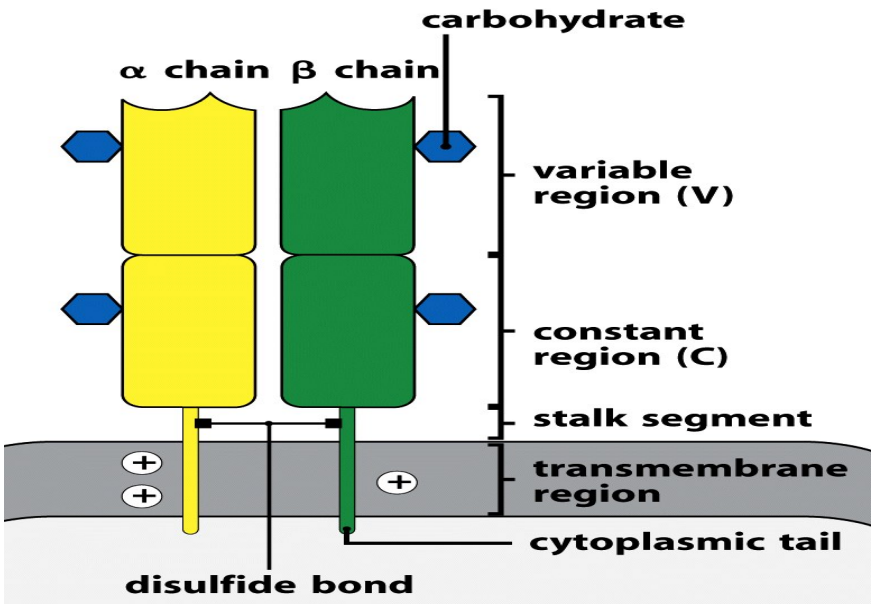

Figure 5: αβ TCR structure. The TCR heterodimer is composed of two transmembrane glycoprotein chains, α and β. The extracellular portion of each chain consists of two domains, resembling immunoglobulin V and C domains, respectively. Both chains have carbohydrate side chains attached to each domain. A short stalk segment, analogous to an immunoglobulin hinge region, connects the immunoglobulin-like domains to the membrane and contains the cysteine residue that forms the inter-chain disulfide bond. The transmembrane helices of both chains are usually containing positively charged (basic) residues within the hydrophobic transmembrane segment. The α chain carries two such residues; the β chain has one. *Adapted from reference* [5].

1.1.5.2 CD4+ αβ TCR+ T cells

This T cell subset, often referred to as the helper T (Th) cell, is mainly responsible for orchestrating the function of other immune cell types and promoting their maturation and proliferation but also exerts direct protective functions. It recognizes MHC class II-presented non-self peptide epitopes on the target cell and responds by producing cytokines or in some cases by killing the target. These cells can be divided into two populations, the Th1 and the Th2 cell subsets, according to their cytokine secretion pattern. The Th1 and Th2 cell subsets play distinct roles in the pathogenesis of autoimmune diseases and in the immunity against cancer and infectious diseases [4-5].

1.1.5.3 DN αβ TCR+ T cells

This is a small subset of T cells that recognizes glycolipids in the context of CD1.While DN T cells are capable of killing bacteria-infected cells by CD90-CD95 interaction; they are incapable of lysing the bacteria. The role of this subset in protective immunity, if any, is unknown [5].

1.1.5.4 γδ TCR+ T cells

This is a DN subset of T cells, although some γδ TCR^+ T cells that reside in the intestines express CD8. γδ T cells recognize non-peptidic antigen from mycobacteria, which is presented by an unknown ligand. Tumor derived γδ T cells recognize the tumor-associated antigens MICA and MICB (the MHC class I related chains A and B) which are overexpressed on epithelial tumors. γδ T cells play an important immunoregulatory role in controlling resistance to intracellular bacteria [11-12].

1.1.5.5 NK Tcells

This T cell subset expresses, in addition to αβ TCRs, markers that are typically expressed on NK cells, such as NK1.1 in the mouse or CD56 in human. This T cell subset is either DN or $CD4^+$, and expresses a restricted set of TCR genes. Mouse NK T cells recognize CD1 molecules on certain cell types. Cytokine secretion seems to be the main role of NK T cells [5].

1.1.6 NK cells

NK cells are TCR- and Ig- negative lymphocytes. They are characterized by a $CD56^+$ $CD3^-$ surface phenotype (Table 1) as well as by the expression of several activating and inhibitory receptors. Activating receptors on NK cells have signaling subunits that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. ITAMs become phosphorylated on tyrosine residues when the receptors bind their ligands. The phosphorylated ITAMs bind and promote the activation of cytoplasmic protein tyrosine kinases, and these enzymes phosphorylate, and thereby activate, other substrates in several different downstream signal transduction pathways, eventually leading to cytotoxic granule exocytosis and production of IFN-γ. On the other hand, inhibitory receptors contain in their cytoplasmic domains structural motifs called immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which become phosphorylated on tyrosine residues when the receptors bind MHC class I molecules. The phosphorylated ITIMs bind and promote the activation of cytoplasmic protein tyrosine phosphataes. These phosphatases remove phosphate groups from the tyrosine residues of various signaling molecules, thereby blocking the activation of NK cells through activating receptors. Therefore, when the inhibitory receptors of NK cells encounter self MHC-peptide complexes, the NK cells are shut off [4].

Table 1. Characteristics of selected cell surface molecules (markers)

Surface markers	**Cell type**	**Function**
CD1	Various human APCs	Presentation of nonpeptide (lipid and glycolipid) antigens to NK T cells
CD3	T cells	Association with the TCR to generate an activation signal in T lymphocytes
CD4	Th cells, monocytes, MØs, DCs	Signaling co-receptor in class II MHC-restricted antigen-induced T cell activation and thymocyte development
CD8	Mainly on cytotoxic T cells, but also on NK cells, DCs	Signaling co-receptor in class I MHC-restricted antigen-induced T cell activation and thymocyte development
CD16	NK cells, MØ, monocytes	Fc receptor binds to the Fc portion of IgG antibodies
CD19	B cells	Co-receptor, facilitates signal transduction upon activation
CD20	B cells	Acting as a calcium channel in the cell membrane
CD28	T cells	Providing co-stimulatory signals, which are required for T cell activation
CD56	NK cells, some subsets of T cells	Adhesion molecule, prototypic marker of human NK cells
CD95 (FAS)	Activated T and B cells	Mediation of apoptosis-inducing signals
CD314 (NKG2D)	NK cells, some subsets of T cells	Killer cell lectin-like receptor
CD335 (NKP46)	NK cells	Natural cytotoxicity-triggering receptor

1.1.7 Soluble molecules of the immune response

A number of soluble molecules produced mainly by the cells of the immune system are involved in orchestrating and mediating the immune response. The most important members of this group of molecules will be described here.

1.1.7.1 Antibodies

They are the effectors of the B cell response. Antibodies are glycoproteins with a unique peptide binding specificity that enables them to bind to their ligand. Effector mechanisms of antibodies include neutralization, agglutination and opsonization, which result in complement fixation, phagocytosis and the triggering of the antibody-dependent cellular cytotoxicity (ADCC). In antibody molecules, the antigen-binding (Fab) regions are spatially separate from the effector (Fc) regions. Different antibody classes or isotypes exist, which are determined by their effector functions and are correlated with the Th type induced by the immune response [3-4].

1.1.7.2 The complement system

This is a set of proteins that can kill cells and bacteria by a pore-forming mechanism and that mediate phagocytosis and inflammation [4]. The complement system can be activated indirectly by binding to an antibody complexed with an antigen (fixation) or by binding to a mannan-binding lectin that is bound to bacteria. Alternatively, the complement system can be activated directly on the surface of a pathogen.

1.1.7.3 Cytokines and chemokines

These are cell-produced proteins that affect other cells. Cytokines produced by lymphocytes are often called interleukins (IL). All cytokines exert their function via binding to a specific receptor on their target cells [3]. Cytokines act directly by killing infected or malignant cells and by inhibiting viral replication or indirectly by activating or inhibiting their target cells. The main properties and characteristics of selected cytokines are listed in Table 2. Small cytokines, which are involved in cell migration and activation, are called chemokines.

Table 2. Characteristics of selected cytokines

Cytokine	**Major source**	**Target cell**	**Main effect**
IL-2	Th1 cells	B cells, NK cells, T cells	Growth, proliferation, activation factor
GM-CSF (granulocyte macrophage colony-stimulating factor)	MØs, T cells	Granulocytes, monocytes, DCs	Growth, differentiation
IFN-γ (interferon)	Th1 cells, CTLs, NK cells	MØs, NK cells, all other tested cell types	Activating MØs and NK cells, increasing MHC class I and MHC class II expression
TNF-α (tumor necrotic factor)	MØs, B cells, NK cells, T cells	B cells, T cells, several other cell types	Cytotoxicity, local inflamation

1.1.7.4 The cytolytic molecules

The killing mediated by T and NK cells represents an important defense mechanism against virus- or bacteria-infected cells and tumors. Cytolytic molecules involved in this killing are described in Table 3.

Table 3. Characteristics of selected cytolytic molecules

Cytolytic molecule	**Major source**	**Target**	**Main effect**
Perforin	NK cells, T cells	Any cell, targets the lipid bilayer membrane	Polymerizion to form a pore in target cell membrane, osmotic lysis
Granzyme A and B	NK cells, T cells	Any cell, target tryptase and Aspase sequences	Proteolysis which leads to the induction of apoptosis
FAS-L	NK cells, T cells	Widespread, FAS-positive cells	Induction of apoptosis via FAS-binding

1.2 Immunotherapy and gene therapy

Immunotherapy in a broad sense is to treat diseases utilizing cells, proteins or genes that belong to or activate the immune system. Gene therapy is the introduction of new genetic material into the cells of an individual with the intention of producing a therapeutic benefit [1-13]. Immuno- therapy and gene therapy are sometimes interactive and the terms are often interchangeably used. Using a gene in an appropriate expression system to induce a protective immune response can be defined both as gene therapy and as immunotherapy. The same is true when using a gene that belongs to the immune system to treat a disease.

Immunotherapy can be divided into three categories: active, passive and adoptive immunotherapy [1]. Each of these categories can be divided further, based on the mechanism of action, into specific and nonspecific immunotherapy. It is worthwhile to note that these divisions are by no means mutually exclusive and often overlap.

1.2.1 Active immunotherapy

Vaccination represents the specific form of active immunotherapy. Whether prophylactic or therapeutic, a vaccine should fulfill several unique requirements to be applied for improving the public health status. Active nonspecific immunotherapy includes immunostimulators of biological origin, and those of synthetic origin that have been used as adjuvants for vaccination and as immunotherapeutics to treat cancer, infections and autoimmune diseases.

1.2.2 Passive immunotherapy

The transfer of antibodies from an immune donor to a non-immune recipient is called passive immunization. In this type of immunotherapy, specific antibodies (mono- or poly-clonal) are used to treat or to prevent infections. The antibody-based therapy of cancer was

recently reported to improve the response rate and to induce regression in women with breast cancer, particularly when combined with chemotherapy [14]. Also, treatment of patients with a monoclonal antibody specific for a tumor antigen expressed on colorectal carcinomas increased their five years overall survival significantly.

1.2.3 Adoptive immunotherapy

This term is used to describe the transfer of immunocompetent cells to a recipient, such as a patient with cancer, virus infection or an immune defect. Adoptive immunotherapy includes bone marrow transplantation (autologous, syngeneic or allogeneic) or transfer of peripheral blood lymphocytes (PBLs), or specific CTL lines and clones [1].

1.2.3.1 Specific TCR-loaded lymphocytes

The isolation and expansion of naturally occurring tumor associated antigens (TAAs)-specific T cell clones used in adoptive cell therapy, is technically difficult, labor-intensive, and time consuming. Therefore, further improvement of current strategies based on the adoptive transfer of tumor-reactive T cells is required. A promising approach to generate large populations of polyclonal T cells with a defined specificity for TAAs in a relatively short time is the genetic modification of T cells using viral or non-viral transduction systems. One strategy uses natural TCR αβ heterodimers of known specificity and affinity for TAAs [15-16]. However, it has been argued that formation of these new TCRs by pairing of endogenous and transgenic TCR chains may lead to *de novo* receptor specificities reacting against autologous MHC-peptide complexes and thereby mediate autoimmune reactions. Another general limitation of this approach is that each transgenic TCR is specific for a certain MHC-peptide complex and therefore is only suitable for MHC-matched tumor patients. Moreover, various immune evasion mechanisms of tumors such as the downregulation of different components of MHC class I and presentation machinery might limit the success of this approach.

1.2.3.2 Chimeric antigen receptor (CAR)-loaded lymphocytes

In an attempt to extend the recognition specificity of T lymphocytes beyond their classical MHC-peptide complexes, a gene-therapeutic strategy has been developed that allows redirecting T cells to defined tumor cell surface antigens [15]. This strategy uses both the cellular and humoral arm of the immune response by assembling an antigen-binding moiety, most commonly a single chain variable fragment (scFv) derived from a monoclonal antibody (mAb), together with an activating immune receptor (Figure 6).

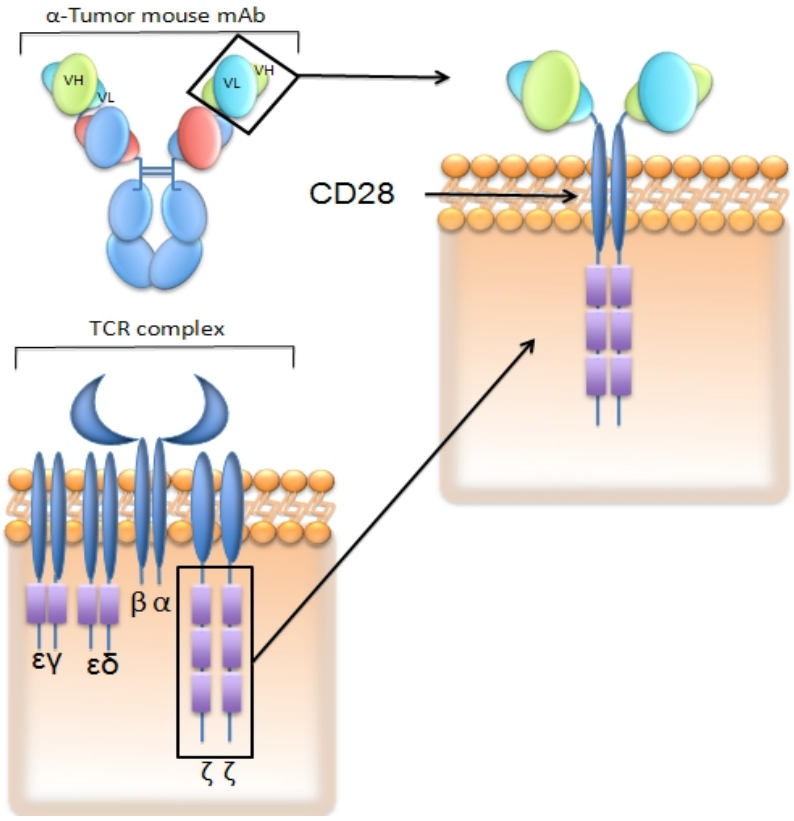

Figure 6: Structure of chimeric antigen receptors (second generation). CARs are made of two components: first, the recognition part consisting of a single chain variable fragment (scFv) containing the heavy (VH, green) and the light (VL, cyan) variable regions of a monoclonal antibody (mAb) targeting the desired TAA, and second, the signal transduction part composed of a cytoplasmic signaling domain of the TCR (CD3ζ chains, violett) and a signaling domain of CD28 (blue) (second generation). First generation-CARs miss the CD28 signaling domain. *Adapted from reference* [19].

Once this artificial immune receptor is expressed at the surface of a modified T lymphocyte, upon binding of the scFv to its antigen an activating signal is transmitted into the lymphocyte, which in turn triggers its effector functions against the target cell. In first attempts to reconfigure T cells with antibody specificity the variable parts of the TCR α and β chains were replaced with scFv fragments derived from monoclonal antibodies. These hybrid T cell receptors were functionally expressed and recognized the corresponding antigens in a non-MHC-restricted manner [17–18].

1.2.3.2.1 First generation of CARs

CD3ζ chain-signaling on its own is sufficient for T cell activation [20, 21]. Consequently, the first "true" chimeric single-chain receptors were created by fusing a scFv directly to the CD3ζ chain [22]. At that time this concept was called the "T body approach" [23]. Nowadays these types of artificial lymphocyte signaling receptors are commonly referred to as chimeric immune receptors (CIRs) or chimeric antigen receptors (CARs) or simply chimeric receptors (CRs). The use of CARs to redirect T cells specifically against TAA-expressing tumor cells has a number of theoretical advantages over classical T cell-based immunotherapies. In contrast to the long-lasting procedure of *in vitro* selection, characterization, and expansion of T cell clones with native specificity for MHC-tumor peptide complexes, genetic modification of polyclonal T cell populations allows to generate TAA-specific T cells during one to two weeks [24]. Engraftment with CARs enables T cells to MHC-independent antigen recognition, thus major immune escape mechanisms of tumors such as downregulation of MHC molecules are efficiently bypassed [25]. Furthermore, proliferation and survival of modified T cells can be improved by the implementation of a multitude of signaling domains from different immune receptors in a single CAR.

1.2.3.2.2 Second generation of CARs

Although first generation CARs could initiate a cytotoxic antitumor response in grafted T cells, it soon became obvious that signals from ITAM-bearing receptors alone can only induce transient cell division and suboptimal cytokine secretion [26, 27] but cannot provide prolonged polyclonal expansion and sustained antitumor reaction *in vivo*. According to the prevailing two-signaling model for lymphocyte activation, full activation and proliferation of T cells require a co-stimulatory signal through CD28-B7 interaction in addition to signaling through the TCR/CD3 complex [28]. Providing the co-stimulatory signal in Trans through B7-expressing cells sustained proliferation of single ITAM-bearing CAR-expressing lymphocytes and lead to enhanced antitumor response in mouse models [29, 30]. A more straight forward strategy comprised the CD28 signaling in the CAR concept, either in two separate molecules [31] or combined in a single CD28-ITAM receptor fusion protein [32, 33]. Placement of the CD28 domain proximal to the CD3ζ chain and immediately distal to the transmembrane domain resulted in good surface expression, whereas in the opposite way the surface expression seems to be inhibited [34]. Therefore, all known second generation CARs follow the general outline transmembrane domain-CD28-ITAM-bearing signaling chain. Subsequently, it has been shown that the synergistic action of the two signaling domains in one single CAR resulted in sustained proliferation of grafted lymphocytes [35, 36], increased levels of IL-2 [37, 38], IFN-γ [37, 38, 39], and granulocyte macrophage colony-stimulating factor (GM-CSF) secretion [39] independent of exogenous B7/CD28 co-stimulation. Due to CD28 co-stimulation the expression of anti-apoptotic proteins like B-cell lymphoma- (Bcl-) 2 is up-regulated and, the induction of activation induced cell death (AICD) is delayed [40, 41].

1.3 CAR-modified NK cell-based therapy

Cancer is a major health problem worldwide and one of the most important causes of mortality in children and adults. Cancers arise from the uncontrolled proliferation and can include the spread of these transformed cells to other parts of the body. The growth of malignant tumors is determined in large part by the proliferative capacity of the tumor cells and by the ability of these cells to invade host tissues and metastasize to distant sites [1]. The immunogenicity of tumors implies that tumor cells express antigens that are recognized as foreign by the adaptive immune system. Active immunotherapy for tumors is designed to augment immune responses against tumors for example by vaccination. Approaches for passive immunotherapy include the administration of anti-tumor antibodies, antibodies conjugated with toxic drugs, and tumor-reactive T cells and NK cells isolated from patients and expanded by culture with growth factors. Nowadays, adoptive immunotherapy represents a promising approach to eradicate tumors.

1.3.1 NK cell-based targeting

Adoptive cell therapy is a form of immunotherapy based on the use of tumor targeting lymphocytes [42, 43]. While being usually considered as a form of T cell therapy, tumor cells with defined antigens have been successfully targeted with adoptively transferred T cells. This type of therapy is often associated with tumor regression as well as the development of MHC class I negative or low tumor-cell variants [43, 44]. In the periphery, self-reactive T cells are suppressed by several tolerance mechanisms. Therefore, expressing a second antigen-specific receptor in T cells might support these cells self-reactive if the introduced targeting receptor stimulates the recognition via an endogenous self-specific TCR.

For that, the utilization of engineered NK cells in this approach is becoming increasingly attractive to target tumors. 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. Since their discovery, NK cells were described as cytotoxic lymphocytes, which are able to destroy certain tumor cells *in vitro* without prior sensitization. The molecular mechanism of tumor cell recognition by NK cells is regulated by a balance of activating and inhibitory signals (Figure 7). Activating receptors, such as the NKp46 [45] and other natural cytotoxicity receptors (NCRs) as well as NKG2D [46], are sometimes able to completely override the inhibitory signal delivered by inhibitory killer immunoglobulin-like receptors (KIRs), which interact with MHC class I molecules.

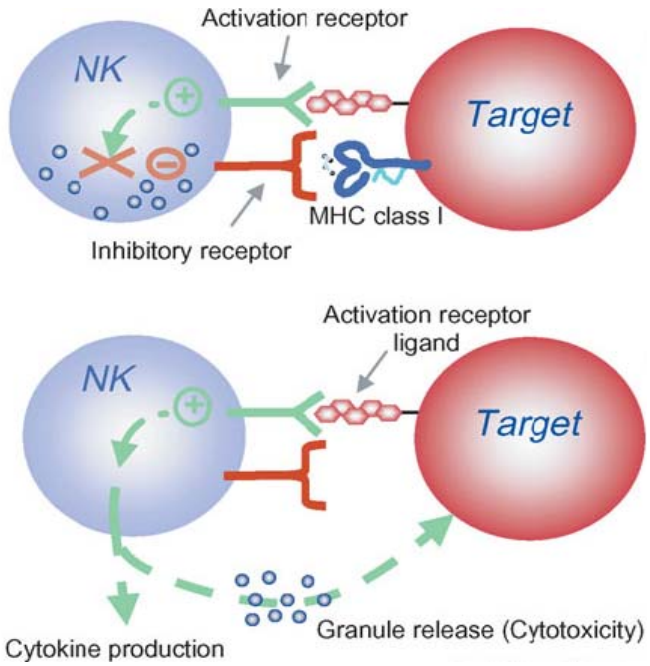

Figure 7: Recognition mechanism of NK cells. NK cell killing depends on the balance between activating and inhibiting (MHC class I) signals (upper panel). Upon activation, NK cells release cytokines and granule contents which induce apoptosis in the target cell (lower panel).

CD16, the low-affinity FcγRIIIa on the surface of NK cells that binds to the Fc part of antibodies [47, 48], also activates NK cells leading to cytokine production or to the killing of coated target cells in a process referred to as ADCC. While loss or downregulation of ligands for NK inhibitory receptors on tumor cells is often associated with NK recognition [49, 50], 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, as is often the case for solid tumors. Various approaches were evaluated to overcome tumor-cell resistance to NK cells. These included the use of *allo* NK cells [44], blocking the inhibitory signal [51], or triggering the activation receptors by using monoclonal antibodies or by genetic modification of tumor cells [52, 53, 54].

In certain tumor models, the role of NKG2D, an activation receptor also expressed on γδ-T cells, NK T cells and activated $CD8^+$ T cells, was investigated by several groups [53, 55]. Induced expression of endogenous NKG2D ligands strongly increased the NK cell ability to recognize tumor cells *in vitro*, and the ectopic expression of NKG2D ligands Rae-1 and H60 by tumor cell lines resulted in their rejection [53, 54, 56].

1.3.2 HER-2 targeting: CAR-NK cell-based therapy versus antibody treatment

The human epidermal growth factor 2 (HER-2), which promotes the growth of cancer cells, is amplified and overexpressed in 30-80% of human breast, ovarian, pancreatic, colon, gastric, lung and prostate carcinomas and melanomas [57-58] and its overexpression correlates with a more aggressive disease [59]. Furthermore, breast cancer lymph node metastatic lesions generally overexpress HER-2 to the same extent as the corresponding primary tumors [60] and the HER-2 gene amplification status in distant metastases is conserved and is not affected by chemotherapy [61, 62]. Therefore, HER-2 represents an attractive target for immunotherapy. The humanized monoclonal antibody trastuzumab targeting HER-2 is believed to manifest its effect through direct antibody binding, by blocking the development of new blood vessels (angiogenesis) through inhibition of Vascular Endothelial Growth Factor (VEGF) expression and by NK-mediated ADCC [57, 63]. However, polymorphisms in Fc receptors have a very significant impact on the ADCC and can predict clinical outcome of trastuzumab therapy [64].

Recombinant CARs combine the antigen-specific binding properties of a monoclonal antibody with the cytotoxic activity of lymphocytes in a single molecule and are currently implemented in a panel of clinical trials using predominantly CAR-modified T cells [43, 65, 66]. Primary human NK cells can be successfully targeted to tumors. In an acute lymphoblastoid leukemia (ALL) model, the leukemia cells were shown to be a good target for NK cells transduced with a CD19 specific CAR and in targeting HER-2 positive carcinomas [67, 68]. This study presents an optimized protocol for the engineering of primary human NK cells with HER-2 specific CARs targeting HER-2 expressing tumor cell lines. While incorporation of a co-stimulatory domain within the CAR improved IL-2 production, the soluble NKG2D ligand MICA did not impact specific HER-2 recognition by these cells. Furthermore, engineered NK cells became activated via the CAR upon co-culture with MCF-7 cells, which is a well-established trastuzumab-resistant carcinoma model [69, 70].

1.3.3 Direct aims of the thesis

The main goals of this project are the following:

• Establishing an optimized protocol to expand human primary NK cells, and to achieve optimal retroviral transduction for them with chimeric antigen receptors (specific for HER-2 antigen).

• Comparing first and second generation CARs including a stimulatory (CD3ζ) signaling domain alone or together with a co-stimulatory (CD28) signaling domain in sense of having different cytokines production patterns.

• Determining the independent recognition pattern achieved via CARs, and showing that, the blocking of NKG2D by its soluble ligand (which is known to reduce NK cell responsiveness) did not impact CAR-mediated HER-2 specific recognition by these cells.

• Comparing CAR targeting of NK cells to soluble antibody (trastuzumab) targeting, and showing that breast carcinomas without HER-2 gene amplification could be uniquely targeted by CAR modified NK cells.

• Determining that the direct coupling of the antibody specificity to NK cell functions mediated by CAR expression resulted in superior NK cell targeting via CD16 binding of soluble monoclonal antibodies.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

The human embryonic kidney cell line **293T** was kindly provided by Prof. Dr. W. Uckert (MDC, Berlin) and was used as a packaging cell line for retroviral vectors as described in [42, 71].It was grown in DMEM medium (Invitrogen, Darmstadt, Germany) supplemented with 10% (v/v) heat-inactivated FBS (PAN-Biotech). **RPMI8866** is a human B cell line that is frequently used as a feeder cell line for generation of NK cell clones. This cell line cultured in RPMI medium 1640, 10% (v/v) FBS. **C1R/A2**, a MHC class I defective lymphoblastoid cell line (LCL) transfected with HLA-A2, and its transfectant **C1R/A2HER2**, which expresses HER-2, were described by Rongcun, Y., et al [72]. Ovarian tumor cells were isolated from the ascitic fluid of patients with advanced epithelial ovarian cancer, the ovarian carcinoma **SKOV3** and the breast carcinoma **MCF-7** cell lines were obtained as described in [72, 73]. The breast carcinoma cell lines were kindly provided by the following investigators from MDC: **MDA-MB-435** (Prof.Dr.W.Uckert), **Cal 51**(Dr.W.Birchmeier), and **ZR-75-1** (Dr.S.Scherneck), all cultured in RPMI medium 1640, 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 medium 1640, 20% (v/v) FBS. The **p815** mouse lymphoblast-like mastocytoma cell line was obtained from ATCC and was cultured in RPMI 1640 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% (v/v) FBS (PAN-Biotech).

2.1.2 pMIG vector

pMIG is a murine stem cell virus (MSCV) based vector capable of transducing mouse and human T cells [42]. It is kindly provided by Dr. L. V. Parijs. It encodes the retroviral packaging signal φ (PSI), the green fluorescence protein (GFP) and also an internal ribosome entry site (IRES) that allows cap-independent translation of the mRNA by using the IRES sequence to direct the mRNA to the ribosome. This vector was used as a control in the transduction experiments (Figure 8).

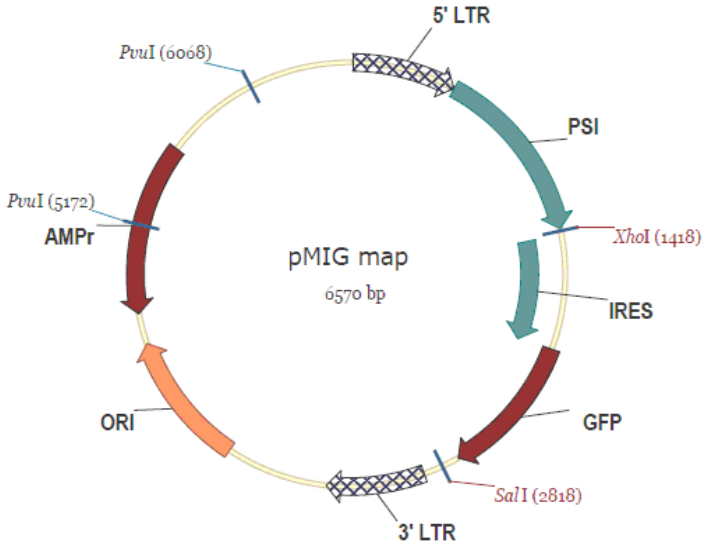

Figure 8: pMIG vector map. pMIG encodes the retroviral packaging signal (PSI), the green fluorescence protein (GFP) and an internal ribosome entry site (IRES), flanked by 3' and 5' long terminal repeats (LTRs). The backbone contains an ampicillin resistance (AMPr) gene and the origin of replication (ORI).

2.1.3 Chimeric antigen receptor constructs

The chimeric receptor constructs specific for HER-2, C6.5-scFv-Fc-CD3ζ and C6.5-scFv-Fc-CD3ζ-CD28 were earlier described by Kruschinski, A., et al. and Hombach, A., et al. [68, 39]. The CARs were cloned into the modified retroviral vector pMIG by replacing the GFP and IRES fragment. The GFP and IRES sequences were deleted by using *Xho*I and *Sal*I. Afterwards, the desired sequences of C6.5-scFv-Fc-CD3ζ and C6.5-scFv-Fc-CD3ζ-CD28 were inserted into *Nco*I and *Bam*HI sites, then the ends were blunted and religated. The resulting constructs were designated pMSCV-CARz and pMSCV-CARz28, respectively (Figure 9). The pMIG vector encoding GFP was used as control. Cloning of the various constructs was confirmed by restriction mapping and partial sequencing as described by Charo, J., et al. and Kruschinski, A., et al. [42, 68].

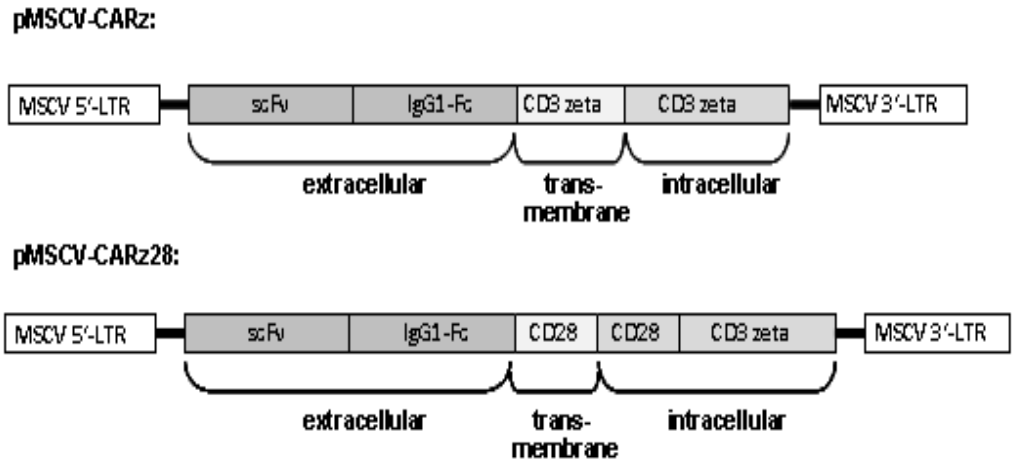

Figure 9: **A schematic representation of the two CAR constructs used in this study.** The chimeric receptor constructs were cloned into pMIG replacing IRES and GFP. The resulting constructs were designated pMSCV-CARz and pMSCV-CARz28. scFv, single-chain fragment variable; hIgG1-Fc, human IgG1 crystallizable fragment.

2.1.4 Restriction enzymes

*Xho*I, *Sal*I, *Nco*I and *Bam*HI were purchased from Amersham Bioscience Europe GmbH (Freiburg, Germany). They were used for plasmids construction and to evaluate afterwards the cloning results.

2.1.5 Antibodies

Antibody	**Abbreviation**	**Conjugates**	**Company**
mouse anti-human CD3 (clone UCHT1)	mαh CD3	FITC, PE-Cy-5	BD Pharmingen™ (Heidelberg, Germany)
mouse anti-human CD16 (clone B73-1)	mαh CD16	PE	BD Pharmingen™ (Heidelberg, Germany)
mouse anti human CD56 (clone NCAM16.2)	mαh CD56	APC, PE	BD Pharmingen™ (Heidelberg, Germany)
F(ab)2 goat anti human Ig polyclonal antibody	gαh Ig	APC, PE	Biozol, Eching, Germany
mouse anti human NKG2D (clone 1D11)	mαh NKG2D	APC, PE	BD Pharmingen™ (Heidelberg, Germany)
anti-MICA/B (clone 6D4)	α MICA/B	PE	BD Pharmingen™ (Heidelberg, Germany)
anti-ULBP-2 (clone BUMO1)	α ULBP-2	PE	BAMOBAM GmbH, Munich, Germany

2.1.6 Reagents

Reagents	Abbreviation	Manufacturer
Agarose		Serva, Heidelberg
Biocoll™		Biochrom AG, Berlin
Bovine serum albumin	BSA	Invitrogen, Karlsruhe
Dimethylsulfoxide	DMSO	Sigma, Taufkirchen
1,5-Dimethyl-1,5-diazaundeca-methylene polymethobromide	Polybrene	Sigma, Taufkirchen
Dimethylsulfoxide	DMSO	Sigma, Taufkirchen
DNA-ladder (1 kb)	1 kb	Invitrogen, Karlsruhe
Dulbecco‘s phosphate buffered saline (+/- CaCl2/ MgCl2)	D-PBS	Gibco, Karlsruhe
Dulbecco‘s modified Eagle Medium	D-MEM	Gibco, Karlsruhe
Ethanol	EtOH	Merck, Darmstadt
Ethidiumbromide	EtBr	Serva, Heidelberg
Ethylenediaminetetraacetic acid	EDTA	Merck, Darmstadt
Fetal calf serum (heat inactivated)	FCS	Greiner, Solingen
Gentamycin		Gibco, Karlsruhe
Geneticin (Neomycin)	G418	Chiron, Emeryville, CA
Interleukin-2, recombinant	IL-2	Roche , Mannheim
LB-Amp (100µg /ml) Agar	LB-Amp	Invitrogen, Karlsruhe
Leucoagglutinin, lectin (Phytohaemagglutinin)	PHA-L	Sigma, Missouri, USA
Lipofectamine™2000		Invitrogen, Karlsruhe
β-Mercaptoethanol	β-ME	Gibco, Karlsruhe
N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)	Hepes	Serva, Heidelberg
NuSieve®GTG® Agarose (low melt agarose)		Biozym, Hess. Oldendorf
Opti-MEM®		Invitrogen, Karlsruhe
RetroNectin®		TaKaRa Biomedicals, Apen
RPMI 1640 (+GlutaMAX™1 + 10% FCS, + 50mg/ml Gentamycin, + 50mM β-ME)	RPMI-All	Gibco, Karlsruhe
S.O.C. medium		Invitrogen, Karlsruhe
Streptavidin œ Phycoerythrin (-PE)	Strep. -PE	Molecular Probes, Oregon, USA
Trypsin-EDTA (1x)		Gibco, Karlsruhe
Trypan blue (25%)	TB	Sigma, Taufkirchen

2.1.7 Kits

Kits	Manufacturer
DNA ligation Kit Version 2.1	TaKaRa Biomedicals, Apen, Germany
QIAquick gel extraction kit	Quiagen, Helden, Germany
QIAGEN plasmid purification mini/ maxi kit	Quiagen, Helden, Germany
HumanIL-2 ELISA kit	BD Clonetech, Heidelberg, Germany
Human IFN-γ ELISA kit	BD Clonetech, Heidelberg, Germany

2.1.8 Buffers and solutions

Buffers and solutions	Ingredients
ACK-lysis buffer (Erythrocyte lysis buffer)	0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA, pH 7,2
DEPC-H2O	500 µl DEPC, in 500 ml distilled water
Ethidiumbromide	10 mg Ethidiumbromide/ml distilled water
Elution buffer	10 mM Tris-Cl; pH 8,5
Fixation solution	50 ml DPBS, 50 µl FCS, 1.4 ml formaldehyde
TAE (50x)	242 g Tris, 57,1 ml concentrated HCL, 100 ml EDTA (0,5 M, pH 8), ad 1 l H2O

2.1.9 Laboratory material

Laboratory material	Manufacturer
Centrifugation tubes, polypropylene (15 ml, 50 ml)	BD-Falcon, Heidelberg, Germany
Cell strainer/ mesh (40 µm)	Becton Dickinson, Heidelberg, Germany
Cuvettes, single use	Roth, Karlsruhe, Germany
Cryotubes	Nunc, Wiesbaden, Germany
Eppendorf tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Filter, sterile (0.2 µm, 0.45 µm)	Schleicher &Schüll, Dassel, Germany
Flow cytometry tubes	Becton Dickinson, Heidelberg, Germany
Needles, single use (0.9 mm)	Sanimed, Berlin, Germany
Pasteur-Pipettes	Brand, Wertheim, Germany
Petri dishes	Greiner, Solingen, Germany
Pipettes, single use, sterile (5 ml, 10 ml, 25 ml)	Costar, Bodenheim, Germany
Pipette tips	Roth, Karlsruhe, Germany
Quartz cuvettes	Hellma, Müllheim, Germany
Round bottom tubes	Greiner, Solingen, Germany
Syringes, single use (0.5 ml, 1 ml, 5 ml, 10 ml, 25 ml)	Sanimed, Berlin, Germany
Tissue culture flasks (T-25, T-75, T-150)	TPP, Trasadingen, Switzerland
Tissue culture plates, 6-, 24-, 48-well, non treated	TPP, Trasadingen, Switzerland
Tissue culture plates, 96-well, round bottom	Costar, Bodenheim, Germany
Tissue culture plates, 96-well, V-bottom	Costar, Bodenheim, Germany

2.1.10 Laboratory devices

Device	Manufacturer
Analytical scale	Sartorius, Göttingen, Germany
Bio-Freezer (-80°C)	Forma Scientific, Cotech, Berlin, Germany
Cell particle counter	Coulter, Krefeld, Germany
Centrifuge 3K12	Sigma, Taufkirchen, Germany
Centrifuge 5415 C	Eppendorf, Hamburg, Germany
Centrifuge RT 6000D	Sorvall, Langenselbold, Germany
Electrophoreses-chamber	Bio-Rad, München, Germany
FACS Calibur flow cytometer	Becton Dickinson, Heidelberg, Germany
Incubator	Labotect, Göttingen, Germany
Laminar flow box (sterile hood)	BDK, Sonnbuhl, Germany
Lysis device	Becton Dickinson, Heidelberg, Germany
Magnetic stiring device	IKA-Labortechnik, Staufen, Germany
Microscope Leitz DM IL	Leica, Wetzlar, Germany
Multichannel pipette	Eppendorf, Hamburg, Germany
Nitrogen tank CHRONOS	Messer Griesheim, Griesheim, Germany
Neubauer-hematocytometer	Roth, Karlsruhe, Germany
PCR machine	Biometra, Göttingen, Germany
Spectrophotometer UV-160A	Shimadzu, Berlin, Germany
super centrifuge	Sorvall, Langenselbold, Germany
UV-table	Appligene, Illkirch, Germany
Vortex device	IKA-Labortechnik, Staufen, Germany
Water bath	GFL, Burgwedel, Germany

2.2 Methods

2.2.1 Isolation of human peripheral blood mononuclear cells (PBMCs)

Buffy coats from the blood of anonymous healthy donors were obtained from the blood bank of Deutsches Rotes Kreuz (Berlin, Germany) and were diluted 1:2 with Phosphate buffered saline (PBS). Mononuclear cells were collected from the diluted samples by centrifugation using a Lymphoprep density separation (Biocoll, Biochrom AG, Berlin, Germany; or PANcoll ready-to-use, PAN-Biotech, Aldenbach, Germany). Biocoll is a Ficoll-based separation solution consisting of carbohydrate polymers of a high density. Upon centrifugation, dead cells that weight more than live cells sank through the Ficoll layer to the bottom of the tube whereas live cells accumulated as a faint band between the upper layer of rest medium and the lower Ficoll solution. The tubes were centrifuged for 10 minutes at room temperature (RT) at 1,000 rpm in a swing centrifuge. During the separation, 50 ml tubes were filled with 25 ml RPMI-All and after centrifugation the faint band of live cells was carefully transferred into the prepared tubes. After another round of centrifugation for 5 minutes, at 10^{o}C and 1,000 rpm, the supernatant was discarded and the cell pellet re-suspended in 5 ml RPMI-All supplemented with 20 IU/ml IL-2. The PBMCs were washed two to four times in PBS and were frozen to maintain standard conditions.

2.2.2 Expansion of primary human NK cells

Thawed PBMCs were washed twice in RPMI 1640 medium. The cells were seeded at 2x10^{7} cells in 20 ml medium 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 with 30 Gy and washed twice. The cells were then co-cultured 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.

2.2.3 Plasmid amplification

The general procedures to increase the amount of plasmid DNA for further transfection experiments involved the following steps, transformation of E.coli bacteria (MAX™DH5α-FT™) with the DNA plasmids, a minipreparation to purify the plasmid DNA followed by a test to identify the plasmids of interest. The bacteria containing the correct plasmid were then cultured to amplify the plasmid, which was purified by maxiprep.

2.2.3.1 Transformation

Competent bacteria (MAX™DH5α-FT™) were thawed on ice, 3 µl of 1 µg/ µl of the plasmid DNA were added to 27 µl of the bacteria in a 1.5 ml Eppendorf tube and mixed gently by flicking the tube. After 30 min incubation on ice, the bacteria were heat shocked for 45 seconds in a 42°C warm water bath and immediately cooled on ice for 2 minutes. 900 µl of room temperature S.O.C. medium was added. Bacteria were cultured on a shaker for 1 hr at 37°C. 100 µl of each bacteria culture was spread onto LB agar plates containing 100µg/ml Ampicillin and incubated over night at 37°C. On the next day 4-6 colonies were inoculated into 5 ml of liquid LB-Amp and cultured on a shaker at 37°C for minipreparation on the following day.

2.2.3.2 Minipreparation (QIAprep Spin Miniprep Kit Protocol)

Before the plasmid DNA was re-isolated from the competent bacteria, glycerol stocks of each clone were prepared. Therefore, 750 µl of the overnight grown bacterial culture were mixed with 150 µl of glycerol and stored at -80°C. 1 ml of the bacterial culture were

centrifuged for 5 min at 4°C and 3000 rpm, the supernatant was discarded and the cells were re-suspended in 250 µl of buffer P1 containing RNase A. The cells were then lysed in 250 µl of lysis buffer P2, containing NaOH and sodiumdodecylsulphate (SDS) to solubilize the bacterial wall under alkaline conditions and the tubes were slowly end-over-end inverted 6 times. 350 µl of neutralizing buffer N3 were added and the tubes were inverted again. This step leads to the precipitation of the cellular proteins and genomic DNA. The samples were then centrifuged for 10 min at full speed (13,200 rpm) in a tabletop centrifuge and the supernatant was applied onto the spin columns that had been inserted into a vacuum manifold. The plasmid DNA bound to the matrix was washed 2 times by adding of 750 µl washing buffer (PB) containing ethanol. Residual buffer was removed by spinning the columns for 1 min at full speed after they were transferred into new Eppendorf tube. The columns were again placed in new tubes, 50 µl of elution buffer (EB) was added and after 1 min the tubes were spun for 1 min at full speed in order to elute the DNA from the columns. To test the plasmid, 5 µl of the eluted DNA were digested over night at 37°C with restriction enzymes. On the next day the DNA was loaded onto an agarose gel and separated for 30-40 min.

2.2.3.3 Maxipreparation (QIAGEN-tip 500 Plasmid Purification Maxi Kit)

Maxipreparation of plasmid DNA was prepared from a "starter culture" using bacteria from frozen glycerol stock that was inoculated into 15 ml LB-Amp and cultured on a shaker for 8 hr at 37°C and 300 rpm. This was necessary to let the frozen bacteria recover. After 8 hr, the bacteria grew dense enough to expand them further (5ml from a starter culture in a 150 ml LB-Amp) in an Erlenmeyer flask on a shaker over night at 37°C. On the following day, the bacteria were harvested and pelleted for 15 min at 4°C and 4,000 rpm in 50 ml centrifugation tubes using Sorvall centrifuge. The supernatant was discarded and the cells were re-suspended in 10 ml buffer P1. They were then lysed with 10 ml of lysing buffer (P2) by inverting the tube gently 6 times followed by 5 mini-incubations at room temperature (RT). The lysis was stopped by adding 10 ml of pre-chilled

neutralization buffer (P3) and inverting the tube again. Afterwards, the tube was put on ice for 20 min. All debris including the genomic DNA were removed by centrifugation for 30 min at 4°C and 9,000 rpm using Sorvall centrifuge. The plasmid containing supernatant was later moved into a new 50 ml BD tube and centrifuged again for 15 min at 9,000 rpm at 4°C. In the meantime the columns were equilibrated with 10 ml equilibration buffer (QBT). Then the plasmid containing supernatant was applied onto the column and bound DNA was washed 3 times with washing buffer (QC). Next, the DNA containing flow through was eluted into new 50 ml BD tube by adding 15 ml of elution buffer (QF) onto the columns. To precipitate the plasmid DNA, 10.5 ml of room temperature isopropanol was added and the eluate tube was inverted 2-3 times. The DNA was pelleted by centrifugation (30 min, 4°C, and 9,000 rpm) and washed afterwards with 5 ml 70% EtOH. Centrifugation was repeated and the DNA pellet was re-suspended in 1 ml 70% EtOH and transferred into a new 1.5 ml Eppendorf tubes. The tube was centrifuged at 13,200 rpm for 5 min in a tabletop centrifuge. Afterwards the supernatant was discarded under a sterile hood and the pellet was air-dried until most traces of EtOH had evaporated. Finally the DNA was dissolved in 200-300 µl elution buffers. An aliquot of each plasmid was diluted in water in order to measure the DNA concentration spectro-photometrically. The following formula was used to calculate the amount of DNA:

DNA (µg/ml) = OD260 x 50 x dilution factor

2.2.4 Retrovirus production by 293T cells

The 10A1-pseudotyped retrovirus supernatants were generated by co-transfecting 293T cells with the CARz, CARz28 or MIG plasmids and the gag, pol and env encoding pCL-10A1 vector [74] using Lipofectamine 2000 according to [68]. The Lipofectamine solution contains cationic lipids that complexes with the plasmid DNA and carries it into the target cell by membrane fusion. 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 manufacturer's protocol. The cells were transfected over night in the incubator at 37°C. The following day, medium was changed to 5 ml RPMI 1640 medium, 10% (v/v) FBS, and 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.

2.2.5 Retroviral transduction

PBLs were cultured (day 1) in described ratios with the irradiated feeder cell line RPMI 8866 and transduced 3 times at 3 successive days. Transduction was performed on day 6 or 8 after co-culture initiation, using either RetroNectin or spinocculation assessed transduction protocol.

For RetroNectin transduction, 6-well tissue culture plates were coated with 50 µg per well RetroNectin (Takara, Saint-Germain-en-Laye, France) 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 (Novartis, Wehr, Germany). 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. This protocol was used in experiments presented in Figures 10-12.

For 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 with 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. In Figure 13, NK cells were transduced only twice on day 7 and 8 after co-culture initiation. Because transduction via spinocculation is more cost-effective and gave good efficacy [65], I switched to this protocol to transduce NK cells in all subsequent experiments.

2.2.6 Cell culture

Cells were cultured in tissue culture flasks in a 95% humidified atmosphere at 37°C and 8% CO_2 in the incubator. For the calculation of the cell number, 10µl of the cells were mixed with 10µl of trypan blue (TB) and counted by hematocytometer. Every 3-4 days cultures were re-suspended and the old medium was exchanged leaving 0.25-0.5 ml of the culture volume and 5 ml fresh medium was added. Thereby they were 10-20 times diluted depending on their growth rate. Adherent growing cells were trypsinized with Trypsin/ EDTA when they had grown to a confluent monolayer. 2-5 ml Trypsin/EDTA were added onto the cells depending on the size of the flask. When they started to be detached from the flask (approx 4-5 min later), fresh medium was added to stop the reaction. The cells were re-suspended and 10% of the cells, transferred into new flasks filled with fresh medium. Cells that were transfected with constructs encoding an antibiotic resistance gene were cultured in medium supplemented with the appropriate antibiotic (Gentamycin 10 µg/ µl).

2.2.7 Cell storage

In order to store cells for later experiments, they were frozen and thawed at a later time point. To freeze cells, they were pelleted by centrifugation and re-suspended in 2 ml of "freezing medium" consisting of 90% FCS and 10% Dimethylsulfoxide (DMSO). The cells were then transferred into cryotubes and gradually cooled down to -80°C in isopropanol-containing boxes. Depending on the duration of the storage they were either

left in the -80°C freezer or stored in liquid nitrogen. The process of thawing cells has to be done very fast, because DMSO is toxic to the cells at higher temperature. Thus, they were thawed with suitable medium and transferred into 14 ml tubes. Afterwards they were pelleted for 5 min at 1,200 rpm and 10°C to remove DMSO. They were then re-suspended in fresh medium and cultured as usual.

2.2.8 Cytotoxicity test

In order to select cells, which were transduced with an antibiotic resistance gene, the lethal concentration of the antibiotic for non-transduced cells had to be tested. Therefore 1x10cells were cultured in RPMI-All containing different antibiotic concentrations (2.5, 5 and 10 µg/µl of Gentamycin) for 10 days. Each day the numbers of live cells in the wells was counted by hematocytometer based trypan blue (TB) exclusion.

2.2.9 Flow cytometry

Fluorescence activated cell sorting (FACS) is a method that is used for quantitative and qualitative analysis of cellular parameters, such as size, granularity or surface molecules (markers). The detection is usually carried out with the help of fluorochrome-labeled monoclonal antibody that bind specifically to surface molecule. Examples for fluorescent molecules are fluorescein isothiocyanate (FITC), allophycocyanin (APC) or R-phycoerthrin (R-PE), which emit light of a definite wavelength upon excitation. Single cells stained with fluorochrome-labeled antibodies are passed through a laser ray within a flow cytometer. As a result of the size and granularity of the cell light becomes dispersed and the fluorochromes get excited and emit light. The light is collected and transformed into electrical signals. On this occasion the intensity of the emitted light is proportional to the amount of fluorochromes bound on the surface of a single cell.

For FACS analysis, 1 million cells were washed 2X with PBS and stained on ice for 30 minutes in 96-well plates with 0.5 µg of their specific fluorochrome-labeled antibody or with the corresponding isotype control antibody on. The following antibodies were used for immunophenotypic characterization of expanded and transduced cells: anti-CD3 (clone UCHT1), anti-CD56 (clone NCAM16.2), anti-CD16 (clone B73.1) and anti-NKG2D (clone 1D11), all purchased from BD biosciences. Surface expression of the chimeric receptors was determined using the $F(ab)_2$ fragment from goat anti-human Ig polyclonal antibody (Biozol, Eching, Germany). Cancer cell lines were stained with an anti-MICA/B (clone 6D4, BD biosicences) or anti-ULBP-2 (clone BUMO1, BAMOMAB GmbH, Munich, Germany). Dead cells were excluded from the analysis by either gating on the size (forward scatter), and granularity (side scatter) of live cells or by propidium iodide (PI) staining. PI incorporates within the DNA of only dead cells that have disrupted membranes. Data acquisition was performed on a FACSCalibur (BD Biosiences) and analyzed using CellQuest Pro (BD Biosiences) and FlowJo (Tree star, Ashland, OR) softwares.

2.2.10 NKG2D modulation assay

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 (R&D, Wiesbaden, Germany) 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 CAR-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 subsequent co-culture experiments.

2.2.11 T cell depletion

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

2.2.12 Cytokine release assay and ELISA

Target cells (5x104) were co-cultured with an equal number of effector cells in 96-well flat bottom plates for 24 hours. For antibodies dependent cytokine production experiments, mock-transduced NK cells ($5x10^4$) were co-cultured with the same number of target cells in the presence or absence of 10 µg/ml of a mouse anti human CD16 antibody, trastuzumab (Roche, Basel, Switzerland) or rituximab (Roche). The supernatants were assayed for IFN-γ and IL-2 by specific enzyme-linked immunosorbent assays (ELISAs) (Mabtech, Hamburg, Germany, or BD Biosciences, Heidelberg, Germany) according to the manufacturer's protocol. ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen or cytokine in a sample. The data represent 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 of IFN-γ or 20 pg/ml of IL-2.

3 Results

3.1 Effect of PBL:feeder cell ratio on NK transduction efficacy

Different culture conditions were employed in order to establish reproducible conditions under which NK cells expressing CAR could be obtained. Using pMIG vector that expresses GFP as a reporter, comparative analysis involving preferential NK cell expansion and transduction efficacy based on the percentage of GFP positive NK cells was performed. Transduction was designed on three subsequent days starting on day 8 after co-culture initiation (day 1), and no IL-2 was added to the co-culture. Culturing PBLs together with RPMI 8866 cells at a 5:1 ratio (Figure 10) 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 was achieved in the co-culture using a 3:1 ratio (89.2% $CD3^{-}CD56^{+}$ NK cells), but here the transduction efficacy was only 5.3% GFP^{+} $CD3^{-}CD56^{+}$ NK cells. Co-culture ratios of 1:3 and 1:5 led to a less efficient NK expansion of 84.8% and 54.2% $CD3^{-}CD56^{+}$ NK cells and accordingly a less efficient transduction of 4.7% and 4.4% $GFP^{+}CD3^{-}CD56^{+}$ NK cells.

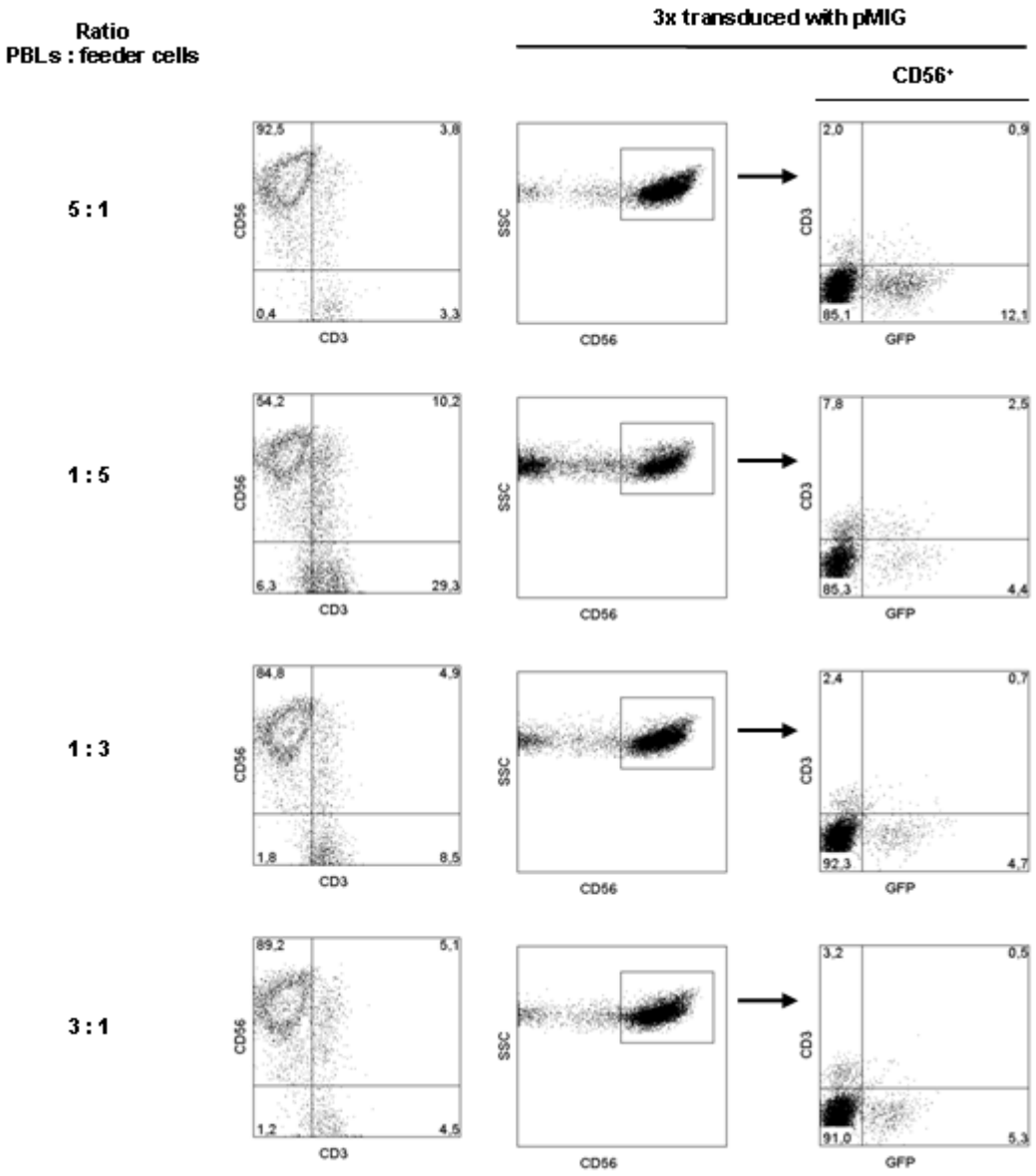

Figure 10: PBL to feeder cell ratio of 5:1 leads to the highest NK expansion and transduction efficacy. PBLs were cultured in described ratios with the irradiated feeder cell line RPMI 8866 and transduced 3 times with pMIG at 3 successive days. The cells were then stained with a mouse anti-human CD3 antibody conjugated to PE-Cy-5 and a mouse anti-human CD56 antibody conjugated to APC (left panels) to determine the percentage of $CD3^{-}CD56^{+}$ NK cells. Cells were then gated on the $CD56^{+}$ population (middle panels) and analyzed for GFP and CD3 expression (right panels) by flow cytometry.

3.2 Role of IL-2 in modulating NK transduction efficacy

To augment the production of transduced NK cells, the effect of IL-2 added during the transduction process on the survival and the transduction efficacy of NK cells was analyzed. Figure 11 shows GFP expression on cells that were transduced three times, starting on day 8 of co-culture initiation (day 1), at a 5:1 ratio either without IL-2 or with 200 IU/ml IL-2. While the presence of IL-2 during the transduction process slightly improved the transduction efficacy from 12.1 % to 14.5 %, it markedly improved NK cell survival as the cell viability was about twice as high (32 % live cells versus 59.1 % live cells) when IL-2 was included during transduction.

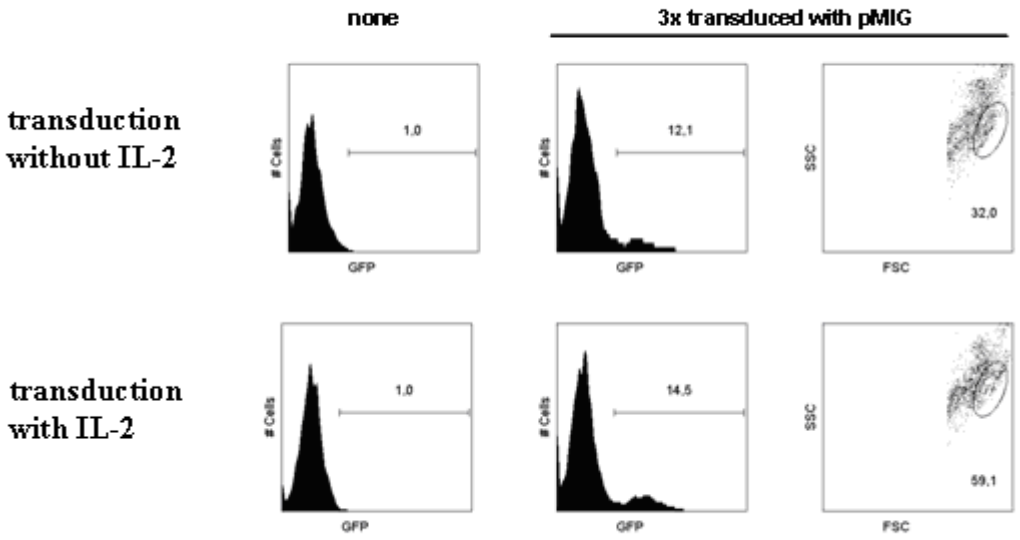

Figure 11: IL-2 addition during transduction increases cell viability, but not transduction efficacy. PBLs were transduced 3 times with pMIG either without IL-2 (upper panel) or with 200 IU/ml IL-2 (lower panel) at three successive days. Cells were analyzed for GFP expression and for viability, evaluated by forward and sideward light scattering, by flow cytometry.

3.3 Effect of timing and number of transduction rounds on transduction efficacy

The optimal time points and frequency of retroviral transduction following NK cell activation was also investigated. Figure 12 shows GFP expression on cells that were transduced starting either on day 6 or on day 8 after co-culture initiation in the presence of IL-2 at a 5:1 ratio. A more efficient transduction was achieved when transduction was performed starting on day 6 after the initiation of co-culture (day 1). This led to a GFP expression of 7.4 %, 21.9 % and 35 % after one, two or three transduction rounds, respectively (Figure 12). Transduction performed on day 8 after initiation of co-culture was less efficient with 1.8 %, 6.1 % and 13.7 % after one, two or three transduction rounds, respectively (Figure 12).

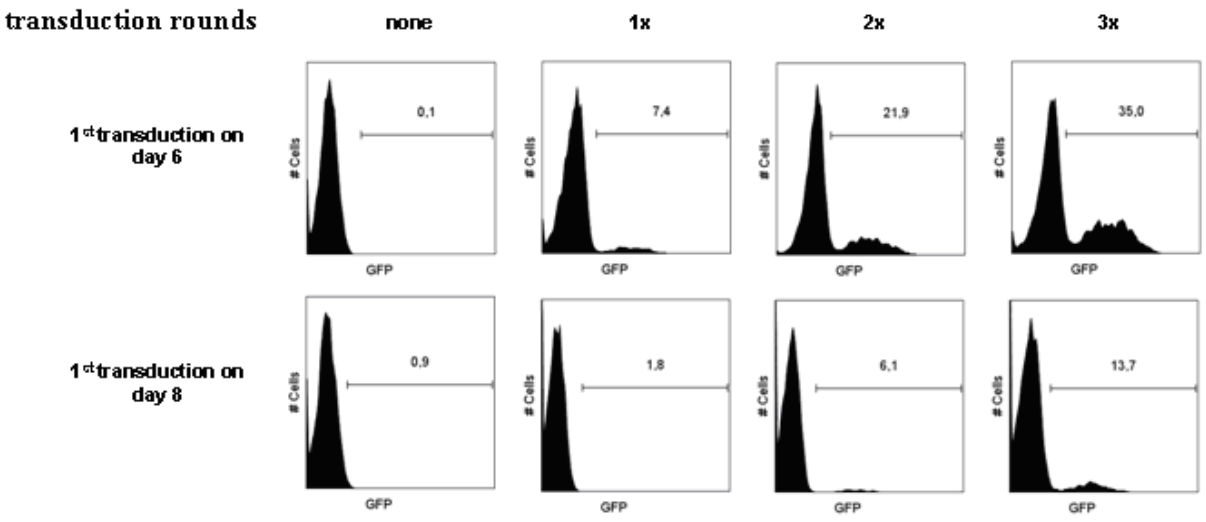

Figure 12: Improved transduction efficacy is achieved when transduction is performed on day 6 as compared to day 8 of co-culture initiation. PBLs were transduced 1, 2 or 3 times with pMIG starting on day 6 (upper panel) or day 8 (lower panel) within three successive days. On day 12, cells were analyzed for GFP expression by flow cytometry.

3.4 Efficient expression of the chimeric antigen receptors on engineered NK cells

To further investigate the feasibility of engineering antigen-specific NK cells, two CAR constructs consisting of a HER-2 specific binding antibody domain fused to CD3ζ for signal transduction, or to joined CD3ζ and CD28 signaling domains were compared. The constructs were cloned into pMIG replacing IRES and GFP. CARs expressed from these constructs were designated CARz and CARz28. Retrovirus supernatants produced from either constructs were used to transduce PBL-derived primary human NK cells (Figure 13). Flow cytometry analysis revealed that the transduction efficacy for the CARz construct was 41.8 % and for the CARz28 construct 53.1 %, respectively, as detected by antibodies specific for human Ig recognizing the extracellular domain of the CAR (Figure 13).

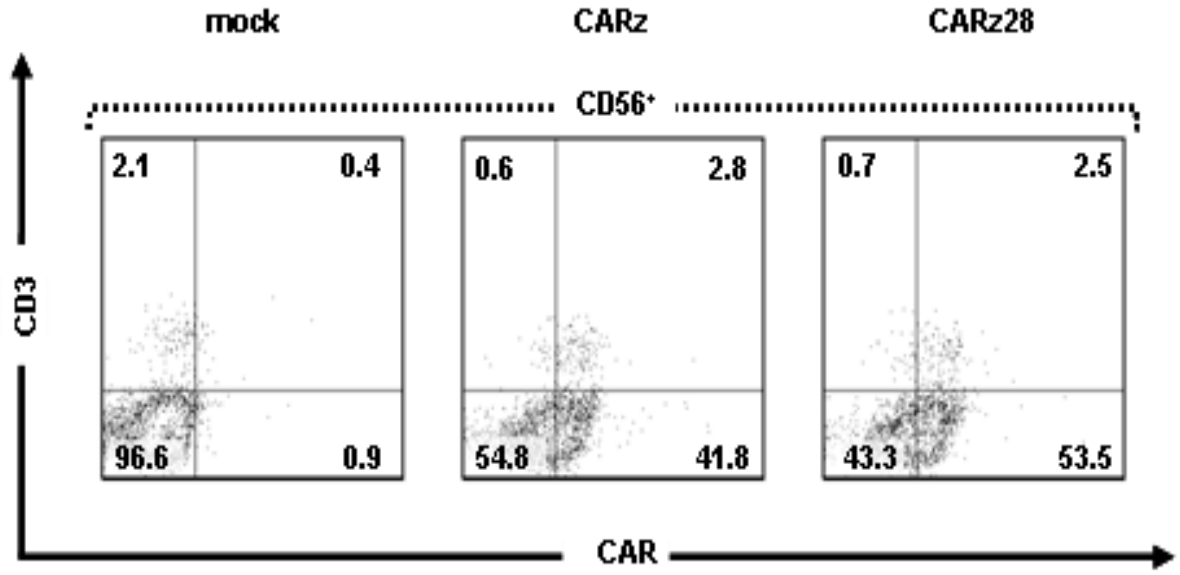

Figure 13: Expression of the transduced CARs on primary human NK cells. PBLs were transduced with pMIG (mock), pMSCV-CARz and pMSCV-CARz28. On day 12, cells were analyzed for the expression of the respective CAR by flow cytometry. Cells were stained with a mouse anti-human CD56 antibody conjugated to APC, a mouse anti-human CD3 antibody conjugated to FITC and a goat anti-human Ig antibody conjugated to PE recognizing the CAR. Cells were gated on the $CD56^+$ population and were then analyzed for the expression of CAR and CD3.

3.5 CAR-engineered NK cells produce cytokines upon HER-2 recognition

To assess the function and specificity of CAR-transduced NK cells, they had to be purified in order to remove residual T and NK T cells. This was achieved by depleting CD3 positive T cells using magnetic beads that resulted in cell cultures with 98.7% ±1.1 % of the cells expressing the NK phenotype $CD3^-$ $CD56^+$ cells (Figure 14).

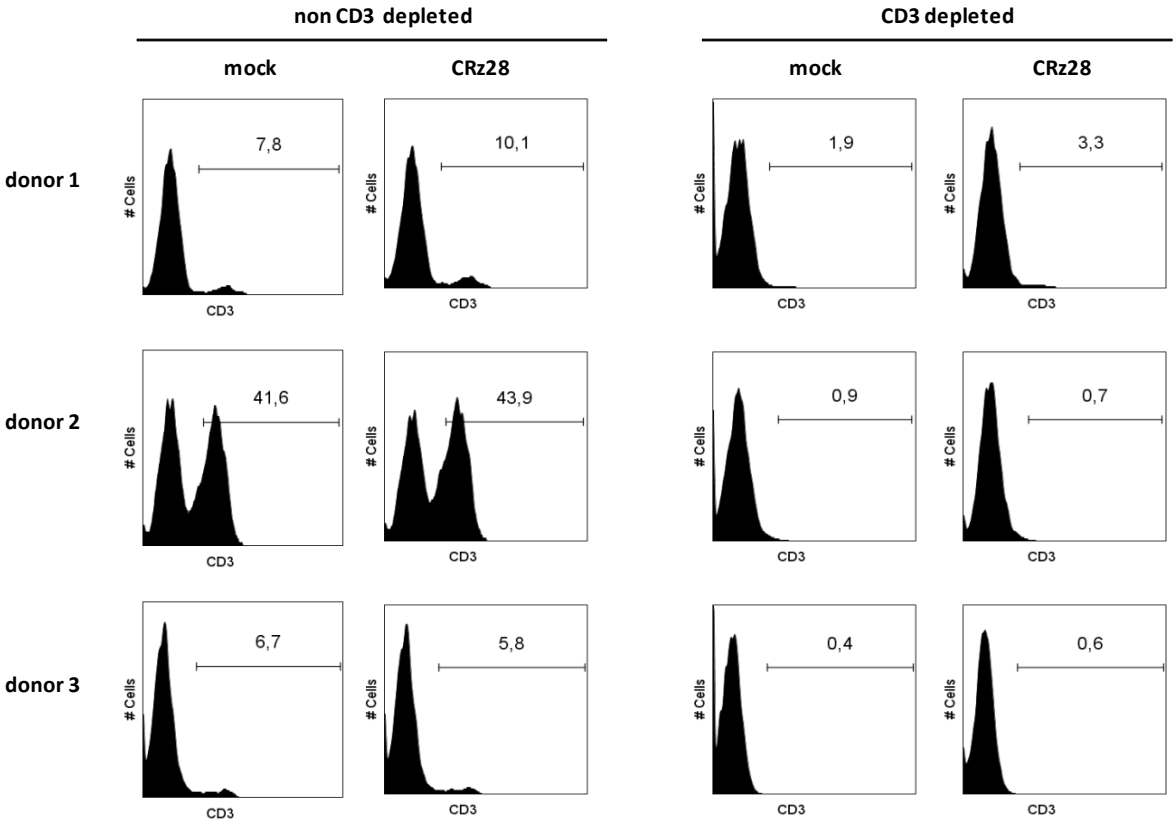

Figure 14: High purity of NK cell lines after CD3 depletion. PBLs were transduced with either the mock or the CAR constructs and were subsequently depleted using magnetic beads conjugated to mouse anti-human CD3 antibodies. Staining with a mouse anti-human CD3 antibody conjugated to PE-Cy-5 of 3 representative donors transduced with the mock- or CARz28 plasmid which were either undepleted (left panels) or from day 6 after depletion (right panels) is depicted. The depletion of CARz-transduced NK cells resulted in similar NK cell purity (data not shown).

Subsequently, the ability of CAR engineered NK cells to recognize HER-2 expressing target cells was investigated in IFN-γ and IL-2 release assays (Figure 15, 16). CARz- and CARz28-engineered NK cells cultures were normalized by adding mock-transduced cells to CARz28-NK cell cultures. This resulted in CAR cultures expressing the CARs on approximately 40 % of the cells in both CARz-NK and CARz28-NK cultures. Subsequently, CD3 depleted mock-, CARz- and CARz28-engineered NK cells were cultured alone (none) or co-cultured with either the HER-2 negative tumor cell line C1R/A2, the HER-2 expressing transfectant of this cell line, C1R/A2HER2, or with the endogenously HER2 expressing ovarian carcinoma cell line SKOV3. Subsequently, an IFN-γ (Figure 15) and IL-2 (Figure 16) specific ELISA were performed. Neither CAR- nor mock-NK cells produced IFN-γ or IL-2 spontaneously. Mock-NK cells were stimulated by target cells to produce some IFN-γ (between 550 and 2800 pg/ml) (Figure 15). CARz- and CARz28-NK cells secreted similar IFN-γ levels (1200 and 1250 pg/ml, respectively) when co-cultured with the HER-2 negative C1R/A2 cell line, however significantly higher levels of IFN-γ were produced by CARz- and CARz28-NK cells in response to stimulation by C1R/A2HER2 cells (7000 and 8100 pg/ml, respectively) and SKOV3 cells (8100 and 9000 pg/ml, respectively, Figure 15).

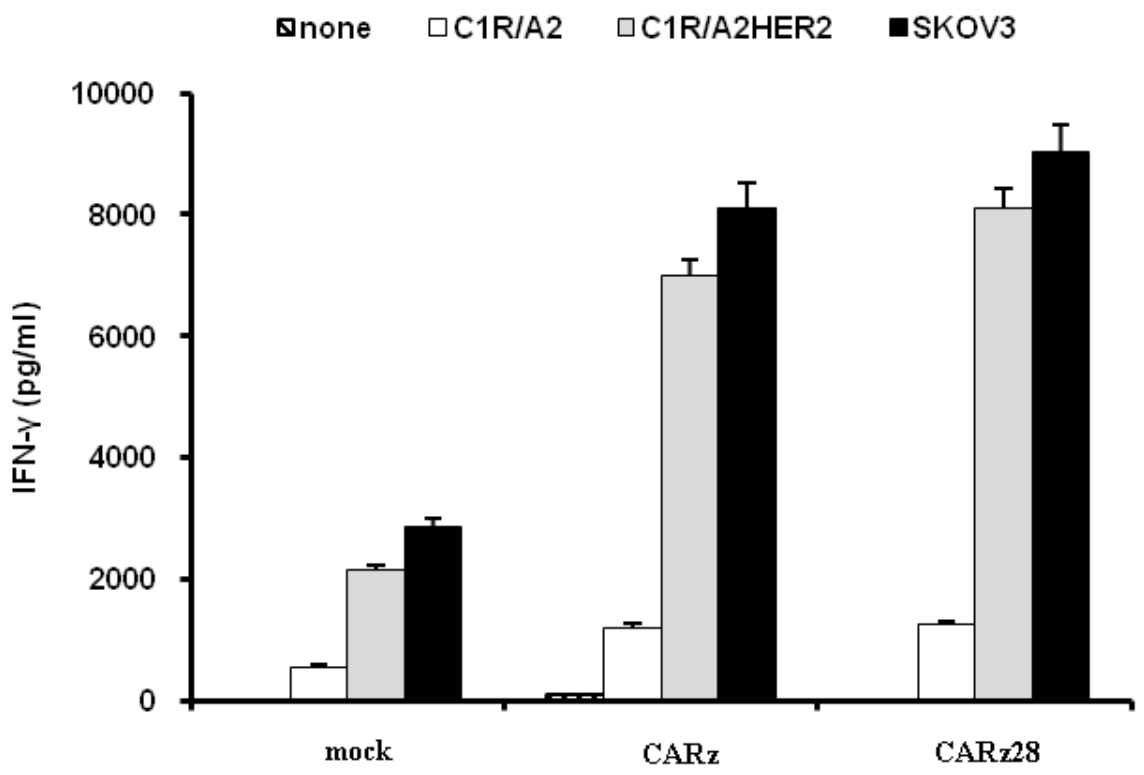

Figure 15: CARz and CARz28-engineered NK cells produce similar levels of IFN-γ upon HER-2 recognition. IFN-γ was measured in the supernatants of stimulated NK cells. Effector cells were mock-, CARz- or CARz28-transduced NK cells normalized to comprise the same percentage (40 %) of CAR^+ cells. They were either cultured without target cells (none) or co-cultured with C1R/A2 cells, C1R/A2HER2 cells, or SKOV3 cells. Values shown represent mean values of triplicates obtained from IFN-γ specific ELISA.

Likewise, some IL-2 was produced by mock-NK cells when co-cultured with target cells (between 250 and 700 pg/ml) (Figure 16), and CARz- and CARz28-NK cells produced similar IL-2 levels when co-cultured with C1R/A2 cells (400 and 1000 pg/ml, respectively) (Figure 16). A modest increase in IL-2 production by CARz-NK cells (1250 and 2000 pg/ml), as compared to markedly higher IL-2 production by CARz28-NK (4800 and 5500 pg/ml, respectively) could be observed when these cells were co-cultured with the HER-2 positive targets C1R/A2HER2 and SKOV3 (Figure 16). Since CARz28-NK cells had better IL-2 response than CARz-NK cells, they were chosen to continue the further analysis of CAR-engineered NK cells in this study.

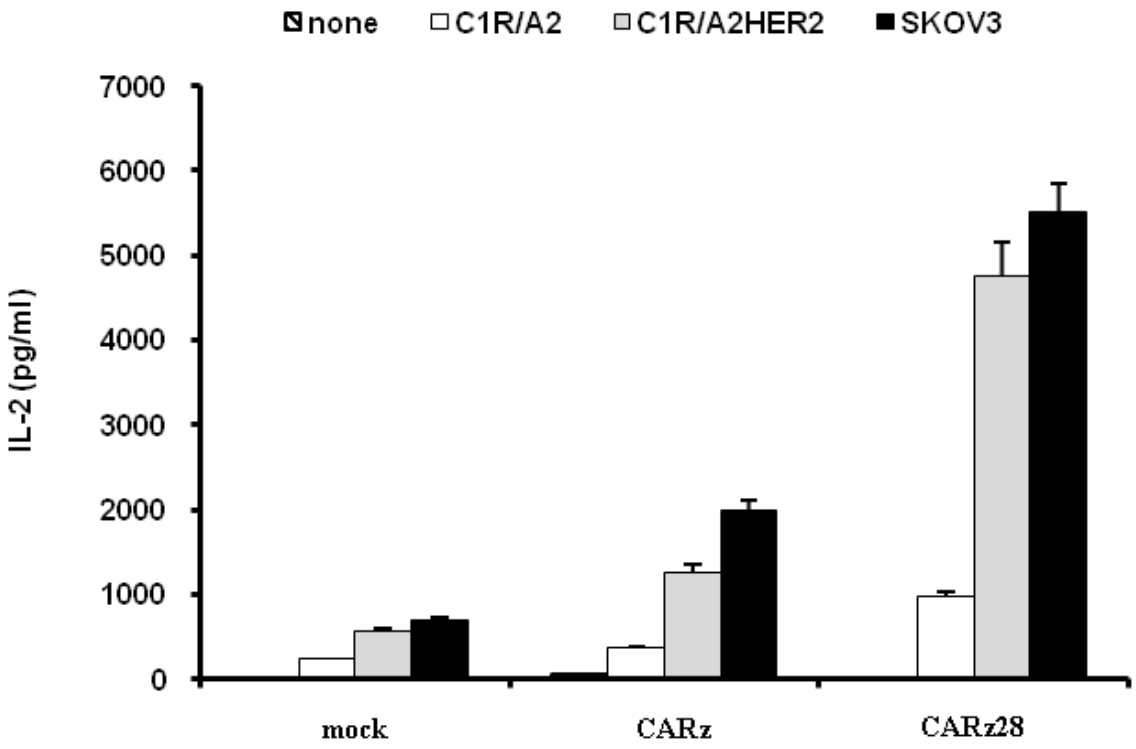

Figure 16: CARz28-NK produces more IL-2 than CARz upon HER-2 recognition. IL-2 was measured in the supernatants of stimulated NK cells. Effector cells were mock-, CARz- or CARz28-transduced NK cells normalized to comprise the same percentage (40 %) of CAR^+ cells. They were either cultured without target cells (none) or co-cultured with C1R/A2 cells, C1R/A2HER2 cells, or SKOV3 cells. Values shown represent mean values of triplicates obtained from IL-2 specific ELISA.

3.6 CARz28-NK cells are resistant to NKG2D blockade

The effect of soluble MICA on HER-2 specific CAR-NK cell activation was investigated. First, binding of a recombinant human MICA protein to NKG2D was confirmed by flow cytometry (Figure 17A). I evaluated the effect of earlier described overnight cultures [75] of NK cells in the presence or absence of the recombinant MICA protein on NKG2D level of expression on CARz28-NK cells. Even if high concentration of MICA protein was included in the overnight NK cell cultures, no effect on NKG2D expression level was detected (Figure 17B).

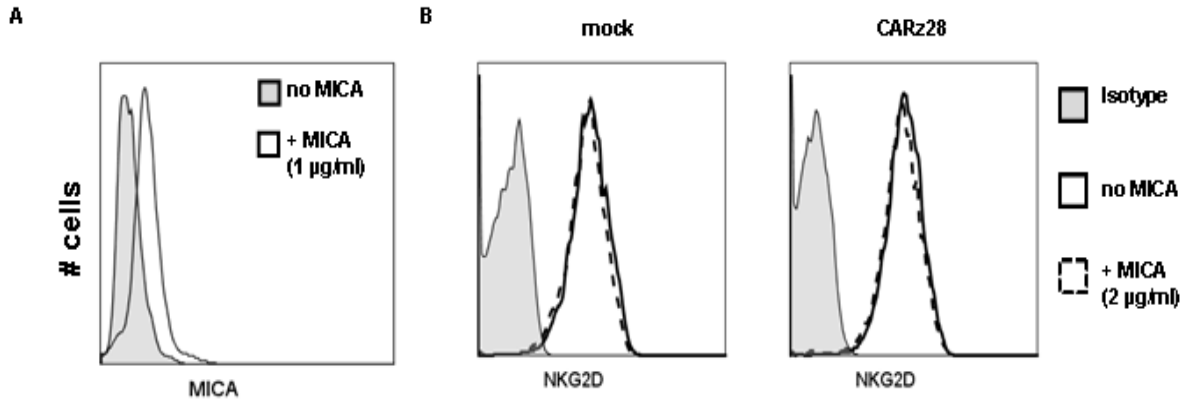

Figure 17: (A) **MICA binds to NKG2D on NK cells**. NK cells were cultured in the presence or absence of 1µg/ml of the recombinant MICA protein for 30 min and the cells were subsequently stained for MICA using a mouse anti-human MICA/B antibody conjugated to PE. Filled histogram represents MICA staining 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. (B) **No NKG2D down-modulation after overnight exposure to MICA**. Mock- and CAR-NK cells were incubated over night either with or without of the recombinant MICA protein 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 of cells cultured overnight with (solid line) or without no soluble MICA (dotted line).

To determine whether soluble NKG2D ligands can block NK recognition of target expressing these ligands, I investigated MICA/B and ULBP-2 expression on cancer cell lines to select suitable targets (Figure 18 and Figure 19). C1R/A2HER2 lymphoblastoid cells do not express NKG2D ligands, and MDA MB 435 and ZR-75-1 express only ULBP-2 (Figure 18).

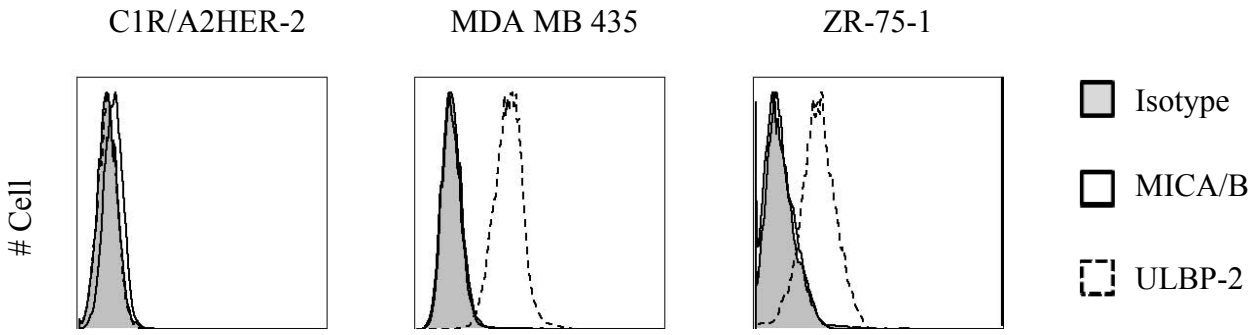

Figure 18: **NKG2D ligand expression on selected tumor cell lines.** Staining of carcinoma cell lines with a mouse anti-human MICA/B antibody conjugated to PE as well as a mouse anti-human ULBP-2 antibody and a secondary anti-mouse IgG1 antibody conjugated to PE. Filled histograms represent isotype control 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.

Therefore, two breast- and one ovarian-carcinoma cell lines were chosen which express both NKG2D ligands MICA/B and ULBP-2 (Figure 19). The breast cancer cell line Cal 51 expressed both MICA/B and ULBP-2 (Figure 19). The ovarian cancer cell line SKOV3 expressed ULBP-2 and to some extent MICA/B (Figure 19). The breast cancer cell line MCF-7 expressed high level of ULBP-2 and low level of MICA/B (Figure 19).

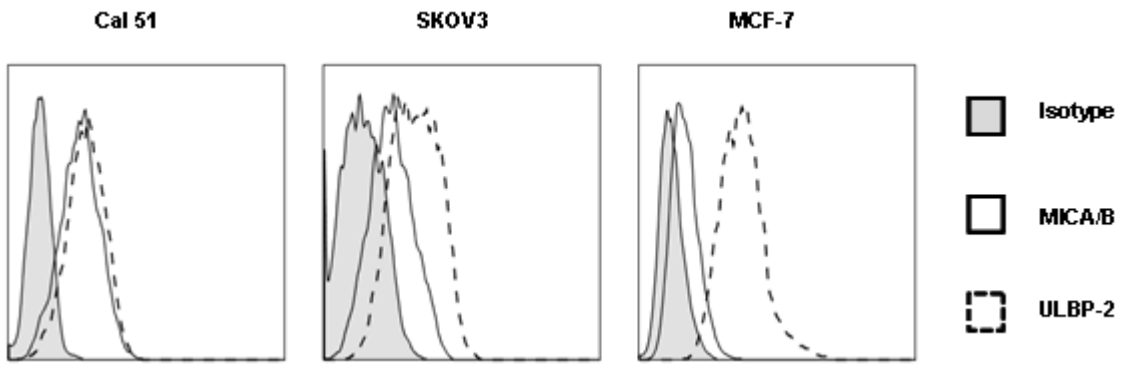

Figure 19: NKG2D ligand expression on selected tumor cell lines. Staining of selected carcinoma cell lines with isotype control (filled histograms), a mouse anti-human MICA/B antibody (open histograms solid line) as well as a mouse anti-human ULBP-2 antibody (open histograms dotted line).

Due to expression of NKG2D ligands, recognition of these cell lines by CAR-engineered NK cells likely involves the interaction of MICA/B and ULBP-2 with NKG2D in addition to CAR-mediated recognition of HER-2. Therefore, I investigated whether NKG2D interaction with its soluble ligands could prevent the HER-2 specific NK cell activation. For that purpose, a co-culture of these three cell lines together with mock- or CARz28-transduced NK cells was initiated, in presence or absence of soluble MICA. Alternatively, effector cells were pre-incubated overnight with plate-coated MICA before being further co-cultured with the three 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 soluble MICA on CARz28-NK function (Figure 20, 21, 22). In absence of recombinant human MICA, significantly higher levels of IFN-γ were produced by CARz28-NK cells, as compared to mock-NK cells, in response to stimulation by all HER2 positive cell lines with an average amount of 3000 pg/ml (Figure 20).

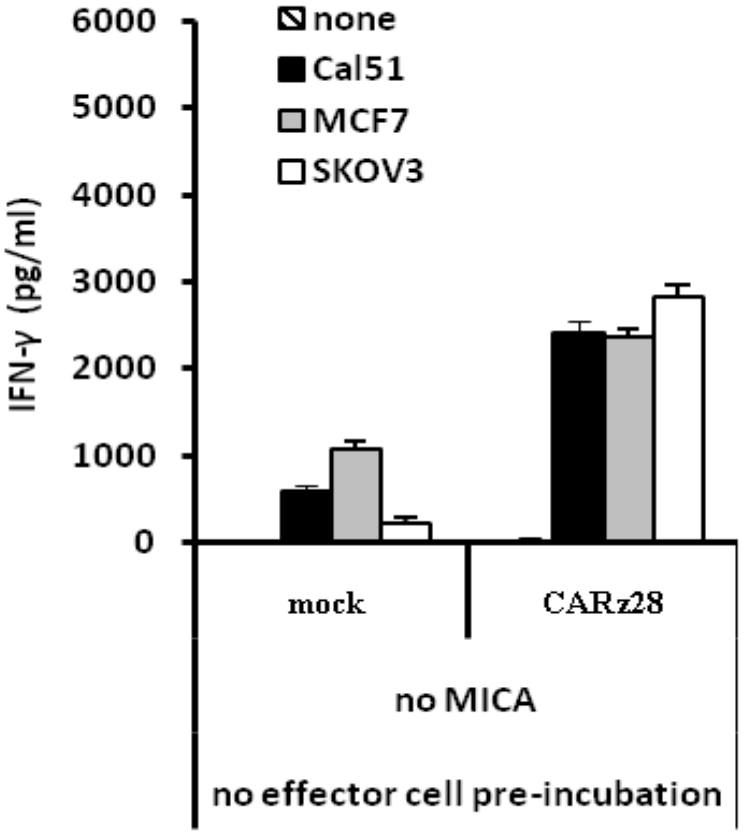

Figure 20: Blocking of NKG2D does not impair HER-2 specific recognition. Effector cells were either mock- or CAR-transduced NK cells cultured in medium alone. The effector cells were cultured alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Mock-NK cells produced some IFN-γ when co-cultured with Cal 51 and MCF-7 cells (500 – 1200 pg/ml), but no IFN-γ when co-cultured with SKOV3 cells. Interestingly, no considerable difference in IFN-γ levels was observed when MICA was present in these co-cultures (Figure 21).

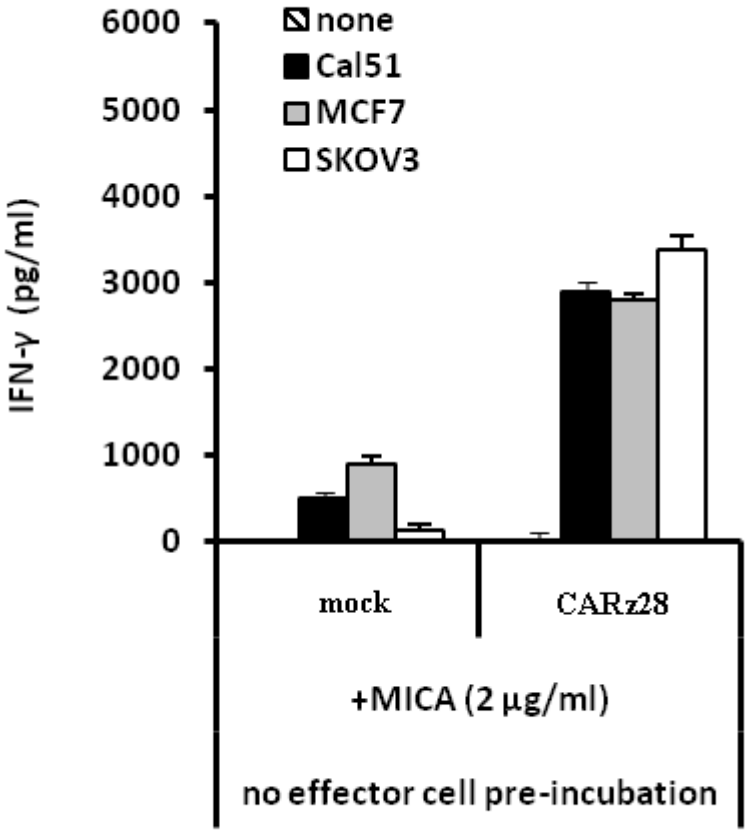

Figure 21: Blocking of NKG2D does not impair HER-2 specific recognition. Effector cells were either mock- or CAR-transduced NK cells cultured in the presence of recombinant MICA protein. The effector cells were cultured alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Higher amounts of IFN-γ were produced by both mock- and CARz28-NK cells that were previously cultured overnight in the presence of plate-bound recombinant MICA, indicating a pre-activation of the NK cells (Figure 22). In this setting, mock-NK cells and CARz28-NK cells produced a little amounts of IFN-γ when cultured alone (410 pg/ml and 220 pg/ml, respectively), but when co-cultured with the three target cell lines, mock-NK

cells produced markedly more IFN-γ (860 – 3000 pg/ml), however an even higher level of IFN-γ was produced by CARz28-engineered NK cells (4300 – 5000 pg/ml).

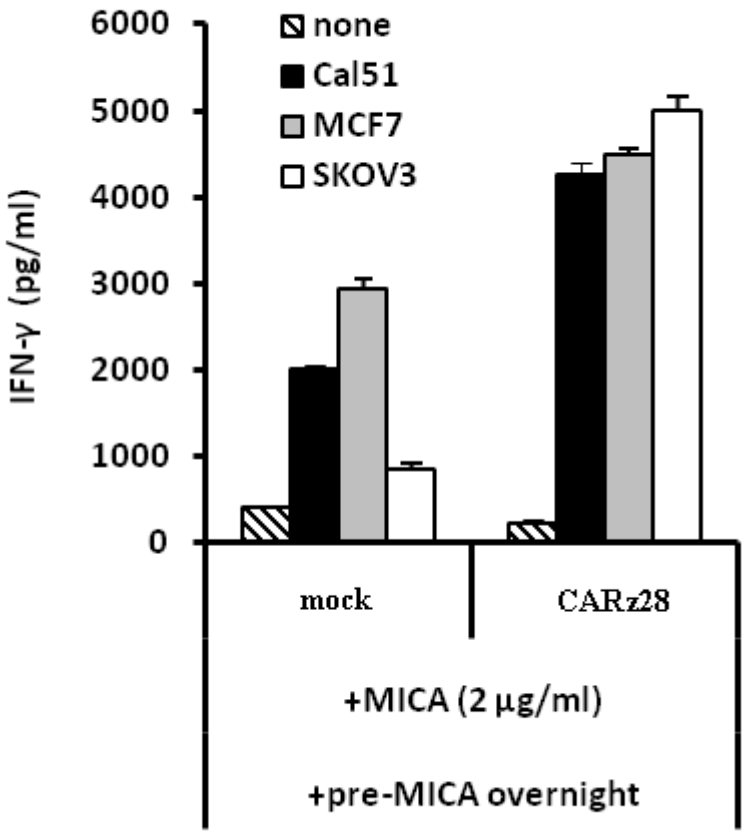

Figure 22: Blocking of NKG2D does not impair HER-2 specific recognition. Effector cells were either mock- or CAR-transduced NK cells cultured in the presence of recombinant MICA protein, and previously cultured overnight with plate-bound recombinant MICA protein (+ MICA pre-incubation). The effector cells were cultured alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

3.7 CAR-mediated recognition compares favorably to trastuzumab mediated NK cell activation.

NK cells also express CD16 (Fc receptors), which have been shown to be critical for anti-HER-2 monoclonal antibody (trastuzumab)-mediated therapy [76] . Therefore, I compared the two recognition systems, monoclonal antibody-based and CAR-based, for their ability to trigger NK cell responses toward HER-2 positive carcinoma cells.

Genetically engineered NK cells from five different donors were tested for the expression of functional CD16, the Fc receptor involved in ADCC. All tested NK cell lines from five donors expressed high levels of CD16 (Figure 23).

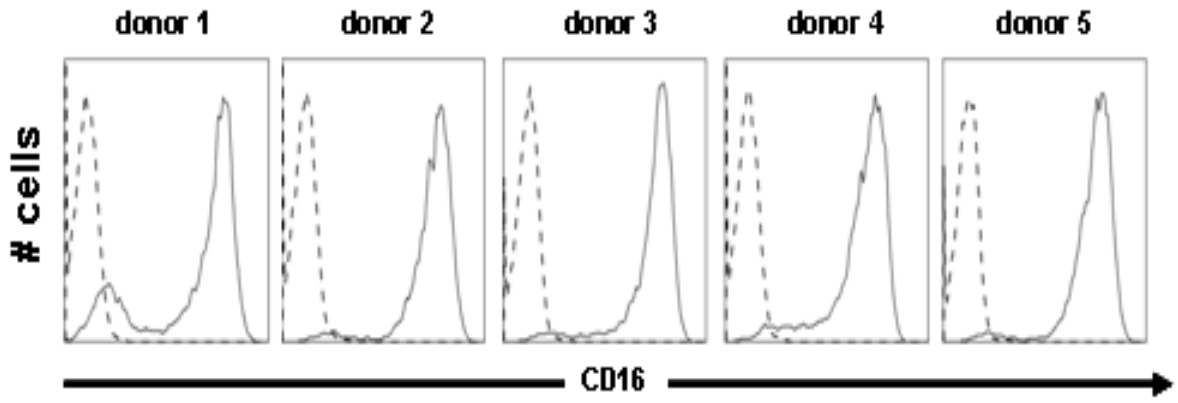

Figure 23: High levels of CD16 are expressed on genetically engineered NK cells. Mock-NK cells from five donors were stained for CD16 and analyzed by flow cytometry. Open histograms dotted line represent isotype control staining, open histograms solid line represent cells stained using a mouse anti-human CD16 antibody conjugated to PE.

Furthermore, to evaluate the functional expression of CD16, an overnight co-culture of mock transduced NK cells with p815 cells (expressing the murine Fc receptor) was initiated in the presence or absence of CD16. 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 (Figure 24). Mock transduced NK cells did not produce any detectable amount of IFN-γ when cultured alone or co-cultured with p815 cells (Figure 24). However, mock-NK cells co-cultured with p815 cells in the presence of 10 µg/ml of mouse anti-human CD16 antibody were activated and produced significant levels of IFN-γ (on average 1500 pg/ml; Figure 24).

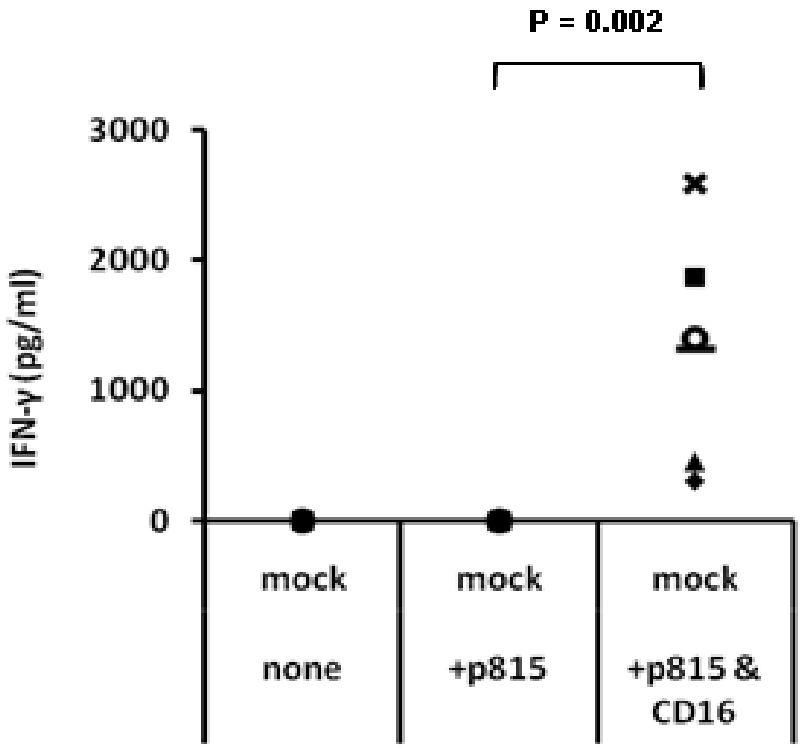

Figure 24: NK cells can be stimulated via CD16 to produce IFN-γ. Effector cells were mock transduced NK cells from five donors. They were either cultured alone or co-cultured with the equal number of p815 cells in the presence or absence of 10 µg/ml of a mouse anti human CD16 antibody. Supernatants were 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.

Next, I confirmed that trastuzumab, a monoclonal antibody specific for HER-2, would be able to activate mock-NK cells via CD16 to recognize the high HER-2 expressing SKOV3 cell line. Therefore, an overnight co-culture of mock-NK cells from two donors with SKOV3 cells in the absence or presence of trastuzumab was performed. Mock-NK cells co-cultured with SKOV3 cells in the absence or presence of the rituximab (an antibody specific for CD20) were used as controls. IFN-γ production was measured in supernatants using a specific ELISA (Figure 25). Mock-NK cells produced little IFN-γ (309 pg/ml and 941 pg/ml) when co-cultured with SKOV3 cells (Figure 25) alone or in the presence of 10 µg/ml rituximab (267 pg/ml IFN-γ and 846 pg/ml). However, if mock-NK cells were co-cultured 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; Figure 25).

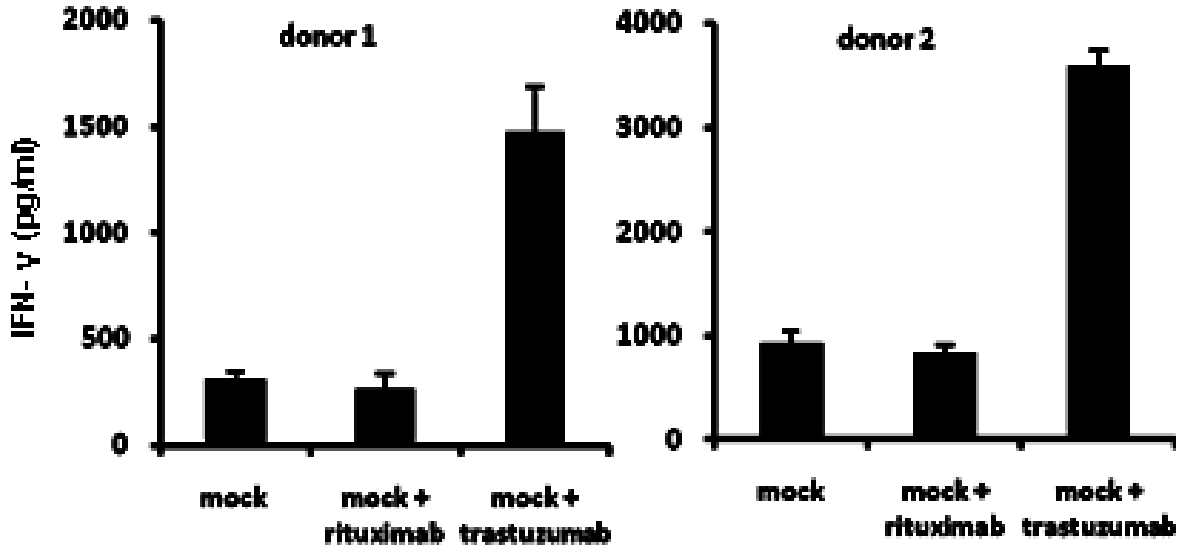

Figure 25: Trastuzumab activates mock-NK cells via CD16. Effector cells were mock transduced NK cells from two $CD16^+$ donors. They were co-cultured with the equal number of SKOV3 cells, which express high levels of HER-2, in the presence or absence of 10 µg/ml of either rituximab or trastuzumab. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Finally, a side-by-side comparison of the trastuzumab-targeted and CAR-targeted NK cells towards a cell line expressing lower level of HER-2 than SKOV3 was performed. Mock-NK cells from five donors were co-cultured with MCF-7 cells alone or presence of rituximab, trastuzumab and compared to CARz28-NK cells co-cultured with MCF-7 cells. In the control co-cultures of MCF-7 cells together with mock-NK cells in the absence or presence of rituximab, on average 1700 pg/ml IFN-γ were detected (Figure 26). No significant increase in IFN-γ production could be measured if the MCF-7 cells were co-cultured with mock-NK cells in the presence of trastuzumab (Figure 26). However, significantly higher levels of IFN-γ production (5700 pg/ml) were produced, when MCF-7 cells were co-cultured with CARz28-NK cells.

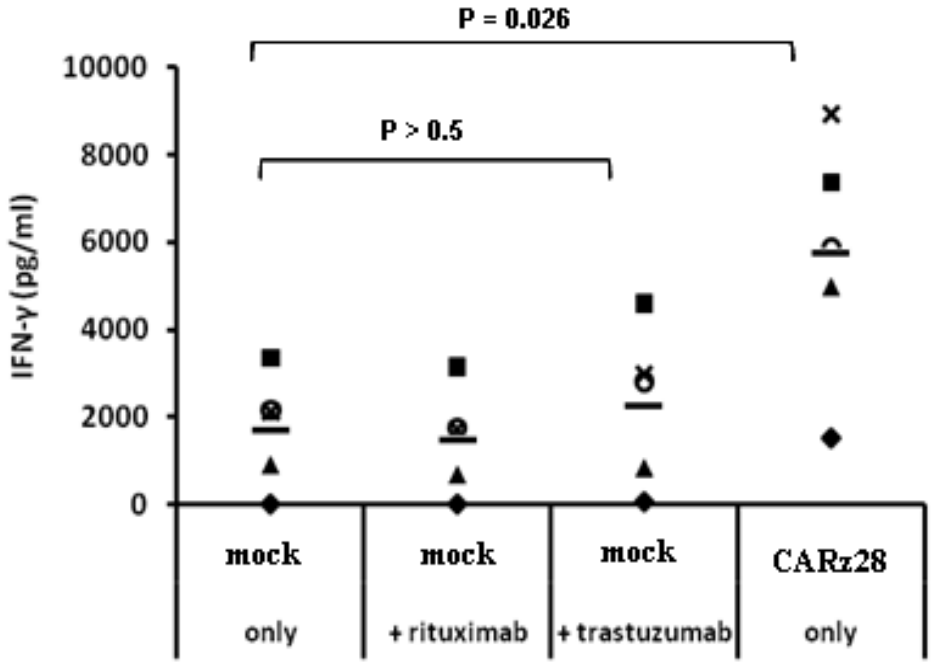

Figure 26: CARz28-NK cells recognize HER-2 low breast carcinomas that are not recognized by trastuzumab targeted NK. CAR-mediated NK recognition is superior to trastuzumab mediated recognition. Effector cells ($5x10^4$) were mock transduced or CARz28 transduced NK cells from five donors. They were co-cultured 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.

4 Discussion

4.1 Design of CAR-loaded human primary NK cells

Unlike transformed cells from hematopoietic origin which express several activation ligands (77, 52), cells derived from solid tumors are rather resistant to NK recognition. To improve solid tumor cell targeting and provide NK effector cells with an additional activation pathway, I have modified human primary NK cells by introducing a CAR which combines antigen specificity and NK cell activating properties in a single fusion molecule as described in [68]. Others have used a similar approach to target human NK cells towards leukemia or used transient transfection to target mouse NK cells toward solid tumors [67, 68, 78]. I analyzed the factors affecting NK cell engineering by retroviral transduction. A relatively high level of retroviral transduction efficiency is achieved when cells are at their maximal proliferative capacity [79]. The *in vitro* expansion of primary NK cells was regulated not only by soluble factors such as cytokines, but also by stimuli from activation molecules, such as those expressed on the B lymphoblastoid cell line RPMI 8866 used in this study as a feeder line [80]. Therefore, the optimal ratio of feeder cells to PBLs to achieve more optimal transduction efficacy reflects the availability of these two factors. During the process, IL-2 did not increase transduction efficacy, but increased cell viability most likely due to the fact that IL-2 was shown not to only induce NK cell proliferation, but also protected the NK cells from death by apoptosis [81]. My findings show that CAR-NK cells have the capacity to respond specifically to HER-2 expressing targets by IFN-γ and IL-2 production which are essential for tumor rejection and NK cell survival. Interestingly, the CARz- and the CARz28-engineered NK cells showed no significant difference in IFN-γ production, but the CARz-engineered NK cells produced much less IL-2 in comparison to the CARz28-engineered NK cells. This is the first description of such an effect on NK cells that can be attributed to CD28 signaling. A similar finding was reported for CAR transduced T cells [74, 36], suggesting the presence of a shared CD28-mediated signaling mechanism in both cell types.

4.2 Impact of NKG2D down-modulation on CAR-modified NK cells recognition

A few studies have pointed out that many tumors of epithelial origin secret soluble NKG2D ligands such as MICA, which can be detected in sera of certain tumor patients at significantly elevated levels. Soluble MICA was shown to down-modulate NKG2D expression on $CD8^+$ T cells and NK cells [76, 82], which might render the NK cells less responsive. To analyze the effect of soluble NKG2D ligands on the function of CARZ28-NK cell function, I used a commercially available recombinant MICA and tested its effect on the recognition of HER-2 carcinomas by these cells. Surprisingly, although MICA binding to NKG2D on NK cells was confirmed by flow cytometry, this binding did not down-modulate the receptor even when used at a high concentration. Furthermore, I did not observe any blocking of the CAR-mediated recognition of the HER-2 positive carcinoma cell lines, expressing NKG2D ligands, in the presence of soluble recombinant MICA. On the contrary, I observed a slightly better recognition of the tumor cell lines by both mock- and CARz28-engineered NK cells, when these cells were pre-incubated with plate-bound recombinant MICA over night. Thus, the exposure to plate-bound MICA, unlike what has been described for T cells [76], had a pre-activating effect, because both NK cell populations produced low but detectable amount of IFN-γ in the absence of any target cells. Furthermore, upon co-culture with the three target cell lines, NK cells pre-incubated with plate-bound MICA produced higher levels of IFN-γ than those observed in the two other conditions employed, without prior pre-incubation with plate-bound MICA. Therefore, the NK cells used in this study are resistant to the negative effect of the soluble NKG2D ligand MICA. This resistance might be due to the fact that the NK cells used in this study were maintained in the presence of IL-2 [82].

4.3 Targeting efficacy of CAR-modified NK cells and trastuzumab

Comparing the CAR-NK approach to trastuzumab or other monoclonal antibody-based strategies for targeting NK cells to HER-2 positive carcinomas is important. This is because if CAR-NK targeting does not compare favorably, the use of monoclonal antibody in any therapy protocol will be advantageous based on their ease of use and administration. Therefore, in addition to the observed resistance of CAR-NK cells to soluble NKG2D ligand inhibition, I evaluated their ability to recognize a representative trastuzumab-resistant target. Only tumor cells that have gene amplified HER-2, accounting for one third of the HER-2 positive tumors [59], represent good targets for trastuzumab treatments, an effect that is perfectly mirrored in *in vitro* studies [83]. The MCF-7 breast carcinoma cell line was described to belong to the group of breast carcinoma that express low but detectable levels HER-2 as judged by flow cytometry. This cell line has no HER-2 amplification and is being used by the US Food and Drug Administration approved immunohistochemistry-based Pathway® kit as a level 0 standard control cell line to exclude patients from trastuzumab therapy [70, 84]. Therefore, I compared the recognition of this line by trastuzumab- and CAR-targeted NK cells. I fist confirmed that genetically engineered NK cells can still be activated via CD16 to produce IFN-γ and that trastuzumab can mediate recognition of the high HER-2 expressing cell line SKOV3 by mock-NK cells. Interestingly, while confirming published observations [69, 85] that MCF-7 did not activate mock-NK cells in the presence of trastuzumab, I was able to show that CAR-NK cells, from five different donors, were significantly activated by this breast carcinoma. In an earlier study, it has been reported that CAR-NK cells can target breast carcinoma that express very low levels of HER-2, which might be useful for targeting cells displaying the breast cancer stem cell phenotype [68, 86].

The use of soluble monoclonal antibodies in cancer therapy has some drawbacks. This was revealed by knock-out studies demonstrating a detrimental effect of the inhibitory FcγRIIb receptor, since its deletion significantly improved the antitumor response to trastuzumab therapy [77]. Moreover, polymorphisms in Fc receptors have a very significant impact on

the ADCC levels, and can predict clinical outcomes of mAb-based therapies. Patients with NK cells bearing an FcγRIIIa with a 158 V/V genotype had a significantly higher response to trastuzumab therapy than the F-carriers patients with a 158 F/V or 158 F/F genotype [64]. Since only 15 - 20 % of the population have the homozygous 158 V/V genotype [64, 87, 88], these studies indicate that most of the treated patients will only moderately benefit from a trastuzumab therapy. In clinical trials using trastuzumab, manageable cardiotoxicity was reported in patients, especially in combination with chemotherapy [89, 90]. Furthermore, a recent case report suggested that pulmonary toxicity and cytokine storm have led to a patient death due to multiple organ failure [91]. The authors suggested that a reduced dose therapy should be evaluated when such a therapy will be tested in the future.

4.4 CAR-modified NK cells provide a promising approach to treat established tumors

The ability to genetically modify primary NK cells, apart from providing an opportunity for further studies of NK cell biology, can represent an effective alternative or a complement to the currently used approaches in cancer immunotherapy. In our group, we have recently used a myc-tagged T cell receptor introduction to genetically modify T cells, which enabled their specific depletion, and prevented the development of a fatal autoimmunity [92]. Such a safety modality could also be introduced into the CAR to eliminate CAR-engineered NK cells *in vivo,* if they become autoreactive or show other adverse effects which might occur upon genetic modification [93]. Altogether, the risk of HER-2 specific autoimmunity needs to be further addressed in a suitable *in vivo* model.

The possibility of using CAR-modified NK cells for the immunotherapy of cancer is an attractive approach. Studies have shown that 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 [94, 95]. The data from this study suggests that primary human NK cells can be efficiently modified to express CARs and that the use of second generation CARs are distinguished from trastuzumab targeting, by being very effective in targeting HER-2 positive carcinomas even those expressing low levels of HER-2. Furthermore, by using CAR signaling domains that are activated independently of the patient's given FcγR genotype, and are not negatively affected by the presence of soluble NKG2D ligand MICA, there is a greater likelihood of efficacious results for the treatment and eradication of established tumors.

5 Summary

Natural killer (NK) cells express an array of activating cell surface receptors that can trigger cytolytic programs, as well as cytokine or chemokine secretion, which suggesting them as promising effectors for tumor adoptive therapy.

I identified the optimal conditions for expanding and transducing human primary NK cells. Next, I engineered NK cells with chimeric antigen receptors (CARs) specific for HER-2, a tumor-associated antigen frequently overexpressed by many tumors of epithelial origin, providing them with tumor-antigen targeting specificity. I compared first and second generation CARs including a stimulatory (CD3ζ) signaling domain alone or together with a co-stimulatory (CD28) signaling domain. I found that co-stimulatory signaling significantly improved IL-2 production by NK cells. Blocking of NKG2D by its soluble ligand, which is known to reduce NK cell responsiveness, did not impact CAR-mediated HER-2 specific recognition by these cells. Comparing CAR targeting of NK cells to soluble antibody (trastuzumab) targeting revealed that breast carcinoma without HER-2 gene amplification can be uniquely targeted by CAR-modified NK cells. Thus, the direct coupling of the antibody specificity to NK cells functions mediated by CAR expression resulted in superior NK cell targeting via CD16 binding of soluble monoclonal antibodies.

6 Deutschsprachige Zusammenfassung

Natürliche Killerzellen (NK-Zellen) exprimieren eine Vielzahl aktivierender Oberflächenrezeptoren, die sowohl zytolytische Prozesse, als auch die Sekretion von Cytokinen und Chemokinen auslösen können. Diese Eigenschaften lassen NK-Zellen als aussichtsreiche Effektoren der adoptiven Tumortherapie erscheinen.

Im Verlauf meiner Arbeit optimierte ich die Bedingungen zu Kultivierung, Expansion und Transduktion primärer humaner NK-Zellen. Im Folgenden konstruierte ich NK-Zellen mit HER-2-spezifischen chimären Antigen-Rezeptoren (CARs). HER-2 ist ein tumor-assoziertes Antigen, welches in vielen Tumoren epithelialen Ursprungs überexprimiert wird und diese dadurch mit einer Targeting-Spezifizität gegen Tumor-Antigen ausstattet. Beim Vergleich der ersten und zweiten CAR-Generation, die entweder lediglich eine CD3ζ-signalstimulierende Domäne oder zusätzlich eine kostimulierende CD28-Domäne enthielten, fand ich eine signifikante Erhöhung der IL-2-Produktion in NK-Zellen durch die Kostimulation. Die Blockierung von NKG2D durch seine löslichen Liganden, die üblicherweise zu einer Reduktion der Ansprechempfindlichkeit der NK-Zellen führt, hatte keinen Einfluss auf die CAR-vermittelte HER-2-spezifische Erkennung dieser Zellen. Der Vergleich zwischen CAR-Targeting von NK-Zellen und löslichen Antikörpern (Trastuzumab) zeigte, dass Brustkarzinome ohne HER-2-Genamplifikation durch CAR-modifizierte NK-Zellen angesprochen werden können. Die direkte Kopplung der Antikörper-Spezifität mit der Funktion der NK-Zellen durch die Expression von CARs resultierte in überlegenem NK-Zell-Targeting mittels der Bindung gelöster monoklonaler Antikörper durch CD16.

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8 Appendix

8.1 Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
AICD	Activation induced cell death
ALL	Acute lymphoblastoid leukemia
APC	Antigen presenting cell
APM	Antigen presenting molecule
BCL	B cell lymphoma
CAR	Chimeric antigen receptor
CARz	Chimeric antigen receptor fused with CD3ζ
CARz28	Chimeric antigen receptor fused with CD3ζ and CD28
$CD8^+$ T cell	Effector T cell
CIR	Chimeric immune receptor
CLIP	Class II-associated invariant chain
CMV	Murine stem cell virus
CR	Chimeric receptor
CTL	cytotoxic T lymphocyte
DC	Denteric cell
DN	Double negative
DM	Class II-like molecule
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay

ER	Endoplasmic reticulum
Fab	Antigen-binding regions
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
Fc	Antibody effector regions
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HER-2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL-2	Interleukine 2
IRES	Internal ribosome entry site
IU/ml	international units per ml
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer immunoglobulin-like receptor
LCL	Lymphoblastoid cell line
LTR	long terminal repeat
mAb	monoclonal antibody
MHC	Major histo compatibility complex

MICA	MHC class I-related chain A
MSCV	Murine stem cell virus
MØ	Macrophage
NCR	Natural killer cell receptor
NK cell	Natural killer cell
PBLs	peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PSI	Packaging signal
rpm	rounds per minute
RT	Room temperature
ScFv	Single chain variable fragment
SDS	Sodium Dodecylsulphate
TAA	Tumor associated antigen
TAP	Transporter associated with antigen presentation
TB	Trypan blue
TCR	T cell receptor
Th cells	Helper T cells
TLR	Toll-like receptor
TNF	Tumor necrotic factor
VEGF	Vascular epidermal growth factor

8.2 List of Figures and Tables

(CD3ζ chains, violett) and a signaling domain of CD28 (blue) (second generation). First generation-CARs miss the CD28 signaling domain. *Adapted from reference* [19].

Page 29 - Figure 7: Recognition mechanism of NK cells.
NK cell killing depends on the balance between activating and inhibiting (MHC class I) signals (upper panel). Upon activation, NK cells release cytokines and granule contents which induce apoptosis in the target cell (lower panel).

Page 34 - Figure 8: pMIG vector map.
pMIG encodes the retroviral packaging signal (PSI), the green fluorescence protein (GFP) and an internal ribosome entry site (IRES), flanked by 3' and 5' long terminal repeats (LTRs). The backbone contains an ampicillin resistance (AMPr) gene and the origin of replication (ORI).

Page 35 - Figure 9: **A schematic representation of the two CAR constructs used in this study.**
The chimeric receptor constructs were cloned into pMIG replacing IRES and GFP. The resulting constructs were designated pMSCV-CARz and pMSCV-CARz28. scFv, single-chain fragment variable; hIgG1-Fc, human IgG1 crystallizable fragment.

Page 51 - Figure 10: PBL to feeder cell ratio of 5:1 leads to the highest NK expansion and transduction efficacy.
PBLs were cultured in described ratios with the irradiated feeder cell line RPMI 8866 and transduced 3 times with pMIG at 3 successive days. The cells were then stained with a mouse anti-human CD3 antibody conjugated to PE-Cy-5 and a mouse anti-human CD56 antibody conjugated to APC (left panels) to determine the percentage of $CD3^{-}CD56^{+}$ NK cells. Cells were then gated on the $CD56^{+}$ population (middle panels) and analyzed for GFP and CD3 expression (right panels) by flow cytometry.

Page 52 - Figure 11: IL-2 addition during transduction increases cell viability, but not transduction efficacy.
PBLs were transduced 3 times with pMIG either without IL-2 (upper panel) or with 200 IU/ml IL-2 (lower panel) at three successive days. Cells were analyzed for GFP expression and for viability, evaluated by forward and sideward light scattering, by flow cytometry.

Page 53 - Figure 12: Improved transduction efficacy is achieved when transduction is performed on day 6 as compared to day 8 of co-culture initiation.
PBLs were transduced 1, 2 or 3 times with pMIG starting on day 6 (upper panel) or day 8

(lower panel) within three successive days. On day 12, cells were analyzed for GFP expression by flow cytometry.

Page 54 - Figure 13: Expression of the transduced CARs on primary human NK cells. PBLs were transduced with pMIG (mock), pMSCV-CARz and pMSCV-CARz28. On day 12, cells were analyzed for the expression of the respective CAR by flow cytometry. Cells were stained with a mouse anti-human CD56 antibody conjugated to APC, a mouse anti-human CD3 antibody conjugated to FITC and a goat anti-human Ig antibody conjugated to PE recognizing the CAR. Cells were gated on the $CD56^+$ population and were then analyzed for the expression of CAR and CD3.

Page 55 - Figure 14: High purity of NK cell lines after CD3 depletion.
PBLs were transduced with either the mock or the CAR constructs and were subsequently depleted using magnetic beads conjugated to mouse anti-human CD3 antibodies. Staining with a mouse anti-human CD3 antibody conjugated to PE-Cy-5 of 3 representative donors transduced with the mock- or CARz28 plasmid which were either undepleted (left panels) or from day 6 after depletion (right panels) is depicted. The depletion of CARz-transduced NK cells resulted in similar NK cell purity (data not shown).

Page 57 - Figure 15: CARz and CARz28-engineered NK cells produce similar levels of IFN-γ upon HER-2 recognition.
IFN-γ was measured in the supernatants of stimulated NK cells. Effector cells were mock-, CARz- or CARz28-transduced NK cells normalized to comprise the same percentage (40 %) of CAR^+ cells. They were either cultured without target cells (none) or co-cultured with C1R/A2 cells, C1R/A2HER2 cells, or SKOV3 cells. Values shown represent mean values of triplicates obtained from IFN-γ specific ELISA.

Page 58 - Figure 16: CARz28-NK produces more IL-2 than CARz upon HER-2 recognition.
IL-2 was measured in the supernatants of stimulated NK cells. Effector cells were mock-, CARz- or CARz28-transduced NK cells normalized to comprise the same percentage (40 %) of CAR+ cells. They were either cultured without target cells (none) or co-cultured with C1R/A2 cells, C1R/A2HER2 cells, or SKOV3 cells. Values shown represent mean values of triplicates obtained from IL-2 specific ELISA.

Page 59 - Figure 17: (A) **MICA binds to NKG2D on NK cells**.
NK cells were cultured in the presence or absence of 1µg/ml of the recombinant MICA protein 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. (B) **No NKG2D down-modulation after overnight exposure to MICA**. Mock- and CAR-NK cells were incubated over night either with or without of the recombinant MICA protein 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 of cells cultured overnight with (solid line) or without no soluble MICA (dotted line).

Page 60 - Figure 18: **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.

Page 61 - Figure 19: NKG2D ligand expression on selected tumor cell lines.
Staining of selected carcinoma cell lines with isotype control (filled histograms), a mouse anti-human MICA/B antibody (open histograms solid line) as well as a mouse anti-human ULBP-2 antibody (open histograms dotted line).

Page 62 - Figure 20: Blocking of NKG2D does not impair HER-2 specific recognition.
Effector cells were either mock- or CAR-transduced NK cells cultured in medium alone. The effector cells were cultured alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Page 63 - Figure 21: Blocking of NKG2D does not impair HER-2 specific recognition.
Effector cells were either mock- or CAR-transduced NK cells cultured in the presence of recombinant MICA protein. The effector cells were cultured alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Page 64 - Figure 22: Blocking of NKG2D does not impair HER-2 specific recognition.
Effector cells were either mock- or CAR-transduced NK cells cultured in the presence of recombinant MICA protein, and previously cultured overnight with plate-bound recombinant MICA protein (+ MICA pre-incubation). The effector cells were cultured

alone (none) or co-cultured with the indicated carcinoma cell line. Supernatants were harvested and measured for IFN-γ using specific ELISA.

Page 65 - Figure 23: High levels of CD16 are expressed on genetically engineered NK cells.
Mock-NK cells from five donors were stained for CD16 and analyzed by flow cytometry. Open histograms dotted line represent isotype control staining, open histograms solid line represent cells stained using a mouse anti-human CD16 antibody conjugated to PE.

Page 66 - Figure 24: NK cells can be stimulated via CD16 to produce IFN-γ.
Effector cells were mock transduced NK cells from five donors. They were either cultured alone or co-cultured with the equal 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.

Page 67 - Figure 25: Trastuzumab activates mock-NK cells via CD16.
Effector cells were mock transduced NK cells from two $CD16^+$ donors. They were co-cultured with the equal 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.

Page 68 - Figure 26: CARz28-NK cells recognize HER-2 low breast carcinomas that are not recognized by trastuzumab targeted NK.
CAR-mediated NK recognition is superior to trastuzumab mediated recognition. Effector cells ($5x10^4$) were mock transduced or CARz28 transduced NK cells from five donors. They were co-cultured 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.

Page 19 - Table 1. Characteristics of selected cell surface molecules (markers)

Page 21 - Table 2. Characteristics of selected cytokines

Page 22 - Table 3. Characteristics of selected cytolytic molecules

Publication

Buschow C, Charo J, Anders K, Loddenkemper C, Jukica A, **Alsamah W**, Perez C, Willimsky G, Blankenstein T, *In vivo imaging of an inducible oncogenic tumor antigen visualizes tumor progression and predicts CTL tolerance*.Journal of Immunology, 2010. 184(6):2930-8.

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