Chimeric antigen receptor (CAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Stefan Grote, M.Sc.
aus Dortmund

Tübingen
2021

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	21.06.2022
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	Prof. Dr. Alexander Weber
2. Berichterstatter:	Prof. Dr. Rupert Handgretinger

TABLE OF CONTENTS

Table of contents

ABBREVIATIONS

AdCAR	Adapter chimeric antigen receptor
ADCC	Antibody-dependent cellular cytotoxicity
AJCC	American Joint Committee on Cancer
ALL	Acute lymphocytic leukemia
AM	Adapter molecule
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
bAb	Biotinylated antibody
AM	Adapter molecule
BCMA	B cell maturation antigen
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CRS	Cytokine release syndrome
CT	Computer tomography
DLBCL	Diffuse large B cell lymphoma
ECM	Extracellular matrix
EMA	European Medicines Agency
E:T ratio	Effector-to-target ratio
FasL	Fas ligand
Fc	Fragment crystallizable
FDA	American Food and Drug administration
GFP	Green fluorescent protein
GvHD	Graft-versus-host disease
HCT	High-dose chemotherapy
HD	Hodgkin’s disease
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase

Abbreviations

IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
iPSC	Induced pluripotent stem cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin-like receptor
KO	Knock-out
Lc	Long chain
LLE	Linker-label-epitope
mAb	Monoclonal antibody
MCL	Mantle cell lymphoma
MDSC	Myeloid-derived suppressor cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
NB	Neuroblastoma
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NO	Nitric oxide
ON	Over night
PBMC	Peripheral blood mononuclear cell
PDX	Patient-derived xenograft
PE	Phycoerythrin
PRR	Pattern-recognition receptor
RTCA	Real-time cell analysis
scFv	Single-chain variable fragment
SM	Small molecule
TAA	Tumor-associated antigen
TCR	T cell receptor
TGF	Transforming growth factor
TIL	Tumor infiltrating lymphocytes
TM	Transmembrane

Abbreviations

TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
TSA	Tumor-specific antigen
UCB	Umbilical cord blood
V_H	Heavy chain variable domain
V_L	Light chain variable domain

1. ABSTRACT

Despite the tremendous clinical success of chimeric antigen receptor (CAR)-expressing T cells, severe side effects have been associated with CAR T cell therapy. Moreover, production for individual patients still requires an extensive and time-consuming manufacturing process, which presses the need for clinical alternatives. The continuously expandable and well-established natural killer (NK) cell line NK-92 might represent such alternative. Irradiated NK-92 cells provide a safe and consistent way to produce NK effector cells in a GMP-compliant and cost-effective way. Furthermore, NK-92 can be redirected by CARs to mediate antigen specific tumor lysis.

We recently developed the universal adapter CAR (AdCAR) system. Utilizing adapter molecules (AM), antigen recognition and CAR-driven immune cell activation were split into a two-step process. The system allows precise quantitative (on-/off-switch) as well as qualitative (change and combination of target antigens) regulation of immune cell function. AdCARs are based on the unique properties of a novel scFv targeting a “neo”-epitope-like structure derived from the endogenous vitamin biotin. Biotinylation of antibodies, which are already approved by the American Food and Drug administration (FDA) and European Medicines Agency (EMA), such as cetuximab or trastuzumab, generates functional AM with a known safety profile for AdCAR NK-92 therapy and facilitates translation into clinical settings.

The primary goal of this thesis was the development of a highly flexible CAR system for universal tumor targeting to overcome the existing limitations of CAR therapy, especially for solid tumors. We used lentiviral vectors for the transduction process and single-cell sorted successfully transduced AdCAR NK-92 cells using flow cytometry. The clone with the highest CAR surface expression and highest viability was chosen for further experiments. We thoroughly characterized AdCAR NK-92 cells for their NK receptor expression as well as for the expression of immune checkpoints and chemokine receptors. For functional assessment we utilized standard calcein release cytotoxicity assays as well as impedance-based real-time live cell analysis technology. AdCAR NK-92 cells successfully induced significant lysis of various hematological malignancies such as lymphoma and leukemia as well as solid tumors such as melanoma and various carcinomas *in vitro* but only in the presence of biotinylated antibodies (bAb) targeting antigens that are sufficiently expressed on the tumor cell surface. Therefore, classic tumor antigen evasion mechanisms such as antigen downregulation can be counteracted by simultaneous or successive addition of different bAb. Specific AdCAR-mediated

cytotoxicity could be demonstrated regardless of tumor cell expression of inhibitory ligands. To bridge the gap between *in vitro* and *in vivo* assessment of AdCAR NK-92 cell function, we generated three-dimensional tumor cell models that are physiologically more relevant and predictive than standard two-dimensional assays. AdCAR NK-92 cells were capable of successful tumor spheroid infiltration and specific long-term tumor cell lysis.

Utilizing CAR-modified NK-92 cells targeting the immune checkpoint molecule B7-H3 (CD276), a tumor-associated antigen severely overexpressed on a variety of solid cancers, we assessed the ability of CAR NK cell-based immunotherapy to overcome a variety of the obstacles exerted by the immunosuppressive tumor microenvironment (TME). Moreover, we used CRISPR/Cas9 technology to generate CAR NK-92 cells with a NKG2A (CD159a) knock-out (KO) and assessed whether we could boost CAR-mediated effector function by eliminating a highly expressed inhibitory NK receptor. The CRISPR-mediated NKG2A KO as well as blocking the NKG2A receptor with an inhibitory antibody did not significantly boost CAR-mediated cytotoxic potential of CD276-CAR NK-92 cells suggesting that CAR-induced tumor lysis is independent of the expression of inhibitory receptors on NK-92 cells.

In conclusion, we generated a universal CAR-engineered and an antigen-specific CAR NK-92 cell line. These cells can be manufactured as an “off-the-shelf, on-demand” standardized product whose powerful effector function can be tightly regulated for tunable, patient-individualized targeting of hematological and solid cancers.

2. ZUSAMMENFASSUNG

Die Immuntherapie und insbesondere chimäre Antigenrezeptoren haben in den letzten Jahren beeindruckende Erfolge verzeichnen können. Trotz der Zulassung mehrerer autologer CAR T-Zell Therapien für die Behandlung von hämatologischen Krebserkrankungen ist es wichtig, dass weitere therapeutische Alternativen erforscht werden, um die noch vorhandenen Problematiken der teils schweren Nebenwirkungen oder des zeit- und kostenintensiven Produktionsprozesses zu beheben. Die gut charakterisierte und von der FDA bereits klinisch zugelassene NK-92 Zelllinie stellt eine vielversprechende Option dar, funktionelle, CAR-modifizierte Immunzellen kostengünstig und nach strengen Qualitätsmanagement-Vorgaben zu generieren. Diese können dann eingesetzt werden, um serienmäßig spezifische Immuntherapie von hämatologischen und soliden Tumoren durchzuführen.

Mit dem im Rahmen der vorliegenden Dissertation generierten und validierten Adapter CAR System ist es möglich, durch den Einsatz verschiedener biotinylierter Adaptermoleküle in einem Zwei-Stufen-Prozess antigenspezifische Immunzellaktivität zu induzieren. Das System ermöglicht dabei die quantitative sowie qualitative Feinregulierung der ausgelösten Immunantwort. Das AdCAR erkennt spezifisch ein Neoepitop, das bei der Biotinylierung von Adaptermolekülen entsteht. Dabei können sowohl monoklonale Antikörper, als auch Antikörperfragmente oder andere kleine Moleküle als Adapter fungieren. Dies ermöglicht auch die Nutzung bereits klinisch zugelassener Antikörper, die schnell und kostengünstig biotinyliert werden können, um so mit patientenindividualisierter Therapie auf tumorspezifische Mechanismen zur Immunevasion zu reagieren.

Das Primärziel dieser Doktorarbeit bestand in der Entwicklung, Generierung, Charakterisierung und funktionellen Testung von AdCAR NK-92 Zellen. Mittels lentiviraler Transduktionssysteme wurden NK-92 Zellen mit dem AdCAR ausgestattet und anschließend mit Hilfe durchflusszytometrischer Verfahren separiert, um Einzelzellklone zu generieren, die eine besonders hohe CAR Expression aufweisen. Die Population mit der höchsten Expression und der höchsten Viabilität wurde im Folgenden ausführlich charakterisiert und dann funktionell in verschiedenen *in vitro* Untersuchungen getestet. Dabei konnte sowohl gegen hämatologische Erkrankungen, wie Lymphome oder Leukämien, als auch gegen verschiedenste solide Tumorentitäten eine signifikant erhöhte und AdCAR-spezifische Tumorlyse gemessen werden. Die Intensität der zytotoxischen Reaktion war dabei proportional zur Antigenexpression auf den Tumorzellen. Zudem

wurde ein neues drei-dimensionales Tumormodell etabliert, welches die Effektivität von AdCAR NK-92 Zellen auch in einem Szenario zeigen konnte, das einer potentiellen *in vivo* Situation mehr ähnelt als klassische zwei-dimensionale Modelle. Darüber hinaus konnte sowohl in 2D als auch in 3D Modellen gezeigt werden, dass die spezifische AdCAR NK-92 Aktivität bei verschiedenen Tumorentitäten ein Nachwachsen der Tumorzellen verhindert.

Weiterhin wurde ein direktes CAR Konstrukt entwickelt und in NK-92 exprimiert, das den Immuncheckpoint CD276 erkennt. Dieses Oberflächenprotein ist auch auf Normalzellen zu finden, wird aber von Krebszellen hochreguliert, da es die T-Zell-induzierte Immunantwort mindert. Die CD276-CAR NK-92 Zellen wurden benutzt, um den Effekt von verschiedenen Faktoren der Umgebung solider Tumoren, das TME, zu untersuchen. Dabei konnte gezeigt werden, dass, durch die Nutzung von NK-92 Zellen als CAR-Vehikel im Vergleich zu primären Immunzellen, viele der immunsuppressiven Faktoren keine Auswirkungen auf die CAR-induzierte Immunantwort haben.

Beim Versuch, die CAR NK-92 Funktionalität durch den CRISPR/Cas9-vermittelten KO des inhibitorischen NK Zell Rezeptors NKG2A noch weiter zu erhöhen, konnte gezeigt werden, dass die Höhe der CAR-spezifischen Tumorlyse unabhängig von der Expression inhibitorischer und aktivierender Rezeptoren der NK-92 Zellen sowie den entsprechenden Liganden auf den Tumorzelloberfläche ist.

Zusammenfassend wurden für diese Arbeit mehrere CAR NK-92 Zelllinien hergestellt und erfolgreich funktionell charakterisiert. CAR NK-92 Zellen bieten viele Vorteile gegenüber der klassischen CAR T-Zell Therapie und könnten in der Zukunft potentiell als universelle und kostengünstige Immuntherapie für die Behandlung verschiedenster Tumorerkrankungen genutzt werden.

3. EMBEDDED PUBLICATIONS AND MANUSCRIPTS

Publication 1:

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting

Stefan Grote, Joerg Mittelstaet, Caroline Baden, Kenneth Chun-Ho Chan, Christian Seitz, Patrick Schlegel, Andrew Kaiser, Rupert Handgretinger, Sabine Schleicher

OncoImmunology **9**, 1825177 (2020), doi:10.1080/2162402X.2020.1825177, PMID: 33457105

Publication 2:

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells for the multiplex targeting of bone metastases

Stefan Grote, Frank Traub, Joerg Mittelstaet, Christian Seitz, Andrew Kaiser, Rupert Handgretinger, Sabine Schleicher

Cancers **13**, 1124 (2021), doi:10.3390/cancers13051124, PMID: 33807875

Publication 3:

CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma

Stefan Grote, Kenneth Chun-Ho Chan, Caroline Baden, Hans Bösmüller, Mihály Sulyok, Leonie Frauenfeld, Martin Ebinger, Rupert Handgretinger, Sabine Schleicher

Adv Cell Gene Ther **4**, 15 (2021), doi:10.1002/acg2.105

Publication 4:

***In vitro* Evaluation of CD276-CAR NK-92 Functionality, Migration and Invasion Potential in the Presence of Immune Inhibitory Factors of the Tumor Microenvironment**

Stefan Grote, Guillermo Ureña-Bailén, Kenneth Chun-Ho Chan, Caroline Baden, Markus Mezger, Rupert Handgretinger, Sabine Schleicher

Cells **10**, 1020 (2021), doi:10.3390/cells10051020, PMID: 33925968

4. PERSONAL CONTRIBUTION

Publication 1:

I was involved in the generation of AdCAR-engineered NK-92 cells. All experiments regarding characterization of AdCAR NK-92 cells as well as lymphoma cell lines and primary lymphoma cells were performed by me. Furthermore, I was directly involved in all experiments assessing cytotoxic effector function of AdCAR NK-92 cells. I was involved in the analysis and interpretation of the data as well as the preparation of the manuscript.

Scientific ideas

Experimental performance

Collection and assembly of data

Data analysis and interpretation

Manuscript writing

Publication 2:

I was involved in the generation and characterization of all newly developed bone metastatic cell lines. I conducted all experiments regarding functional analysis of the AdCAR NK-92 cell line. Moreover, I analyzed and interpreted the data and was involved in the preparation of the manuscript.

Scientific ideas

Experimental performance

Collection and assembly of data

Data analysis and interpretation

Manuscript writing

Personal contribution

Publication 3:

I was involved in the generation of CD276-CAR NK-92 cells. All experiments regarding characterization of CD276-CAR NK-92 cells as well as neuroblastoma cell lines were performed by me. Furthermore, I was directly involved in all experiments assessing cytotoxic effector function of CD276-CAR NK-92 cells. I was involved in the analysis and interpretation of the data as well as the preparation of the manuscript.

Scientific ideas

Experimental performance

Collection and assembly of data

Data analysis and interpretation

Manuscript writing

Publication 4:

I was involved in characterization of melanoma cell lines and I personally conducted all experiments regarding functional analysis of CD276-CAR NK-92 cells. Moreover, I assessed NK-92 cell migration and tumor infiltration. I was involved in the generation of CRISPR-mediated KO of the NKG2A gene in CD276-CAR NK-92 cells. Furthermore, I was involved in the analysis and interpretation of the data and in the preparation of the manuscript.

Scientific ideas

Experimental performance

Collection and assembly of data

Data analysis and interpretation

Manuscript writing

5. PUBLICATIONS NOT EMBEDDED IN THIS THESIS

Publication 5:

Establishment and characterization of a sclerosing spindle cell rhabdomyosarcoma cell line with a complex genomic profile

Sabine Schleicher*****, **Stefan Grote***, Elke Malenke, Kenneth Chun-Ho Chan, Martin Schaller, Birgit Fehrenbacher, Rosa Riester, Torsten Kluba, Leonie Frauenfeld, Hans Boesmueller, Gudrun Goehring, Brigitte Schlegelberger, Rupert Handgretinger, Hans-Georg Kopp, Frank Traub, Karen A. Boehme

*****SS and **SG** contributed equally.

Cells **9**, 2668 (2020), doi:10.3390/cells9122668, PMID: 33322555

Publication 6:

Novel adapter CAR T-cell (AdCAR-T) technology for precisely controllable multiplex targeting

Christian Seitz, Joerg Mittelstaet, Daniel Atar, Jana Hau, Selina Reiter, Clara Illi, Verena Kieble, Fabian Engert, Britta Drees, Giulia Bender, Ann-Christin Krahl, Philipp Knopf, Sarah Schroeder, Nikolas Paulsen, Alexander Rokhvarguer, Sophia Scheuermann, Elena Rapp, Anna-Sophia Mast, Armin Rabsteyn, Sabine Schleicher, **Stefan Grote**, Karin Schilbach, Manfred Kneilling, Bernd Pichler, Dominik Lock, Bettina Kotter, Sandra Dapa, Stefan Miltenyi, Andrew Kaiser, Peter Lang, Rupert Handgretinger, Patrick Schlegel

OncoImmunology **10**, 2003532 (2021), doi:10.1080/2162402X.2021.2003532, PMID: 35686214

6. INTRODUCTION

6.1 Cancer

Accounting for around one in six deaths worldwide, cancer is the second leading cause of death.[1] In many middle- and high-income countries, cancer even tops cardiovascular disease for highest mortality.[2] Worldwide, approximately 19.3 million new cancer cases and almost 10 million cancer deaths occurred in 2020.[3] Cancerous cells are characterized by abnormal proliferation and their ability to invade healthy tissue or migrate to other parts of the body via the circulatory or lymphatic systems.[4] Usually, cell growth and function is tightly regulated. The development of cancer is a multi-step process in which cells gradually lose their regulation due to the accumulation of a series of genetic mutations. These mutations occur as gain of function of proto-oncogenes as well as loss of function mutations in tumor suppressor or DNA repair genes and are caused by a variety of physical factors such as exposure to radiation, chemical components including tobacco and alcohol as well as biological factors such as infections with certain viruses.[5,6] During this process of tumor progression, cells acquire more mutations with favorable traits that offer selective advantages.[4] These advantages not only include an increased growth rate with a sustained proliferative potential enabling replicative immortality but also evasion of growth suppressors, resistance to cell death, elevated levels of invasive and metastatic as well as angiogenetic signaling and the reprogramming of energy metabolism.[7] Furthermore, cancer cells can develop mechanisms to evade destruction by the innate and adaptive immune system.[8]

6.1.1 Hematologic malignancies

Hematologic malignancies are cancers that affect the hematopoietic and lymphoid systems. Leukemia arises from aberrant hematopoietic stem cells (HSC) and can be subdivided into different types including acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myeloid (AML) and chronic myeloid (CML) leukemia. A malignant lymphoma is characterized by neoplastic lymphocytes and the most common lymphomas are the Hodgkin's (HD) and non-Hodgkin's lymphoma (NHL).[9] Together, leukemia and lymphoma account for around 9% of all newly diagnosed cancers.[10]

Lymphoma cells are malignant precursor lymphocytes arrested at a specific stage of lymphocytic differentiation. The accumulation of various genetic mutations results in the clonal expansion of aberrant T or B cells, and, subsequently, the formation of either a solid or leukemic malignancy.[11] These tumor cells show a similar antigen expression profile and home to similar sites compared to their physiological counterparts.[12] Approximately 95% of all lymphomas are of B cell origin and about 20 new cases of lymphoma are diagnosed per 100,000 people per year.[13]

Standard therapeutic approaches include radio- and/or systemic chemotherapy. Novel antibody-based therapies have increased long-term survival rates for patients with HD and high-grade NHL to 90% and 50%, respectively. Relapsing patients can be treated with second-line salvage treatments, including high-dose chemotherapy (HCT) followed by autologous stem cell transplantation (ASCT).[14]

6.1.2 Solid tumors

A solid cancer is a discrete tumor mass that forms within a specific type of tissue and can occur anywhere in the body. With approximately 320,000 newly diagnosed cases in 2020, melanoma represents ~1.7% of all cancer entities.[3] The majority of cases occur in developed countries where melanoma ranks as the sixth most frequently diagnosed cancer overall.[15] Following the introduction of novel therapeutic concepts, such as immune checkpoint inhibitors and targeted therapies, melanoma-related death rates have steadily declined since. In only three years, from 2013 to 2016, overall mortality decreased by 17.9%.[16] Currently, the five-year survival rate for patients with primary melanoma of the skin from the time of initial diagnosis is 93%. However, early detection is the most important factor for beneficial outcome. The five-year survival rate for patients with late-stage or metastatic melanoma is only 10-30%, even with modern immunotherapeutic approaches.[17] Melanoma can be classified into four main stages depending on various factors according to the official Tumor-Node-Metastasis (TNM) staging system by the American Joint Committee on Cancer (AJCC). The staging system describes characteristics of the primary tumor, such as thickness and mitotic rate, the involvement of nearby lymph nodes and development of distant metastases.[18] Standard of care treatment for small, early-stage melanoma (thickness <1 mm) is surgical removal. Moreover, targeted therapy with BRAF and MEK inhibitors in BRAF V600 mutated melanomas has shown to be more impactful than chemotherapy with dacarbazine or

temozolomide. Immunotherapy using high-dose interleukin 2 (IL-2) is used to prolong survival for late-stage melanoma.

Neuroblastoma (NB) is by far the most common extracranial tumor in infants and young children. While NB represent 6-10% of all childhood cancers, it accounts for ca. 15% of all cancer-related deaths in the pediatric population.[19,20] NB can be classified into low-, intermediate-, and high-risk groups based on clinical and molecular features.[21] The average five-year survival rate for NB is 81% and for children with low-risk NB, the five-year survival rate is higher than 95%. Since NB is characterized by a major clinical heterogeneity which ranges from spontaneous regression or differentiation in low-risk NB to cases of aggressive, metastatic disease. Overall survival for patients with late-stage or metastatic NB is below 50%, despite intensive multimodal therapy including induction chemotherapy, surgical resection, radiotherapy, high-dose chemotherapy, which is often followed by autologous stem cell transplantation and immunotherapy with monoclonal antibodies (mAb) targeting the cell-surface disialoganglioside GD2 in combination with cytokines and retinoic acid.[22,23] Patient age plays a pivotal role since overall outcome for younger patients is usually better.[24] To help classify NB, the International Neuroblastoma Risk Group Staging System (INRGSS) uses results from computer tomography (CT) or magnetic resonance imaging (MRI), while the International Neuroblastoma Staging System (INSS) uses results from the surgery. Since the INRGSS stage can be determined before a potential therapy and surgery is part of many initial treatment plans, the INRGSS is utilized more widely. Staging includes assessment of tumor growth into vital structures such as nearby organs or around important blood vessels.

6.2 Metastasis

Metastases are defined as secondary tumors that manifest in a part of the body far from the original cancer.[25] Tumor cells can metastasize to soft tissue organs such as the lung and the liver, which are the most frequent sites of metastasis. The third most common site is the bone, often including the spine or the ribs.[26] Metastatic development, although severe improvement has been made over the last decades, remains an incurable disease and is the main cause of cancer-related mortality.[27]

Patients with solid tumors regularly release large amounts of cancer cells to the circulation, however, only very few cells are able to form solid metastases since they need

to overcome various obstacles including successful invasion of tissues and survival of immune surveillance.[28] Moreover, secondary sites are primed by the primary tumor to generate a pre-metastatic niche. This priming process involves soluble factors and extracellular vesicles that induce vascular leakage and form a microenvironment that is more susceptible for tumor cell colonization.[29]

6.3 Tumor microenvironment

Solid tumors are surrounded by an immunosuppressive microenvironment that consists of a variety of cells including tumor-associated fibroblasts, macrophages, dendritic cells, neutrophils, regulatory T cells (T_{regs}) and myeloid derived suppressor cells (MDSCs) as well as blood and lymph vessels, extracellular matrix and multiple secreted or cell membrane-bound molecules.[30,31] The immunosuppressive properties of the TME enables tumor cells to escape immune surveillance by impairing immune cell infiltration and effector function leading to tumor growth and metastatic development.[32]

Tumor infiltration with T lymphocytes has been demonstrated throughout various tumor entities. Intratumoral infiltration with cytotoxic $CD8^+$ T cells was correlated to a more beneficial overall outcome and is therefore more predictive than the classical tumor staging system.[33–35] However, a good prognosis is highly dependent on the composition of tumor infiltrating lymphocytes (TILs). MDSCs and T_{regs} can suppress effector function of cytotoxic immune cells from both the adaptive and innate immune system. Soluble factors such as transforming growth factor (TGF) beta, which are highly secreted within the TME, have been shown to facilitate metastatic development as well as tumor escape mechanisms due to inhibition of cytotoxic signaling pathways in immune cells as well as downregulation of activating receptors and upregulation of inhibitory receptors on tumor cells.[36–38] Furthermore, metabolic dysregulation by catabolizing enzymes such as indoleamine 2,3-dioxygenase promotes an acidic TME which is linked to inhibition of cytotoxic TIL proliferation and tumor cells exposed to an acidic pH demonstrate elevated levels of invasiveness and metastatic development.[39–41]

To elicit a strong anti-tumor response against solid tumors, immune cells need to overcome the challenges posed by the TME including physiological barriers, immunosuppressive cells and cytokines as well as oxidative stress.[36,42]

6.4 Immune system and cancer

The characteristics of the human immune system have been highly debated for a long time. Currently it is accepted that the human body can react to foreign pathogens and tumors in two different ways, namely with the adaptive and the innate immune response. Both, the innate and the adaptive immune system depend on the activation of leukocytes.[12]

The innate immune system, including NK cells, macrophages and granulocytes, relies on the recognition of germline-encoded pattern-recognition receptors (PRR) to fight pathogens immediately after exposure. The adaptive immunity is mostly composed of highly specialized cells, like B and T cells, that act as an adaptation to pathogen infection.[43]

Tumor cells are malignant cells that are only able to grow uncontrollably if they manage to evade the surveillance of the innate and adaptive immune system.[7,44] Due to genetic mutations that these malignant cells acquire over time, they often show an altered protein expression profile compared to their physiological state.[45] These mutations can lead to the expression of foreign proteins that are not expressed in healthy cells and are called tumor-specific antigens (TSA).[46] In contrast, tumor cells can show an enhanced protein expression of tumor-associated antigens (TAA).[47] TAA expression, even within the same tumor entity, can be very heterogeneous in regards to intensity and distribution, which causes difficulties for targeted therapy of solid tumors.[48] Moreover, tumor cells have been frequently shown to alter their antigen expression profile as part of various immune evasion strategies.[49]

6.4.1 Natural killer cell-based immune response

While the adaptive immune response relies on time-consuming clonal expansion and differentiation of specialized B and T cells, the innate immune response can act instantly upon recognition of foreign particles. The immediate immune reaction is carried out within the first four hours by non-specific effector cells. It is followed by the early induced response that promotes recruitment and activation of immune cells such as macrophages, dendritic cells and natural killer cells.[12] Only if a foreign organism or tumor cell can overcome these early defense mechanisms, the adaptive immune response is activated.[12]

NK cells, as innate lymphocytes, recognize cancerous cells by a condition known as “missing self”, which describes abnormally low levels of major histocompatibility complex (MHC) class I surface expression.[50,51] In humans, MHC is known as human leukocyte antigen (HLA). NK cell activation is tightly regulated by the balance of binding of activating receptors, such as NKG2D (CD314) or DNAM-1 (CD226), to “induced self” ligands - molecules that are expressed at low levels on healthy cells but are abnormally upregulated on tumor cells - as well as inhibitory receptors, such as NKG2A (CD159a) and HLA class I molecules, including the classical HLA-A, -B and –C or the non-classical HLA-E and -G.[52] This “altered self” hypothesis describes NK cell activation as the result of the combination of the “missing self” and “induced self” concepts. Only if the activating signals overpower the inhibitory signaling, NK cell-mediated cytotoxicity is induced.[53,54]

NK cells account for 5-15% of human blood leukocytes and can be subdivided by their CD56 expression level.[55] $CD56^{bright}$ NK cells make up the majority of NK cells and are responsible for the secretion of immunomodulatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and beta (TNF-β), which further stimulate the endogenous immune system and can indirectly enhance anti-tumor activity.[56,57] $CD56^{dim}$ NK cells are characterized by their powerful cytotoxic function. Upon activation, these NK cells release lytic granules containing perforin, granulysin, granzyme A and B as a result of an intracellular signaling cascade. This cascade is carried out by tyrosine kinases that can phosphorylate tyrosine residues on certain molecules. A major component of this tyrosine kinase signaling is the immunoreceptor tyrosine-based activation motif (ITAM) which can be found on the cytoplasmic tails of e.g. CD3 or various Fcγ receptors as well as activating NK cell receptors such as NKG2D. On the other hand, immune checkpoint receptors, such as TIGIT, TIM-3 or LAG-3, carry immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that, upon binding to their ligands, phosphorylate different cytoplasmic molecules within the NK cell, thus inhibiting effector activation.

When the activating cascade overpowers the inhibitory signals, the released perforin forms transmembrane pores by polymerization after attaching to the membrane-bound phosphorylcholine on target cells. Granzymes belong to the family of serine proteases which are capable of cleaving proteins.[58] Together with granulysin they induce apoptosis once they reach the cytoplasm of the target cell through the perforin-induced pores. The interaction of all three effector proteins is needed for effective cytotoxicity.[12] While most of the NK cell-dependent cytotoxicity is due to the release of cytolytic granules, other mechanisms of apoptosis induction have been discovered as well such as the expression

of Fas ligand (FasL or CD178) or TNF-related apoptosis-inducing ligand (TRAIL, CD253).[59,60] Moreover, $CD56^{dim}$ NK cells express the Fcγ receptor III (CD16 or FcγRIII), a key mediator of antibody-dependent cellular cytotoxicity (ADCC).[57] CD16 recognizes the Fc (Fragment, crystallizable) part of the antibody subclasses immunoglobulin G (IgG) 1 and IgG3, representing an antigen-specific mode of action for NK cells.[12]

6.4.2 The NK-92 cell line

NK-92 is a stable, immortalized cell line with the characteristics of primary NK cells. It was isolated from a patient with NHL and characterized in 1994 by Hans Klingemann and colleagues at the British Columbia Cancer Agency.[61] It is one of only few clonal cell lines consisting of pure NK cells with consistent proliferative characteristics and the only NK cell line with robust cytotoxic effector function comparable to primary NK cells, although they resemble the immunomodulatory $CD56^{bright}$ blood NK cell population.[62] NK-92 cells are negative for the surface expression of CD16 and are therefore not able to induce ADCC.

To date, NK-92 has been utilized in various phase I and phase II clinical studies examining its efficacy in treating hematological as well as solid malignancies. Due to their origin as tumor cell line, NK-92 cells have to be γ-irradiated with 10 Gy prior to application to induce double-strand DNA breaks and, in turn, completely nullify proliferation and prohibit long-term persistence.[63] In various clinical studies NK-92 cells could be safely administered in multiple doses of up to 10^{10} cells per m^2 without the induction of short- and long-term side effects.[63–65]

6.5 Chimeric antigen receptors

Genetic transfer of synthetic antigen-specific T cell receptors (TCRs) or CARs is used to increase the cytotoxic efficacy of lymphocytes.[66] The TCR is directly dependent on association with the CD3 complex, expression of either CD8 or CD4 and antigen-presentation on the MHC molecule. Contrarily, CAR-mediated cytotoxicity is independent thereof.[67] This can lead to a more effective recognition of tumor cells which often show a dysregulated MHC expression.[66]

The human TCR is a disulfide-linked heterodimer of the four distinct T cell receptor glycoproteins α, β, γ and δ. These can form the two different dimers α:β and γ:δ.[68] Their

primary protein sequences are similar to the Fab fragment of immunoglobulins and they contain clonally variable regions that determine antigen specificity. However, in contrast to antibodies, the α:β TCR can only function on a cell surface and can only recognize peptides that are presented on the MHC of other cells.[69] After binding to the MHC molecules, the TCR non-covalently associates with invariant CD3 subunits, CD3γ, CD3δ, CD3ε or CD3ζ (Figure 1). These subunits carry ITAMs, which are phosphorylated by Src-kinases and then serve as docking sites for other kinases.[70] They are therefore responsible for coupling antigen recognition to multiple signal transduction pathways.[71]

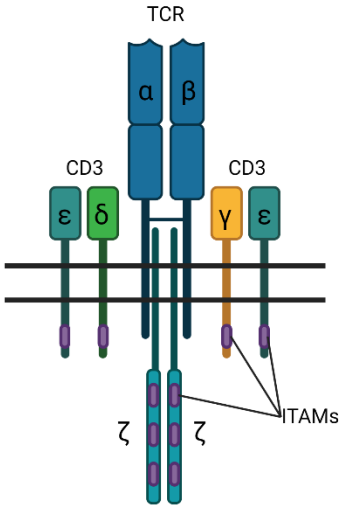


Figure 1: CD3-TCR complex with their respective subunits and ITAMs

CARs combine an antigen-recognition domain with an intracellular domain into a single chimeric protein.[66] The antigen-binding domain usually derives from the variable regions of the heavy (V_H) and light (V_L) chain of monoclonal antibodies (mAb), which are fused to generate the single-chain variable fragment (scFv).[72] They exhibit similar antigen specificity when compared to complete mAb while being much smaller. For intracellular signal transduction the antigen-binding domain is connected to a CD3ζ chain derived from the TCR complex.[73] The scFv and the intracellular signaling domain are linked by an extracellular hinge region and a transmembrane (TM) domain.

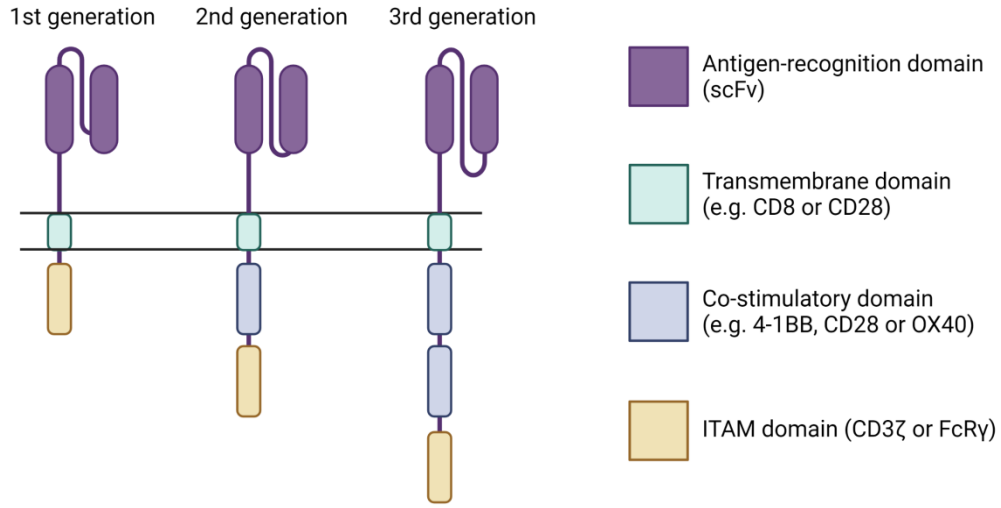


Figure 2: Structural composition of chimeric antigen receptors

Over the years, CARs underwent constant development. The first generation of CARs only carries intracellular ITAMs, a CD3ζ- or FcRγ-domain. The second and third generation of CARs exhibit one or, respectively, two additional co-stimulatory domains such as CD28, CD134 (OX40) or CD137 (4-1BB). These intracellular signaling domains increase the *in vivo* persistence and proliferation of CAR-modified T cells.[66,74] Which of these domains is clinically superior has yet to be determined. So far CD28-based CAR constructs have shown higher cytotoxic potency while 4-1BB-based CARs usually persist longer in the *in vivo* setting (Figure 2).[75] Fourth and fifth generation CARs incorporate a third stimulatory signal or even an additional membrane receptor. They combine signaling of third generation CARs with various immunomodulatory cytokines that augment immune cell activation or provide resistance to the TME.[76]

6.5.1 CAR T cells

CAR-engineered T cells have demonstrated remarkable results in clinical and pre-clinical trials and, so far, CAR T cells are the only approved cellular CAR therapeutic. Their invention dates back to 1989, where Zelig Eshhar and colleagues performed the first redirection of T cell specificity through genetic engineering.[77] They replaced the extracellular α and β chain of a TCR with immunoglobulin variable heavy and variable light chains and demonstrated that this “T-body” showed effector function specific to the introduced immunoglobulin chains.[78] The next big milestone was crossed in 2017 when two autologous CD19-redirected CAR T cell therapies against diffuse large B cell

lymphoma (DLBCL), B-ALL and follicular lymphoma, namely tisagenlecleucel (Kymriah®, Novartis) and axicabtagene-ciloleucel (Yescarta®, Gilead), were approved by the FDA after merely completing phase II clinical trials. While tisagenlecleucel comprises a 4-1BB co-stimulatory domain, axicabtagene-ciloleucel utilizes a CD28 intracellular signaling domain. Both drugs produced outstanding clinical results inducing long-lasting remission in 80% of lymphoma patients.[79] To date, two more anti-CD19 CARs have been approved, Tecartus® (Gilead) and Breyanzi® (Juno Therapeutics), for the treatment of mantle cell lymphoma (MCL) and DLBCL, respectively. The most recent achievements in the CAR T cell field were the approvals of the first non-CD19-targeted therapeutics. Idecabtagene vicleucel (Abecma®, Bluebird Bio) and ciltacabtagene autoleucel (Carvykti®, Janssen), second generation CARs targeting the B cell maturation antigen (BCMA), are utilized to treat patients with relapsed and refractory multiple myeloma.[80,81]

Table 1: FDA-approved CAR T cell therapies

Brand name	Target	Signal domain	Indication	Approval date
Kymriah	CD19	41BB - CD3ζ	Diffuse large B cell lymphoma Follicular lymphoma B cell precursor ALL	Aug 30, 2017
Yescarta	CD19	CD28 - CD3ζ	Diffuse large B-cell lymphoma Follicular lymphoma	Oct 18, 2017
Tecartus	CD19	CD28 - CD3ζ	Mantle cell lymphoma B cell precursor ALL	Jul 24, 2020
Breyanzi	CD19	41BB - CD3ζ	Diffuse large B-cell lymphoma	Feb 05, 2021
Abecma	BCMA	41BB - CD3ζ	Multiple myeloma	Mar 26, 2021
Carvykti	BCMA	41BB - CD3ζ	Multiple myeloma	Feb 28, 2022

Despite astonishing success rates, T cell-based CAR therapy still shows major drawbacks. Severe side effects such as neurotoxicity, affecting neural tissues, and cytokine release syndrome (CRS), a systemic inflammatory response affecting all organs, are frequently observed in patients receiving CAR T cell therapy. These potentially lethal conditions can develop due to overstimulation of immune cells and the resulting production of large amounts of cytokines such as IL-6 and IFN-γ.[82,83] CRS can be subdivided into five grades of severity and typical symptoms include but are not limited to hypotension, high fever and tachycardia. Standard treatment involves the application of competitive IL-6R agonists and corticosteroids for non-responsive patients.[84]

The manufacturing process of autologous CAR T cells requires the isolation of peripheral mononuclear blood cells (PBMCs) of, to some extent, heavily pre-treated patients. This pre-treatment with cytostatic drugs or radio therapy can significantly impair PBMC yield in terms of quantity as well as quality. Therefore, patients need to be treatment-free for two weeks before the isolation process.[85] In combination with the additional four weeks of CAR T cell manufacturing, survival time of patients is an important limitation. Moreover, production cost for a single CAR T cell therapy is extensive with prices of up to $350,000. Currently, these costs are just too high to be sustainable even for a socialized healthcare system.[86] On the other hand, generation of “off-the-shelf” allogeneic CAR T cells, CAR-engineered T cells from healthy donors, would always carry the risk of inducing graft-versus-host disease (GvHD) because the donor T cells could misidentify the host as “non-self” and cause potentially lethal side effects.[87]

6.5.2 CAR NK cells

In the context of the major drawbacks of CAR-engineered T cells, alternative cell sources for CAR modification are direly needed. NK cells provide an interesting approach as they are less likely to induce IL-6-mediated CRS or neurotoxic events, they provide multiple, antigen-independent mechanisms of cytotoxic function as well as the feasibility of “off-the-shelf” manufacturing, where the final therapy product does not have to be produced individually for each patient.[55]

NK cells usually display a different cytokine phenotype compared to T cells. While CAR T cells secrete a broad variety of interleukins, including the aforementioned IL-6, studies have shown that CAR NK cell activation predominantly leads to the production of IFN-γ or GM-CSF and is therefore far less likely to induce CRS.[88,89] Since these severe side effects significantly affect CAR T cell treatment costs, the lack of lengthy post-treatment hospitalization severely lowers associated expenditure.[90] Moreover, the risk of on-target/off-tumor toxicity is low due to limited persistence of CAR NK cells and *ex vivo* expanded NK cells from an allogeneic source, in contrast to T cells, do not carry the risk of GvHD induction.[91,92] This enables CAR NK cells to be generated patient-independently from various sources such as PBMCs, umbilical cord blood (UCB) cells, hematopoietic progenitor cells (HPCs) and induced pluripotent stem cells (iPSCs) to be readily available for treatment.[93]

However, major obstacles for the application of CAR-engineered primary NK cells remain. Transduction efficiency with either, lentiviral as well as retroviral vectors has not generated satisfying results. Single-cell sorting and subsequent cultivation and expansion of primary immune cells is difficult and recovery after cryopreservation, which is mandatory for an “off-the-shelf” product, is poor with low cytotoxic function.[63] Thus, the majority of CAR NK cell studies, to date, have been performed with NK-92 cells which have demonstrated good transduction efficiencies with lentiviral CAR vectors. NK-92 cells are easily cryopreserved and expanded upon thawing without showing impairment of cytotoxic effect even after multiple freeze/thaw cycles. CAR-modified NK-92 cells can be easily generated in a GMP-compliant manner and expanded to high cell numbers to meet the clinical need, thus, severely reducing manufacturing time and associated costs.[94] Irradiation of CAR NK-92 cells impairs *in vivo* proliferation and persistence. Therefore, usually multiple infusions with an NK-92-based CAR product are required for treatment.[95]

6.6 Adapter CAR technology

To broaden the spectrum of possible immunotherapeutic targets for flexible CAR-engineered immune cell therapy, a universal adapter CAR was developed and evaluated during this thesis. The AdCAR is based on the unique properties of a novel scFv, mBio3, targeting a “neo”-epitope-like structure derived from the endogenous vitamin biotin, which can be linked to the Fc part of a mAb, mAb fragments or small molecules (SM).[96] Biotin, also known as vitamin H or B_7, is a hydrophilic coenzyme which is essential to all lifeforms.[97] It is involved in gluconeogenesis, the synthesis of fatty acids and metabolism of amino acids.[98] Biotinylation of adapter molecules (AM) forms an irreversible amide bond with the antibody, thus, creating the “linker-label-epitope” (LLE) targeted by the AdCAR. Therefore, AdCAR-driven immune cell activation is the result of a two-step process: Specific target antigen recognition utilizing biotinylated antibodies (bAb) and CAR-mediated intracellular signal transduction upon LLE binding (Figure 2). The system allows precise quantitative (on-/off switch) as well as qualitative (change and combination of target antigen) fine-tuning of effector cell function. Other, comparable universal CAR approaches with T and also NK cells based on a FITC tag or the biotin-streptavidin interaction have already demonstrated promising pre-clinical results.[99–101] These CAR systems, however, utilize the scFv-mediated recognition of non-physiological

AMs that are foreign to the human body and, thus, can potentially cause severe immunogenic reactions.

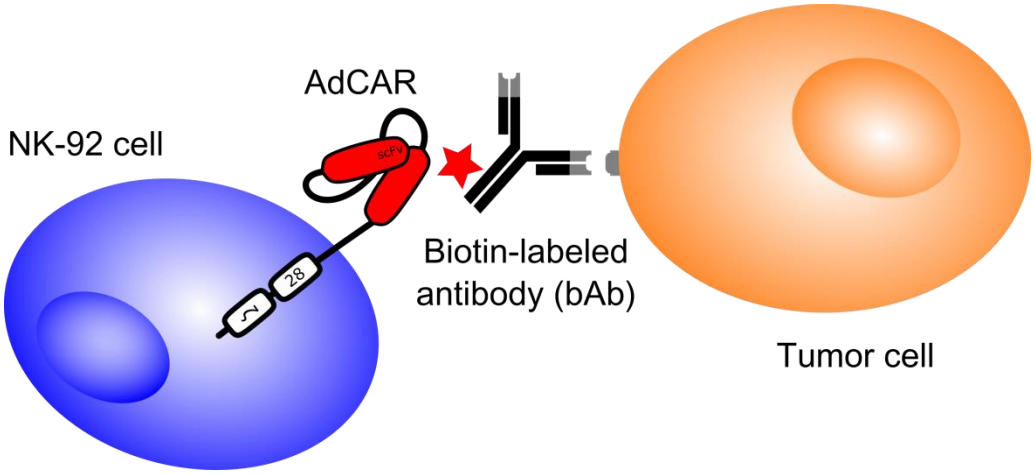


Figure 3: Schematic representation of AdCAR NK-92 function and structure. (Grote et al., 2021)

6.7 Data analysis

All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). While for the comparison of two experimental groups a standard t-test was utilized, the analysis of statistical significance between multiple groups was conducted using a one-way ANOVA test. Flow cytometry data were analyzed using FlowJo software V10.0.8 (FlowJo LLC., BD Biosciences, Franklin Lakes, NJ, USA).

7. AIM OF THE THESIS

Personalized therapy of cancers has become increasingly attractive with deeper understanding of tumor biology. The recent clinical success of chimeric antigen receptor-modified T cells has demonstrated that CARs can be a robust tool to redirect and stimulate immune responses. In order to overcome the obstacles posed by classical CAR T cell therapy, an adapter CAR was developed that can tackle tumor immune evasion mechanisms with its inherent ability to target multiple antigens sequentially or even simultaneously.

The primary aim of the present thesis was the successful generation, characterization and functional assessment of a powerful AdCAR-engineered NK cell product. In combining the universal applicability of the AdCAR with the flexibility of the first FDA-approved “off-the shelf” NK cell line NK-92, we aimed at generating a strong, universal “on-demand” cellular anti-cancer product. In the present thesis we sought to identify antigen structures on hematologic malignancies as well as solid tumors that can be targeted using already FDA-approved and clinically relevant antibodies. These antibodies can be easily modified to generate non-immunogenic adapter molecules that we utilized for the in-detail evaluation of AdCAR NK-92 cell therapy efficacy.

The secondary aim of this thesis was to further characterize the benefits of NK-92 cells as CAR drivers. CD276-specific CAR-engineered NK-92 cells were generated to study CAR-mediated cytotoxicity in 3-dimensional tumor models that better mimic the more complex *in vivo* situation. Additionally, influences exerted by the TME, which have been demonstrated to hamper primary immune cell function, were assessed for the effect on CAR NK-92 cell efficacy. Finally, novel CRISPR/Cas9 technology was implemented in order to evaluate a potential enhancement of CAR NK-92 cell cytotoxicity.

8. RESULTS AND DISCUSSION

8.1 Publication 1

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting

Adapter chimeric antigen receptor (AdCAR)-modified NK-92 cells can be manufactured as an “off-the-shelf, on-demand” standardized product whose effector function can be tightly regulated to provide a universal and cost-effective therapeutic option for safe and tunable targeting of lymphoma cells.

In cooperation with Miltenyi Biotec, a special second generation adapter CAR for the transduction of the continuously expandable cell line NK-92 was generated that is based on the mAb “mBio3”-derived scFv targeting a “neo”-epitope-like structure consisting of the endogenous vitamin biotin in the context of monoclonal antibodies as linkers whose context is introduced in the patent application EP3315511A1.[96] It comprises an IgG4 hinge domain, a CD8 transmembrane domain, CD28 co-stimulatory as well as CD3ζ signaling domains. For detection and enrichment of AdCAR-transduced NK-92 cells a truncated CD34 (tCD34) domain is co-expressed after a furin P2A site (Figure 1b). The P2A peptide is derived from the porcine teschovirus. In this case, it separates the chimeric antigen receptor and the CD34 tag during translation and leads to an equimolar expression. The AdCAR technology utilizes biotin-labeled antibodies (bAb) as adapter molecules (AM) to translate tumor specific antigen recognition into immune effector cell activation.

The present study included the characterization of the newly generated AdCAR NK-92 cell line as well as its functional analysis. As a proof of principle, various commercially available lymphoma cell lines as well as primary patient-derived lymphoma cells were co-cultivated with AdCAR NK-92 cells in various settings to assess optimal AdCAR functionality. Furthermore, the secondary aim of this study was to examine the potential of AdCAR NK-92 cells to counteract tumor evasion strategies.

Successfully transduced NK-92 cells were single-cell sorted and, since we found that CAR expression was proportional to CAR-mediated cytotoxic efficacy, the clone that was screened for the highest CAR expression as well as for highest viability was utilized for further experiments. AdCAR NK-92 cells showed stable AdCAR surface expression for at

least 150 days after cell sorting (Figure 2b) with an average viability of >90%. Importantly, cell proliferation rate was not impaired by the transduction process (Figure 2c).

Further characterization of AdCAR NK-92 cells was conducted by flow cytometric analysis of the NK cell receptor profile as well as important immune checkpoint receptors in comparison to untransduced NK-92 parental cells as well as primary NK cells from three healthy donors. Compared to primary NK cells, the NK-92 and AdCAR NK-92 cells used in this thesis showed lower expression of DNAM-1 (CD226), one of the main activating NK receptors. Another important activating receptor, NKG2D (CD314), was almost completely absent on AdCAR NK-92 cells. Furthermore, AdCAR-transduced NK-92 cells displayed very high expression of KIR2DL2/DL3 (CD158b), a member of the killer cell immunoglobulin-like receptor (KIR) family recognizing the human HLA-C1/2 peptide, and thus, inhibiting NK effector function. Interestingly, NKp80, an activation receptor on all activated NK cells, which induces NK cell mediated cytotoxicity and cytokine production, was only present on the isolated NK cells and not on NK-92 cells (Figure 2d). AdCAR NK-92 immune checkpoint receptor expression profile was similar compared to primary NK cells and parental NK-92 cells (Figure 2e).

For functional analysis of AdCAR NK-92 cells, lymphoma cell lines Raji, Daudi and JeKo-1 were immunophenotyped using flow cytometry. They were screened for the expression of various lymphoma antigens (Table 1) and, subsequently, utilized as target cells in cytotoxicity assays and co-incubated with AdCAR-transduced as well as parental NK-92 cells in various effector-to-target ratios. AdCAR-mediated cytolysis of all lymphoma cell lines was significantly increased within two hours upon addition of bAb targeting highly expressed antigens at different titrated concentrations (Figure 3d & f). The absence of either the bAb or its correspondent target antigen completely prevented AdCAR activity. NK-92 cytotoxicity and antigen expression levels (median fluorescence intensity, MFI) correlated significantly (Figure 3e). Since AdCAR NK-92 cells induced cellular cytotoxicity in a bAb dose-dependent manner (Figure 3a), a bAb concentration of 100 ng/ml, which delivered the best results, was chosen for all functional AdCAR NK-92 experiments. Altering antibody concentration decreased the respective AdCAR NK-92-mediated cytolysis. Also, contrary to primary NK cells, AdCAR NK-92 cells lack CD16 (FcγRIII) expression and cannot be retargeted by IgG1 antibodies.[62] Therefore, AdCAR-mediated cytotoxic effect is specific and tunable, and formation of the immunological synapse is directly controlled by bAb titration. Unbound human biotin, even at supraphysiological levels did not interfere with AdCAR function (Figure 3b). AdCAR NK-92 cells also showed

increased cytolysis of patient-derived tumor cells that had been previously isolated from blood samples from one patient diagnosed with a hematogenously disseminated MCL and three patients with CLL. MCL and CLL cells were immunophenotyped (Table 2) and co-incubated with AdCAR NK-92 cells in the presence or absence of a bCD19 or bCD20 AM (Figure 6a-d). Irradiation of NK-92 cells with 10 Gy prior to testing, as required in all clinical trials, had no significant effect on target cell lysis when AdCAR NK-92 cells were used immediately after irradiation. However, AdCAR NK-92 cells gradually lost effector function and underwent apoptosis over the course of 24-72 hours (Figure 3g), thus, preventing permanent engraftment of NK-92 cells without the need of a safety switch as suggested for CAR T cells in the clinical setting.[102] It has been shown that irradiated CAR NK-92 cells can safely be administered multiple times to further increase efficiency.[103] Importantly, AdCAR NK-92 cells demonstrated no fratricide of parental NK-92 cells upon co-incubation with a biotinylated CD56 antibody.

Next, AdCAR NK-92 cells were screened for the secretion of a variety of cytokines released during cytolysis. Multiple cytokines, such as IL-10 (36-fold; $p<0.0004$), granulysin (27-fold; $p<0.002$), IFN-γ (65-fold; $p<0.01$), MCP-1 (40-fold; $p<0.04$) and TNF-α (28-fold; $p<0.0002$), were significantly increased after co-incubation of AdCAR NK-92 cells with lymphoma cells. Elevated levels were only detectable upon AdCAR induction via specific biotinylated antibodies. NK-92 cells alone showed medium basal secretion of the NK cell effector molecules granzyme B and perforin which was further augmented after AdCAR activation (2.2-fold and 1.3-fold, respectively) but did not reach statistical significance (Figure 3h). TNF-related apoptosis-inducing ligand (TRAIL, CD253) upregulation on AdCAR NK 92 cells could be observed after co-incubation with Raji cells and bCD19 for 6 hours using flow cytometry (MFI: AdCAR NK-92 only: 2.08 ± 0.18; AdCAR NK-92 + Raji: 2.36 ± 0.06; AdCAR NK-92 + Raji + bCD19: 4.48 ± 0.29) while expression of Fas ligand (FasL, CD178), another important apoptosis-inducing transmembrane protein, was not elevated (Figure 3i).

We also tested whether AdCAR activation by co-incubation with lymphoma cells would permanently decreases cytotoxic effector function of AdCAR NK-92 cells due to immune exhaustion. AdCAR NK-92 cells were co-incubated with Raji cells and a bCD19 AM for various time points and screened for the expression of immune checkpoint receptors, whose increased expression has been shown to be responsible for decreased immune effector function, at indicated time points (Figure 5b). Only CD96 (TACTILE), CD223 (LAG-3) and CD366 (TIM-3) were significantly elevated with peak expression levels after

24 hours of stimulation. AdCAR NK-92 cells were then subjected to continuous re-stimulation by addition of fresh lymphoma cells every 24 hours for a total of 72 hours and again screened for immune checkpoint receptor expression. Slightly elevated CD96 expression levels could be observed, while CD223 and CD366 were moderately increased. For functional testing, we compared the continuously re-stimulated AdCAR NK-92 cells with unstimulated control AdCAR NK-92 cells using Raji cells as target and bCD19 as well as bCD20 as adapter molecules (Figure 5c). We found no significant decrease in CAR-mediated cytotoxic effector function after re-stimulation. Thus, AdCAR NK 92 cells did not show relevant signs of exhaustion even after multiple killing events.

In previous CAR T cell studies targeting CD19 in leukemia and lymphoma, patients tumor cells reacted to therapy by down-regulation of CD19.[82,104,105] Using a JeKo-1 antigen-loss model AdCAR NK-92 cells demonstrated their ability to target multiple antigens simultaneously and react to tumor antigen loss by switching target structures simply utilizing a different bAb while retaining therapeutic efficacy. JeKo-1 CD19 and/or CD20 knockout (KO) cell lines were mixed at an equal ratio and subsequently co-incubated with AdCAR NK-92 cells in the presence or absence of bCD19 and/or bCD20 antibodies. Addition of AdCAR NK-92 cells alone to the JeKo-1 target cell mix did not decrease target cell count or alter the ratio of the respective subpopulations. However, co-incubation with specific biotinylated antibodies induced AdCAR-mediated lysis of respectively antigen positive subpopulations while the antigen negative subpopulations were spared (Figure 4a). With the combination of bCD19 and bCD20, AdCAR NK-92 cells almost completely eradicated $CD19^+$ and $CD20^+$ JeKo-1 cells (Figure 4b). Thus, AdCAR NK-92 cells specifically lyse tumor cells only in the presence of a bAb targeting an antigen that is expressed on the surface of the tumor cell. If tumor cells down-regulate antigen expression levels in response to immune therapy, AdCAR NK-92-mediated activity can easily be retained by switching target antigens through the addition of a different bAb.

AdCAR NK-92 cells can be manufactured as an “off-the-shelf, on-demand” standardized product whose effector function can be tightly regulated. They may provide a universal and cost-effective therapeutic option for safe and tunable targeting of malignant cells. This study proved that biotinylation of already FDA-approved antibodies such as rituximab (anti-CD20) is capable of creating functional AM for AdCAR NK-92 therapy, thus, facilitating translation into clinical settings.

8.2 Publication 2

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells for the multiplex targeting of bone metastases

CAR T cells have demonstrated tremendous clinical success in treating hematological malignancies. However, CAR-mediated immunotherapy of solid tumors, so far, has only had limited effectivity due to the lack of reliable target structures and phenotypic tumor plasticity. “Off-the-shelf” AdCAR NK-92 cells display robust anti-tumor activity against cancers metastasizing to the bone. They can overcome typical obstacles in solid tumor immunotherapy through the utilization of clinically approved, therapeutic antibodies as exchangeable adapter molecules which, in turn, facilitates clinical translation.

The present study assesses AdCAR NK-92-mediated anti-tumor efficacy against solid tumors and is a direct extension of the previous AdCAR study that was focused on the generation and characterization of the AdCAR-modified NK-92 cell line (Publication 1). To this day, standard therapeutic approaches, such as surgery, chemotherapy or radiation therapy, only show mediocre success in preventing or treating bone metastatic disease. The complexity and heterogeneity of solid tumors in conjunction with therapeutic resistance of dormant tumor cells make bone metastasis one of the most common causes of cancer-related mortality.[27] The difficulty for modern immunotherapy remains finding suitable target structures that enable a specific anti-tumor response while sparing healthy tissue.

Tumor cell lines were newly established utilizing outgrowth cultures of tumor material from resected bone metastases that were provided by the Department of Orthopedic Surgery, University Hospital Tuebingen (UKT) (Table 1). The cell line panel comprises a mammary, renal cell and colon carcinoma as well as a melanoma cell line. They were authenticated, characterized and subsequently utilized as target cells for evaluation of AdCAR NK-92 cell-mediated targeting of solid tumors. The secondary aim of the study was to develop and assess ideas for clinical implementation of AdCAR NK-92 therapy.

For the present study, we utilized a small panel of potential target antigens that are commonly found in solid tumors and are targeted by already FDA-approved therapeutic antibodies. We utilized respective bAb against these antigens for flow cytometric characterization of the four different bone metastatic cell lines (Figure 1 and Table 2).

Results and discussion

Phenotypes varied greatly throughout the different tumor entities. Interestingly, all cell lines shared ubiquitous expression of CD276, an immune checkpoint molecule of the B7 family. For each cell line, the four different antigens with the highest respective surface expression levels were chosen to serve as target structures for AdCAR NK-92 cell treatment since we have previously shown that antigen expression correlated with AdCAR-mediated cytolysis.[106] Next, we further characterized the tumor cell lines by flow cytometric assessment of NK ligand expression (Table 3). Equivalently, cell lines demonstrated a very heterogeneous expression profile. The NK ligand HLA-ABC, for instance, was completely absent on the colon carcinoma MCK83 while the renal cell carcinoma MAC, the mammary carcinoma MAM and the melanoma MeGa17 expressed it abundantly.

For functional evaluation of AdCAR NK-92 cells, they were co-incubated with calcein-labeled tumor cell lines for two hours (Figure 2). AdCAR-mediated cytotoxicity was significantly increased against all bone metastatic cell lines, but only in the presence of a bAb targeting one of the previously selected antigens. AdCAR NK-92 cells without bAb or in combination with a bAb targeting antigens that were not expressed by the tumor cells did not show considerable tumor lysis. Findings were further underscored by the use of the xCELLigence real-time cell analysis system. Tumor cell lines were seeded and impedance was measured continuously, thereby tracking tumor cell growth or death over time (Figure 3). The dimensionless cell index is proportional to adherent and therefore viable tumor cells. Addition of AdCAR NK-92 cells alone to the tumor cells did not significantly impair tumor growth. Only the addition of specific bAb elicited a strong AdCAR-mediated immune cell response and significant tumor cell lysis could be demonstrated within four hours. Importantly, no long-term tumor regrowth was observed with adapter molecules targeting highly expressed antigens. As a next step, we established a cytokine secretion profile to screen for NK cell effector molecules and to further characterize AdCAR NK-92 cell-mediated tumor lysis (Figure 4). Therefore, AdCAR-modified as well as parental NK-92 cells were co-incubated with the renal cell carcinoma cell line MAC in the presence or absence of a specific bAb for 6 hours and the supernatant was harvested and subsequently analyzed using a Bio-Plex Pro human cytokine 17-plex assay. Among others, enhanced secretion of granulysin (24-fold; $p<0.0006$) and granzyme B (6-fold, $p<0.0001$) as well as IFN-γ (10-fold; $p<0.0009$) and TNF-α (32-fold; $p<0.0001$) was detectable, but only in the presence of a biotinylated EGFR antibody. The secretion of granulysin and granzyme B may directly account for

increased tumor lysis. IFN-γ and TNF-α stimulate the endogenous immune system and can indirectly enhance anti-tumor activity in the clinical setting.

Cellular immunotherapy *in vitro* studies mainly focus on tumor cell monolayer culture systems, thus, only allowing for limited validity in the translation process to the *in vivo* situation. We, therefore, established a three-dimensional spheroid tumor model using GFP-transduced tumor cells to evaluate AdCAR-specific intratumoral functionality (Figure 5). Tumor spheroids were co-incubated with AdCAR NK-92 cells and fluorescence signal was measured using the Celigo S imaging cytometer. Compared to control spheroids, AdCAR NK-92 cells in the presence of specific biotinylated antibodies demonstrated significantly increased cytotoxicity of up to 81% after 48 hours and up to 93% after 96 hours, thus proving AdCAR NK-92 cell functionality and effectivity in a three-dimensional setting that can help bridge the gap towards *in vivo* studies.

In this study, we demonstrated that CAR-modification of the NK-92 cell line with our AdCAR system can enable promising therapeutic opportunities for the treatment of a variety of metastatic tumor entities. Although future research needs to evaluate the therapeutic potential of AdCAR NK-92 cells in pre-clinical *in vivo* and clinical settings, results reported throughout the present study seem promising. For clinical translation, tumor samples from bone marrow biopsies or surgery can be screened for possible target structures using a standardized flow cytometric panel, as presented in this study, or by ultra-high content imaging. AdCAR NK-92 cells can be expanded as a well-characterized GMP-compliant master cell bank in a batch culture and individually scaled according to treatment doses and number of patients. Biotinylation of clinically approved antibodies provides adapter molecules with a known safety profile. They can be applied simultaneously or consecutively to treat highly heterogeneous tumors and counteract tumor antigen evasion strategies.

In summary, AdCAR NK-92 cells combine “off-the-shelf” availability with personalized and controllable elimination of tumor cells, thus, establishing a potent cellular product with universal applicability and quick clinical translation potential for the treatment of solid tumors.

8.3 Publication 3

CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma

Results and discussion

The immune checkpoint molecule B7-H3 (CD276) is a promising target structure for CAR-engineered NK-92 cell-based immunotherapy of pediatric NB. CD276-CAR NK-92 cells can be produced as a readily available “off-the-shelf” cellular product in a GMP-compliant and affordable fashion for the treatment of high-risk NB and potentially other solid cancers.

NB is a pediatric tumor derived from sympathoadrenal progenitor cells within the neural crest. As the most common extracranial neoplasm in childhood it accounts for 8-10% of all pediatric malignancies and more than 15% of cancer-related deaths in children are caused by NB.[107,108] Standard of care treatment for NB includes surgical removal, radio- and chemotherapy as well as adjuvant autologous stem cell transplantation.[22] Modern immunotherapeutic approaches include the use of mAb targeting the disialoganglioside GD2.[23] However, despite multi-factor therapy, overall survival of patients suffering from high-risk NB, which is characterized by spontaneous relapse, metastatic development and drug resistance, is below 50%.[109] Novel therapeutic options that tackle the major obstacles of solid tumor, and especially NB, therapy are urgently needed.

For solid tumors, one of the major challenges is the lack of a real tumor-specific antigen. Abundant surface expression of an antigen that is associated with a tumor entity but is also expressed on normal tissue could increase the risk of unwanted on-target off-tumor toxicities.[110] Furthermore, antigen expression within a tumor is often heterogeneous and most antigens are not broadly expressed across different tumor entities.

CD276 is an immune checkpoint molecule that plays a pivotal role in regulation of the innate and adaptive immune response and recently emerged as a prognostic marker for various solid tumors.[111–114] It was previously reported that CD276 mRNA is ubiquitously expressed in a variety of cells. Immunohistochemical (IHC) analysis, however, only showed weak or absent CD276 protein expression in normal tissue but high expression levels in solid tumors.[115] In this study, we analyzed various commercially available NB cell lines for the expression of CD276 using flow cytometry (Figure 2a). We found that all examined NB cell lines abundantly expressed CD276 on their surface. Next, formalin-fixed, paraffin-embedded NB tissue sections of patients with aggressive and/or recurrent disease were stained for CD276 expression using IHC. Four out of five samples showed a moderate or high cytoplasmic as well as surface membrane expression of CD276 (Figure 1).

To date, CD276 has been examined as target structure for various therapeutic approaches in pre-clinical and early phase clinical trials. One of the most promising antibody candidates is the radioactive, 131 iodine-labeled anti-CD276 mAb omburtamab that received FDA's breakthrough therapy designation in 2017 for the treatment of NB associated CNS metastases.

CD276-specific CAR T cells have shown promising results in pre-clinical trials assessing efficacy for the treatment of a variety of adult as well as pediatric tumors, including brain tumors, gastrointestinal carcinoma and melanoma.[116–119] Clinical CD276-CAR T cell studies are mainly in their early stages and no active trial focuses specifically on the treatment of NB. The majority of clinical CAR T cell studies on NB spotlight disialoganglioside GD2 as target structure. While GD2-CAR T cells have shown promising results in treatment of NB, several obstacles remain.[120] One of the major drawbacks of CAR T cell therapy is the occurrence of severe side effects such as neurotoxicity or cytokine release syndrome (CRS). Pre-clinical *in vivo* studies showed the induction of fatal neurotoxic events after treatment of NB with GD2-specific CAR T cells.[121,122] Moreover, autologous CAR T cells are generated from isolated peripheral blood lymphocytes. Here, isolation yield, transduction efficiency, T cell subtype distribution, and activation state can vary greatly, affecting overall product composition and quality.

As an alternative effector cell source, NK cells have become increasingly popular due to their innate potent anti-tumor activity and distinct safety profile in the allogeneic setting. Recently, CAR-modified primary NK cells targeting CD19 in patients with relapsed or refractory NHL or CLL demonstrated good clinical response without the induction of typical CAR T cell-associated side effects.[123] However, since genetic modification of primary NK cells is problematic with low transfection efficiencies and varying CAR functionality, the majority of CAR NK cell studies have been performed with the NK-92 cells.[124]

In the present study, we introduced a second generation CD276-targeting chimeric antigen receptor with a CD28 co-stimulatory domain to the NK-92 cell line. The anti-CD276 scFv is derived from clone "m851". Truncated CD34 is co-expressed as marker gene (Figure 3a). After transduction, cells were sorted using flow cytometry and multiple single-cell clones were cultivated and expanded. The population with the highest CAR expression was chosen for further experiments. Over the course of more than six months, CAR expression levels as well as NK-92 cell proliferation and viability remained stable (Figure 3b-d).

For further characterization CD276-CAR NK-92 cells were screened for the expression of a variety of activating and inhibitory NK cell receptors, as well as immune checkpoint molecules and chemokine receptors using flow cytometry, and compared to parental NK-92 cells as well as isolated primary NK cells (Figure 4a-c).

Compared to primary NK cells, CD276-CAR NK-92 cells showed slightly lower expression levels of DNAM-1 (CD226) and similar levels of NKG2D (CD314), two of the main activating NK receptors. Moreover, CD276-CAR NK-92 cells displayed high expression of CD94 and NKG2A (CD159a), two membrane receptors that form an immune checkpoint complex recognizing the human HLA-E peptide, which is often elevated in solid tumors, including the NB cell lines used in this study (Figure 2b).[125] Binding of CD94/NKG2A to HLA-E was shown to inhibit NK effector function in various tumor entities.[126] Expression of other immune checkpoint molecules, such as PD-1, TIM-3 or TIGIT, was not elevated in CD276-CAR NK-92 cells, compared to isolated NK cells. Also, CD276-CAR NK-92 cells as well as primary NK cells showed the expression of three major chemokine receptors, CD183 (CXCR3), CD184 (CXCR4) and CD197 (CCR7), which play a pivotal role in immune cell mobilization and trafficking.

For functional analysis CD276-CAR NK-92 cells were co-incubated with NB cell lines. Compared to parental NK-92 cells, specific lysis of NB cells significantly increased up to 80% after two hours (Figure 5a). Importantly, CD276-CAR NK-92 cells demonstrated target specificity since they did not induce any CAR-mediated lysis of the CD276-negative control cell line KG1a. Irradiation with 10 Gy immediately prior to application, as required in all clinical trials with NK-92 cells to fully inhibit proliferation, did not show impairment of CAR-mediated NB cell lysis. Over time, CD276-CAR NK 92 cells gradually underwent apoptosis and, subsequently, lost effector function (Figure 5b). Applicability of irradiated CAR NK-92 cells was already demonstrated in various *in vivo* xenograft models.[103,127,128] Using the xCELLigence real-time live cell analysis system, we confirmed that CD276-CAR NK-92 cells were able to induce strong, specific cytotoxicity of NB cell lines within the first two hours of application (Figure 5c). Moreover, CD276-CAR NK-92 cells were capable of eliminating a sufficient amount of NB cells to fully prevent tumor regrowth. Here, utilization of irradiated CD276-CAR NK-92 cells did not impair their cytotoxic efficacy and, likewise, did not allow for tumor regrowth. To further characterize CD276-CAR NK-92 function, a cytokine secretion profile was established to screen for the secretion of important cytokines during cytolysis, including NK cell effector molecules (Figure 5d). Upon addition of NB cells, compared to parental NK-92 cells, CD276-CAR NK-92 cells showed elevated

secretion of various interleukins as well as the pro-inflammatory molecules IFN-γ and TNF-α, which stimulates the immune system and indirectly enhance anti-tumor activity.[92] CAR-mediated NK cell effector function was based on perforin secretion but not granulysin or granzyme B release. Importantly, no elevated levels of IL-6, one of the driving forces of CRS induction, were detectable, thus, underscoring previous *in vivo* and clinical studies that found no induction of severe side effects, such as neurotoxicity or CRS, upon CAR NK-92 cell application.[64]

Since the assessment of NK effector function in classical *in vitro* assays is usually based on tumor cell monolayer culture systems, we developed a three-dimensional NB cell culture model, which is physiologically more relevant and predictive, thus, bridging the gap between *in vitro* and *in vivo* models.[129] Three-dimensional cultures of GFP-transduced NB cell lines were grown for four days and spheroid fluorescence was tracked using the Celigo S imaging cytometer. To evaluate cytotoxic potential of CD276-CAR NK-92 cells, they were co-incubated with NB spheroids and monitored for over 96 hours (Figure 6a-c). Compared to parental NK-92 cells, CAR NK-92 cells were able to efficiently lyse three-dimensional NB spheroids within 48-72 hours. Only for one cell line, CAR-mediated cytotoxicity was not sufficient to eradicate the spheroid over the given time period. In addition, we assessed whether γ-irradiation of CD276-CAR NK-92 cells prior to the experiment had a negative impact on NB spheroid lysis. Integrated fluorescence intensity of spheroids co-incubated with irradiated effector cells was decreased significantly less after 24 hours, compared to a non-irradiated control. However, by increasing effector-to-target (E:T) ratio, irradiated CD276-CAR NK-92 cells were able to completely lyse NB spheroids in a time frame comparable to non-irradiated CD276-CAR NK-92 cells (Figure 6d). Furthermore, introduction of fresh CD276-CAR NK-92 cells by sequential addition of irradiated effector cells increased CAR-mediated cytotoxic effector function and expanded the therapeutic window (Figure 6e). Subsequent steps for evaluation of CD276-CAR NK-92 cell function will include pre-clinical *in vivo* models and the assessment of CAR NK-92 cells in the context of the immunosuppressive TME.

In this study, we have generated CAR-modified NK-92 cells targeting the pan-cancer antigen CD276. The cells have shown efficient eradication of NB cell lines in *in vitro* monolayer cell cultures as well as three-dimensional spheroid models. CD276-CAR NK-92 cells may provide a powerful, novel “off the shelf” treatment option for high-risk NB and potentially other solid tumor entities.

8.4 Publication 4

In vitro Evaluation of CD276-CAR NK-92 Functionality, Migration and Invasion Potential in the Presence of Immune Inhibitory Factors of the Tumor Microenvironment

Genetic engineering of primary immune cells with CARs has generated powerful tools for adjuvant immunotherapy of hematological as well as solid malignancies. One of the major obstacles of solid tumor therapy, however, is the immunosuppressive tumor microenvironment that significantly impairs efficacy of CAR T as well as CAR NK cell treatment approaches.

In the present study, we report that combining the positive features of a CD276-CAR with the well-characterized NK-92 cell line generates a powerful cellular product with a strong resilience to some of the most prominent negative factors exerted by the TME that can be easily manufactured under GMP guidelines as a readily available, “off-the-shelf” treatment option for melanoma.

Malignant cutaneous melanoma is a highly aggressive tumor that, although it only accounts for around 4% of all skin cancers, is more lethal than any other skin cancer.[1,130] Clinical prognosis for patients diagnosed with early-stage melanoma is generally favorable. Outcome for patients with metastatic disease, however, is very poor with a five-year survival rate of approximately 10%.[17] Novel therapy options with protein kinase inhibitors targeting specific driver mutations and immune checkpoint inhibitors blocking the CTLA-4 and/or PD-1 signaling axis have revolutionized treatment of patients with advanced melanoma.[131] Non-responsiveness or acquired resistance to targeted therapy can be attributed to various tumor evasion mechanisms that also involve the highly immunosuppressive TME.[132,133] The TME consists of a variety of cells such as tumor-associated fibroblasts, macrophages, dendritic cells, neutrophils, T_{regs} and MDSCs as well as extracellular matrix (ECM) and secreted or cell membrane-presented molecules. The immunosuppressive properties of the TME allow tumor cells to escape immune-surveillance by impairing immune cell infiltration and cytotoxic effector function, thus supporting tumor growth and metastatic development.[30] CAR modification of primary immune cells has been shown to significantly boost immune effector function in solid malignancies, including melanoma.[134,135] However, major obstacles, such as the lack of

tumor specific antigens, the aforementioned hostile TME as well as insufficient immune cell homing and infiltration, still remain. Therefore, new advanced treatment strategies for therapy-resistant or metastatic melanoma are urgently needed.

CD276 (B7-H3) has recently emerged as a potential target structure. The immune checkpoint molecule is reported to be aberrantly overexpressed in solid tumors, including melanoma, while expression in normal tissue is rather weak.[112] In the present study, we have analyzed multiple commercially available melanoma cell lines for their CD276 expression levels using flow cytometry. All melanoma cell lines that were screened showed uniform abundance of CD276 surface expression (Figure 1).

In an earlier study, we have generated a CAR-modified NK-92 cell line targeting CD276 and successfully demonstrated its strong cytotoxic effector function against NB cells *in vitro*.[136] The primary goal of the present study was to broaden the potential therapeutic spectrum of CD276-CAR NK-92 cells and to evaluate whether melanoma cells can be successfully targeted as well. Secondly, we assessed whether CAR NK-92 cell-based immunotherapy can overcome obstacles posed by the TME in melanoma and lastly, we sought to increase cytotoxic immune cell function of CD276-CAR NK-92 cells by knocking out one of the few inhibitory NK cell receptors expressed by the NK-92 cell line, NKG2A (CD159a), using CRISPR/Cas9 technology.

Three out of the eight melanoma cell lines that were screened for CD276 expression, FM-3, Mel-Juso and WM115, were chosen as proof of principle for functional analysis of CD276-CAR NK-92 cells. The cell lines were first characterized for the surface expression of a variety of important NK cell ligands (Table 1) and, subsequently, co-incubated with the CAR NK-92 cells. After two hours upon co-incubation, significantly elevated CD276-CAR-mediated cytotoxicity could be demonstrated *in vitro* (Figure 2a-c). Even at low E:T ratios, up to 70% of the melanoma cells were lysed. Monitoring of tumor cell growth with the xCELLigence RTCA system demonstrated that upon addition of CD276-CAR NK-92 cells, immune effector function that occurred within the first two to three hours was sufficient to fully prevent tumor regrowth over a much longer period of time (Figure 2d-f). Moreover, irradiation of NK-92 cells did not significantly impact cytotoxicity. Furthermore, we underscored earlier studies by demonstrating that NK effector function was mainly carried out by release of cytotoxic granules with granulysin, granzyme B and perforin, whereas no induction of apoptosis occurred via FasL and TRAIL upregulation (Figure 2g-h).[106,136]

Utilizing a spheroid model, we assessed whether CD276-CAR NK-92 cells are equally effective in targeting three-dimensional melanoma cell cultures that can better mimic the *in vivo* situation and are therefore physiologically more relevant and predictive than standard two-dimensional cell culture assays (Figure 4).[129,137] Compared to parental NK-92 cells, CAR-engineered NK-92 cells were able to significantly increase tumor lysis of all melanoma spheroids and full eradication of one of the cell lines could be observed after only 48 hours. Interestingly, untransduced NK-92 cells seem to be able to effectively infiltrate tumor spheroids without the induction of specific tumor cell lysis. CD276-CAR NK-92 cells, however, demonstrated their high cytotoxic potential by lysing tumor spheroids from the outside, indicating that lysis of the spheroids is not dependent on complete infiltration (Figure 6a).

During melanoma progression, elevated levels of growth factor secretion and the subsequent growth of additional fibrous tissue surrounding the tumor site impair immune cell migration. To enhance immune cell migration, melanoma spheroids were supplemented with soluble C-X-C motif chemokine 12 (CXCL12), a highly effective chemoattractant expressed in, among others, melanoma cells which binds the C-X-C chemokine receptor type 4 (CXCR-4) that is expressed in NK-92 cells.[136,138] We could demonstrate that CD276-CAR NK-92 cells are able to successfully migrate through small pores of a trans-well system while retaining their cytotoxic efficacy (Figure 6b). The pre-treatment of NK-92 cells with prostaglandin E_2 (PGE2), in contrast to primary NK cells, did not impair migration or cytotoxic potential.[139]

The second aim of this study was to evaluate whether CD276-CAR NK-92 cells can overcome known factors of the TME, which have been shown to negatively affect cytotoxic potential of immune cells, including primary NK cells. The predominantly hypoxic and acidic conditions in the TME in combination with an anti-inflammatory cytokine milieu consisting of, among others, TGFβ can significantly impair CAR-mediated effector function.[140–142]

The hypoxic environment within solid tumors has been shown to downregulate activating NK cell receptors as well as the secretion of NK effector molecules such as perforin and granzyme B.[143,144] Hypoxic culture conditions for a short period of time reduced CD276-CAR NK-92 cell viability by approximately 30%. When NK-92 cells were deprived of oxygen for longer time periods, NK-92 cell viability was decreased up to 80%. Decreased viability of CAR NK-92 cells was proportional to reduced cytotoxic effector function (Figure 3a-c). Since NK-92 cells have to be irradiated for clinical use to prevent

uncontrolled *in vivo* proliferation and effective cytotoxicity is restricted to a 48-72 hour time window after irradiation, decreased immune effector function induced by long-term exposure to hypoxia (>72 hours) should not be a critical issue.

Soluble factors such as TGFβ not only play a pivotal role in metastasis formation and tumor escape during immunotherapy.[145,146] Elevated levels of TGFβ have been linked to upregulated expression of inhibitory receptors, downregulated expression of activating receptors and reduced immune effector function in primary NK cells.[36,37,147] CD276-CAR NK-92 cells did not show any significant decrease of CAR-mediated cytotoxicity after treatment with high concentrations of TGFβ even at low E:T ratios, which suggests that, in contrast to CAR NK cells, TGFβ seems to have no negative effect on CD276-CAR NK-92-mediated tumor lysis (Figure 3d-f). The acidic environment within tumors is caused by the induction of metabolic enzymes such as indoleamine 2,3-dioxygenase (IDO) and the increased secretion of lactate. Acidity in tumors has been associated with inhibited proliferation and reduced cytotoxicity of NK cells.[148,149] In contrast to primary NK cells, CD276-CAR NK-92 cells did not show impaired cytotoxicity or proliferation after pre-treatment with high concentrations of sodium L-lactate (Figure 3g-i). Lastly, we assessed whether treatment with soluble factors secreted by melanoma cells, such as TGFβ, IL-10, IDO, nitric oxide (NO) and prostaglandins, could also weaken immune effector function of NK-92 cells. These molecules are regularly secreted by tumor cells, especially melanoma, and have been shown to induce an immunosuppressive phenotype in primary immune cells.[150–152] Cultivation of NK-92 cells in cell culture supernatants that were harvested from fibroblasts and cancer-associated fibroblasts (CAF), that play a pivotal role in tumor neo-vascularization, had no significant impact on CAR-mediated tumor lysis while melanoma supernatants only slightly affected CD276-CAR NK-92 cell function (Figure 3j-o).[153,154]

Lastly, we assessed whether a pre-incubation under hypoxic conditions, in low pH environment, in melanoma cell culture supernatant or various combinations thereof could impair CD276-CAR NK-92 cytotoxic function against melanoma spheroids. Fluorescence images of the melanoma spheroids were taken regularly over 96 hours (Figure 5a-b) and integrated fluorescence intensity of spheroids co-incubated with NK-92 cells was compared to untreated control spheroids (Figure 5c-d). Interestingly, incubation in low pH slightly delayed CAR-mediated cytotoxicity. However, after 48 to 72 hours all spheroids co-incubated with CD276-CAR NK-92 cells, regardless of the respective pre-incubation, were completely eradicated and no tumor regrowth could be observed afterwards.

In the context of various TME-mediated immune escape strategies, HLA-E and HLA-G expression is often elevated in melanoma and all three melanoma cell lines showed surface expression of HLA-E (Table 1).[155] Since its respective receptor NKG2A is one of the few inhibitory receptors expressed by NK-92 cells, we generated five different NKG2A KO variants of the CD276-CAR NK-92 and parental NK-92 cell line using CRISPR/Cas9 technology (Figure 7a-c). The respective NK-92 cells with the most efficient KO were subsequently single-cell sorted to generate pure cell lines. However, no significant improvement of CAR-mediated cytotoxicity could be observed with the NKG2A KO cells or with CD276-CAR NK-92 cells that had been pre-treated with a blocking anti-NKG2A antibody suggesting that CAR-mediated effector function in CD276-CAR NK-92 cells is independent of inhibitory NK ligands.

The next step in the pre-clinical evaluation of CD276-CAR NK-92 cell function will be the establishment of various *in vivo* models, including patient-derived xenograft (PDX) and humanized mouse models, to validate the preliminary results acquired throughout this and earlier studies.

In summary, CAR-modified NK-92 cells could overcome the majority of the most prominent negative factors of the TME in two-dimensional as well as three-dimensional assays, which suggests that NK-92 cells might represent a superior CAR vehicle for the treatment of melanoma and solid tumors compared to primary immune cells. Combining the positive features of a CD276-CAR with the NK-92 cell line and its strong resilience to influences exerted by the TME generated a powerful cellular product that can be easily manufactured under GMP guidelines as a readily available, “off-the-shelf” treatment option for melanoma and other solid tumors.

9. ACKNOWLEDGEMENTS

I am extremely thankful to Prof. Rupert Handgretinger and Dr. Sabine Schleicher for the opportunity to pursue such incredible research for my PhD in their group. Their persistent support over the years has allowed me to grow personally as well as professionally. It has enabled me to present my research to other scientists at various international conferences and publish in scientific journals.

I would very much like to acknowledge Prof. Alexander Weber for agreeing to be my supervisor in such as spontaneous and straightforward manner.

Moreover, I am very grateful to all the laboratory staff, research assistants and various students that I have worked with during this thesis for their professional and personal input over the years. I value every second of it. Particular appreciation goes to Caroline Baden who, I am sure, will be an incredible physician in a few years.

Thankfully, the hole that was left by the death of my racing donkey Mickey has now been filled. Apparently, rumors of his demise have been greatly exaggerated. We came in search of answers but, this time, we did not leave empty-handed again.

Finally, I want to thank all my family, my dear girlfriend Kerstin and my friends. I will be eternally grateful for all the unconditional support and encouragement I have received ever since I can remember. I would not be the person I am without you. Vielen Dank für alles!

10. REFERENCES

1. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2020. *CA. Cancer J. Clin.* **70,** 7–30 (2020).
2. Dagenais, G. R., Leong, D. P., Rangarajan, S., Lanas, F., Lopez-Jaramillo, P., Gupta, R., Diaz, R., Avezum, A., Oliveira, G. B. F., Wielgosz, A., Parambath, S. R., Mony, P., Alhabib, K. F., Temizhan, A., Ismail, N., Chifamba, J., Yeates, K., Khatib, R., Rahman, O., Zatonska, K., Kazmi, K., Wei, L., Zhu, J., Rosengren, A., Vijayakumar, K., Kaur, M., Mohan, V., Yusufali, A. H., Kelishadi, R., Teo, K. K., Joseph, P. & Yusuf, S. Variations in common diseases, hospital admissions, and deaths in middle-aged adults in 21 countries from five continents (PURE): a prospective cohort study. *Lancet* **395,** 785–794 (2020).
3. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **0,** 1–41 (2021).
4. Cooper, G. M. in *The Cell: A Molecular Approach* (Sinauer Associates, 2000). at <https://www.ncbi.nlm.nih.gov/books/NBK9963/>
5. Ames, B. N., Gold, L. S. & Willett, W. C. The causes and prevention of cancer. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 5258–5265 (1995).
6. Doll, R. & Peto, R. The Causes of Cancer: Quantitative Estimates of Avoidable Risks of Cancer in the United States Today. *JNCI J. Natl. Cancer Inst.* **66,** 1192–1308 (1981).
7. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–674 (2011).
8. Fouad, Y. A. & Aanei, C. Revisiting the hallmarks of cancer. *American Journal of Cancer Research* **7,** 1016–1036 (2017).
9. Nabi, J. & Hohl, R. J. in *Pharmacology and Therapeutics* 945–949 (Elsevier, 2009). doi:10.1016/B978-1-4160-3291-5.50070-6
10. Banerjee, S., Parasramka, M. A. & Paruthy, S. B. in *Role of Nutraceuticals in Chemoresistance to Cancer* 297–324 (Elsevier, 2018). doi:10.1016/b978-0-12-812373-7.00015-2
11. Fisher, S. G. & Fisher, R. I. The epidemiology of non-Hodgkin's lymphoma. *Oncogene* **23,** 6524–6534 (2004).
12. Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. J. *Immunobiology*. (2005).
13. Küppers, R. Mechanisms of B-cell lymphoma pathogenesis. *Nature Reviews Cancer* **5,** 251–262 (2005).
14. Jerusalem, G., Hustinx, R., Beguin, Y. & Fillet, G. Evaluation of therapy for lymphoma. *Seminars in Nuclear Medicine* **35,** 186–196 (2005).
15. Erdmann, F., Lortet-Tieulent, J., Schüz, J., Zeeb, H., Greinert, R., Breitbart, E. W. & Bray, F. International trends in the incidence of malignant melanoma 1953-2008-are recent generations at higher or lower risk? *Int. J. Cancer* **132,** 385–400 (2013).
16. Berk-Krauss, J., Stein, J. A., Weber, J., Polsky, D. & Geller, A. C. New systematic therapies and trends in cutaneous melanoma deaths among US whites, 1986–

2016. *American Journal of Public Health* **110,** 731–733 (2020).

17. Sandru, A., Voinea, S., Panaitescu, E. & Blidaru, A. Survival rates of patients with metastatic malignant melanoma. *J. Med. Life* **7,** 572–576 (2014).

18. Balch, C. M., Gershenwald, J. E., Soong, S. J., Thompson, J. F., Atkins, M. B., Byrd, D. R., Buzaid, A. C., Cochran, A. J., Coit, D. G., Ding, S., Eggermont, A. M., Flaherty, K. T., Gimotty, P. A., Kirkwood, J. M., McMasters, K. M., Mihm, M. C., Morton, D. L., Ross, M. I., Sober, A. J. & Sondak, V. K. Final version of 2009 AJCC melanoma staging and classification. *J. Clin. Oncol.* **27,** 6199–6206 (2009).

19. Park, J. R., Eggert, A. & Caron, H. Neuroblastoma: Biology, Prognosis, and Treatment. *Hematology/Oncology Clinics of North America* **24,** 65–86 (2010).

20. Colon, N. C. & Chung, D. H. Neuroblastoma. *Advances in Pediatrics* **58,** 297–311 (2011).

21. Cohn, S. L., Pearson, A. D. J., London, W. B., Monclair, T., Ambros, P. F., Brodeur, G. M., Faldum, A., Hero, B., Iehara, T., Machin, D., Mosseri, V., Simon, T., Garaventa, A., Castel, V. & Matthay, K. K. The International Neuroblastoma Risk Group (INRG) classification system: An INRG task force report. *J. Clin. Oncol.* **27,** 289–297 (2009).

22. Yu, A. L., Gilman, A. L., Ozkaynak, M. F., London, W. B., Kreissman, S. G., Chen, H. X., Smith, M., Anderson, B., Villablanca, J. G., Matthay, K. K., Shimada, H., Grupp, S. A., Seeger, R., Reynolds, C. P., Buxton, A., Reisfeld, R. A., Gillies, S. D., Cohn, S. L., Maris, J. M. & Sondel, P. M. Anti-GD2 Antibody with GM-CSF, Interleukin-2, and Isotretinoin for Neuroblastoma. *N. Engl. J. Med.* **363,** 1324–1334 (2010).

23. Pinto, N. R., Applebaum, M. A., Volchenboum, S. L., Matthay, K. K., London, W. B., Ambros, P. F., Nakagawara, A., Berthold, F., Schleiermacher, G., Park, J. R., Valteau-Couanet, D., Pearson, A. D. J. & Cohn, S. L. Advances in risk classification and treatment strategies for neuroblastoma. *Journal of Clinical Oncology* **33,** 3008–3017 (2015).

24. Brady, S. W., Liu, Y., Ma, X., Gout, A. M., Hagiwara, K., Zhou, X., Wang, J., Macias, M., Chen, X., Easton, J., Mulder, H. L., Rusch, M., Wang, L., Nakitandwe, J., Lei, S., Davis, E. M., Naranjo, A., Cheng, C., Maris, J. M., Downing, J. R., Cheung, N. K. V., Hogarty, M. D., Dyer, M. A. & Zhang, J. Pan-neuroblastoma analysis reveals age- and signature-associated driver alterations. *Nat. Commun.* **11,** 1–13 (2020).

25. Fares, J., Fares, M. Y., Khachfe, H. H., Salhab, H. A. & Fares, Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduction and Targeted Therapy* **5,** 1–17 (2020).

26. Mundy, G. R. Metastasis to bone: Causes, consequences and therapeutic opportunities. *Nature Reviews Cancer* **2,** 584–593 (2002).

27. Chaffer, C. L. & Weinberg, R. A. A perspective on cancer cell metastasis. *Science* **331,** 1559–1564 (2011).

28. Massagué, J. & Obenauf, A. C. Metastatic colonization by circulating tumour cells. *Nature* **529,** 298–306 (2016).

29. Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., Psaila, B., Kaplan, R. N., Bromberg, J. F., Kang, Y., Bissell, M. J., Cox, T. R., Giaccia, A. J., Erler, J. T., Hiratsuka, S., Ghajar, C. M. & Lyden, D. Pre-metastatic niches: organ-specific homes for metastases. *Nat. Publ. Gr.* **17,** (2017).

30. Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19,** 1423–1437 (2013).

31. Hanahan, D. & Coussens, L. M. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell* **21,** 309–322 (2012).

32. Weber, C. E. & Kuo, P. C. The tumor microenvironment. *Surgical Oncology* **21,** 172–177 (2012).

33. Fridman, W. H., Pagès, F., Sautès-Fridman, C. & Galon, J. The immune contexture in human tumours: Impact on clinical outcome. *Nature Reviews Cancer* **12,** 298–306 (2012).

34. Pagès, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R., Mlecnik, B., Kirilovsky, A., Nilsson, M., Damotte, D., Meatchi, T., Bruneval, P., Cugnenc, P.-H., Trajanoski, Z., Fridman, W.-H. & Galon, J. Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. *N. Engl. J. Med.* **353,** 2654–2666 (2005).

35. Al-Shibli, K. I., Donnem, T., Al-Saad, S., Persson, M., Bremnes, R. M. & Busund, L. T. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin. Cancer Res.* **14,** 5220–5227 (2008).

36. Melaiu, O., Lucarini, V., Cifaldi, L. & Fruci, D. Influence of the Tumor Microenvironment on NK Cell Function in Solid Tumors. *Frontiers in Immunology* **10,** 3038 (2020).

37. Trotta, R., Col, J. D., Yu, J., Ciarlariello, D., Thomas, B., Zhang, X., Allard, J., Wei, M., Mao, H., Byrd, J. C., Perrotti, D. & Caligiuri, M. A. TGF-β Utilizes SMAD3 to Inhibit CD16-Mediated IFN-γ Production and Antibody-Dependent Cellular Cytotoxicity in Human NK Cells. *J. Immunol.* **181,** 3784–3792 (2008).

38. Castriconi, R., Carrega, P., Dondero, A., Bellora, F., Casu, B., Regis, S., Ferlazzo, G. & Bottino, C. Molecular mechanisms directing migration and retention of natural killer cells in human tissues. *Front. Immunol.* **9,** 1–14 (2018).

39. Martínez-Zaguilán, R., Seftor, E. A., Seftor, R. E. B., Chu, Y. W., Gillies, R. J. & Hendrix, M. J. C. Acidic pH enhances the invasive behavior of human melanoma cells. *Clin. Exp. Metastasis* **14,** 176–186 (1996).

40. Rofstad, E. K., Mathiesen, B., Kindem, K. & Galappathi, K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res.* **66,** 6699–6707 (2006).

41. Uyttenhove, C., Pilotte, L., Théate, I., Stroobant, V., Colau, D., Parmentier, N., Boon, T. & Van den Eynde, B. J. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med.* **9,** 1269–1274 (2003).

42. Nayyar, G., Chu, Y. & Cairo, M. S. Overcoming resistance to natural killer cell based immunotherapies for solid tumors. *Frontiers in Oncology* **9,** 51 (2019).

43. Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural innate and adaptive immunity to cancer. *Annu. Rev. Immunol* **29,** 235–71 (2011).

44. Burnet, F. M. The concept of immunological surveillance. *Prog Tumor Res.* **13,** 1–27 (1970).

45. Disis, M. L., Knutson, K. L., Schiffman, K., Rinn, K. & Mcneel, D. G. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res. Treat.* **62,** 245–252 (2000).

46. Van Der Bruggen, P., Zhang, Y., Chaux, P., Stroobant, V., Panichelli, C., Schultz, E. S., Chapiro, J., Van Den Eynde, B. J., Brasseur, F. & Boon, T. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol. Rev.* **188,** 51–64 (2002).

47. Hakomori, S. Tumor-associated carbohydrate antigens. *Ann. Rev. Immunol.* **2,** 103–126 (1984).

48. Chen, N., Li, X., Chintala, N. K., Tano, Z. E. & Adusumilli, P. S. Driving CARs on the uneven road of antigen heterogeneity in solid tumors. *Current Opinion in Immunology* **51,** 103–110 (2018).

49. Drake, C. G., Jaffee, E. & Pardoll, D. M. Mechanisms of Immune Evasion by Tumors. *Advances in Immunology* **90,** 51–81 (2006).

50. Raulet, D. H. Missing self recognition and self tolerance of natural killer (NK) cells. *Seminars in Immunology* **18,** 145–150 (2006).

51. Ljunggren, H. G. & Kärre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11,** 237–244 (1990).

52. Sidney, J., Peters, B., Frahm, N., Brander, C. & Sette, A. HLA class I supertypes: A revised and updated classification. *BMC Immunol.* **9,** 1–15 (2008).

53. Terunuma, H., Deng, X., Dewan, Z., Fujimoto, S. & Yamamoto, N. Potential Role of NK Cells in the Induction of Immune Responses: Implications for NK Cell–Based Immunotherapy for Cancers and Viral Infections. *Int. Rev. Immunol.* **27,** 93–110 (2008).

54. Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K. & Kershaw, M. H. Activating and inhibitory receptors of natural killer cells. *Immunology and Cell Biology* **89,** 216–224 (2011).

55. Xie, G., Dong, H., Liang, Y., Ham, J. D., Rizwan, R. & Chen, J. CAR-NK cells: A promising cellular immunotherapy for cancer. *EBioMedicine* **59,** 102975 (2020).

56. Becker, J. C., Dummer, R., Hartmann, A., Burg, G. & Schmidt, R. E. Shedding of ICAM-1 from human melanoma cell lines induced by IFN-gamma and tumor necrosis factor-alpha. Functional consequences on cell-mediated cytotoxicity. *J. Immunol.* **147,** 4398–4401 (1991).

57. Wu, S. Y., Fu, T., Jiang, Y. Z. & Shao, Z. M. Natural killer cells in cancer biology and therapy. *Molecular Cancer* **19,** 120 (2020).

58. Darmon, A. J., Nicholson, D. W. & Bleackley, R. C. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* **377,** 446–448 (1995).

59. Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K. & Nagata, S. Expression of the Fas ligand in cells of T Cell lineage. *J Immunol* **154,** 3806–3813 (2017).

60. Alderson, M. G., Tough, T. W., Davis-Smith, T., Braddy, S., Falk, B., Schooley, K. A., Goodwin, R. G., Smith, C. A., Ramsdell, F. & Lynch, D. H. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J. Exp. Med* **181,** 71–77 (1995).

61. Gong J H, Maki G & Klingemann H G. Characterization of a Human Cell Line (NK-92) With Phenotypical and Functional Characteristics of Activated Natural Killer Cells. *Leukemia* **4,** 652–658 (1994).

62. Klingemann, H., Boissel, L. & Toneguzzo, F. Natural killer cells for immunotherapy -

Advantages of the NK-92 cell line over blood NK cells. *Front. Immunol.* **7,** 1–7 (2016).

63. Tonn, T., Schwabe, D., Klingemann, H. G., Becker, S., Esser, R., Koehl, U., Suttorp, M., Seifried, E., Ottmann, O. G. & Bug, G. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* **15,** 1563–1570 (2013).

64. Tang, X., Yang, L., Li, Z., Nalin, A. P., Dai, H., Xu, T., Yin, J., You, F., Zhu, M., Shen, W., Chen, G., Zhu, X., Wu, D. & Yu, J. First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. *Am. J. Cancer Res.* **8,** 1899 (2018).

65. Arai, S., Meagher, R., Swearingen, M., Myint, H., Rich, E., Martinson, J. & Klingemann, H. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: A phase I trial. *Cytotherapy* **10,** 625–632 (2008).

66. Gross, G., Waks, T. & Eshhar, Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity (chimeric genes/antibody variable region). *Immunology* **86,** 10024–10028 (1989).

67. Schumacher, T. N. M. T-cell receptor gene therapy. *Nature* **2,** 512–519 (2002).

68. Clevers, H., Alarcon, B., Wileman, T. & Terhorst, C. The T-cell receptor/CD3 complex: a dynamic protein ensemble. *Ann. Rev. Immunol* **6,** 629–62 (1988).

69. Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334,** 395–402 (1988).

70. Palacios, E. H. & Weiss, A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23,** 7990–8000 (2004).

71. Wegener, A.-M. K., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F. & Malissen, B. The T cell receptor/CD3 complex Is composed of at least two autonomous transduction modules. *Cell* **66,** 63–95 (1992).

72. Winter, G. & Milstein, C. Man-made antibodies. *Nature* **349,** 293 (1991).

73. Eshhar, Z. Tumor-specific T-bodies: towards clinical application. *Cancer Immunol Immunother* **45,** 131–136 (1997).

74. Letourneur, F. & Klausner, R. D. T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor zeta family proteins. *Immunology* **88,** 8905–8909 (1991).

75. Salter, A. I., Ivey, R. G., Kennedy, J. J., Voillet, V., Rajan, A., Alderman, E. J., Voytovich, U. J., Lin, C., Sommermeyer, D., Liu, L., Whiteaker, J. R., Gottardo, R., Paulovich, A. G. & Riddell, S. R. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Sci. Signal.* **11,** 6753 (2018).

76. Chmielewski, M. & Abken, H. TRUCKs: The fourth generation of CARs. *Expert Opinion on Biological Therapy* **15,** 1145–1154 (2015).

77. Gross, G., Gorochov, G., Waks, T. & Eshhar, Z. Generation of effector T cells expressing chimeric T cell receptor with antibody type-specificity. *Transplant. Proc.* **21,** 127–130 (1989).

78. Dunbar, C. E. Blood's 70th anniversary: CARs on the Blood highway. *Blood* **128,** 1 (2016).

79. Iyer, R. K., Bowles, P. A., Kim, H. & Dulgar-Tulloch, A. Industrializing autologous adoptive immunotherapies: Manufacturing advances and challenges. *Frontiers in*

Medicine **5,** 1–9 (2018).

80. Munshi, N. C., Anderson, L. D., Shah, N., Madduri, D., Berdeja, J., Lonial, S., Raje, N., Lin, Y., Siegel, D., Oriol, A., Moreau, P., Yakoub-Agha, I., Delforge, M., Cavo, M., Einsele, H., Goldschmidt, H., Weisel, K., Rambaldi, A., Reece, D., Petrocca, F., Massaro, M., Connarn, J. N., Kaiser, S., Patel, P., Huang, L., Campbell, T. B., Hege, K. & San-Miguel, J. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. *N. Engl. J. Med.* **384,** 705–716 (2021).

81. Berdeja, J. G., Madduri, D., Usmani, S. Z., Jakubowiak, A., Agha, M., Cohen, A. D., Stewart, A. K., Hari, P., Htut, M., Lesokhin, A., Deol, A., Munshi, N. C., O'Donnell, E., Avigan, D., Singh, I., Zudaire, E., Yeh, T. M., Allred, A. J., Olyslager, Y., Banerjee, A., Jackson, C. C., Goldberg, J. D., Schecter, J. M., Deraedt, W., Zhuang, S. H., Infante, J., Geng, D., Wu, X., Carrasco-Alfonso, M. J., Akram, M., Hossain, F., Rizvi, S., Fan, F., Lin, Y., Martin, T. & Jagannath, S. Ciltacabtagene autoleucel, a B-cell maturation antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. *Lancet* **398,** 314–324 (2021).

82. Maude, S. L., Laetsch, T. W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M. R., Stefanski, H. E., Myers, G. D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K. L., Martin, P. L., Nemecek, E. R., Yanik, G. A., Peters, C., Baruchel, A., Boissel, N., Mechinaud, F., Balduzzi, A., Krueger, J., June, C. H., Levine, B. L., Wood, P., Taran, T., Leung, M., Mueller, K. T., Zhang, Y., Sen, K., Lebwohl, D., Pulsipher, M. A. & Grupp, S. A. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N. Engl. J. Med.* **378,** 439–448 (2018).

83. Pfefferle, A. & Huntington, N. D. You have got a fast car: Chimeric antigen receptor nk cells in cancer therapy. *Cancers (Basel).* **12,** 1–23 (2020).

84. Gust, J., Taraseviciute, A. & Turtle, C. J. Neurotoxicity Associated with CD19-Targeted CAR-T Cell Therapies. *CNS Drugs* **32,** 1091–1101 (2018).

85. Santomasso, B., Bachier, C., Westin, J., Rezvani, K. & Shpall, E. J. in *American Society of Clinical Oncology Educational Book* **39,** 433–444 (American Society of Clinical Oncology (ASCO), 2019).

86. Fiorenza, S., Ritchie, D. S., Ramsey, S. D., Turtle, C. J. & Roth, J. A. Value and affordability of CAR T-cell therapy in the United States. *Bone Marrow Transplantation* **55,** 1706–1715 (2020).

87. Depil, S., Duchateau, P., Grupp, S. A., Mufti, G. & Poirot, L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nature Reviews Drug Discovery* **19,** 185–199 (2020).

88. Hunter, B. D. & Jacobson, C. A. CAR T-Cell Associated Neurotoxicity: Mechanisms, Clinicopathologic Correlates, and Future Directions. *JNCI J. Natl. Cancer Inst.* **111,** 646–654 (2019).

89. Klingemann, H. Are natural killer cells superior CAR drivers? *Oncoimmunology* **3,** e28147 (2014).

90. Oberschmidt, O., Kloess, S. & Koehl, U. Redirected primary human chimeric antigen receptor natural killer cells as an 'off-the-shelf immunotherapy' for improvement in cancer treatment. *Frontiers in Immunology* **8,** 654 (2017).

91. Lupo, K. B. & Matosevic, S. Natural Killer Cells as Allogeneic Effectors in Adoptive Cancer Immunotherapy. *Cancers (Basel).* **11,** 769 (2019).

92. Zhang, C., Oberoi, P., Oelsner, S., Waldmann, A., Lindner, A., Tonn, T. & Wels, W. S. Chimeric antigen receptor-engineered NK-92 cells: An off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. *Front. Immunol.* **8,** 1–17 (2017).

93. Shimasaki, N., Jain, A. & Campana, D. NK cells for cancer immunotherapy. *Nature Reviews Drug Discovery* **19,** 200–218 (2020).

94. Burger, M. C., Zhang, C., Harter, P. N., Romanski, A., Strassheimer, F., Senft, C., Tonn, T., Steinbach, J. P. & Wels, W. S. CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy. *Frontiers in Immunology* **10,** 2683 (2019).

95. Zhang, C., Burger, M. C., Jennewein, L., Genßler, S., Schönfeld, K., Zeiner, P., Hattingen, E., Harter, P. N., Mittelbronn, M., Tonn, T., Steinbach, J. P. & Wels, W. S. ErbB2/HER2-Specific NK Cells for Targeted Therapy of Glioblastoma. *J. Natl. Cancer Inst.* **108,** 1–12 (2016).

96. Kaiser, A., Mittelstaet, J., Huppert, V., Miltenyi, S., Schlegel, P., Seitz, C., Lang, P. & Handgretinger, R. Adapter Chimeric Antigen Receptor Expressing Cells for Targeting of Multiple Antigens - EP3315511A1. *Eur. Pat. Off.* (2018).

97. Chapman-Smith, A. & Cronan, J. E. The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. *Trends Biochem. Sci.* **24,** 359–363 (1999).

98. Báez-Saldaña, A., Zendejas-Ruiz, I., Revilla-Monsalve, C., Islas-Andrade, S., Cárdenas, A., Rojas-Ochoa, A., Vilches, A. & Fernandez-Mejia, C. Effects of biotin on pyruvate carboxylase, acetyl-CoA carboxylase, propionyl-CoA carboxylase, and markers for glucose and lipid homeostasis in type 2 diabetic patients and nondiabetic subjects. *Am. J. Clin. Nutr.* **79,** 238–43 (2004).

99. Darowski, D., Kobold, S., Jost, C. & Klein, C. Combining the best of two worlds: highly flexible chimeric antigen receptor adaptor molecules (CAR-adaptors) for the recruitment of chimeric antigen receptor T cells. *MAbs* **11,** 621–631 (2019).

100. Mitwasi, N., Feldmann, A., Arndt, C., Koristka, S., Berndt, N., Jureczek, J., Loureiro, L. R., Bergmann, R., Máthé, D., Hegedüs, N., Kovács, T., Zhang, C., Oberoi, P., Jäger, E., Seliger, B., Rössig, C., Temme, A., Eitler, J., Tonn, T., Schmitz, M., Hassel, J. C., Jäger, D., Wels, W. S. & Bachmann, M. "UniCAR"-modified off-the-shelf NK-92 cells for targeting of GD2-expressing tumour cells. *Sci. Rep.* **10,** 1–16 (2020).

101. Lohmueller, J. J., Ham, J. D., Kvorjak, M. & Finn, O. J. mSA2 affinity-enhanced biotin-binding CAR T cells for universal tumor targeting. *Oncoimmunology* **7,** 1–6 (2017).

102. Gargett, T. & Brown, M. P. The inducible caspase-9 suicide gene system as a 'safety switch' to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. *Front. Pharmacol.* **5,** 235 (2014).

103. Oelsner, S., Waldmann, A., Billmeier, A., Röder, J., Lindner, A., Ullrich, E., Marschalek, R., Dotti, G., Jung, G., Große-Hovest, L., Oberoi, P., Bader, P. & Wels, W. S. Genetically engineered CAR NK cells display selective cytotoxicity against FLT3-positive B-ALL and inhibit in vivo leukemia growth. *Int. J. Cancer* **145,** 1935–1945 (2019).

104. Topp, M. S., Gökbuget, N., Zugmaier, G., Degenhard, E., Goebeler, M. E., Klinger, M., Neumann, S. A., Horst, H. A., Raff, T., Viardot, A., Stelljes, M., Schaich, M., Köhne-Volland, R., Brüggemann, M., Ottmann, O. G., Burmeister, T., Baeuerle, P.

A., Nagorsen, D., Schmidt, M., Einsele, H., Riethmüller, G., Kneba, M., Hoelzer, D., Kufer, P. & Bargou, R. C. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. *Blood* **120,** 5185–5187 (2012).

105. Topp, M. S., Gökbuget, N., Zugmaier, G., Klappers, P., Stelljes, M., Neumann, S., Viardot, A., Marks, R., Diedrich, H., Faul, C., Reichle, A., Horst, H.-A., Brüggemann, M., Wessiepe, D., Holland, C., Alekar, S., Mergen, N., Einsele, H., Hoelzer, D., Bargou Max S Topp, R. C., Bargou, R. C. & Würzburg, U. Phase II Trial of the Anti-CD19 Bispecific T Cell-Engager Blinatumomab Shows Hematologic and Molecular Remissions in Patients With Relapsed or Refractory B-Precursor Acute Lymphoblastic Leukemia. *J Clin Oncol* **32,** 4134–4140 (2014).

106. Grote, S., Mittelstaet, J., Baden, C., Chan, K. C.-H., Seitz, C., Schlegel, P., Kaiser, A., Handgretinger, R. & Schleicher, S. Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting. *Oncoimmunology* **9,** 1825177 (2020).

107. Matthay, K. K., Maris, J. M., Schleiermacher, G., Nakagawara, A., Mackall, C. L., Diller, L. & Weiss, W. A. Neuroblastoma. *Nat. Rev. Dis. Prim.* **2,** (2016).

108. Maris, J. M. Recent advances in neuroblastoma. *New England Journal of Medicine* **362,** 2202 (2010).

109. Morgenstern, D. A., London, W. B., Stephens, D., Volchenboum, S. L., Simon, T., Nakagawara, A., Shimada, H., Schleiermacher, G., Matthay, K. K., Cohn, S. L., Pearson, A. D. J. & Irwin, M. S. Prognostic significance of pattern and burden of metastatic disease in patients with stage 4 neuroblastoma: A study from the International Neuroblastoma Risk Group database. *Eur. J. Cancer* **65,** 1–10 (2016).

110. Newick, K., O'Brien, S., Moon, E. & Albelda, S. M. CAR T Cell Therapy for Solid Tumors. *Annu. Rev. Med.* **68,** 139–152 (2017).

111. Flem-Karlsen, K., Tekle, C., Andersson, Y., Flatmark, K., Fodstad, Ø. & Nunes-Xavier, C. E. Immunoregulatory protein B7-H3 promotes growth and decreases sensitivity to therapy in metastatic melanoma cells. *Pigment Cell Melanoma Res.* **30,** 467–476 (2017).

112. Flem-Karlsen, K., Fodstad, O. & Nunes-Xavier, C. E. B7-H3 immune checkpoint protein in human cancer. *Curr. Med. Chem.* **26,** 1–20 (2019).

113. Castellanos, J. R., Purvis, I. J., Labak, C. M., Guda, M. R., Tsung, A. J., Velpula, K. K. & Asuthkar, S. B7-H3 role in the immune landscape of cancer. *Am. J. Clin. Exp. Immunol.* **6,** 66–75 (2017).

114. Lee, Y. H., Martin-Orozco, N., Zheng, P., Li, J., Zhang, P., Tan, H., Park, H. J., Jeong, M., Chang, S. H., Kim, B. S., Xiong, W., Zang, W., Guo, L., Liu, Y., Dong, Z. J., Overwijk, W. W., Hwu, P., Yi, Q., Kwak, L., Yang, Z., Mak, T. W., Li, W., Radvanyi, L. G., Ni, L., Liu, D. & Dong, C. Inhibition of the B7-H3 immune checkpoint limits tumor growth by enhancing cytotoxic lymphocyte function. *Cell Res.* **27,** 1034–1045 (2017).

115. Zhang, H., Zhang, J., Li, C., Xu, H., Dong, R., Chen, C. C. & Hua, W. Survival Association and Cell Cycle Effects of B7H3 in Neuroblastoma. *J. Korean Neurosurg. Soc.* **86,** 1–10 (2020).

116. Huang, B., Luo, L., Wang, J., He, B., Feng, R., Xian, N., Zhang, Q., Chen, L. & Huang, G. B7-H3 specific T cells with chimeric antigen receptor and decoy PD-1 receptors eradicate established solid human tumors in mouse models. *Oncoimmunology* **9,** 1684127 (2020).

117. Majzner, R. G., Theruvath, J. L., Nellan, A., Heitzeneder, S., Cui, Y., Mount, C. W., Rietberg, S. P., Linde, M. H., Xu, P., Rota, C., Sotillo, E., Labanieh, L., Lee, D. W., Orentas, R. J., Dimitrov, D. S., Zhu, Z., Croix, B. S., Delaidelli, A., Sekunova, A., Bonvini, E., Mitra, S. S., Quezado, M. M., Majeti, R., Monje, M., Sorensen, P. H. B., Maris, J. M. & Mackall, C. L. CAR T cells targeting B7-H3, a pan-cancer antigen, demonstrate potent preclinical activity against pediatric solid tumors and brain tumors. *Clin. Cancer Res.* **25,** 2560–2574 (2019).

118. Nehama, D., Di Ianni, N., Musio, S., Du, H., Patané, M., Pollo, B., Finocchiaro, G., Park, J. J. H., Dunn, D. E., Edwards, D. S., Damrauer, J. S., Hudson, H., Floyd, S. R., Ferrone, S., Savoldo, B., Pellegatta, S. & Dotti, G. B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres. *EBioMedicine* **47,** 33–43 (2019).

119. Theruvath, J., Sotillo, E., Mount, C. W., Graef, C. M., Delaidelli, A., Heitzeneder, S., Labanieh, L., Dhingra, S., Leruste, A., Majzner, R. G., Xu, P., Mueller, S., Yecies, D. W., Finetti, M. A., Williamson, D., Johann, P. D., Kool, M., Pfister, S., Hasselblatt, M., Frühwald, M. C., Delattre, O., Surdez, D., Bourdeaut, F., Puget, S., Zaidi, S., Mitra, S. S., Cheshier, S., Sorensen, P. H., Monje, M. & Mackall, C. L. Locoregionally administered B7-H3-targeted CAR T cells for treatment of atypical teratoid/rhabdoid tumors. *Nat. Med.* **26,** 712–719 (2020).

120. Sait, S. & Modak, S. Anti-GD2 immunotherapy for neuroblastoma. *Expert Review of Anticancer Therapy* **17,** 889–904 (2017).

121. Richman, S. A., Nunez-Cruz, S., Moghimi, B., Li, L. Z., Gershenson, Z. T., Mourelatos, Z., Barrett, D. M., Grupp, S. A. & Milone, M. C. High-Affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. *Cancer Immunol. Res.* **6,** 36–46 (2018).

122. Majzner, R. G., Weber, E. W., Lynn, R. C., Xu, P. & Mackall, C. L. Neurotoxicity associated with a high-affinity GD2 CAR. *Cancer Immunology Research* **6,** 494–495 (2018).

123. Liu, E., Marin, D., Banerjee, P., Macapinlac, H. A., Thompson, P., Basar, R., Nassif Kerbauy, L., Overman, B., Thall, P., Kaplan, M., Nandivada, V., Kaur, I., Nunez Cortes, A., Cao, K., Daher, M., Hosing, C., Cohen, E. N., Kebriaei, P., Mehta, R., Neelapu, S., Nieto, Y., Wang, M., Wierda, W., Keating, M., Champlin, R., Shpall, E. J. & Rezvani, K. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. *N. Engl. J. Med.* **382,** 545–553 (2020).

124. Shimasaki, N., Fujisaki, H., Cho, D., Masselli, M., Lockey, T., Eldridge, P., Leung, W. & Campana, D. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy* **14,** 830–840 (2012).

125. Allard, M., Oger, R., Vignard, V., Percier, J. M., Fregni, G., Périer, A., Caignard, A., Charreau, B., Bernardeau, K., Khammari, A., Dréno, B. & Gervois, N. Serum soluble HLA-E in Melanoma: A new potential immune-related marker in cancer. *PLoS One* **6,** e21118 (2011).

126. Kamiya, T., Seow, S. V., Wong, D., Robinson, M. & Campana, D. Blocking expression of inhibitory receptor NKG2A overcomes tumor resistance to NK cells. *J. Clin. Invest.* **129,** 2094–2106 (2019).

127. Nowakowska, P., Romanski, A., Miller, N., Odendahl, M., Bonig, H., Zhang, C., Seifried, E., Wels, W. S. & Tonn, T. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. *Cancer Immunol. Immunother.* **67,** 25–38 (2018).

128. Tonn, T., Becker, S., Esser, R., Schwabe, D. & Seifried, E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. *J. Hematotherapy Stem Cell Res.* **10,** 535–544 (2001).

129. Vinci, M., Gowan, S., Boxall, F., Patterson, L., Zimmermann, M., Court, W., Lomas, C., Mendiola, M., Hardisson, D. & Eccles, S. A. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol.* **10,** 29 (2012).

130. Gershenwald, J. E. & Guy, G. P. Stemming the Rising Incidence of Melanoma: Calling Prevention to Action. *JNCI J. Natl. Cancer Inst.* **108,** 381 (2016).

131. Ott, P. A., Hodi, F. S. & Robert, C. CTLA-4 and PD-1/PD-L1 blockade: New immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin. Cancer Res.* **19,** 5300–5309 (2013).

132. Koyama, S., Akbay, E. A., Li, Y. Y., Herter-Sprie, G. S., Buczkowski, K. A., Richards, W. G., Gandhi, L., Redig, A. J., Rodig, S. J., Asahina, H., Jones, R. E., Kulkarni, M. M., Kuraguchi, M., Palakurthi, S., Fecci, P. E., Johnson, B. E., Janne, P. A., Engelman, J. A., Gangadharan, S. P., Costa, D. B., Freeman, G. J., Bueno, R., Hodi, F. S., Dranoff, G., Wong, K. K. & Hammerman, P. S. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat. Commun.* **7,** 1–9 (2016).

133. Pitt, J. M., Vétizou, M., Daillère, R., Roberti, M. P., Yamazaki, T., Routy, B., Lepage, P., Boneca, I. G., Chamaillard, M., Kroemer, G. & Zitvogel, L. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. *Immunity* **44,** 1255–1269 (2016).

134. Du, H., Hirabayashi, K., Ahn, S., Kren, N. P., Montgomery, S. A., Wang, X., Tiruthani, K., Mirlekar, B., Michaud, D., Greene, K., Herrera, S. G., Xu, Y., Sun, C., Chen, Y., Ma, X., Ferrone, C. R., Pylayeva-Gupta, Y., Yeh, J. J., Liu, R., Savoldo, B., Ferrone, S. & Dotti, G. Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 via Chimeric Antigen Receptor T Cells. *Cancer Cell* **35,** 221-237.e8 (2019).

135. Zhang, Z., Jiang, C., Liu, Z., Yang, M., Tang, X., Wang, Y., Zheng, M., Huang, J., Zhong, K., Zhao, S., Tang, M., Zhou, T., Yang, H., Guo, G., Zhou, L., Xu, J. & Tong, A. B7-H3-Targeted CAR-T Cells Exhibit Potent Antitumor Effects on Hematologic and Solid Tumors. *Mol. Ther. - Oncolytics* **17,** 180–189 (2020).

136. Grote, S., Chan, K. C., Baden, C., Bösmüller, H., Sulyok, M., Frauenfeld, L., Ebinger, M., Handgretinger, R. & Schleicher, S. CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma. *Adv. Cell Gene Ther.* **4,** 15 (2021).

137. Grote, S., Traub, F., Mittelstaet, J., Seitz, C., Kaiser, A., Handgretinger, R. & Schleicher, S. Adapter Chimeric Antigen Receptor (AdCAR)-Engineered NK-92 Cells for the Multiplex Targeting of Bone Metastases. *Cancers (Basel).* **13,** 1124 (2021).

138. Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A. & Springer, T. A. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* **184,** 1101–1109 (1996).

139. Singh, T. & Katiyar, S. K. Honokiol Inhibits Non-Small Cell Lung Cancer Cell Migration by Targeting PGE2-Mediated Activation of β-Catenin Signaling. *PLoS One* **8,** e60749 (2013).

140. Ivey, J. W., Bonakdar, M., Kanitkar, A., Davalos, R. V. & Verbridge, S. S. Improving cancer therapies by targeting the physical and chemical hallmarks of the tumor

microenvironment. *Cancer Lett.* **380,** 330–339 (2016).

141. Kato, Y., Ozawa, S., Miyamoto, C., Maehata, Y., Suzuki, A., Maeda, T. & Baba, Y. Acidic extracellular microenvironment and cancer. *Cancer Cell Int.* **13,** 1 (2013).

142. Almeida, F. V., Douglass, S. M., Fane, M. E. & Weeraratna, A. T. Bad company: Microenvironmentally mediated resistance to targeted therapy in melanoma. *Pigment Cell and Melanoma Research* **32,** 237–247 (2019).

143. Sceneay, J., Chow, M. T., Chen, A., Halse, H. M., Wong, C. S. F., Andrews, D. M., Sloan, E. K., Parker, B. S., Bowtell, D. D., Smyth, M. J. & Möller, A. Primary tumor hypoxia recruits CD11b+/Ly6Cmed/ Ly6G+ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche. *Cancer Res.* **72,** 3906–3911 (2012).

144. Petrova, V., Annicchiarico-Petruzzelli, M., Melino, G. & Amelio, I. The hypoxic tumour microenvironment. *Oncogenesis* **7,** 10 (2018).

145. Xie, F., Ling, L., Van Dam, H., Zhou, F. & Zhang, L. TGF-β signaling in cancer metastasis. *Acta Biochim. Biophys. Sin. (Shanghai).* **50,** 121–132 (2018).

146. Wu, Y., Kuang, D. M., Pan, W. D., Wan, Y. Le, Lao, X. M., Wang, D., Li, X. F. & Zheng, L. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology* **57,** 1107–1116 (2013).

147. Castriconi, R., Cantoni, C., Della Chiesa, M., Vitale, M., Marcenaro, E., Conte, R., Biassoni, R., Bottino, C., Moretta, L. & Moretta, A. Transforming growth factor β1 inhibits expression of NKP30 and NKG2d receptors: Consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4120–4125 (2003).

148. Xie, H., Hanai, J. I., Ren, J. G., Kats, L., Burgess, K., Bhargava, P., Signoretti, S., Billiard, J., Duffy, K. J., Grant, A., Wang, X., Lorkiewicz, P. K., Schatzman, S., Bousamra, M., Lane, A. N., Higashi, R. M., Fan, T. W. M., Pandolfi, P. P., Sukhatme, V. P. & Seth, P. Targeting lactate dehydrogenase-A inhibits tumorigenesis and tumor progression in mouse models of lung cancer and impacts tumor-initiating cells. *Cell Metab.* **19,** 795–809 (2014).

149. Husain, Z., Huang, Y., Seth, P. & Sukhatme, V. P. Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells. *J. Immunol.* **191,** 1486–1495 (2013).

150. Enk, A. H. Dendritic cells in tolerance induction. *Immunology Letters* **99,** 8–11 (2005).

151. Clark, D. A. & Coker, R. Molecules in focus Transforming growth factor-beta (TGF-β). *Int. J. Biochem. Cell Biol.* **30,** 293–298 (1998).

152. Umansky, V. & Sevko, A. Melanoma-induced immunosuppression and its neutralization. *Seminars in Cancer Biology* **22,** 319–326 (2012).

153. Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H. & Takeyama, H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers (Basel).* **7,** 2443–2458 (2015).

154. Hutchenreuther, J., Vincent, K., Norley, C., Racanelli, M., Gruber, S. B., Johnson, T. M., Fullen, D. R., Raskin, L., Perbal, B., Holdsworth, D. W., Postovit, L. M. & Leask, A. Activation of cancer-associated fibroblasts is required for tumor neovascularization in a murine model of melanoma. *Matrix Biol.* **74,** 52–61 (2018).

155. Borst, L., van der Burg, S. H. & van Hall, T. The NKG2A–HLA-E Axis as a Novel

References

Checkpoint in the Tumor Microenvironment. *Clin. Cancer Res.* **26,** 5549–5556 (2020).

11. FIGURES AND TABLES

11.1 LIST OF FIGURES

11.2 LIST OF TABLES

12. APPENDICES

12.1 Curriculum vitae

12.2 Publications

OncoImmunology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/koni20

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting

Stefan Grote , Joerg Mittelstaet , Caroline Baden , Kenneth Chun-Ho Chan , Christian Seitz , Patrick Schlegel , Andrew Kaiser , Rupert Handgretinger & Sabine Schleicher

To cite this article: Stefan Grote , Joerg Mittelstaet , Caroline Baden , Kenneth Chun-Ho Chan , Christian Seitz , Patrick Schlegel , Andrew Kaiser , Rupert Handgretinger & Sabine Schleicher (2020) Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting, OncoImmunology, 9:1, 1825177, DOI: 10.1080/2162402X.2020.1825177

To link to this article: https://doi.org/10.1080/2162402X.2020.1825177

Published online: 29 Sep 2020.

ONCOIMMUNOLOGY
2020, VOL. 9, NO. 1, e1825177 (11 pages)
https://doi.org/10.1080/2162402X.2020.1825177

Taylor & Francis
Taylor & Francis Group

BRIEF REPORT

Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: an off-the-shelf cellular therapeutic for universal tumor targeting

Stefan Grote[a], Joerg Mittelstaet[b], Caroline Baden[a], Kenneth Chun-Ho Chan[a], Christian Seitz[a], Patrick Schlegel[a], Andrew Kaiser[b], Rupert Handgretinger[a], and Sabine Schleicher[a]

[a]Department of Hematology and Oncology, University Hospital Tuebingen, Children's Hospital, Tuebingen, Germany; [b]Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

ABSTRACT

Despite the recent success of CAR T cells targeting CD19 and CD22 in hematological malignancies, the production of CAR T cells still requires an extensive manufacturing process. The well-established NK-92 cell line provides a promising alternative to produce CAR-modified effector cells in a GMP-compliant, cost-effective way. NK-92 can be redirected against a variety of surface antigens by our adapter CAR (AdCAR) system utilizing biotinylated antibodies (bAb) as adapter molecules. Selected bAb were capable of inducing significant AdCAR NK-92-mediated lysis of non-Hodgkin lymphoma (NHL) and mantle-cell lymphoma (MCL) cell lines as well as primary MCL and chronic lymphocytic leukemia (CLL) cells. AdCAR specificity was proven using a JeKo-1 CD19/CD20 knockout antigen-loss model. Moreover, through combinations of bAb, AdCAR NK-92 cells are capable of combatting tumor antigen evasion mechanisms. In conclusion, we successfully generated the AdCAR NK-92 cell line which can be manufactured as an "off-the-shelf, on-demand" product allowing universal and tunable tumor targeting.

ARTICLE HISTORY
Received 10 June 2020
Revised 10 September 2020
Accepted 10 September 2020

KEYWORDS
NK-92; chimeric antigen receptors; universal tumor targeting; adapter

Introduction

First-generation chimeric antigen receptors (CARs) are synthetically designed receptors that connect the antigen-binding region of a monoclonal antibody (mAb), typically a single-chain variable fragment (scFv), with an intracellular CD3ζ signaling domain through a T cell receptor transmembrane domain.[1–5] Second- and third-generation CARs consist of additional intracellular co-stimulatory domains, such as 4–1BB (CD137), CD28 or OX40.[6] When a CAR binds to its respective target antigen, T cell activation is initiated leading to immune effector cell proliferation, cytokine secretion and target cell lysis in an MHC-independent manner.[2] Currently, all FDA-approved CAR products are second-generation CAR T cells used to treat a variety of high-grade or refractory hematological malignancies.[4,7–9] Despite the recent clinical success of CAR T cell therapies, several obstacles and challenges remain.

The manufacturing process of CAR T cell products is very time-consuming and costly. Furthermore, the quality of patient PBMC can vary greatly and the amount required for production cannot always be met.[10,11] Especially for pediatric or heavily pretreated patients, it is often difficult to collect enough lymphocytes with a single round of leukapheresis.[12–15] Long-term persistence of CAR T cells can lead to severe neurotoxicity and cytokine release syndrome (CRS).[16,17] It was recently reported that NK cells as an allogeneic product with a better safety profile could prove to be a potent alternative effector cell source for CAR therapy.[18,19] CAR modification of peripheral blood as well as umbilical cord NK cells has been extensively studied recently with promising results for the targeting of hematological malignancies, first and foremost using CD19 as target structure.[20–23]

However, similar to primary T cells, expansion capacity varies greatly for NK cells from peripheral blood, and generally, transfection efficiency is especially low which makes CAR NK production challenging.[24,25] Although it was shown that retroviral modification of primary NK cells to express genes that encode interleukin-15 increased *in vivo* expansion as well as persistence, Jeff Miller and colleagues demonstrated that persistence of adoptively transferred NK cells, which is usually very low, is strongly dependent on the conditioning regiment that the patients received.[19,26,27]

In our study, we used a well-established NK cell line, NK-92, for modification with a CAR. Continuously expandable NK-92 cells can be easily manufactured in a GMP-compliant manner. Irradiated NK-92 cells were shown to be safe in clinical trials even at high intravenous doses of up to 10^{10} cells.[28] Additionally, the efficacy of CAR-engineered NK-92 cells in various pre-clinical studies has been previously shown, and, to date, only few active clinical trials for the treatment of cancer involve CAR NK-92 cells.[20,28–30] Although *in vivo* persistence due to irradiation safety requirements is low, the superb *ex vivo* expansion potential to high cell numbers makes NK-92 an ideal platform for the generation of CAR-engineered immune cells.[26,31]

The majority of current CAR therapy strategies target a single-specific tumor antigen which restricts CAR T or CAR NK cells as a potential therapy option for heterogeneous

CONTACT Stefan Grote stefan.grote@med.uni-tuebingen.de Department of Hematology and Oncology, University Hospital Tuebingen, Children's Hospital, Tuebingen, Germany

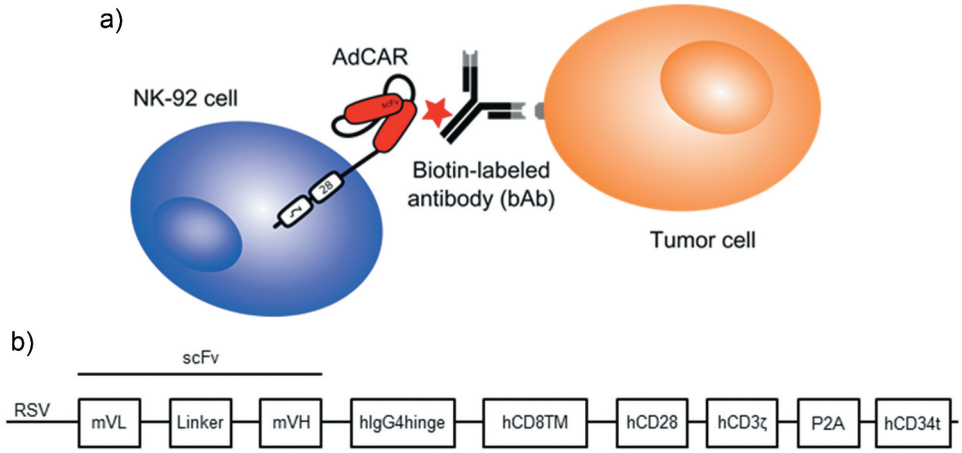


Figure 1. Schematic representation of AdCAR NK-92 function and structure. The NK-92 cells were generated with an adapter CAR recognizing a neoepitope on biotinylated antibodies which are directed against surface antigens on tumor cells (a). Schematic representation of the lentiviral transfer plasmid encoding the AdCAR construct comprising a CD28 co-stimulatory domain and a CD34 tag sequence (b), which was provided by Miltenyi Biotec.

tumor entities and also leaves them vulnerable to classic tumor antigen evasion mechanisms.[32,33] Patients with B cell malignancies, for example, being treated with anti-CD19 CAR T cells show high initial response rates, however, also high relapse rates with reemerging CD19$^-$ tumor cell subpopulations.[34] In order to tackle these obstacles, the idea of modular CARs targeting multiple tumor-specific antigens was proposed.[32]

Our recently developed adapter CAR (AdCAR) technology utilizes biotin-labeled antibodies (bAb) as adapter molecules (AM) to translate tumor-specific antigen recognition into immune effector cell activation. The AdCAR is based on the unique properties of a novel scFv targeting a "neo"-epitope-like structure consisting of the endogenous vitamin biotin in the context of monoclonal antibodies as linkers, referred to as linker-label-epitope (LLE), whose context is introduced in the patent application EP3315511A1.[35–37]

NK-92 cell-mediated target cell lysis is the result of a two-step process: antibody-specific binding to the target cell surface and binding of the AdCAR-modified NK-92 cells to the bAb (Figure 1a). AdCAR specificity relies solely on the bAb used, leading to almost unlimited possibilities in tumor antigen targeting.

Combining the flexible targeting and controllability of the AdCAR with the "off-the-shelf" properties of the NK-92 cells led to the creation of a universal, on-demand cellular CAR NK product which was tested with primary lymphoma cells, different lymphoma cell lines and a lymphoma antigen-loss model.

Methods

Design of the AdCAR system

The second-generation adapter CAR is based on the mAb "mBio3"-derived single-chain variable fragment (scFv). Further, it comprises an IgG4 hinge domain, a CD8 transmembrane domain, CD28 co-stimulatory, as well as CD3ζ signaling domains. Truncated CD34 (tCD34) is co-expressed after a furin P2A site for detection and enrichment.

Generation of AdCAR-construct and lentiviral vectors

Lentiviral particles (LVP) were produced in Lenti-X 293 T cells (Clontech) after lipofection (Lipofectamine 3000, Thermo Fisher) with a second-generation packaging plasmid, a VSV-G envelope plasmid, and the respective AdCAR transfer plasmid. LVP containing supernatants were concentrated using Lenti-X concentrator (TaKaRa) and stored at −80°C until further use.

Generation of AdCAR-engineered NK-92 cells

NK-92 cells were seeded at a concentration of 1.25×10^6 cells/ml of MEM Alpha Medium (Thermo Fisher Scientific), supplemented with 8 ng/µl of protamine sulfate (Sigma-Aldrich) and 2.5 µM of BX-795 (Cayman Chemical Company). Subsequently, cells were transduced with AdCAR lentiviral particles for 16 h. Transduced cells were cultivated in NK-92 complete medium. Transduction efficiency was determined by flow cytometric analysis of CD34 marker gene surface expression using a BD FACSCanto II flow cytometer. Cells were subsequently single-cell sorted and screened for highest CAR expression. Results were analyzed using FlowJo software V10.0.8 (BD Biosciences).

Cell lines and culturing conditions

NHL cell lines Raji and Daudi and mantle-cell lymphoma (MCL) cell line JeKo-1 were purchased from the European Collection of Authenticated Cell Cultures (ECACC) or the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) containing stable L-glutamine (Glutamax, Thermo Fisher Scientific), referred to as RPMI complete medium. Lenti-X 293 T cells (Clontech) were cultivated in DMEM high glucose (4.5 g/L) medium (Thermo Fisher Scientific) containing GlutaMAX supplemented with 10% FBS and 1 mM

sodium pyruvate (Thermo Fisher Scientific). NK-92 cells were purchased from ATCC and maintained at a concentration of 10^5 cells/ml in MEM Alpha Medium containing GlutaMAX supplemented with 20% FBS and 100 U/ml IL-2 (PROLEUKIN, Aldesleukin Chiron) referred to as NK-92 complete medium. Primary MCL cells were isolated from a blood sample from a patient diagnosed with a hematogenously disseminated mantle-cell lymphoma. Primary CLL cells were isolated from blood samples from three patients diagnosed with chronic lymphocytic leukemia. All primary cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% pooled AB-serum from healthy donors (Transfusion Medicine, University Hospital Tuebingen). The study was approved by the ethics committee at the Medical Faculty of the Eberhard Karls University and the University Hospital Tuebingen (reference number 13/2007 V). Human material was collected after obtaining informed consent in accordance with the Helsinki protocol.

All media contained 1x antibiotic-antimycotic solution (Thermo Fisher Scientific) consisting of 100 U/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/ml amphotericin B. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere and regularly tested for mycoplasma contamination.

Generation of CD19/CD20 JeKo-1 knockout variants using CRISPR/Cas9 technology

The JeKo-1 wildtype ($CD19^+/CD20^+$) cell line was used to generate three distinct knockout variants ($CD19^-/CD20^+$, $CD19^+/CD20^-$ and $CD19^-/CD20^-$) using CRIPR/Cas9 technology and kindly provided by Miltenyi Biotec.

Antibody biotinylation

The FDA-approved CD20 antibody Rituximab was acquired from Hoffmann-La Roche, Basel, Switzerland. The chimeric CD19 antibody 4G7SDIE was acquired from SYNIMMUNE GmbH, Tuebingen, Germany. It was approved by the local ethics committee for experimental settings. Both, Rituximab and 4G7SDIE, were biotinylated by Davids Biotechnologie GmbH, Regensburg, Germany. All other bAb were acquired from Miltenyi Biotec, Bergisch Gladbach, Germany.

Flow cytometry

Staining of lymphoma and CLL cells was conducted using primary biotinylated or fluorophore-labeled mAb with antigen specificity of interest. Cells were co-incubated with antibodies at 4°C in flow cytometry buffer containing PBS (Sigma-Aldrich) supplemented with 2% FBS and 0.5 M EDTA (Sigma-Aldrich) for 15 min. Unbound antibody was washed off by centrifugation (4°C, 350 g, 5 min) and, for cells stained with bAb, a secondary, fluorophore-labeled anti-biotin antibody was added for 15 min followed by another washing step. Surface antigen expression was analyzed using a BD FACSCanto II flow cytometer.

Calcein release-based cytotoxicity assay (CRA)

Target cells were labeled with 10 µM of calcein acetoxymethyl (Calcein AM) (Thermo Fisher Scientific) at a concentration of 10^6 cells/ml in RPMI medium supplemented with 2% FBS. AdCAR NK-92 cells were thoroughly washed, resuspended in RPMI medium supplemented with 2% FBS and co-incubated with target cells with and without biotinylated antibodies for 2 h at various effector-to-target (E:T) ratios. AdCAR-specific cytotoxicity was determined by fluorescence measurement using the Spark microplate reader (Tecan).

Real-time label-free live cell analysis

Lymphoma cell lines were adjusted to a concentration of 10^5 cells/ml in RPMI complete medium and seeded in E-Plate 96 VIEW (OLS) micro-well plates that had been coated with 170 µg/ml of fibronectin (Corning) for 1 h. Effector AdCAR NK-92 cells were adjusted to an E:T ratio of 5:1 in NK-92 complete medium without IL-2 and co-incubated with the target cells. Utilizing the xCELLigence real-time cell analysis (RTCA) system, cells were monitored for over 24 h. Lymphoma cell viability was calculated using the RTCA 2.0 software and AdCAR-mediated cytotoxicity was subsequently determined.

Flow cytometry-based cytotoxicity assays using an antigen loss model

To visualize antigen-specific killing, JeKo-1 wildtype, JeKo-1 CD19 knockout (KO), JeKo-1 CD20 KO and JeKo-1 CD19/CD20 double KO variants were incubated at a 1:1:1:1 ratio with 25.000 cells per JeKo-1 variant (in total 100.000 cells) plus 500.000 AdCAR NK-92 cells (E:T 5:1) in equal parts of RPMI complete medium and NK-92 complete medium without IL-2 at 37°C. Furthermore, bCD19 or bCD20 mAb and combinations thereof were added. Cells were incubated for indicated time intervals and then analyzed using a BD CantoFACS II flow cytometer. AdCAR NK-92 cells were identified by CD34 CAR marker gene expression, JeKo-1 in total by GFP expression and JeKo-1 subpopulations by CD19 and CD20 expression. Trucount Tubes (BD Biosciences) were used for quantification and acquisition, with a stopping gate at 20.000 beads. Normalized viability of target cells was calculated using FlowJo software.

Quantification of cytokine release

Target and effector cells were co-incubated in equal parts of target cell complete medium and NK-92 complete medium without IL-2 at an E:T ratio of 5:1 at 37°C for 6 h, supernatants were collected and stored at −80°C until further use. AdCAR NK-92 maximum degranulation was achieved using the cell activation cocktail (BioLegend) containing PMA/Ionomycin.

Cytokine release was determined using the Bio-Plex Pro human cytokine 17-plex assay (Bio-Rad), the human perforin ELISA kit (Thermo Fisher Scientific) and the LEGEND MAX human granulysin and granzyme B ELISA kits (BioLegend).

Data analysis

All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc.). Flow cytometry data were analyzed using FlowJo software V10.0.8 (FlowJo LLC).

Results

NK-92 cells were transduced with lentiviral vectors encoding the adapter CAR with a CD28 co-signaling domain (Figure 1b). Transduction efficiency of NK-92 was determined via flow cytometric analysis of CD34 marker gene surface expression which correlates with CAR expression. We found that the AdCAR was efficiently expressed on the surface of transduced NK-92 cells which were subsequently single-cell-sorted by FACS for high CAR expression (Figure 2a). Ten individual NK-92 clones were tested for CAR expression using flow cytometry. The NK-92 clone with the highest CAR expression and highest viability was chosen for further experiments. AdCAR NK-92 cells showed stable AdCAR surface expression for at least 150 d after cell sorting (Figure 2b) with an average viability of >90%. Notably, the proliferation rate was not impaired by the transduction process compared to untransduced, parental NK-92 cells (Figure 2c).

To compare the NK cell receptor profile of NK-92 cells and primary NK cells, as well as analyzing the effects of CAR transduction on the NK receptor expression of NK-92 cells, flow cytometry was used. Compared to primary NK cells, NK-92 and AdCAR NK-92 cells showed lower expression of DNAM-1 (CD226), one of the main activating NK receptors. Another important activating receptor, NKG2D (CD314) was almost completely absent on AdCAR NK-92 cells. Furthermore, AdCAR-transduced NK-92 cells displayed very high expression of KIR2DL2/DL3 (CD158b), a member of the killer-cell immunoglobulin-like receptor (KIR) family recognizing the human HLA-C1/2 peptide, and thus, inhibiting NK effector function. Interestingly, NKp80, an activation receptor on all activated NK cells, which induces NK cell-mediated cytotoxicity and cytokine production, was only present on the isolated NK cells and not on NK-92 cells (Figure 2d). Next, AdCAR NK-92 cells were characterized for the expression of important immune checkpoint receptors via flow cytometry and compared to parental NK-92 as well as isolated NK cells from three healthy donors (Figure 2e). AdCAR NK-92 cells showed a similar immune checkpoint receptor expression profile, compared to primary NK cells and parental NK-92 cells apart from a slight decrease in expression of TIGIT, a protein that directly inhibits NK cell cytotoxicity.

We then tested whether AdCAR NK-92 cells combined with different biotinylated antibodies targeting lymphoma cells could induce AdCAR-mediated antigen-specific cytotoxicity. As proof of principle for the functionality and specificity of AdCAR NK-92 cells *in vitro*, the lymphoma cell lines Raji, Daudi (both NHL) and JeKo-1 (MCL) were used. The cell lines were immunophenotyped via flow cytometry for lymphoma antigen expression (Table 1) and used as target cells for functional analyses.

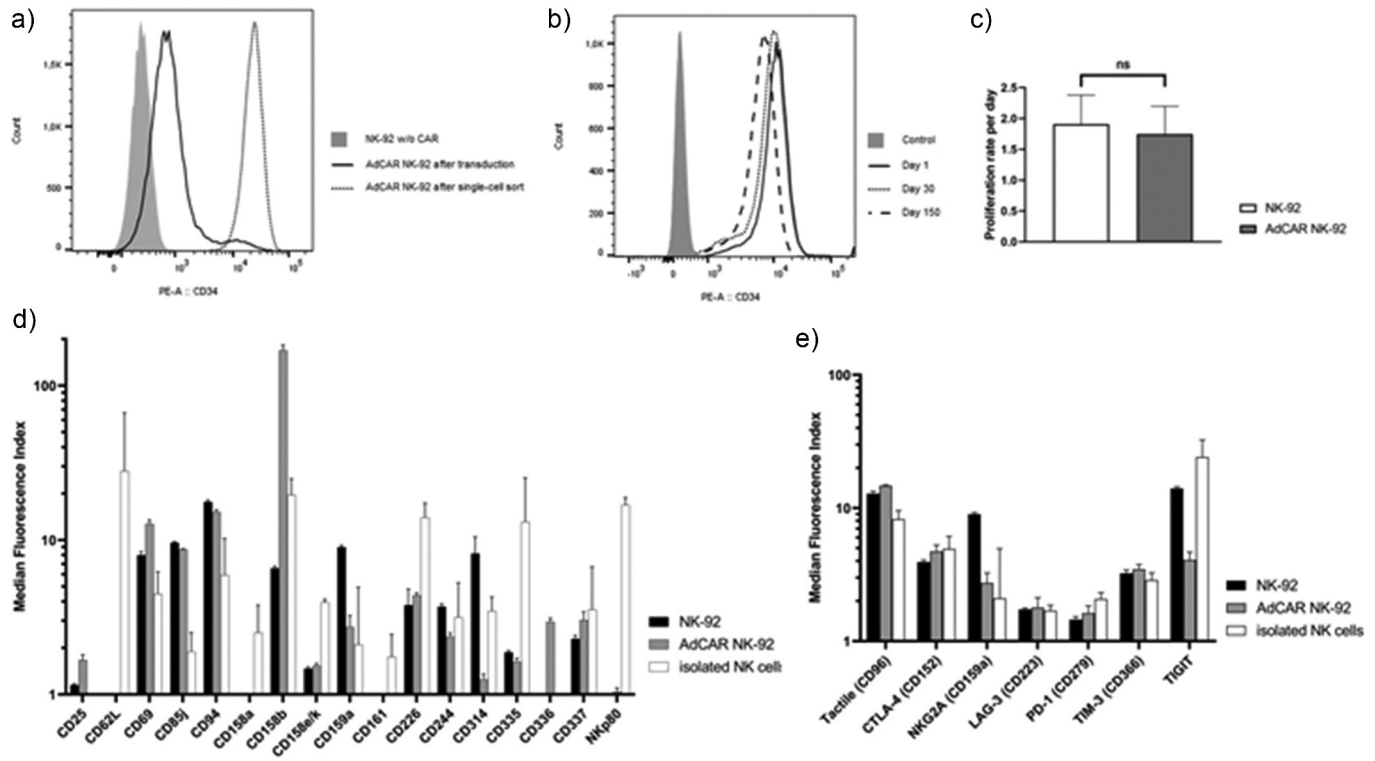


Figure 2. Generation and characterization of AdCAR NK-92 cells. CD34 marker gene expression was measured via flow cytometry on untransduced (gray, filled) and transduced NK-92 cells before (black line) and after (gray line) single-cell sort (a) and monitored for 150 d (b). Average proliferation rate of parental NK-92 and sorted AdCAR NK-92 was measured over the same time frame and calculated as mean ± SD (c). AdCAR-transduced and parental NK-92 cells were characterized for the expression of activating and inhibitory NK receptors as well as immune checkpoint receptors via flow cytometry. Results were compared to flow cytometric analysis of isolated NK cells from three healthy donors. Median fluorescence intensity ± SD was calculated using FlowJo software, n = 3 (d, e).

Table 1. Surface antigen expression of lymphoma cell lines. Raji, Daudi and JeKo-1 cell lines were screened for target antigen expression using flow cytometry. Lymphoma cells were co-incubated with primary biotinylated antibodies for 15 min at 4°C. Antigen expression was detected using a secondary PE-coupled anti-biotin antibody. Mean positivity and mean fluorescence index (MFI) values ± SD were calculated using staining with the secondary antibody alone as a negative control, n = 3.

	Raji		Daudi		JeKo-1	
	Positive cells	MFI	Positive cells	MFI	Positive cells	MFI
CD19	99.97 ± 0.06%	107.72 ± 22.13	99.90 ± 0.00%	74.08 ± 4.50	97.93 ± 1.90%	21.93 ± 3.51
CD20	95.8 ± 3.99%	44.57 ± 15.78	98.5 ± 0.69%	85.85 ± 40.91	97.07 ± 3.35%	40.42 ± 19.28
CD22	99.1 ± 0.35%	47.90 ± 6.80	98.60 ± 0.69%	77.32 ± 26.56	24.07 ± 3.48%	1.80 ± 0.09
CD30	2.77 ± 0.92%	1.10 ± 0.10	0.57 ± 0.21%	1.04 ± 0.01	1.49 ± 0.49%	1.07 ± 0.02
CD38	98.43 ± 1.59%	57.50 ± 20.20	95.07 ± 1.16%	191.70 ± 11.45	81.07 ± 2.35%	17.69 ± 4.47
CD70	98.83 ± 1.68%	92.02 ± 23.64	41.97 ± 8.38%	2.93 ± 0.52	98.47 ± 0.29%	35.11 ± 8.53
CD79b	66.23 ± 9.82%	5.24 ± 2.08	98.30 ± 1.01%	76.67 ± 24.13	96.30 ± 0.56%	27.66 ± 0.59

Using a standard calcein release cytotoxicity assay (CRA), AdCAR NK-92 cells were co-incubated with calcein-labeled lymphoma cells with and without bAb for 2 h. For determination of the optimal antibody concentration lymphoma cells were co-incubated with varying amounts of bCD19 and AdCAR NK-92 cells. The AdCAR NK-92 cells induced cellular cytotoxicity of Raji cells in a bCD19-dose-dependent manner (Figure 3a). A concentration of 100 ng/ml was chosen for all biotinylated antibodies for further functional assays. Altering antibody concentration decreased the AdCAR NK-92-mediated cytotoxicity. Thus, the AdCAR NK-92 effect is tunable, directly depending on the concentration of bAb used. Since biotin, which is conjugated to the mAb, is an endogenous vitamin, we tested whether free biotin decreases AdCAR-mediated cytotoxicity. Notably, AdCAR NK-92 cells retained their specific cytotoxic ability even in the presence of unbound biotin that exceeded physiological concentrations which proves that the AdCAR specifically binds to the neoepitope on biotinylated antibodies and not biotin itself (Figure 3b). Raji cells were incubated with bCD19 and varying concentrations of NK-92 effector cells (Figure 3c). Interestingly, titration of effector cell concentration showed that at low E:T ratios the AdCAR still reliably enhanced NK-92 effector function in the presence of a specific bAb.

Most importantly, AdCAR NK-92 but not untransduced NK-92 cells significantly induced cellular cytotoxicity against all lymphoma cell lines but only in the presence of bAb targeting antigens expressed on lymphoma cell lines (Figure 3d). Biotinylated antibodies targeted against antigens that were not expressed on the cell surface were not capable of inducing AdCAR-mediated responses, thus proving AdCAR specificity. Moreover, NK-92 cytotoxicity and antigen expression levels (MFI) correlated significantly for the NHL cell line Raji (R^2 = 0.695; 95%-CI: 0.2166–0.9748; P = .0198) and the MCL cell line JeKo-1 (R^2 = 0.8942; 95%-CI: 0.6689–0.9922; P = .0013) (Figure 3e). Utilizing the xCELLigence real-time cell analysis (RTCA) assay, kinetics of NK-mediated cytotoxicity following co-incubation of AdCAR NK-92 cells and Raji cells were assessed (Figure 3f). The dimensionless cell index is indicative of target cell viability and therefore anti-proportional to tumor lysis. The assay demonstrated a rapid decrease in cell index of Raji cells following the addition of AdCAR NK-92 cells but only in the presence of the respective biotinylated antibodies bCD19 and/or bCD20 while no considerable AdCAR-effect was detectable in the absence of bAb.

Essentially, irradiation of NK-92 cells prior to testing, as required in all active clinical trials using NK-92, had no significant effect on target cell lysis when AdCAR NK-92 cells were used immediately after irradiation; however, AdCAR NK-92 cells gradually lost effector function and underwent apoptosis over time (Figure 3g).

Furthermore, an NK-92 cytokine secretion profile was established to screen for the secretion of a variety of cytokines, including NK cell effector molecules. Various cytokines were significantly increased after co-incubation of AdCAR-transduced NK-92 cells with Raji cells. IL-10 (36-fold; $p < .0004$), granulysin (27-fold; $p < .002$), IFN-γ (65-fold; $p < .01$), MCP-1 (40-fold; $p < .04$) and TNF-α (28-fold; $p < .0002$) showed significantly elevated levels but only upon AdCAR induction via specific biotinylated antibodies. Notably, NK-92 cells alone showed medium basal secretion of granzyme B, MIP-1b and perforin which was further augmented after AdCAR activation (2.2-fold, 1.5-fold and 1.3-fold, respectively) but did not reach statistical significance (Figure 3h). TNF-related apoptosis-inducing ligand (TRAIL, CD253) upregulation on AdCAR NK-92 cells could be observed after co-incubation with Raji cells and bCD19 for 6 h (MFI: AdCAR NK-92 only: 2.08 ± 0.18; AdCAR NK-92 + Raji: 2.36 ± 0.06; AdCAR NK-92 + Raji + bCD19: 4.48 ± 0.29) while expression of Fas ligand (FasL, CD178), another important apoptosis-inducing transmembrane protein, was not elevated (Figure 3i).

In order to determine whether AdCAR activation also induced NK-92 fratricide, AdCAR-engineered NK-92 cells were co-incubated with calcein-labeled, parental NK-92 cells. Favorably, co-incubation of bCD56 with NK-92 target cells and AdCAR-transduced NK-92 effector cells did not show a significant increase in lysis of parental NK-92 cells (Figure 3j).

To prove the specificity of our AdCAR system, a JeKo-1 CD19 and/or CD20-knockout (KO) model was used. JeKo-1 wildtype, JeKo-1 CD19 KO, JeKo-1 CD20 KO and JeKo-1 CD19/CD20 double KO variants were mixed at an equal ratio and subsequently co-incubated with AdCAR NK-92 cells at an E:T ratio of 5:1 in the presence or absence of bCD19 and/or bCD20 antibodies for 4 h and 24 h. Subsequently, cells were analyzed via flow cytometry.

Addition of AdCAR NK-92 cells alone to the JeKo-1 target cell mix did not decrease target cell count or alter the ratio of the respective subpopulations. However, co-incubation with specific biotinylated antibodies induced AdCAR-mediated lysis of respective antigen positive subpopulations while the antigen-negative subpopulations were spared (Figure 4a). With the combination of bCD19 and bCD20, AdCAR NK-92 cells eradicated $CD19^+$ and $CD20^+$ JeKo-1 cells almost completely (Figure 4b). Thus, the AdCAR NK-92 cells specifically lyse

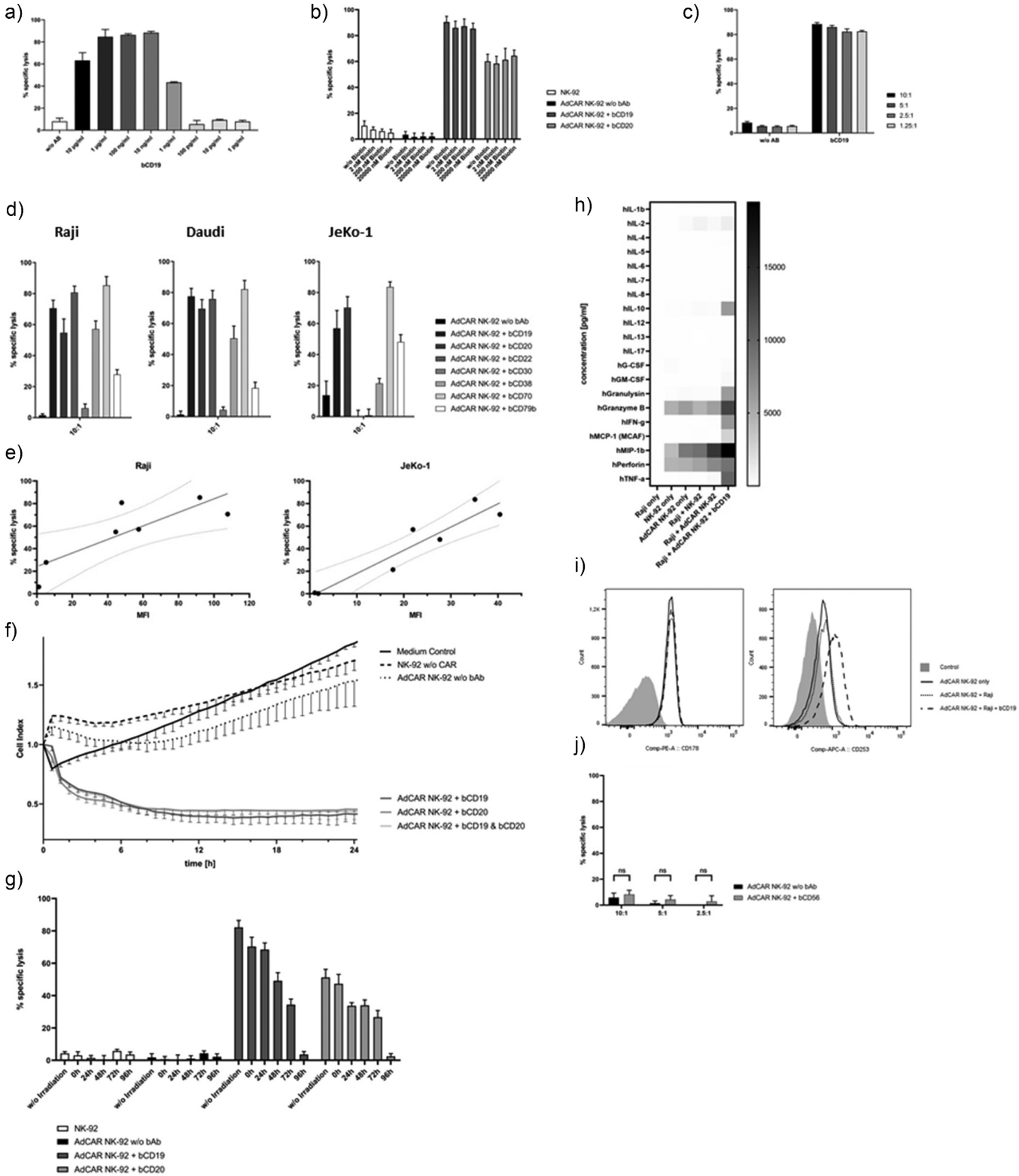


Figure 3. AdCAR NK-92-mediated tumor cell lysis. AdCAR NK-92 cells were co-incubated with calcein-labeled lymphoma cell lines in the presence or absence of indicated biotinylated antibodies for 2 h. Specific lysis is shown as mean ± SD, n = 3. Using the Raji cell line as target, an optimal bAb concentration of 100 ng/ml was chosen after titration of bCD19 (a). Free biotin added in excess of physiological concentrations showed no impairment of AdCAR-mediated lysis of lymphoma cells (b). Various E:T ratios utilizing AdCAR NK-92 cells as effectors and Raji cells as target were analyzed using a calcein release assay (c). Biotinylated antibodies, already utilized for the flow cytometry screening panel (Table 1), were tested with Raji, Daudi and JeKo-1 cells as target in a CRA (d). Target antigen expression levels were correlated with their respective AdCAR NK-92-mediated tumor cell lysis (e). Kinetics of AdCAR-mediated lysis of Raji cells was assessed using the xCELLigence real-time cell-analysis system at an E:T ratio of 5:1 (f). Cytotoxic effector function of AdCAR NK-92 cells irradiated with 10 Gy at indicated time points was assessed using Raji cells as target (g). The release of cytokines by NK-92 cells was measured using the Bio-Plex Pro human cytokine 17-plex assay and is shown as a heatmap (h). AdCAR NK-92 cells were co-incubated with Raji cells in the presence or absence of bCD19 for 6 h and screened for expression of FasL (CD178) and TRAIL (CD253) via flow cytometry (i). Finally, AdCAR NK-92-mediated lysis of calcein-labeled parental NK-92 cells using bCD56 as adapter molecule (j) was tested for the assessment of possible fratricide.

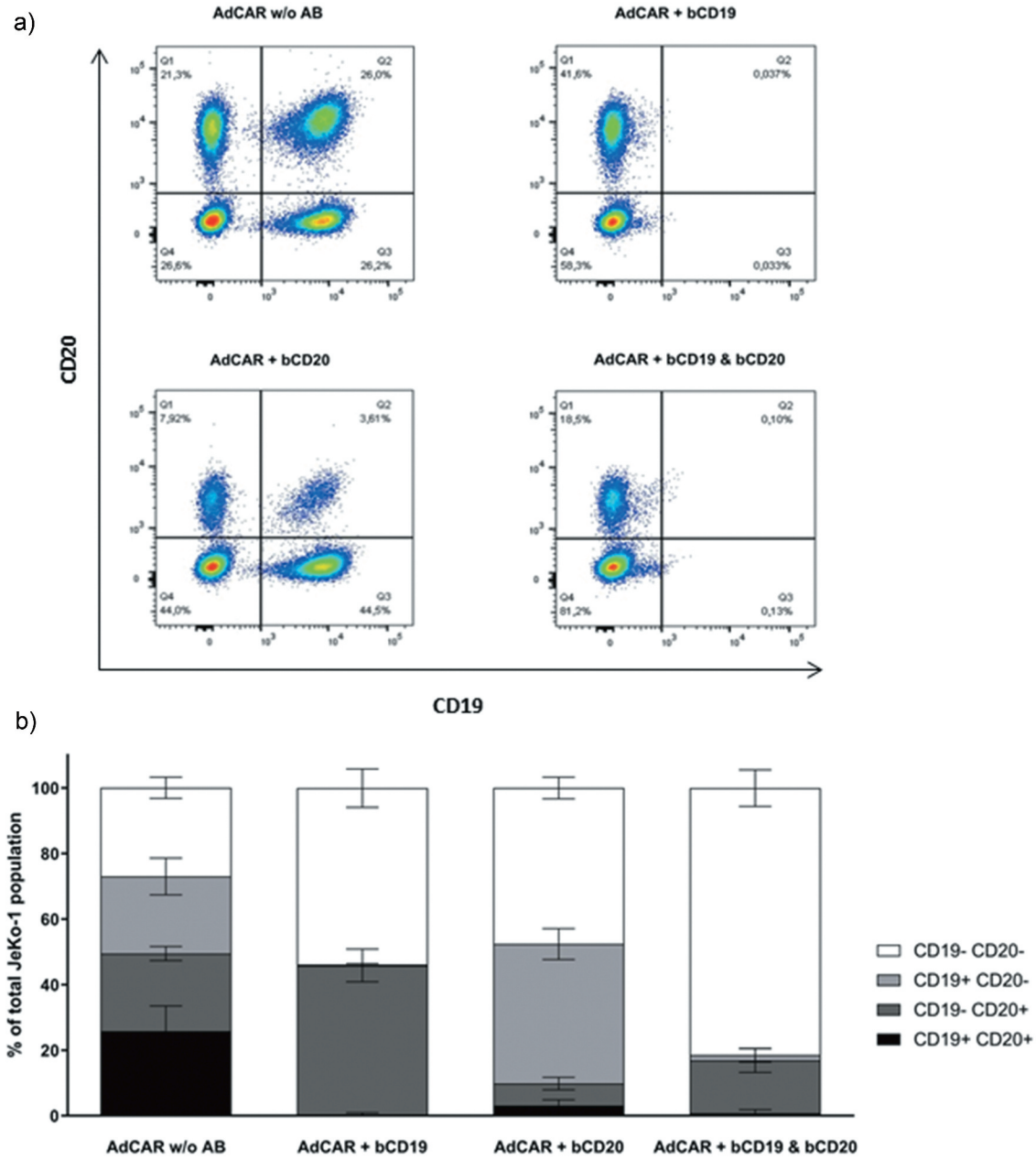


Figure 4. JeKo-1 cell line target antigen-loss model. JeKo-1 CD19 and/or CD20 knock-out (KO) cell lines were mixed at an equal 1:1:1:1 ratio and co-incubated with AdCAR NK-92 cells at an E:T ration of 5:1 in the presence and/or absence of indicated bAb for 24 h and analyzed using flow cytometry (a). Ratio of KO variants after cytotoxicity assay is shown as a mean of three independent experiments (b).

tumor cells only in the presence of a bAb targeting an antigen that is expressed on the surface of the tumor cell. If tumor cells down-regulate antigen expression levels in response to immune therapy, AdCAR NK-92-mediated activity is easily retained by switching target structures through the addition of a different bAb.

Next, we tested whether AdCAR activation by co-incubation with lymphoma cells would permanently decrease the cytotoxic effector function of AdCAR NK-92 cells due to immune exhaustion. Therefore, AdCAR NK-92 cells were co-incubated with Raji cells and bCD19 for 4, 24 or 48 h at an E:T ratio of 5:1 and screened for expression of the immune checkpoint receptors at indicated time points (Figure 5b). Notably, only CD96 (TACTILE), CD223 (LAG-3) and CD366 (TIM-3) were significantly elevated with peak expression levels after 24 h of stimulation. We then subjected AdCAR NK-92 cells to continuous re-stimulation by the addition of fresh Raji cells every 24 h for a total of 72 h and again screened for immune checkpoint receptor expression. Only slightly elevated CD96 expression levels could be observed, while CD223 and CD366 were moderately increased. For functional testing, we compared the continuously re-stimulated AdCAR NK-92 cells with unstimulated control AdCAR NK-92 cells using Raji cells as target and bCD19 as well as bCD20 as adapter molecules (Figure 5c). We found no significant decrease in CAR-mediated cytotoxic effector function after re-stimulation. Thus, AdCAR NK-92 cells did not show relevant signs of exhaustion after multiple killing events.

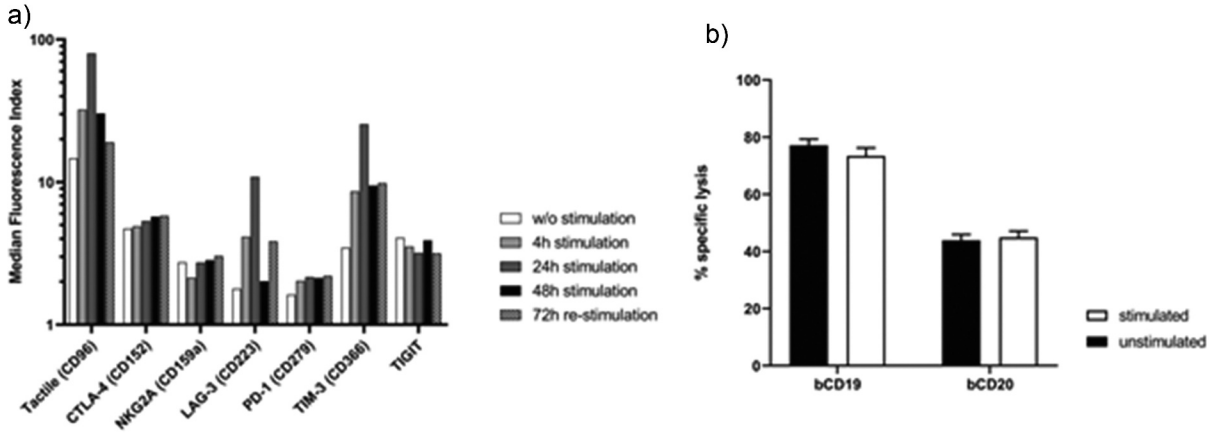


Figure 5. Immune checkpoint receptor expression profile and cytotoxic effector function of AdCAR NK-92 cells upon re-stimulation with Raji cells. AdCAR NK-92 cells were co-incubated with Raji cells at an E:T ratio of 5:1 either for indicated time points (filled bars) or re-stimulated every 24 h with fresh Raji cells for 72 h (checkered pattern) and screened for immune checkpoint receptor expression (a). Re-stimulated AdCAR NK-92 cells were co-incubated with calcein-labeled Raji cells at an E:T ratio of 5:1 for 2 h. Specific cytotoxicity was calculated and compared to un-stimulated control AdCAR NK-92 cells (b). Specific lysis is shown as mean ± SD, n = 3.

Table 2. Surface antigen expression of primary cells derived from different B cell malignancies. Patient-derived, primary MCL and CLL cells were co-incubated with primary biotinylated antibodies for 15 min at 4°C. Antigen expression was detected using a secondary, PE-coupled anti-biotin antibody. Mean positivity and mean fluorescence index (MFI) values ± SD were calculated using staining with the secondary antibody alone as a negative control.

	Primary MCL (W)		Primary CLL (AWK)		Primary CLL (FA)		Primary CLL (M)	
	Positive cells	MFI	Positive cells	MFI	Positive cells	MFI	Positive cells	MFI
CD19	91.67%	46.81	90.05%	184.92	95.30%	254.32	90.70%	153.47
CD20	94.27%	300.12	90.90%	41.04	66.07%	12.1	81.11%	12.42

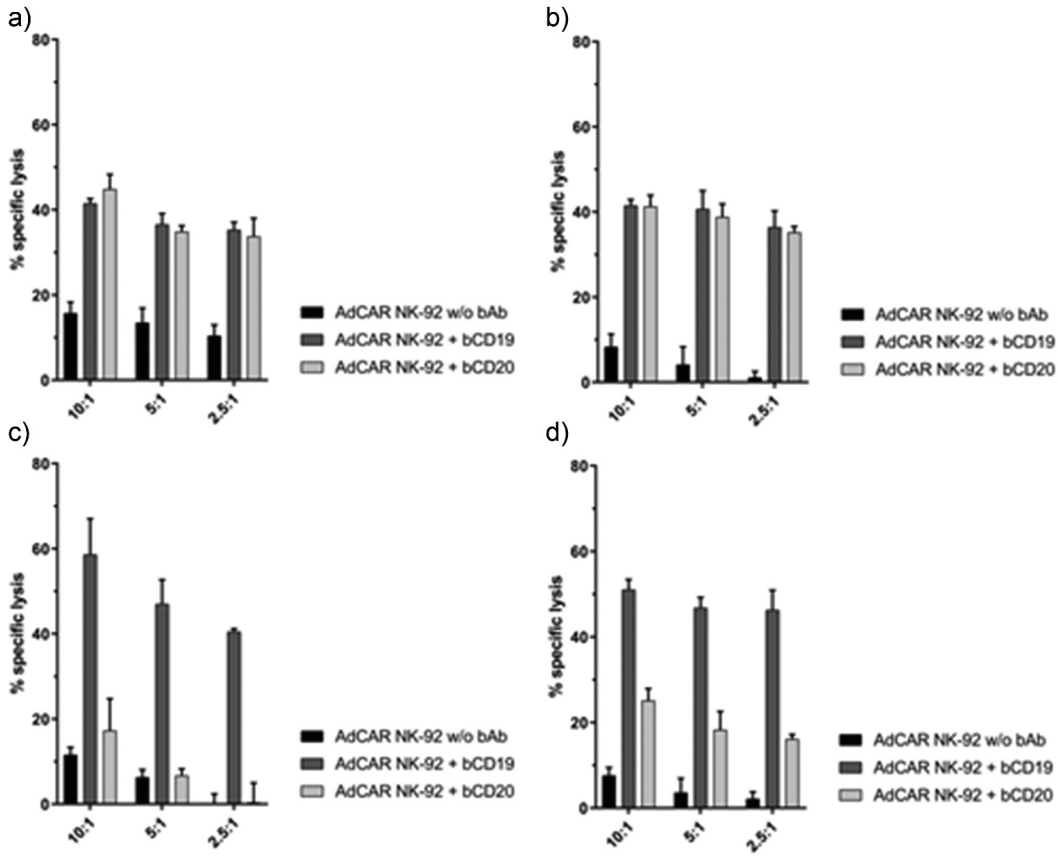


Figure 6. AdCAR NK-92-mediated lysis of primary cells of different B cell malignancies. AdCAR NK-92 cells were co-incubated with calcein-labeled patient-derived, primary MCL cells (one patient, (a) and CLL cells (three different patients, (b-d) in the presence or absence of indicated biotinylated antibodies for 2 h. Specific lysis is shown as mean ± SD, n = 3.

Further, we wanted to examine whether AdCAR NK-92 cells were also capable of inducing CAR-mediated cytotoxicity against patient-derived, primary tumor cells. Therefore, we used tumor cells that had been previously isolated from a blood sample from one patient diagnosed with a hematogenously disseminated mantle-cell lymphoma and three patients with chronic lymphocytic leukemia. These cells were immunophenotyped (Table 2) and used as target cells in a standard CRA. AdCAR NK-92 cells were able to induce significant cellular cytotoxicity in the presence of specific bAb (Figure 6). Likewise, the addition of bAb targeting non- or very low expressed antigens did not induce any AdCAR-related lysis. Evidently, AdCAR NK-92 cells are effective in specifically targeting various patient-derived, primary tumor cells.

Discussion

Tumor heterogeneity remains a major obstacle even for advanced immunotherapeutic options such as CAR T cells. NK cells provide an interesting approach as an alternative cell source for CAR therapy.[38,39] Most current pre-clinical and clinical trials work with either allogeneic donor NK cells or NK-derived cell lines since primary, autologous NK cells can easily be silenced by tumor cells through self-antigens.[21,40,41] NK-92 is a continuously expandable cell line that lacks the expression of most inhibitory receptors.[31] It constitutively expresses a variety of activating receptors.[42] The lack of KIR expression and constitutive expression of activating receptors allows for the high cytotoxic nature of NK-92 cells which have been shown to be effective and safe in various pre-clinical trials.[28,40,43–46] To avoid malignant expansion through *in vivo* proliferation of the NHL-derived NK-92, cells need to be γ-irradiated prior to administration.[28,43] Irradiation with 10 Gy blocks NK-92 proliferation limiting its life span to only a few days while retaining most of their cytolytic activity. This increases safety since without permanent engraftment NK-92 cells there is no need for active elimination after application using a CAR T-like safety switch.[47] Conversely, CAR-transduced NK-92 cells could safely be administered multiple times to further increase efficiency.[48]

In the present study, we demonstrated that the combination of the well-established NK-92 cell line with the controllability of our newly developed Adapter CAR system enables distinct therapeutic options for a multitude of malignancies while counteracting drawbacks of conventional CAR T cell therapy such as tumor evasion strategies like antigen loss. By targeting extracellular antigen structures using specific biotinylated antibodies (bAb) NK-mediated cell lysis could be successfully induced in multiple lymphoma cell lines as well as various primary tumor cells. The absence of either the bAb or its corresponding target antigen prevents AdCAR activity. Additional controllability of AdCAR NK-92 cells is demonstrated by the fact that cytolytic activity occurred in a concentration-dependent fashion. Contrary to primary NK cells, AdCAR NK-92 cells lack the CD16 (FcγRIII) expression and cannot be retargeted by IgG1 antibodies.[40] Therefore, the formation of the immunological synapse is simply controlled by bAb titration.

Transduction of NK-92 cells with the AdCAR was shown to be stable for at least 150 d without limiting the NK-92 cells proliferative or cytotoxic activity. Thus, "off-the-shelf" AdCAR NK-92 cells provide a cost-effective alternative to standard CAR T cells which have to be produced individually for each patient.[49] These direct CAR-mediated immune cell approaches also lack the flexibility of a universal CAR. In previous CAR T cell studies targeting CD19 tumor cells reacted to therapy by down-regulation of CD19.[49–52] Using a JeKo-1 antigen-loss model AdCAR NK-92 cells demonstrated their ability to target multiple antigens simultaneously. They were able to react to tumor antigen loss by switching target structures simply utilizing a different bAb while retaining therapeutic efficacy. Other universal CAR approaches with T cells, including the presented adapter CAR, as well as NK cells have already shown promising results.[32,35,36,53,54] However, CAR systems using adapter molecules that are foreign to the human body can cause immunogenic reactions and make clinical translation difficult. Even CAR systems utilizing endogenous molecules as tag such as biotin may face challenges due to the potential immunogenicity of avidin and streptavidin as well as their high affinity for physiological biotin in the clinical setting.[54,55] Here, the AdCAR is based on an scFv targeting a "neo"-epitope-like structure, the linker-label-epitope, consisting of biotin in the context of a mAb, instead of biotin itself.[37] Specificity was proven since we saw no interference of unbound biotin even at supraphysiological levels. Thus, using AdCAR NK-92 cells is a promising approach to circumvent adverse immunogenic reactions of other CAR systems and therefore decrease the risk of severe side effects during therapy. Moreover, this study proved that biotinylation of already FDA-approved antibodies such as Rituximab is capable of creating functional adapter molecules for AdCAR NK-92 therapy thus facilitating translation into clinical settings. Our data represent preliminary results which have to be further investigated *in vivo* using different mouse models of hematological malignancies as well as solid tumors to evaluate AdCAR efficacy and predict the response to AdCAR NK-92 immunotherapy in patients.

To conclude, AdCAR NK-92 cells can be manufactured as an "off-the-shelf, on demand" standardized product whose effector function can be tightly regulated. They may provide a universal and cost-effective therapeutic option for safe and tunable targeting of malignant cells.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

ORCID

Christian Seitz http://orcid.org/0000-0002-0520-7866

References

1. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak Ö, Brogdon JL, Pruteanu-Malinici I, Bhoj V, Landsburg D, *et al.* Chimeric antigen receptor T cells in refractory B-cell lymphomas. N Engl J Med. 2017;377:2545–2554. doi:10.1056/NEJMoa1708566.
2. Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. Cancer Discov. 2013;3(4):388–398. doi:10.1158/2159-8290.CD-12-0548.
3. Davila ML, Kloss CC, Gunset G, Sadelain M. CD19 CAR-targeted T cells induce long-term remission and B cell aplasia in an immunocompetent mouse model of B cell acute lymphoblastic leukemia. PLoS One. 2013;8:1–14. doi:10.1371/journal.pone.0061338.
4. June CH, Sadelain M. Chimeric antigen receptor therapy. N Engl J Med. 2018;379:64–73. doi:10.1056/NEJMra1706169.
5. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. Science. 2018;359:1361–1365. doi:10.1126/science.aar6711.
6. Weinkove R, George P, Dasyam N, McLellan AD. Selecting costimulatory domains for chimeric antigen receptors: functional and clinical considerations. Clin Transl Immunol. 2019;8. doi:10.1002/cti2.1049.
7. Mohty M, Gautier J, Malard F, Aljurf M, Bazarbachi A, Chabannon C, Kharfan-Dabaja MA, Savani BN, Huang H, Kenderian S, *et al.* CD19 chimeric antigen receptor-T cells in B-cell leukemia and lymphoma: current status and perspectives. Leukemia. 2019;2767–2778. doi:doi:10.1038/s41375-019-0615-5.
8. Subklewe M, Von Bergwelt-Baildon M, Humpe A. Chimeric antigen receptor T cells: A race to revolutionize cancer therapy. Transfus Med Hemotherapy. 2019;46:15–24. doi:10.1159/000496870.
9. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, Braunschweig I, Oluwole OO, Siddiqi T, Lin Y, *et al.* Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-Cell lymphoma. N Engl J Med. 2017;377:2531–2544. doi:10.1056/NEJMoa1707447.
10. Allen ES, Stroncek DF, Ren J, Eder AF, West KA, Fry TJ, Lee DW, Mackall CL, Conry-Cantilena C. Autologous lymphapheresis for the production of chimeric antigen receptor T cells. Transfusion. 2017;57(5):1133–1141. doi:10.1111/trf.14003.
11. You F, Wang Y, Jiang L, Zhu X, Chen D, Yuan L, An G, Meng H, Yang L. A novel CD7 chimeric antigen receptor-modified NK-92MI cell line targeting T-cell acute lymphoblastic leukemia. Am J Cancer Res. 2019;9:64–78.
12. Ceppi F, Rivers J, Annesley C, Pinto N, Park JR, Lindgren C, Mgebroff S, Linn N, Delaney M, Gardner RA, *et al.* Lymphocyte apheresis for chimeric antigen receptor T-cell manufacturing in children and young adults with leukemia and neuroblastoma. Transfusion. 2018;58(6):1414–1420. doi:10.1111/trf.14569.
13. Mackall CL, Fleisher TA, Brown MR, Magrath IT, Shad AT, Horowitz ME, Wexler LH, Adde MA, McClure LL, Gress RE, *et al.* Lymphocyte depletion during treatment with intensive chemotherapy for cancer. Blood. 1994;84(7):2221–2228. doi:10.1182/blood.V84.7.2221.2221.
14. Mackall CL. T-cell immunodeficiency following cytotoxic antineoplastic therapy: A review. Stem Cells. 2000;18:10–18. doi:10.1634/stemcells.18-1-10.
15. Verma R, Foster RE, Horgan K, Mounsey K, Nixon H, Smalle N, Hughes TA, Carter CR. Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer. Breast Cancer Res. 2016;18(1):1–12. doi:10.1186/s13058-015-0669-x.
16. Feldmann A, Arndt C, Koristka S, Berndt N, Bergmann R, Bachmann MP. Conventional CARs versus modular CARs. Cancer Immunol Immunother. 2019;68:1713–1719. doi:10.1007/s00262-019-02399-5.
17. Bachmann M. The UniCAR system: A modular CAR T cell approach to improve the safety of CAR T cells. Immunol Lett. 2019;211:13–22.
18. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer. 2016;16:7–19.
19. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, Nassif Kerbauy L, Overman B, Thall P, Kaplan M, *et al.* Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N Engl J Med. 2020;382:545–553. doi:10.1056/NEJMoa1910607.
20. Wang W, Jiang J, Wu C. CAR-NK for tumor immunotherapy: clinical transformation and future prospects. Cancer Lett. 2020;472:175–180. doi:10.1016/j.canlet.2019.11.033.
21. Rezvani K. Adoptive cell therapy using engineered natural killer cells. Bone Marrow Transplant. 2019;54:785–788. doi:10.1038/s41409-019-0601-6.
22. Shimasaki N, Fujisaki H, Cho D, Masselli M, Lockey T, Eldridge P, Leung W, Campana D. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. Cytotherapy. 2012;14:830–840. doi:10.3109/14653249.2012.671519.
23. Oelsner S, Wagner J, Friede ME, Pfirrmann V, Genßler S, Rettinger E, Buchholz CJ, Pfeifer H, Schubert R, Ottmann OG, *et al.* Chimeric antigen receptor-engineered cytokine-induced killer cells overcome treatment resistance of pre-B-cell acute lymphoblastic leukemia and enhance survival. Int J Cancer. 2016;139:1799–1809. doi:10.1002/ijc.30217.
24. Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res. 2009;33:1255–1259. doi:10.1016/j.leukres.2008.11.024.
25. Sutlu T, Nyström S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther. 2012;23:1090–1100.
26. Zhang C, Oberoi P, Oelsner S, Waldmann A, Lindner A, Tonn T, Wels WS. Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. Front Immunol. 2017;8:1–17. doi:10.3389/fimmu.2017.00533.
27. Cooley S, He F, Bachanova V, Vercellotti GM, DeFor TE, Curtsinger JM, Robertson P, Grzywacz B, Conlon KC, Waldmann TA, *et al.* First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. Blood Adv. 2019;3:1970–1980. doi:10.1182/bloodadvances.2018028332.
28. Nowakowska P, Romanski A, Miller N, Odendahl M, Bonig H, Zhang C, Seifried E, Wels WS, Tonn T. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. Cancer Immunol Immunother. 2018;67:25–38. doi:10.1007/s00262-017-2055-2.
29. Merker M, Pfirrmann V, Oelsner S, Fulda S, Klingebiel T, Wels WS, Bader P, Rettinger E. Generation and characterization of ErbB2-CAR-engineered cytokine-induced killer cells for the treatment of high-risk soft tissue sarcoma in children. Oncotarget. 2017;8(39):66137–66153. doi:10.18632/oncotarget.19821.
30. Jiang H, Zhang W, Shang P, Zhang H, Fu W, Ye F, Zeng T, Huang H, Zhang X, Sun W, *et al.* Transfection of chimeric anti-CD138 gene enhances natural killer cell activation and killing of multiple myeloma cells. Mol Oncol. 2014;8:297–310. doi:10.1016/j.molonc.2013.12.001.
31. Klingemann HG, Wong E, Maki G. A cytotoxic NK-cell line (NK-92) for ex vivo purging of leukemia from blood. Biol Blood Marrow Transplant. 1996;2:68–75.
32. Darowski D, Kobold S, Jost C, Klein C. Combining the best of two worlds: highly flexible chimeric antigen receptor adaptor molecules (CAR-adaptors) for the recruitment of chimeric antigen receptor T cells. MAbs. 2019;11(4):621–631. doi:10.1080/19420862.2019.1596511.
33. Minutolo NG, Hollander EE, Powell DJ. The emergence of universal immune receptor t cell therapy for cancer. Front Oncol. 2019;9. doi:10.3389/fonc.2019.00176.

34. Li X, Chen W. Mechanisms of failure of chimeric antigen receptor T-cell therapy. Curr Opin Hematol. 2019;26:427–433. doi:10.1097/MOH.0000000000000548.
35. Seitz CM, Schlegel P, Hau J, Krahl AC, Schroeder S, Bender G, Reiter S, Schleicher S, Schilbach K, Ebinger M, *et al*. Novel adapter chimeric antigen receptor (aCAR) T cells for temporally controllable targeting of single and multiple tumor antigens. Blood. 2017;130:1912–1912.
36. Seitz CM, Kieble V, Illi C, Reiter S, Grote S, Mittelstaet J, Lock D, Kaiser A, Schleicher S, Handgretinger R, *et al*. Combinatorial targeting of multiple shared antigens by adapter-CAR-T cells (aCAR-Ts) allows target cell discrimination and specific lysis based on differential expression profiles. Blood. 2018;132:4543–-4543. doi:10.1182/blood-2018-99-115630.
37. Kaiser A, Mittelstaet J, Huppert V, Miltenyi S, Schlegel P, Seitz C, Lang P, Handgretinger R, Inventors; Miltenyi Biotec GmbH, assignee. Adapter chimeric antigen receptor expressing cells for targeting of multiple antigens - EP3315511A1. Eur Pat Off. 2018.
38. Rezvani K, Rouce RH. The application of natural killer cell immunotherapy for the treatment of cancer. Front Immunol. 2015;6:578. doi:10.3389/fimmu.2015.00578.
39. Rezvani K, Rouce R, Liu E, Shpall E. Engineering natural killer cells for cancer immunotherapy. Mol Ther. 2017;25:1769–1781. doi:10.1016/j.ymthe.2017.06.012.
40. Klingemann H, Boissel L, Toneguzzo F. Natural killer cells for immunotherapy - advantages of the NK-92 cell line over blood NK cells. Front Immunol. 2016;7:91. doi:10.3389/fimmu.2016.00091.
41. Zhang J, Zheng H, Diao Y. Natural killer cells and current applications of chimeric antigen receptor-modified NK-92 cells in tumor immunotherapy. Int J Mol Sci. 2019;20:317.
42. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, Suttorp M, Seifried E, Ottmann OG, Bug G, *et al*. Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy. 2013;15:1563–1570. doi:10.1016/j.jcyt.2013.06.017.
43. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematotherapy Stem Cell Res. 2001;10:535–544. doi:10.1089/15258160152509145.
44. Uherek C, Tonn T, Uherek B, Becker S, Schnierle B, Klingemann H-G, Wels W. Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood. 2002;100:1265–1273. doi:10.1182/blood.V100.4.1265.h81602001265_1265_1273.
45. Maki G, Klingemann HG, Martinson JA, Tam YK. Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J Hematotherapy Stem Cell Res. 2001;10:369–383. doi:10.1089/152581601750288975.
46. Klingemann H. Are natural killer cells superior CAR drivers? Oncoimmunology. 2014;3:e28147. doi:10.4161/onci.28147.
47. Gargett T, Brown MP. The inducible caspase-9 suicide gene system as a 'safety switch' to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. Front Pharmacol. 2014;5:235. doi:10.3389/fphar.2014.00235.
48. Oelsner S, Waldmann A, Billmeier A, Röder J, Lindner A, Ullrich E, Marschalek R, Dotti G, Jung G, Große-Hovest L, *et al*. Genetically engineered CAR NK cells display selective cytotoxicity against FLT3-positive B-ALL and inhibit in vivo leukemia growth. Int J Cancer. 2019;145:1935–1945.
49. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, *et al*. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med. 2018;378:439–448. doi:10.1056/NEJMoa1709866.
50. Topp MS, Gökbuget N, Zugmaier G, Degenhard E, Goebeler M-E, Klinger M, Neumann SA, Horst HA, Raff T, Viardot A, *et al*. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. Blood. 2012;120:5185–5187. doi:10.1182/blood-2012-07-441030.
51. Topp MS, Gökbuget N, Zugmaier G, Klappers P, Stelljes M, Neumann S, Viardot A, Marks R, Diedrich H, Faul C, *et al*. Phase II trial of the anti-CD19 bispecific T cell-engager blinatumomab shows hematologic and molecular remissions in patients with relapsed or refractory B-precursor acute lymphoblastic leukemia. J Clin Oncol. 2014;32:4134–4140. doi:10.1200/JCO.2014.56.3247.
52. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF, *et al*. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014;371:1507–1517. doi:10.1056/NEJMoa1407222.
53. Mitwasi N, Feldmann A, Arndt C, Koristka S, Berndt N, Jureczek J, Loureiro LR, Bergmann R, Máthé D, Hegedüs N, et al. "UniCAR"-modified off-the-shelf NK-92 cells for targeting of GD2-expressing tumour cells. Sci Rep. 2020;10:1–16.
54. Lohmueller JJ, Ham JD, Kvorjak M, Finn OJ. mSA2 affinity-enhanced biotin-binding CAR T cells for universal tumor targeting. Oncoimmunology. 2017. doi:10.1080/2162402X.2017.1368604.
55. Urbanska K, Lanitis E, Poussin M, Lynn RC, Gavin BP, Kelderman S, Yu J, Scholler N, Powell DJ. A universal strategy for adoptive immunotherapy of cancer through use of a novel T-cell antigen receptor. Cancer Res. 2012;72(7):1844–1852. doi:10.1158/0008-5472.CAN-11-3890.

cancers

MDPI

Article

Adapter Chimeric Antigen Receptor (AdCAR)-Engineered NK-92 Cells for the Multiplex Targeting of Bone Metastases

Stefan Grote [1,*], Frank Traub [2], Joerg Mittelstaet [3], Christian Seitz [1], Andrew Kaiser [3], Rupert Handgretinger [1] and Sabine Schleicher [1]

[1] Department of Hematology and Oncology, University Hospital Tuebingen, Children's Hospital, 72076 Tuebingen, Germany; christian.seitz@med.uni-tuebingen.de (C.S.); rupert.handgretinger@med.uni-tuebingen.de (R.H.); sabine.schleicher@med.uni-tuebingen.de (S.S.)
[2] Department of Orthopedics and Traumatology, University Medical Center Mainz, Johannes Gutenberg University Mainz, 55131 Mainz, Germany; frank.traub@unimedizin-mainz.de
[3] Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany; joergmi@miltenyi.com (J.M.); andrewk@miltenyi.com (A.K.)
* Correspondence: stefan.grote@med.uni-tuebingen.de

Simple Summary: Metastatic disease remains one of the biggest challenges for tumor therapy. The aim of our study was the preclinical evaluation of adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cell efficacy as a possible treatment strategy for various types of bone metastatic cancers. We confirmed that AdCAR NK-92 cells successfully induces tumor cell lysis in bone metastasis cell lines derived from mammary, renal cell and colorectal carcinoma as well as melanoma in a specific and controllable manner, thus, establishing a potent cellular product with universal applicability and quick clinical translation potential for the treatment of solid tumors, including metastases.

Abstract: Background: Since metastatic spreading of solid tumor cells often leads to a fatal outcome for most cancer patients, new approaches for patient-individualized, targeted immunotherapy are urgently needed. Methods: Here, we established cell lines from four bone metastases of different tumor entities. We assessed AdCAR NK-92-mediated cytotoxicity in vitro in standard cytotoxicity assays as well as 3D spheroid models Results: AdCAR-engineered NK-92 cells successfully demonstrated distinct and specific cytotoxic potential targeting different tumor antigens expressed on cell lines established from bone metastases of mammary, renal cell and colorectal carcinoma as well as melanomas. In that process AdCAR NK-92 cells produced a multitude of NK effector molecules as well as pro inflammatory cytokines. Furthermore, AdCAR NK-92 showed increased cytotoxicity in 3D spheroid models which can recapitulate in vivo architecture, thereby bridging the gap between in vitro and in vivo models. Conclusions: AdCAR NK-92 cells may provide an interesting and promising "off-the-shelf" cellular product for the targeted therapy of cancers metastasizing to the bone, while utilization of clinically approved, therapeutic antibodies, as exchangeable adapter molecules can facilitate quick clinical translation.

Keywords: NK-92; adapter; chimeric antigen receptor; bone metastasis; solid tumors

Citation: Grote, S.; Traub, F.; Mittelstaet, J.; Seitz, C.; Kaiser, A.; Handgretinger, R.; Schleicher, S. Adapter Chimeric Antigen Receptor (AdCAR)-Engineered NK-92 Cells for the Multiplex Targeting of Bone Metastases. *Cancers* **2021**, *13*, 1124. https://doi.org/10.3390/cancers13051124

Academic Editors: Paola Vacca, Laura Chiossone and William J. Murphy

Received: 10 January 2021
Accepted: 4 March 2021
Published: 5 March 2021

1. Introduction

Cancer is one of the leading causes of death worldwide [1]. Despite the development of new therapeutic approaches and significant improvement of survival rates in the last 20 years, metastatic disease, primarily to the bone, lungs and brain, remains incurable and is the main cause of cancer-associated mortality [2]. Bone is the third most common site for tumor metastasis after the lungs and the liver [3,4]. Depending on the origin of the primary tumor, bone metastases are diagnosed in approximately 75% of patients with breast or prostate cancer and to a lesser extent in other cancers, including lung, kidney, liver and melanoma [5].

The most frequent site affected by bone metastasis is spine, including thoracic spine (63.6%) and lumbar spine (53.8%), followed by ribs (57.5%), pelvis (54.1%), sternum (44.3%), scapula (25.1%), and femur (24.8%) [6]. Bone metastases are often associated with hypercalcemia, severe bone pain, pathological fractures and spinal cord compression, leading to increased morbidity in cancer patients [7].

The treatment of bone metastases in patients with solid tumors is generally palliative, with very limited opportunities for complete eradication. Given the limited success of standard therapies at preventing or treating bone metastatic cancer novel therapeutic strategies designed to destroy dormant disseminated tumor cells and existing cancer metastases is an objective of paramount importance. Adoptive immunotherapy may represent such an innovative treatment option for bone metastases but has not been assessed in detail yet.

Chimeric antigen receptor (CAR)-modified lymphocytes represent a promising immunotherapeutic approach that involves the genetic modification of immune cells to express synthetic recombinant receptors on the cell surface, leading to predefined target specificity [8]. The CAR fusion protein typically comprises an extracellular single-chain variable fragment (scFv) of an antibody for target recognition, a hinge region to provide flexibility, a transmembrane region, and an intracellular activation domain for signal transduction. The core component of the CAR endodomain contains either the CD3ζ portion of the TCR complex or the γ-chain of the high-affinity IgE Fc receptor (first generation CAR), whereas the addition of one or two costimulatory domains derived from CD28, 4-1BB, OX40, ICOS or CD27 for example resulted in second and third generation CAR T cells with sustained activation, persistence and improved functions. Upon expression in lymphocytes, the CAR can engage its target antigen and thereby activating a variety of effector responses resulting in targeted cell killing [9].

Immunotherapy using autologous CD19 CAR T cells has resulted in impressive clinical response rates in patients with relapsed or refractory B cell malignancies [10–12]. Recently, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved two CD19 CAR T cell therapeutics, Kymriah (Tisagenlecleucel) and Yescarta (Axicabtagene ciloleucel), for patients with acute lymphoblastic leukemia and certain types of relapsed or refractory large B cell lymphoma. However, the patient-specific nature of this cell therapy, complex manufacturing workflows and the substantial risk of severe side effects, including cytokine release syndrome (CRS) and immune effector cell–associated neurotoxicity syndrome (ICANS) have led to concerns over costs and safety [13]. In addition, difficulties in obtaining sufficient numbers of autologous T cells for CAR production from heavily pretreated, lymphopenic patients may pose a further problem, illustrating the clinical need for alternative CAR effector cell sources.

There is a rapidly growing interest in NK cells for CAR engineering due to their potent anti-tumor activity and safety in an allogeneic, "off-the-shelf" format which could overcome some of the limitations associated with autologous CAR T cell therapies. In a recent phase 1 and 2 trials, CD19-specific CAR-engineered primary NK cells have shown a tremendous clinical response in patients with relapsed or refractory CD19-positive non-Hodgkin's lymphoma (NHL) or chronic lymphocytic leukemia (CLL) without the induction of typical CAR T cell-associated side effects such as cytokine release syndrome, neurotoxicity or graft-versus-host disease [14]. The majority of CAR NK cell studies to date, however, have been performed with NK-92 cells, a FDA-approved human cell line, which can be effectively expanded to high cell numbers and easily manufactured in a GMP-compliant manner. More importantly, early phase clinical trials have demonstrated the safety of irradiated NK-92 cells as an allogeneic cell therapeutic in patients with advanced hematological malignancies and solid tumors [15–17]. These properties make NK-92 cells an interesting option for CAR engineering and the development of standardized "off-the-shelf" cell products with enhanced antitumor activity for adoptive cancer immunotherapy [18]. Despite the progress in treating hematological malignancies, CAR T cells in patients with solid tumors have demonstrated only limited antitumor activity [19]. Antigen escape is a key barrier for

expanding the use of CAR-modified immune effector cells towards solid cancers with their more diverse surface antigen repertoires which are likely to fail single-targeted CAR therapy [20].

Since antigen heterogeneity and phenotypic plasticity of tumor cells present additional obstacles to the current development of CAR-based immunotherapies, efforts are being made to boost flexibility and improve the effectiveness by engineering modular chimeric antigen receptors so that the antigen recognition domain is split from the signaling domain of a conventional CAR, hence the target antigen can be switched or re-directed more readily without the requirement of re-engineering the CAR-modified immune effector cells [21].

We previously established a modular adapter CAR (AdCAR) platform which consists of AdCAR-expressing NK-92 cells that cannot recognize target antigens directly but are redirected to a target structure referred to as linker-label epitope, which consists of the endogenous vitamin biotin, conjugated to an adapter molecule (AM), e.g., a monoclonal antibody, in the context of special linker moiety, thereby allowing an on/off switch of CAR activity, and facilitating flexible targeting of various tumor antigens depending on the presence and specificity of the biotinylated AM [22–26]. Since novel or preexisting therapeutic antibodies can be easily labeled with biotin, there are almost unlimited possibilities in tumor antigen targeting using the AdCAR technology.

Bringing together the advantages of NK-92 cells as an "off-the-shelf" therapeutic and the controllable multiplex targeting capacity of the AdCAR system, led to the generation of a universal, on-demand CAR NK product which can be maintained and expanded at low cost in a GMP compliant manner for clinical use. Here, we outline the preclinical approach using AdCAR NK-92 cells in combination with therapeutic antibodies for targeting and elimination of bone metastatic cells in vitro using newly established bone metastatic cancer cell lines from different tumor entities, including mammary carcinoma, colorectal carcinoma, renal cell carcinoma and melanoma.

2. Results

2.1. Establishment and Characterization of Newly Developed Bone Metastasis Cell Lines

Tumor material from resected bone metastases and blood samples of the patients were provided by the Department of Orthopedic Surgery, University Hospital Tuebingen (UKT) (Table 1).

Table 1. Patient data for tumor cells from bone metastasis resections.

	Patient Age [years]	Patient Sex	Metastatic Site	Tumor Entity	Designation
Patient 1	63	f	Scapula	Mammary carcinoma	**MAC**
Patient 2	17	m	Spine L3/L4	Renal cell carcinoma	**MAM**
Patient 3	54	m	Spine C7	Colorectal carcinoma	**MCK83**
Patient 4	47	m	Acetabulum	Melanoma	**MeGa17**

Cell lines were established from outgrowth cultures and successfully cultivated for more than 30 passages. All assays conducted throughout the present study were performed with cell lines in early passages between 5 and 7 to prevent potential, cell culture-induced mutational changes. Cell line authentication was performed by Eurofins Scientific using short tandem repeat (STR) analysis. Tumor cells were immunophenotyped for extracellular expression of tumor antigens that can be targeted by therapeutic antibodies using flow cytometry (Figure 1 and Table 2).

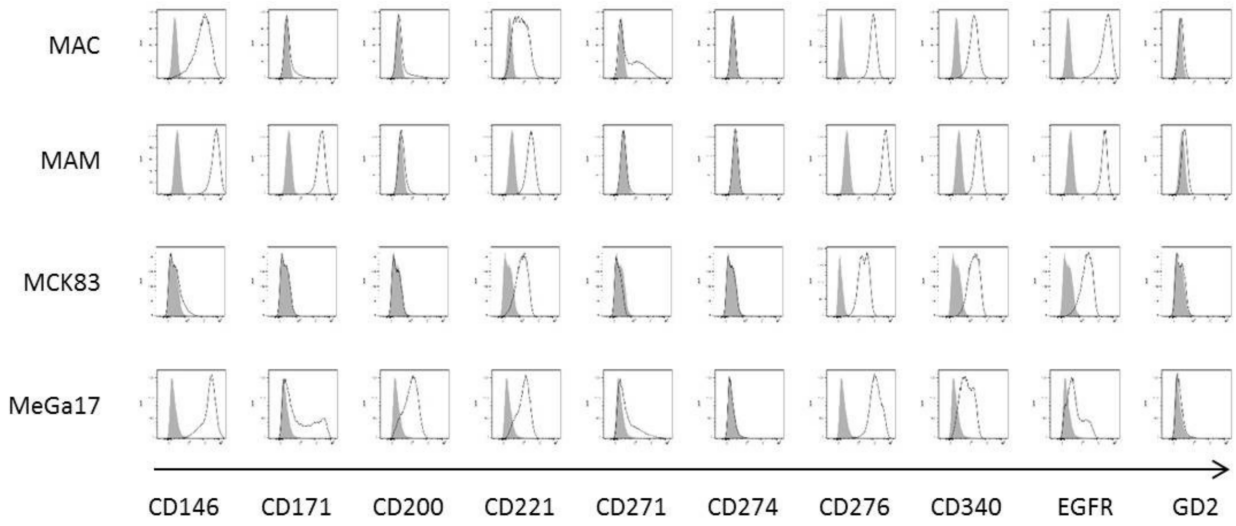


Figure 1. Cell line immunophenotyping. Bone metastatic cell lines were screened for tumor antigen surface expression using flow cytometry. Cells were co-incubated with primary biotinylated antibodies for 15 min at 4 °C. Antigen expression was detected using a secondary PE-coupled anti-biotin antibody (black line) using staining with the secondary antibody alone as negative control (grey area).

Table 2. Cell line immunophentoyping. Tumor cells were co-incubated with primary biotinylated antibodies. Antigen expression was detected using a secondary PE-coupled anti-biotin antibody. Percentage of stained cells and median fluorescence index (MFI) values were calculated using staining with the secondary antibody alone as negative control.

Cell Line	Percentage of Stained Cells									
	CD146	**CD171**	**CD200**	**CD221**	**CD271**	**CD274**	**CD276**	**CD340**	**EGFR**	**GD2**
MAC	97.70%	11.80%	13.90%	68.50%	44.60%	0.40%	100.00%	97.90%	99.90%	1.78%
MAM	99.80%	99.90%	5.29%	99.40%	2.06%	0.65%	99.90%	99.70%	100.00%	4.04%
MCK83	5.62%	0.45%	0.31%	59.60%	0.13%	0.46%	99.50%	81.20%	85.80%	0.44%
MeGa17	99.20%	42.90%	62.10%	70.40%	22.80%	1.23%	99.00%	44.10%	27.20%	1.91%
	Median Fluorescence Index (MFI)									
	CD146	**CD171**	**CD200**	**CD221**	**CD271**	**CD274**	**CD276**	**CD340**	**EGFR**	**GD2**
MAC	45.48	1.15	1.23	4.10	1.91	1.03	106.65	9.39	183.42	1.21
MAM	194.20	79.54	1.16	11.27	0.99	1.03	113.92	11.59	92.86	1.35
MCK83	1.22	1.00	0.96	6.26	0.77	0.97	31.39	10.08	12.41	1.14
MeGa17	172.52	3.49	7.71	9.06	1.96	0.96	67.70	5.00	2.78	1.18

All cell lines shared high expression of B7-H3 (CD276), a recently emerging immune checkpoint molecule of the B7 superfamily. The epithelial growth factor receptor (EGFR), an important oncogene in the development of lung and colorectal cancer, is expressed in the mammary carcinoma MAC, the renal cell carcinoma MAM and the colorectal carcinoma MCK83 but not in the melanoma MeGa17. Interestingly, the melanoma cell adhesion molecule MCAM (CD146) was not only expressed in the MeGa17 cell line but also in the MAM cell line.

Further characterization of the tumor cell lines was conducted by flow cytometric assessment of cell surface expression of well-known NK cell ligands (Table 3). The colorectal carcinoma MAC, renal cell carcinoma MAM and melanoma MeGa17 showed comparable NK ligand expression profiles. Uniformly, all cell lines expressed human leukocyte antigen E (HLA-E), a major ligand for the inhibitory receptor complex CD94/NKG2A on NK cells, as well as the major histocompatibility complex (MHC) class I chain-related protein A and B (MICA/B), a protein that acts as an activating signal for NK cells through the natural killer group 2, member D (NKG2D or CD314) receptor. Moreover, all four cell

lines expressed death receptor 5 (CD262), Nectin 2 (CD112) and PVR (CD155). CD262 is a cell surface receptor of the TNF-receptor superfamily that binds the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and mediates apoptosis. CD262 and CD112 are important ligands for DNAM-1 (CD226) triggering activating signaling cascades. They can also trigger an inhibitory NK response by activation of the immune checkpoint receptor TIGIT. Interestingly, the NK ligand HLA-ABC was completely absent on MCK83 cells while MAC, MAM and MeGa17 cells expressed it widely.

Table 3. Characterization of tumor cell lines for NK ligand expression. Tumor cells were co-incubated with fluorescently labeled antibodies. MFI values were calculated using staining with the respective isotype control antibody.

Cell Line	CD48	CD50	CD54	CD58	CD95	CD102	CD112	CD155	CD261	CD262
MAC	1.47	1.69	5.46	73.22	23.35	1.27	34.41	128.96	3.01	16.87
MAM	1.36	1.78	7.31	30.55	9.54	1.13	69.73	192.65	6.06	59.84
MCK83	1.84	1.75	1.29	5.54	4.26	1.27	15.32	89.62	4.15	14.91
MeGa17	1.25	1.58	9.66	31.32	2.18	1.13	16.27	50.20	1.22	15.03
	HLA-ABC		**HLA-DR**	**HLA-E**	**HLA-G**	**MICA/B**	**ULBP1**	**ULBP2/5/6**	**ULBP3**	**ULBP4**
MAC	52.41		0.90	9.18	0.98	6.53	3.45	1.26	0.68	0.75
MAM	19.07		0.87	8.07	0.81	21.25	3.15	1.00	1.67	1.53
MCK83	0.47		0.92	10.01	0.82	9.07	6.09	1.09	1.25	1.41
MeGa17	23.33		12.10	7.55	1.34	7.27	2.45	1.37	0.92	0.77

2.2. AdCAR NK-92 Cells Specifically Lyse Bone Metastasis Cell Lines In Vitro

NK-92 cells were transduced with lentiviral vectors encoding the second generation adapter CAR with a CD28 co-signaling domain and an intracellular immunoreceptor tyrosine-based activation motif (ITAM) from the CD3 zeta chain (CD3ζ). Cells were subsequently single-cell sorted for highest CAR expression and functionality. Transduction process and AdCAR NK-92 cell characterization was previously described [23]. AdCAR NK-92 cells showed stable AdCAR surface expression for at least 150 days after cell sorting with an average viability of >90%. Proliferation rate was not impaired by the transduction process compared to untransduced, parental NK-92 cells.

Functional assessment of AdCAR NK-92-mediated cytotoxicity was conducted using the previously established metastatic tumor cell lines earlier described in this manuscript as target cells. Calcein-labeled tumor cells were co-incubated with AdCAR NK-92 as well as parental NK-92 cells in the presence and/or absence of biotinylated antibodies (bAb) targeting antigens highly expressed on the tumor cells. AdCAR NK-92 but not untransduced NK-92 cells significantly induced cellular cytotoxicity against all tumor cell lines but only in the presence of a bAb targeting antigens sufficiently expressed on the cell surface (Figure 2a–d), thus, underscoring the controllability of the AdCAR system. Correlation of median fluorescence index (MFI) as an indicator of antigen density on tumor cell surface and specific cell lysis of MAC, MAM, MCK83 and MeGa17 cells resulted in R^2 values of 0.5157, 0.1161, 0.7650 and 0.5375, respectively.

Next, we examined the kinetics of AdCAR-mediated cytotoxicity after addition of specific biotinylated antibodies. Utilizing the xCELLigence real-time label-free live cell analysis (RTCA) system based in cell impedance measurement, tumor cells were co-incubated with AdCAR and parental NK-92 cells with and without bAb and monitored for over 12 h. The dimensionless cell index is proportional to the amount of live tumor cells. NK-mediated cytotoxicity is assessed by measurement of cell index decrease. AdCAR NK-92 cells but not parental NK-92 cells successfully lysed the tumor cells of renal cell carcinoma MAM and melanoma MeGa17 in less than 4 h, but only in the presence of a specific bAb (Figure 3a,b). Specific tumor cell lysis correlated with surface expression of the respective antigen and no long-term tumor regrowth was observed with adapter molecules targeting highly expressed antigens.

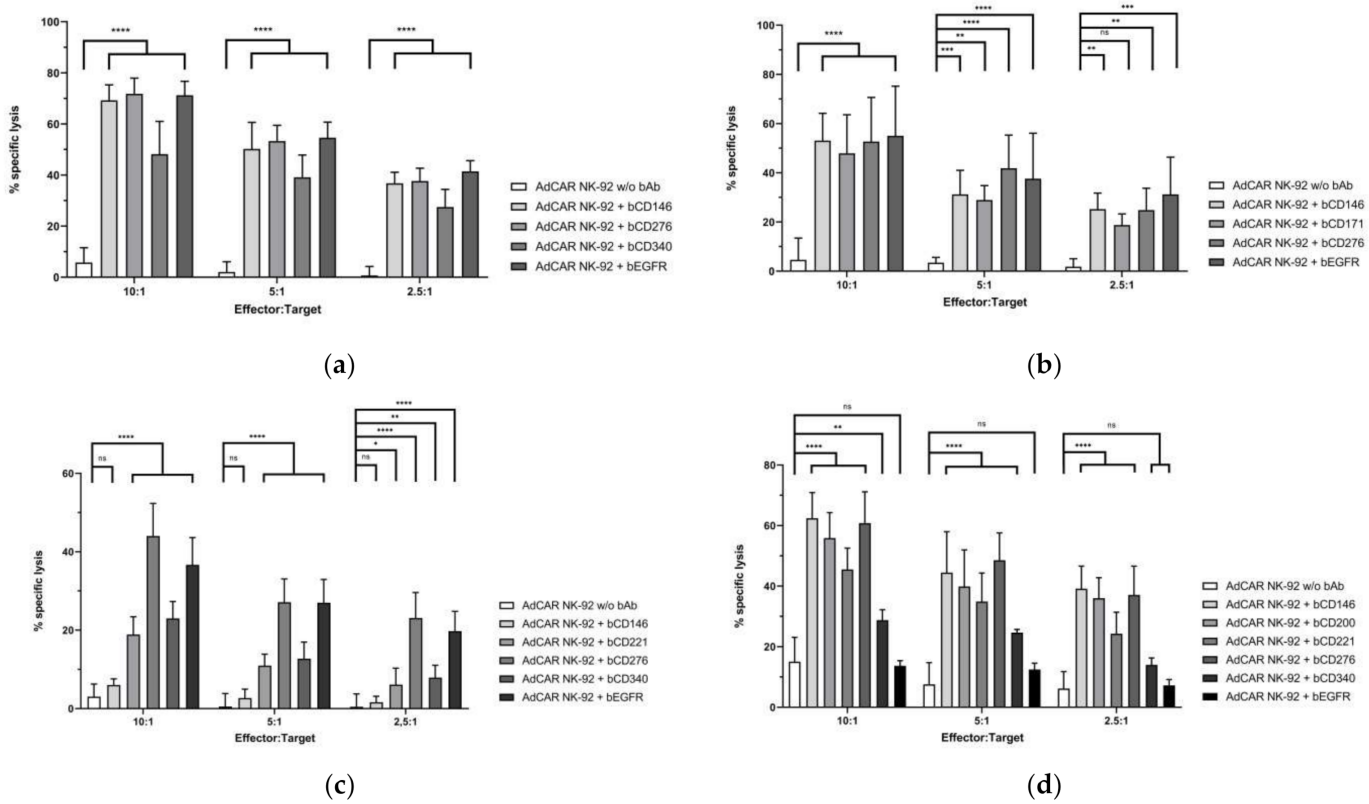


Figure 2. AdCAR NK-92-mediated metastatic tumor cell lysis. AdCAR NK-92 cells were co-incubated with calcein-labeled tumor cell lines MAC (**a**), MAM (**b**), MCK83 (**c**) and MeGa17 (**d**) in the presence or absence of indicated biotinylated antibodies for 2 h at indicated E:T ratios. Specific lysis is shown as mean ± SD, n = 3. ****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.1$; ns: $p \geq 0.1$.

To further examine NK-92-mediated lysis, a cytokine secretion profile was established to screen for secretion of a variety of cytokines, including NK cell effector molecules. Various cytokines were significantly increased after co-incubation of AdCAR-transduced NK-92 cells with MAC cells (Figure 4). GM-CSF (22-fold; $p < 0.002$), IL-10 (10-fold, $p < 0.0002$), granulysin (24-fold; $p < 0.0006$), granzyme B (6-fold, $p < 0.0001$), IFN-γ (10-fold; $p < 0.0009$), MIP-1b (2-fold; $p < 0.008$) and TNF-α (32-fold; $p < 0.0001$) showed significantly elevated levels but only upon AdCAR induction via specific biotinylated antibodies. While enhanced secretion of granulysin and granzyme B directly account for increased tumor lysis, IFN-γ and TNF-α stimulate the endogenous immune system and indirectly enhance anti-tumor activity. Secretion of MCP-1 and perforin was not significantly augmented after AdCAR activation (1.7-fold and 1.4-fold, respectively).

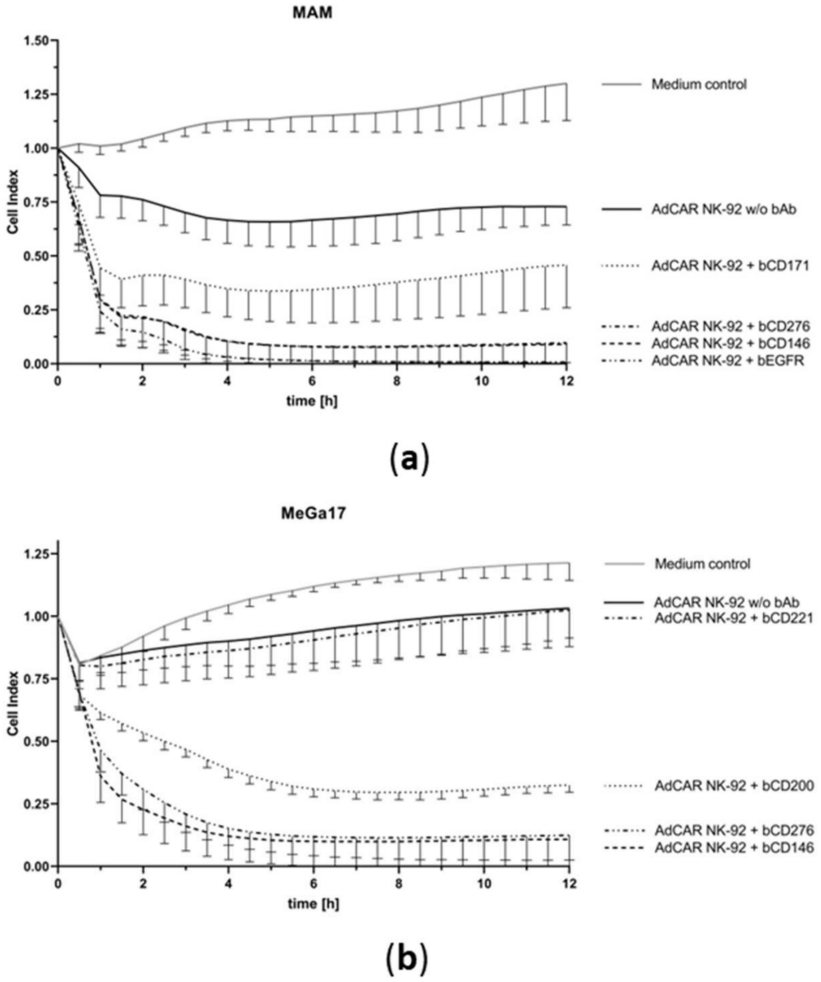


Figure 3. Kinetics of AdCAR-mediated tumor cell lysis. AdCAR NK-92 cells were co incubated with unlabeled tumor cell lines MAM (**a**) and MeGa17 (**b**) in the presence or absence of indicated biotinylated antibodies and constantly monitored over time using the xCELLigence real time cell analysis system. NK-mediated tumor cell lysis is depicted as decrease in the dimensionless "cell index", $n = 3$.

2.3. NK-92 Cells Exhibit Successful AdCAR-Mediated Cytotoxicity in a Three-Dimensional Tumor Cell Model

While the majority of in vitro studies about cellular immunotherapy are still based on tumor cell monolayer culture systems, examination of three-dimensional (3D) tumor models allows for limited translation to the in vivo situation. Thus, we generated multicellular spheroids of the GFP-transduced cell lines. Since just one out of the four cell lines successfully grew as a solid spheroid, only the renal cell carcinoma MAM was used to assess cytotoxic potential of AdCAR NK-92 cells in a 3D model.

After four days of culture tumor spheroids were co-incubated with either AdCAR-transduced or parental NK-92 cells in the presence or absence of biotinylated antibodies and monitored for over 96 h. Fluorescence signals of MAM spheroids co-incubated with NK-92 cells were correlated with untreated control spheroids (Figure 5a,b). After 48 h AdCAR NK-92 cells in combination with bCD146, bCD276 or bEGFR successfully increased NK-mediated lysis of MAM tumor cells to 76.9%, 81.1% and 80.3%, while AdCAR NK-92 cells in combination with bCD171 showed specific lysis of 51.4% of tumor cells. After 96 h AdCAR-mediated cell lysis increased to 82.3% (bCD146), 57.0% (bCD171), 83.5% (bCD276), 93.3% (bEGFR).

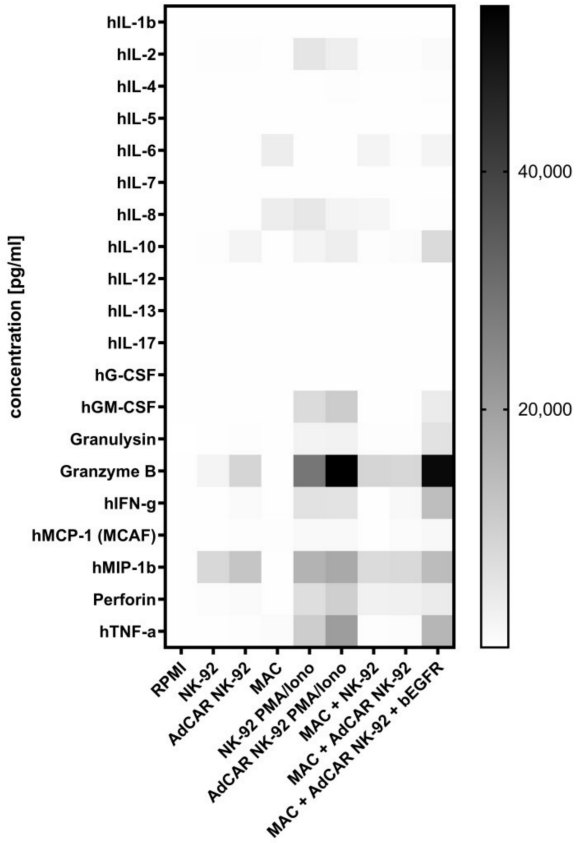


Figure 4. Cytokine secretion profile of AdCAR NK-92 cells. AdCAR NK-92 cells as well as parental NK-92 cells were co-incubated with the tumor cell line MAC in the presence and absence of bEGFR for 6 h at an E:T ratio oif 5:1. The release of cytokines was measured using the Bio-Plex Pro human cytokine 17-plex assay and is shown as a heatmap. PMA/Ionomycin was used as control to induce maximum cytokine secretion.

3. Discussion

To date, conventional therapies have limited success in preventing or treating bone metastasis due to the complex nature of the bone microenvironment, tumor heterogeneity, and the therapeutic resistance of dormant tumor cells. Despite significant advancement of conventional therapies, such as surgery, chemotherapy, hormone or radio therapy, metastatic disease remains virtually incurable and still is one of the most common causes for cancer-associated mortality [2].

Our recently developed AdCAR platform combines a modular adapter chimeric antigen receptor with the universal applicability of the NK-92 cell line, thus creating an "off-the-shelf" cellular product for the treatment of cancer [23]. Currently, universal CAR approaches with T or NK cells using adapter molecules that are foreign to the human body like the affinity-enhanced monomeric streptavidin 2 (mSA2) can potentially cause immunogenic reactions and make clinical translation difficult [27,28]. Even adapter molecules tagged with endogenous molecules such as biotin could potentially cause adverse reactions due to the immunogenicity of avidin and streptavidin [29]. The adapter system used herein is based on a scFv targeting a "neo"-epitope-like structure, the linker label epitope, consisting of biotin in the context of a mAb, instead of biotin itself [26]. Hence, application of AdCAR NK-92 cells can circumvent adverse immunogenic reactions of other CAR systems and decrease the risk of severe side effects during therapy.

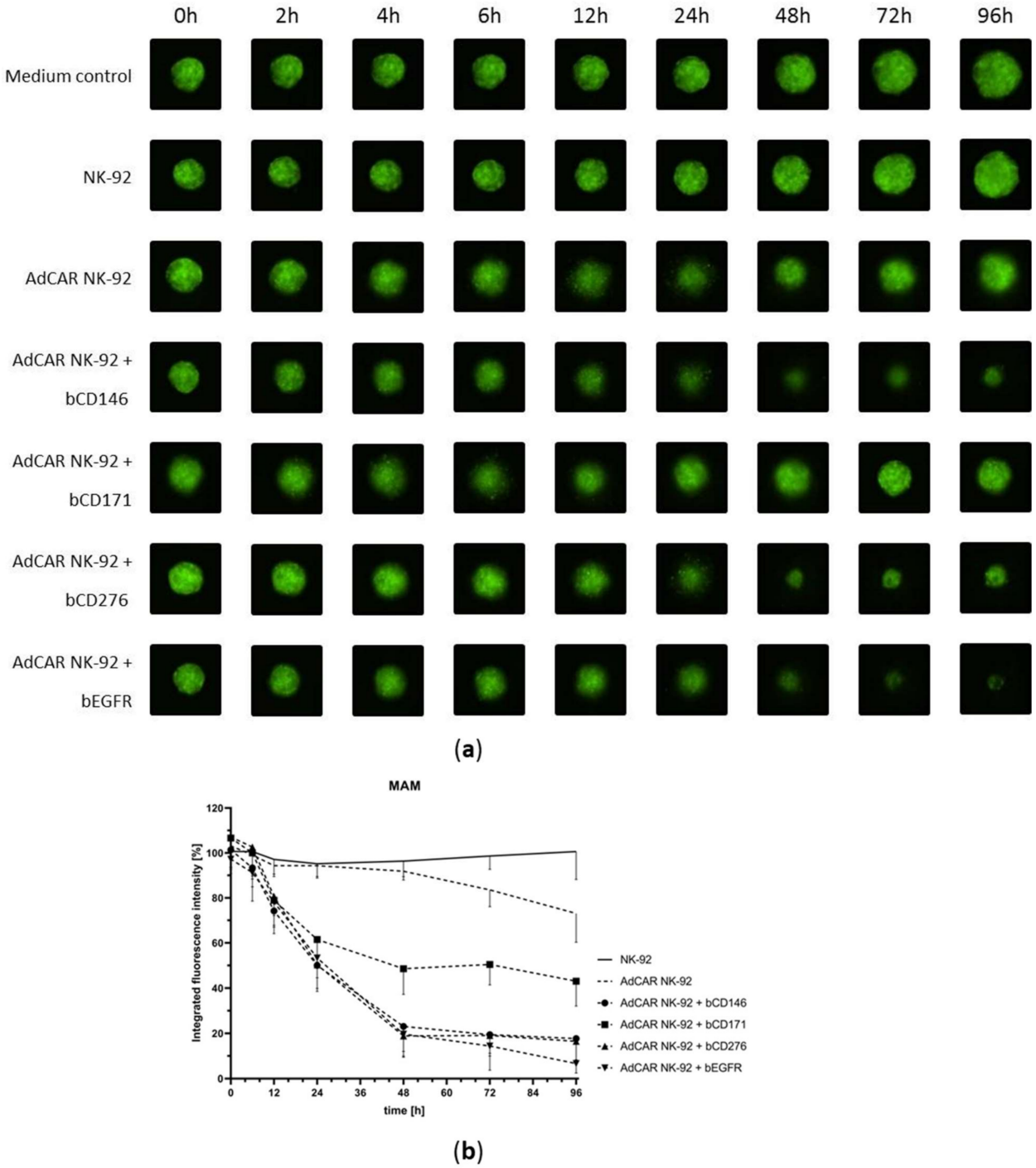


Figure 5. AdCAR NK-92-mediated lysis of 3D tumor spheroids. GFP-transduced cell lines MAM was grown as 3D spheroids in ultra-low attachment plates and subsequently co-incubated with AdCAR NK-92 or parental NK-92 cells and indicated biotinylated antibodies for 96 h in at least three individual experiments. Fluorescence images show representative MAM spheroids at indicated time points (**a**). Integrated fluorescence intensity of tumor spheroids was measured regularly using the Celigo S Imaging Cytometer (Nexcelom, Lawrence, MA, USA), compared to untreated control spheroids and is shown as mean ± SD, $n = 3$ (**b**).

The flexibility of the presented AdCAR system can further counteract drawbacks of conventional CAR T cell therapy such as off-tumor toxicity or tumor evasion strategies. Previous CAR T cell studies showed downregulation of the target antigen as a reaction to therapy which ultimately leads to immunotherapy resistance [30–32]. Additionally,

since only few antigens are uniquely specific for solid tumors, eradication of tumor cells while simultaneously sparing healthy tissue is a major concern of CAR T cell therapy [33]. Utilizing a standardized flow cytometry screening panel enables target options that are tailored on a patient-individualized basis. In case of tumor antigen evasion during therapy, the target structure can be easily switched by application of a different bAb while still retaining therapeutic efficacy. Moreover, simultaneous or consecutive use of different biotinylated antibodies in combination with AdCAR NK-92 cells may provide a possible treatment strategy for highly heterogeneous tumors and is able to counteract tumor antigen loss and off-tumor/on-target toxicity. Utilization of biotinylated antibodies, which are already approved by the FDA, such as cetuximab or trastuzumab generates functional adapter molecules with a known safety profile for AdCAR NK-92 therapy and facilitates translation into clinical settings.

In this study, we demonstrated that CAR-modification of the NK-92 cell line with our AdCAR system can enable promising therapeutic opportunities for the treatment of a variety of metastatic tumor entities. AdCAR NK-92 cells effectively eliminated tumor cells from the newly established cell lines derived from bone metastases of renal cell, mammary and colorectal carcinomas, as well as melanomas in standard in vitro cytotoxicity assays within two hours. Specificity of cytolytic activity was achieved by prior screening for suitable target antigen structures on tumor cell lines and utilization of respective biotinylated antibodies as adapter molecules. Due to the lack of FcγRIII (CD16) expression on NK-92 cells, the formation of the immunological synapse is simply dependent on bAb titration [34]. AdCAR NK-92 cells that were co-incubated without bAb or with bAb without specificity of interest lead to little to no tumor lysis. Additional controllability of AdCAR NK-mediated cytotoxicity is underscored by the fact that cytolytic activity occurred in a concentration-dependent manner [23]. Moreover, AdCAR NK-92 cells are functionally independent of the target cells' NK ligand profile. Specific AdCAR-mediated cytotoxicity could be demonstrated regardless of tumor cell surface expression of inhibitory ligands such as HLA-E which was shown to have negative effects cytotoxic activity of primary NK cells [35,36].

As shown in first in vivo and clinical CAR NK-92 trials the NK cell line was less likely to induce severe side effects such as neurotoxicity and cytokine release syndrome (CRS) [37,38]. Likewise, in the present study, IL-6, a driving causation of CRS, was not produced by AdCAR NK-92 cells after co-incubation with either tumor cell line or a specific bAb. The secretion of the pro-inflammatory cytokines IFN-γ and TNF-α, as shown by AdCAR NK-92 cells co-incubated with the target cell line and a specific bAb, may, additionally, stimulate the endogenous immune system and enhance anti-tumor activity [18].

One of the key necessities of CAR-immune cell therapies to be successful for the treatment of solid tumors and, especially, in the metastatic setting is their capability of immune cell homing and tumor infiltration [39–41]. Establishment of three-dimensional in vitro spheroids enables accurate assessment of a range of in vivo biological processes [42]. Here, AdCAR NK-92 cells are clearly able to efficiently lyse tumor spheroids in an antibody-dependent manner. Furthermore, they have been shown to be resistant to the tumor microenvironmental influences such as the secretion of transforming growth factor beta (TGFβ) which was reported to account for tumor resistance to immunotherapy [18,43,44]. Future research needs to evaluate the therapeutic potential of AdCAR NK-92 cells in pre-clinical in vivo and clinical settings. Particularly, mouse bone metastasis models will be of major importance to assess AdCAR NK-92 biodistribution, homing and infiltration.

As previously described, frozen CAR-engineered NK-92 cells can be thawed and expanded as a batch culture in gas permeable cell culture bags. Doubling time of CAR NK-92 cells is approximately 32 to 36 h and culture yields can be individually scaled according to treatment doses and number of patients treated [45]. Safety was proven in a recent phase I clinical trial with CD33-specific CAR NK-92 cells. No dose-limiting toxicities were observed upon repeated intravenous infusions of up to 5×10^9 irradiated cells per

dose [37]. Several other early phase clinical trials with CAR-engineered NK-92 cells are also currently being carried out in Europe, China and the US (clinicaltrials.gov; NCT 02742727, 03383978, 04050709).

For clinical translation, metastatic tumor cells isolated from bone marrow biopsies or surgery can be quickly screened for their target antigen expression profile with the implemented, standardized antibody panel using flow cytometry or ultra-high content imaging techniques. AdCAR-engineered NK-92 cells could be utilized to eradicate disseminated, dormant metastatic tumor cells within the bone marrow as well as micrometastases. Furthermore, for the treatment of large bone metastases, AdCAR NK-92 cells could be given as intratumoral injections or also be applied after surgery at the resection site to eliminate non-resectable tumor residues. A well-characterized, GMP-compliant qualified master cell bank of CAR NK-92 cells as a reliable source for subsequent production of patient doses, enables therapeutic translation within hours of the surgery [38,46]. Together with biotinylated therapeutic antibodies, universal "off-the-shelf" AdCAR NK-92 cells can be utilized for flexible and patient-individualized therapy, thus, enabling broadly available and more affordable immunotherapy for cancer patients independent of specialized facilities. Their specific properties make AdCAR NK-92 cells a promising treatment option for bone metastases.

4. Materials and Methods

4.1. Cell lines and Culturing Conditions

NK-92 cells were purchased from ATCC and maintained at a concentration of 10^5 cells/mL in MEM Alpha Medium containing stable L-glutamine (GlutaMAX, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% FBS and 100 U/mL IL 2 (Proleukin, Aldesleukin, Chiron, Emeryville, CA, USA) referred to as NK-92 complete medium. MAC, MAM, MCK83 and MeGa17 cell lines were established from biopsy tumor samples from resected bone metastases provided by the Department of Orthopedic Surgery, University Hospital Tuebingen (UKT). Newly established cell lines and matched tumor samples, as well as patient's blood lymphocytes for comparison, were authenticated by short-tandem-repeat analysis (Eurofins Scientific, Luxembourg City, Luxembourg) to verify cell origin and identity. Tumor cell lines were cultivated in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) containing GlutaMAX, referred to as RPMI complete medium, maintained at 37 °C in a humidified 5% CO_2 atmosphere and regularly tested for mycoplasma contamination. The present study was approved by the ethics committee at the Medical Faculty of the Eberhard Karls University and the University Hospital Tuebingen (reference number 008/20114/BO2). Human material was collected after obtaining informed consent in accordance with the Helsinki protocol. All media contained 1x antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA).

4.2. Design of the AdCAR System

The second-generation adapter CAR is based on the mAb "mBio3"-derived single-chain variable fragment (scFv) targeting a "neo"-epitope-like structure, the linker label epitope, consisting of biotin in the context of a mAb. Exact constitution of the AdCAR construct and generation of AdCAR-engineered NK-92 cells was previously described [23].

4.3. Biotinylated Antibodies

Antibodies were either purchased as bAb from Miltenyi Biotec (Bergisch-Gladbach, Germany) or acquired from the UKT pharmacy and biotinylated by Davids Biotechnologie (Regensburg, Germany).

Antigen	Clone	Antibody	Order #	Lot	Supplier
CD146	541-10B2	n/a	130-092-852	5190627154	Miltenyi Biotec, Bergisch Gladbach, Germany
CD171	REA163	n/a	130-100-702	5190607129	Miltenyi Biotec, Bergisch Gladbach, Germany
CD200	OX-104	n/a	130-106-064	5191021606	Miltenyi Biotec, Bergisch Gladbach, Germany
CD221	REA271	n/a	130-103-973	5190627184	Miltenyi Biotec, Bergisch Gladbach, Germany
CD271	REA844	n/a	130-112-608	5190627191	Miltenyi Biotec, Bergisch Gladbach, Germany
CD274	n/a	Atezolizumab	n/a	n/a	Hoffmann-La Roche, Basel, Switzerland
CD276	FM276	n/a	130-095-514	5190627174	Miltenyi Biotec, Bergisch Gladbach, Germany
CD340	n/a	Trastuzumab	n/a	n/a	Hoffmann-La Roche, Basel, Switzerland
EGFR	n/a	Cetuximab	n/a	n/a	Merck KgaA, Darmstadt, Germany
GD2	n/a	Dinutuximab beta	n/a	n/a	Eusa Pharma, Hertfordshire, Great Britain

4.4. Flow Cytometry

Staining of cells was conducted using primary biotinylated mAb with antigen specificity of interest. Cells were incubated with specific antibodies at 4 °C in flow cytometry buffer containing PBS (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% FBS and 0.5 M EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Unbound antibody was washed off by centrifugation (4 °C, 350 g, 5 min) and stained with a secondary, fluorophore-labeled anti-biotin antibody (Anti-Biotin PE, clone: REA746, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min followed by another washing step. Surface antigen expression was analyzed with the secondary antibody alone as a negative control using a BD FACSCanto II flow cytometer (BD, Franklin Lakes, NJ, USA).

4.5. Calcein Release-Based Cytotoxicity Assay (CRA)

Target cell staining with Calcein AM (Thermo Fisher Scientific, Waltham, MA, USA) as well as the protocol for the calcein release-based cytotoxicity assay (CRA) was described previously [23,45].

4.6. Real-Time Label-Free Live Cell Analysis

Bone metastasis cell lines were adjusted to a concentration of 10^5 cells/mL in RPMI complete medium and seeded in E-Plate 96 VIEW (OLS, Bremen, Germany) micro-well plates. Effector AdCAR NK-92 cells were adjusted to an E:T ratio of 5:1 in NK-92 complete medium without IL-2 and co-incubated with the target cells in the presence or absence of specific bAb. Utilizing the xCELLigence real-time cell analysis (RTCA, OLS, Bremen, Germany) system, cells were monitored for over 12 h. Tumor cell viability was calculated using the RTCA 2.0 software (OLS, Bremen, Germany) and AdCAR-mediated cytotoxicity was subsequently determined.

4.7. Quantification of Cytokine Release

Cytokine release of AdCAR NK-92 cells upon AdCAR induction was determined using the Bio-Plex Pro human cytokine 17-plex assay (Bio Rad, Hercules, CA, USA). The respective protocol was described previously [23].

4.8. 3D Spheroid Cytotoxicity Assay

GFP-transduced metastatic tumor cells were grown as three-dimensional spheroids and co-incubated with NK-92 as well as AdCAR NK-92 cells in the presence or absence of biotinylated antibodies. The respective protocol was described previously [23].

4.9. Data Analysis

All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Flow cytometry data were analyzed using FlowJo software V10.0.8 (FlowJo LLC, Ashford, OR, USA).

5. Conclusions

Innovative immunotherapy for the treatment of solid cancers and especially metastatic disease is urgently needed. Adapter CAR-engineered NK-92 cells are able to combine their "off-the-shelf" availability with personalized and controllable elimination of metastatic tumor cells, thus, establishing a potent cellular product with universal applicability and quick clinical translation potential for the treatment of solid tumors, including metastases.

Author Contributions: Conceptualization, S.G. and S.S.; validation, S.G. and S.S.; formal analysis, S.G.; investigation, S.G., F.T., J.M., C.S. and A.K.; writing—original draft preparation, S.G. and S.S.; writing—review and editing, S.G. and S.S.; visualization, S.G.; supervision, S.S.; project administration, S.G. and S.S.; funding acquisition, R.H. All authors have read and agreed to the published version of the manuscript.

Funding: The employing institution of S.G., C.S., R.H. and S.S. (University Children's Hospital Tuebingen) received research funding by Miltenyi Biotec GmbH on the basis of a collaboration research agreement.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by ethics committee at the Medical Faculty of the Eberhard Karls University and the University Hospital Tuebingen (reference number 008/2014BO2).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: J.M., C.S., A.K. and R.H. are co-inventors of a patent application focusing on adapter CAR technology.

References

1. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2019. *Cancer J. Clin.* **2019**, *69*, 7–34. [CrossRef]
2. Chaffer, C.L.; Weinberg, R.A. A perspective on cancer cell metastasis. *Science* **2011**, *331*, 1559–1564. [CrossRef] [PubMed]
3. Hage, W.D.; Aboulafia, A.J.; Aboulafia, D.M. Incidence, location, and diagnostic evaluation of metastatic bone disease. *Orthop. Clin. North Am.* **2000**, *31*, 515–528. [CrossRef]
4. Mundy, G.R. Metastasis to bone: Causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer* **2002**, *2*, 584–593. [CrossRef] [PubMed]
5. Macedo, F.; Ladeira, K.; Pinho, F.; Saraiva, N.; Bonito, N.; Pinto, L.; Gonçalves, F. Bone metastases: An overview. *Oncol. Rev.* **2017**, *11*, 321. [CrossRef] [PubMed]
6. Chen, W.-Z.; Shen, J.-F.; Zhou, Y.; Chen, X.-Y.; Liu, J.-M.; Liu, Z.-L. Clinical characteristics and risk factors for developing bone metastases in patients with breast cancer. *Sci. Rep.* **2017**, *7*, 1–7. [CrossRef]
7. Coleman, R.E. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin. Cancer Res.* **2006**, *12*, 6243s–6249s. [CrossRef]
8. Gross, G.; Waks, T.; Eshhar, Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity (chimeric genes/antibody variable region). *Immunology* **1989**, *86*, 10024–10028.
9. Sadelain, M.; Brentjens, R.; Rivière, I. The basic principles of chimeric antigen receptor design. *Cancer Discov.* **2013**, *3*, 388–398. [CrossRef]
10. Maude, S.L.; Frey, N.; Shaw, P.A.; Aplenc, R.; Barrett, D.M.; Bunin, N.J.; Chew, A.; Gonzalez, V.E.; Zheng, Z.; Lacey, S.F.; et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N. Engl. J. Med.* **2014**, *371*, 1507–1517. [CrossRef]

11. Lee, D.W.; Kochenderfer, J.N.; Stetler-Stevenson, M.; Cui, Y.K.; Delbrook, C.; Feldman, S.A.; Fry, T.J.; Orentas, R.; Sabatino, M.; Shah, N.N.; et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: A phase 1 dose-escalation trial. *Lancet* **2015**, *385*, 517–528. [CrossRef]
12. Gardner, R.A.; Finney, O.; Annesley, C.; Brakke, H.; Summers, C.; Leger, K.; Bleakley, M.; Brown, C.; Mgebroff, S.; Kelly-Spratt, K.S.; et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood* **2017**, *129*, 3322–3331. [CrossRef] [PubMed]
13. Srivastava, S.; Riddell, S.R. Chimeric antigen receptor T cell therapy: Challenges to bench-to-bedside efficacy. *J. Immunol.* **2018**, *200*, 459–468. [CrossRef] [PubMed]
14. Liu, E.; Marin, D.; Banerjee, P.; Macapinlac, H.A.; Thompson, P.; Basar, R.; Kerbauy, L.N.; Overman, B.; Thall, P.; Kaplan, M.; et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. *N. Engl. J. Med.* **2020**, *382*, 545–553. [CrossRef]
15. Tonn, T.; Schwabe, D.; Klingemann, H.G.; Becker, S.; Esser, R.; Koehl, U.; Suttorp, M.; Seifried, E.; Ottmann, O.G.; Bug, G. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* **2013**, *15*, 1563–1570. [CrossRef] [PubMed]
16. Williams, B.A.; Law, A.D.; Routy, B.; Denhollander, N.; Gupta, V.; Wang, X.-H.; Chaboureau, A.; Viswanathan, S.; Keating, A. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. *Oncotarget* **2017**, *8*, 89256–89268. [CrossRef] [PubMed]
17. Boyiadzis, M.; Agha, M.; Redner, R.L.; Sehgal, A.; Im, A.; Hou, J.-Z.; Farah, R.; Dorritie, K.A.; Raptis, A.; Lim, S.H.; et al. Phase 1 clinical trial of adoptive immunotherapy using "off-the-shelf" activated natural killer cells in patients with refractory and relapsed acute myeloid leukemia. *Cytotherapy* **2017**, *19*, 1225–1232. [CrossRef]
18. Zhang, C.; Oberoi, P.; Oelsner, S.; Waldmann, A.; Lindner, A.; Tonn, T.; Wels, W.S. Chimeric antigen receptor-engineered NK-92 cells: An off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. *Front. Immunol.* **2017**, *8*, 533. [CrossRef]
19. Bin, H.; Tang, Y.; Li, W.; Zeng, Q.; Chang, D. Efficiency of CAR-T therapy for treatment of solid tumor in clinical trials: A meta-analysis. *Dis. Markers* **2019**. [CrossRef]
20. O'Rourke, D.M.; Nasrallah, M.P.; Desai, A.; Melenhorst, J.J.; Mansfield, K.; Morrissette, J.J.D.; Martinez-Lage, M.; Brem, S.; Maloney, E.; Shen, A.; et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci. Transl. Med.* **2017**. [CrossRef]
21. Darowski, D.; Kobold, S.; Jost, C.; Klein, C. Combining the best of two worlds: Highly flexible chimeric antigen receptor adaptor molecules (CAR-adaptors) for the recruitment of chimeric antigen receptor T cells. *mAbs* **2019**, *11*, 621–631. [CrossRef] [PubMed]
22. Grote, S.; Seitz, C.M.; Diepold, S.; Buchner, M.; Baden, C.; Malenke, E.; Dieckmann, S.M.; Schwaemmle, H.; Mittelstaet, J.; Kaiser, A.; et al. Adapter chimeric antigen receptor (aCAR)-engineered NK-92 cells: An off-the-shelf cellular therapeutic for universal tumor targeting. *Blood* **2018**, *132*, 3331. [CrossRef]
23. Grote, S.; Mittelstaet, J.; Baden, C.; Chan, K.C.-H.; Seitz, C.; Schlegel, P.; Kaiser, A.; Handgretinger, R.; Schleicher, S. Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: An off-the-shelf cellular therapeutic for universal tumor targeting. *Oncoimmunology* **2020**, *9*, 1825177. [CrossRef]
24. Seitz, C.M.; Schlegel, P.; Hau, J.; Krahl, A.-C.; Schroeder, S.; Bender, G.; Reiter, S.; Schleicher, S.; Schilbach, K.; Ebinger, M.; et al. Novel adapter chimeric antigen receptor (aCAR) T cells for temporally controllable targeting of single and multiple tumor antigens. *Blood* **2017**, *130*, 1912.
25. Seitz, C.M.; Kieble, V.; Illi, C.; Reiter, S.; Grote, S.; Mittelstaet, J.; Lock, D.; Kaiser, A.; Schleicher, S.; Handgretinger, R.; et al. Combinatorial targeting of multiple shared antigens by adapter-CAR-T cells (aCAR-Ts) allows target cell discrimination and specific lysis based on differential expression profiles. *Blood* **2018**, *132*, 4543. [CrossRef]
26. Kaiser, A.; Mittelstaet, J.; Huppert, V.; Miltenyi, S.; Schlegel, P.; Seitz, C.; Lang, P.; Handgretinger, R. Adapter chimeric antigen receptor expressing cells for targeting of multiple antigens. Europe Patent No. 3315511A1, 29 October 2016.
27. Mitwasi, N.; Feldmann, A.; Arndt, C.; Koristka, S.; Berndt, N.; Jureczek, J.; Loureiro, L.R.; Bergmann, R.; Máthé, D.; Hegedüs, N.; et al. "UniCAR"-modified off-the-shelf NK-92 cells for targeting of GD2-expressing tumour cells. *Sci. Rep.* **2020**, *10*, 1–16. [CrossRef]
28. Lohmueller, J.J.; Ham, J.D.; Kvorjak, M.; Finn, O.J. mSA2 affinity-enhanced biotin-binding CAR T cells for universal tumor targeting. *OncoImmunology* **2017**, *7*, e1368604. [CrossRef] [PubMed]
29. Urbanska, K.; Lanitis, E.; Poussin, M.; Lynn, R.C.; Gavin, B.P.; Kelderman, S.; Yu, J.; Scholler, N.; Powell, D.J. A universal strategy for adoptive immunotherapy of cancer through use of a novel T-cell antigen receptor. *Cancer Res.* **2012**, *72*, 1844–1852. [CrossRef]
30. Topp, M.S.; Gökbuget, N.; Zugmaier, G.; Degenhard, E.; Goebeler, M.-E.; Klinger, M.; Neumann, S.A.; Horst, H.A.; Raff, T.; Viardot, A.; et al. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. *Blood* **2012**, *120*, 5185–5187. [CrossRef] [PubMed]
31. Topp, M.S.; Gökbuget, N.; Zugmaier, G.; Klappers, P.; Stelljes, M.; Neumann, S.; Viardot, A.; Marks, R.; Diedrich, H.; Faul, C.; et al. Phase II trial of the anti-CD19 bispecific T cell–engager blinatumomab shows hematologic and molecular remissions in patients with relapsed or refractory B-precursor acute lymphoblastic leukemia. *J. Clin. Oncol.* **2014**, *32*, 4134–4140. [CrossRef] [PubMed]
32. Maude, S.L.; Laetsch, T.W.; Buechner, J.; Rives, S.; Boyer, M.; Bittencourt, H.; Bader, P.; Verneris, M.R.; Stefanski, H.E.; Myers, G.D.; et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N. Engl. J. Med.* **2018**, *378*, 439–448. [CrossRef]

33. Guo, Y.; Wang, Y.; Han, W. Chimeric antigen receptor-modified T cells for solid tumors: Challenges and prospects. *J. Immunol. Res.* **2016**, *2016*, 1–11. [CrossRef] [PubMed]
34. Gong, J.H.; Maki, G.; Klingemann, H.G. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* **1994**, *4*, 652–658.
35. Ward, J.P.; Bonaparte, M.I.; Barker, E. HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts. *AIDS* **2004**, *18*, 1769–1779. [CrossRef]
36. Sarkar, S.; Van Gelder, M.; Noort, W.; Xu, Y.; Rouschop, K.M.A.; Groen, R.; Schouten, H.C.; Tilanus, M.G.J.; Germeraad, W.T.V.; Martens, A.C.M.; et al. Optimal selection of natural killer cells to kill myeloma: The role of HLA-E and NKG2A. *Cancer Immunol. Immunother.* **2015**, *64*, 951–963. [CrossRef] [PubMed]
37. Tang, X.; Yang, L.; Li, Z.; Nalin, A.P.; Dai, H.; Xu, T.; Yin, J.; You, F.; Zhu, M.; Shen, W.; et al. First-in-man clinical trial of CAR NK-92 cells: Safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. *Am. J. Cancer Res.* **2018**, *8*, 1899. [PubMed]
38. Nowakowska, P.; Romanski, A.; Miller, N.; Odendahl, M.; Bonig, H.; Zhang, C.; Seifried, E.; Wels, W.S.; Tonn, T. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. *Cancer Immunol. Immunother.* **2017**, *67*, 25–38. [CrossRef] [PubMed]
39. Schürch, C.M. CARving up colorectal cancer organoids in vitro. *Genes Immun.* **2019**, *21*, 1–3. [CrossRef]
40. Wallstabe, L.; Göttlich, C.; Nelke, L.C.; Kühnemundt, J.; Schwarz, T.; Nerreter, T.; Einsele, H.; Walles, H.; Dandekar, G.; Nietzer, S.L.; et al. ROR1-CAR T cells are effective against lung and breast cancer in advanced microphysiologic 3D tumor models. *JCI Insight* **2019**, *4*. [CrossRef]
41. Hart, A.L.; Ng, S.C.; Mann, E.; Al-Hassi, H.O.; Bernardo, D.; Knight, S.C. Homing of immune cells: Role in homeostasis and intestinal inflammation. *Inflamm. Bowel Dis.* **2010**, *16*, 1969–1977. [CrossRef]
42. Fatehullah, A.; Tan, S.H.; Barker, N. Organoids as an in vitro model of human development and disease. *Nat. Cell Biol.* **2016**, *18*, 246–254. [CrossRef] [PubMed]
43. Zhang, C.; Burger, M.C.; Jennewein, L.; Genβler, S.; Schönfeld, K.; Zeiner, P.; Hattingen, E.; Harter, P.N.; Mittelbronn, M.; Tonn, T.; et al. ErbB2/HER2-specific NK cells for targeted therapy of glioblastoma. *J. Natl. Cancer Inst.* **2015**, *108*. [CrossRef]
44. Jiao, S.; Subudhi, S.K.; Aparicio, A.; Ge, Z.; Guan, B.; Miura, Y.; Sharma, P. Differences in tumor microenvironment dictate T helper lineage polarization and response to immune checkpoint therapy. *Cell* **2019**, *179*, 1177–1190. [CrossRef] [PubMed]
45. Grote, S.; Chan, K.C.; Baden, C.; Bösmüller, H.; Sulyok, M.; Frauenfeld, L.; Ebinger, M.; Handgretinger, R.; Schleicher, S. CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma. *Adv. Cell Gene Ther.* **2020**, *4*. [CrossRef]
46. Burger, M.C.; Zhang, C.; Harter, P.N.; Romanski, A.; Strassheimer, F.; Senft, C.; Tonn, T.; Steinbach, J.P.; Wels, W.S. CAR-Engineered NK cells for the treatment of glioblastoma: Turning innate effectors into precision tools for cancer immunotherapy. *Front. Immunol.* **2019**, *10*, 2683. [CrossRef] [PubMed]

Received: 1 July 2020 | Revised: 5 October 2020 | Accepted: 11 October 2020

DOI: 10.1002/acg2.105

ADVANCES IN CELL AND GENE THERAPY

WILEY

ORIGINAL ARTICLE

CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma

Stefan Grote[1] | Kenneth Chun-Ho Chan[1] | Caroline Baden[1] | Hans Bösmüller[2] | Mihály Sulyok[2] | Leonie Frauenfeld[2] | Martin Ebinger[1] | Rupert Handgretinger[1] | Sabine Schleicher[1]

[1]Department of Hematology/Oncology, Children's University Hospital Tuebingen, Tuebingen, Germany

[2]Institute of Pathology, University Hospital Tuebingen, Tuebingen, Germany

Correspondence
Stefan Grote, Department of Hematology/Oncology, Children's University Hospital Tuebingen, Tuebingen, Germany.
Email: stefan.grote@med.uni-tuebingen.de

Abstract

Despite advanced understanding of its biology and improvements in standard of care treatment, the outcome for children with neuroblastoma (NB), the most common solid extracranial tumor in pediatrics, remains poor. Particularly, frequent relapse and high mortality rates of high-risk NB patients necessitate new therapeutic approaches such as chimeric antigen receptor (CAR)-modified immune cells. CAR T cells recently showed incredible clinical response targeting CD19 and CD22 in hematological malignancies. However, targeting solid cancers remains a difficult challenge and production of autologous CAR T-cell products still requires an extensive manufacturing process. The well-established natural killer (NK)-92 cell line provides a promising alternative to produce "off-the-shelf" CAR-modified effector cells. In the present study, we demonstrate that the immune checkpoint molecule B7-H3 (CD276) is aberrantly expressed on NB cells. Second generation CD276-CAR-engineered but not parental NK-92 cells were capable of specific and long-term elimination of NB cells in vitro while sparing CD276-negative cancer cells. Furthermore, CD276-CAR NK-92 cells showed increased cytotoxicity in a three-dimensional NB spheroid model which can recapitulate in vivo morphology as well as cell connectivity, polarity, gene expression, and tissue architecture, thereby, bridging the gap between in vitro and in vivo models. CD276-CAR NK-92 cells produced a multitude of NK effector molecules as well as pro-inflammatory cytokines that can stimulate the immune system. CD276-CAR surface expression and cytotoxic effector function remained stable for more than 6 months. Data show that CD276-CAR NK-92 may be a promising treatment option for patients with high-risk NB.

KEYWORDS

B7-H3, CD276, chimeric antigen receptor, immune therapy, neuroblastoma, NK-92

1 | INTRODUCTION

Neuroblastoma (NB) is a pediatric tumor derived from sympathoadrenal progenitor cells within the neural crest and is the most common extracranial solid neoplasm in childhood, accounting for 8%-10% of all pediatric malignancies but more than 15% of deaths from cancer in children.[1,2] This tumor entity is characterized by an extreme clinical heterogeneity, which ranges from spontaneous regression or differentiation to cases of aggressive metastatic disease for which patient survival is below 50% despite intensive multimodal therapy.[3] Treatment of high-risk NB currently includes induction chemotherapy, surgical resection, radiotherapy, high-dose chemotherapy, which is often followed by autologous stem cell transplantation and immunotherapy with monoclonal antibodies (mAb) targeting the cell-surface disialoganglioside GD2 in combination with cytokines and retinoic acid.[4,5] However, drug resistance and disease recurrence as well as late effects remain significant challenges in patients with high-risk NB. Thus, novel targeted therapies to improve cure rates while minimizing toxicities are urgently needed.

Chimeric antigen receptor (CAR)-modified lymphocytes represent a promising immunotherapeutic approach that involves the genetic modification of immune cells to express synthetic recombinant receptors which induce lymphocyte activation upon antigen binding.[6] CAR T cells have gained enormous clinical recognition with impressive response rates reported in patients receiving autologous CD19-redirected T cells for the treatment of relapsed or refractory B-cell malignancies.[7-9] Two autologous CD19 CAR T cell products were recently approved by the US Food and Drug Administration (FDA) and the European Medicines Agency for patients with acute lymphoblastic leukemia and certain types of relapsed or refractory large B-cell lymphoma. However, CAR T cells can induce severe side effects, including cytokine release syndrome (CRS) and neurotoxicity, and the fact that this treatment must be produced on a patient-individual basis makes the manufacturing process complex and expensive.[10] Since autologous CAR-T cells are made from the patients' own peripheral blood lymphocytes, yield, transduction efficiency, T-cell subtype distribution, and activation state can vary, affecting overall product composition and quality.

Although current CAR-based immunotherapeutic strategies focus on T cells as effector cells, natural killer (NK) cells have become an increasingly attractive alternative because of their potent anti-tumor activity and safety in an allogeneic setting which could overcome some of the obstacles of autologous CAR T cell therapies. The majority of CAR NK cell studies to date have been performed with NK-92 cells, an IL-2 dependent, continuously expanding human NK cell line, which exhibits phenotypic and functional characteristics of activated NK cells, except the expression of FcγRIII (CD16).[11] In preclinical studies, NK-92 cells exhibited persistent antitumor activity against different hematological malignancies and solid tumors.[12,13] In addition, the safety of irradiated NK-92 cells was demonstrated in phase I clinical trials, with some of the patients experiencing long-lasting treatment responses.[14-16] These properties make NK-92 cells an interesting option for CAR-engineering, thus developing a standardized "off-the-shelf" therapeutic for adoptive cancer immunotherapy.[17]

Whereas CAR T cells have proven highly effective in the treatment of hematological malignancies, they have shown only limited activity in solid tumors.[18] A major obstacle for immunotherapy in solid tumors is the lack of real tumor-specific antigens, which translates to the targeting of tumor-associated antigens overexpressed on tumors but shared with normal tissues which increases the potential risk of significant on-target off-tumor toxicity.[19] In addition, antigen expression is often heterogeneous within a tumor and most antigens are not broadly expressed across different tumor types.

CD276 (B7-H3) belongs to the B7 family of immune checkpoint molecules, which are important regulators of the adaptive immune response and emerging key players in human cancer.[20-24] While it appears that CD276 mRNA is ubiquitously expressed in many tissues and cell types, immunohistochemical (IHC) analysis demonstrates that the expression of CD276 protein is either absent or weak in normal.[25,26] In contrast, CD276 protein is frequently overexpressed in the majority of solid human tumors such as prostate cancer, non-small-cell lung cancer, pancreatic cancer, breast cancer, ovarian cancer, and colorectal cancer and within the tumor vasculature as well.[27] This disparity may in large part be due to the reduced levels of microRNA-29 which are inversely related to the upregulation of CD276 expression in malignant cells.[28] Overexpression of CD276 contributes to tumor immune evasion, an increased potential for metastasis and frequently correlates with rapid cancer progression and poor clinical outcome in several malignancies, such as pancreatic adenocarcinoma, prostate cancer, ovarian cancer, lung cancer, and renal carcinoma.[29-33] In a recent publication Zhang et al analyzed the transcriptional profiles of five Gene Expression Omnibus datasets of clinically annotated NB cases which revealed that high CD276 expression was associated with poor overall survival.[25,34-36] Within both primary and metastatic tumors, CD276 may be expressed on multiple cell types, including differentiated tumor cells, tumor-initiating or cancer stem cells, cancer-associated fibroblasts, and cells of the tumor vasculature.[27,37-39] Compared with other immune checkpoints, CD276 not only regulates innate and adaptive immunity but also promotes cancer progression and metastasis through various non-immunologic functions. These diverse properties in combination with the differential expression in tumors vs healthy tissues make CD276 a prime target for novel immunotherapeutic strategies.[40] CD276-specific mAb and antibody-drug conjugates were evaluated in xenograft mouse models and phase I clinical trials, showing specific antitumor activity and a good safety profile.[41-44] In addition, CAR T cells targeting CD276 have been reported to mediate significant antitumor effects in preclinical studies of different solid tumors, including NB.[45-51]

In this study, we evaluate the benefit of CD276-CAR-engineered NK-92 cells as a potential "off-the-shelf" cellular therapeutic for the recognition and targeting of NB cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines and culturing conditions

Neuroblastoma cell lines LAN-1, SH-SY5Y, SK-N-AS, SK-N-BE, and IMR-32 were purchased from the European Collection of Authenticated Cell Cultures. The NB cell line Kelly and the acute myeloid leukemia (AML) cell line KG-1a were purchased from Cell Line Services. The NB cell line LS was established by Rudolph et al and kindly provided by Prof. Handgretinger, University Hospital Tuebingen, Children's Hospital, Germany.[52] All tumor cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific), 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Biochrom), referred to as RPMI complete medium.

Lenti-X 293T cells (Clontech) were cultivated in DMEM high glucose (4.5 g/L) medium (Thermo Fisher Scientific) containing GlutaMAX supplemented with 10% FBS and 1 mmol/L sodium pyruvate (Thermo Fisher Scientific). NK-92 cells were purchased from the American Type Culture Collection and maintained at a concentration of 10^5 cells/mL in Minimal Essential Medium (MEM) Alpha Medium containing GlutaMAX (Thermo Fisher Scientific) supplemented with 20% FBS and 100 U/mL IL-2 (PROLEUKIN, Aldesleukin, Chiron) referred to as NK-92 complete medium. Primary NK cells were isolated from healthy donors using the EasySep human NK cell isolation kit (STEMCELL Technologies) and maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% pooled AB-serum from healthy donors (Transfusion Medicine, University Hospital Tuebingen, Germany).

All media contained 1× antibiotic-antimycotic solution (Thermo Fisher Scientific) consisting of 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL amphotericin B. All cells and cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere and were regularly tested for mycoplasma contamination.

2.2 | Flow cytometry

Cells were stained at 4°C using the indicated antibodies in flow cytometry buffer containing PBS (Sigma-Aldrich) supplemented with 2% FBS and 0.5 mol/L EDTA (Sigma-Aldrich) for 15 minutes. Using a BD FACSCanto II flow cytometer live cells were gated based on forward and side scatter. CD276-CAR expression on NK-92 cells was determined by CD34 marker gene expression.

2.3 | Immunohistochemistry

Immunohistochemistry for B7-H3 was performed on formalin-fixed, paraffin-embedded tissue sections. Staining was performed using an automated immunostainer (BenchMark ULTRA; Ventana Medical Systems) according to the manufacturer's protocol with a murine anti-human-CD276 (6A1) mAb (Abcam) as primary antibody. Cytoplasmic B7-H3 expression was independently evaluated by two investigators and was assessed as percentage of stained tumor cells compared to total tumor cells. A semiquantitative score of the cytoplasmic staining intensity (IHC grade) was calculated as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).[33]

2.4 | Generation of lentiviral vectors

A 2nd generation lentiviral vector plasmid encoding the CD276-specific CAR (CD276-CAR) construct was acquired from Creative Biolabs. The CD276-CAR was generated by conjugating a B7-H3 clone m851-derived scFv on a 2nd generation CAR backbone incorporating an EF1α promotor, a CD8 hinge domain, a CD28 transmembrane domain, and the cytoplasmic CD28 co-stimulatory as well as CD3-ζ signaling domains. Truncated CD34 (tCD34) is co-expressed after a T2A site for detection and enrichment. The transfer plasmid encoding a green fluorescent protein (GFP)-luciferase construct was obtained from Dr Irmela Jeremias, Helmholtz Center, Munich, Germany.

Lentiviral particles (LVP) were produced in Lenti-X™ 293T (Clontech) after lipofection (Lipofectamine 3000, Thermo Fisher) of a 2nd generation packaging plasmid, a VSV-G envelope plasmid and the indicated transfer plasmid. LVP containing supernatants were concentrated using Lenti-X concentrator (TaKaRa) and stored at −80°C until further use.

2.5 | Lentiviral transduction

NK-92 cells were seeded at a concentration of 1.25×10^6 cells/mL of MEM Alpha Medium, supplemented with 8 ng/µL of protamine sulfate (Sigma-Aldrich) and 2.5 µmol/L of BX-795 (Cayman Chemical Company) and transduced with CD276-CAR LVP for 16 hours. Transduced cells were cultivated in NK-92 complete medium. Transduction efficiency was determined by flow cytometric analysis of CD34 surface expression. CD276-CAR NK-92 cells were subsequently single-cell sorted and screened for the highest CAR-expression.

Neuroblastoma cells were seeded at 5.0×10^4 or 1.25×10^5 cells/mL in RPMI medium without supplements and subsequently transduced with GFP-luciferase LVP for 16 hours. Cells were maintained in RPMI complete medium. Transduction efficiency was determined by flow cytometry.

2.6 | Calcein-release cytotoxicity assay

Target cells were labeled with 10 µmol/L of calcein acetoxymethyl (Calcein AM) (Thermo Fisher Scientific) at a concentration of 10^6 cells/ml in RPMI medium supplemented with 2% FBS.

CD276-CAR NK-92 cells were thoroughly washed, resuspended in RPMI medium supplemented with 2% FBS and co-incubated with target cells for 2 hours. CD276-CAR-specific cytotoxicity was determined by fluorescence measurement using the Spark multimode microplate reader (Tecan).

2.7 | Real-time label-free live cell analysis

Neuroblastoma cell lines were adjusted to a concentration of 10^5 cells/mL in RPMI complete medium and seeded in E-Plate 96 VIEW (OLS) micro-well plates. Effector CAR NK-92 cells were adjusted to an E:T ratio of 5:1 in NK-92 complete medium without IL-2 and co-incubated with the target cells. Utilizing the xCELLigence real-time cell analysis (RTCA) system, cells were monitored for 72 hours. NB cell viability was calculated using the RTCA 2.0 software and CAR-mediated cytotoxicity was subsequently determined.

2.8 | Neuroblastoma three-dimensional spheroid cytotoxicity assay

GFP-positive NB cells were adjusted to a concentration of 5.0×10^3 cells/mL and seeded in a 96-well low-attachment U-bottom plate (Nexcelom). Spheroids were grown for 72 hours and were subsequently co-incubated with CD276-CAR NK-92 cells. Fluorescence was measured using the Celigo S imaging cytometer (Nexcelom) at indicated time points over a period of at least 96 hours. CAR-mediated cytotoxicity was calculated using the average integrated fluorescence intensity of NB spheroids.

2.9 | Quantification of cytokine release

Target and effector cells were co-incubated in equal parts of target cell complete medium and NK-92 complete medium without IL-2 at an E:T ratio of 5:1 at 37°C for 6 hours, supernatants were collected and stored at −80°C until further use. CD276-CAR NK-92 maximum degranulation was achieved using the cell activation cocktail (BioLegend) containing phorbol 12-myristate 13-acetate (PMA)/ionomycin.

Cytokine release was determined using the Bio-Plex Pro human cytokine 17-plex assay (Bio-Rad), the human perforin ELISA kit (Thermo Fisher Scientific), and the LEGEND MAX human granulysin and granzyme B ELISA kits (Biolegend).

2.10 | Data analysis

All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc). Flow cytometry data were analyzed using FlowJo software V10.0.8 (FlowJo LLC).

3 | RESULTS

3.1 | B7-H3 is aberrantly expressed on NB cells

To determine whether B7-H3 (CD276) is a suitable target antigen for CAR-based immunotherapy of NB, we analyzed CD276 expression on NB cell lines and patient tumor samples. Formalin-fixed, paraffin-embedded NB tissue sections of patients with aggressive and/or recurrent disease were stained for CD276 expression using immunohistochemistry with 4 of 5 samples showing a moderate or high cytoplasmic and membrane expression of CD276 (Figure 1). Next, the established high-grade NB cell lines LAN-1, Kelly, and LS were screened for CD276 surface expression by flow cytometry. All NB cell lines abundantly expressed CD276 which makes the antigen ideal for targeted, CAR-based NB therapy (Figure 2A).

	Age (y)	Gender	Clinical stage	IHC grade	Stained tumor cells (%)
Patient 1	3	M	NB IV relapsed	1	70
Patient 2	5	F	NB III	3	90
Patient 3	7	F	NB IV relapsed	3	100
Patient 4	5	F	NB IV relapsed	2	80
Patient 5	6	M	NB IV relapsed	2	80

For further characterization of the NB cell lines they were screened for the expression of known NK cell ligands by flow cytometry (Figure 2B). All three cell lines showed similar NK ligand expression profiles. Uniformly, they expressed human leukocyte antigen E (HLA-E) which is a major ligand for the inhibitory receptor complex CD94/NKG2A on NK cells. Furthermore, LAN-1, Kelly and LS cells shared a relatively low expression of major histocompatibility complex (MHC) class I chain-related protein A and B, a protein that acts as an activating signal for NK cells through the natural-killer group 2, member D (NKG2D or CD314) receptor on NK-92 cells. Moreover, all three cell lines expressed Nectin-2 (CD112) and PVR (CD155). These two receptors are known targets to DNAM-1 (CD226) triggering an activating signaling cascade, as well as the immune checkpoint receptor TIGIT triggering an inhibitory NK response. Interestingly, the inhibitory NK ligand HLA-ABC was completely absent on Kelly cells and LAN-1 cells only showed mediocre expression while HLA-ABC expression was elevated on LS cells.

3.2 | Lentiviral transduction of NK-92 cells results in surface expression of CD276-specific CARs

A CAR construct with an anti-CD276 scFv derived from clone "m851" was subcloned into a second generation lentivirus vector. The CD276-CAR is encoded under the control of the human elongation factor 1 alpha promoter and it is co-expressed with truncated

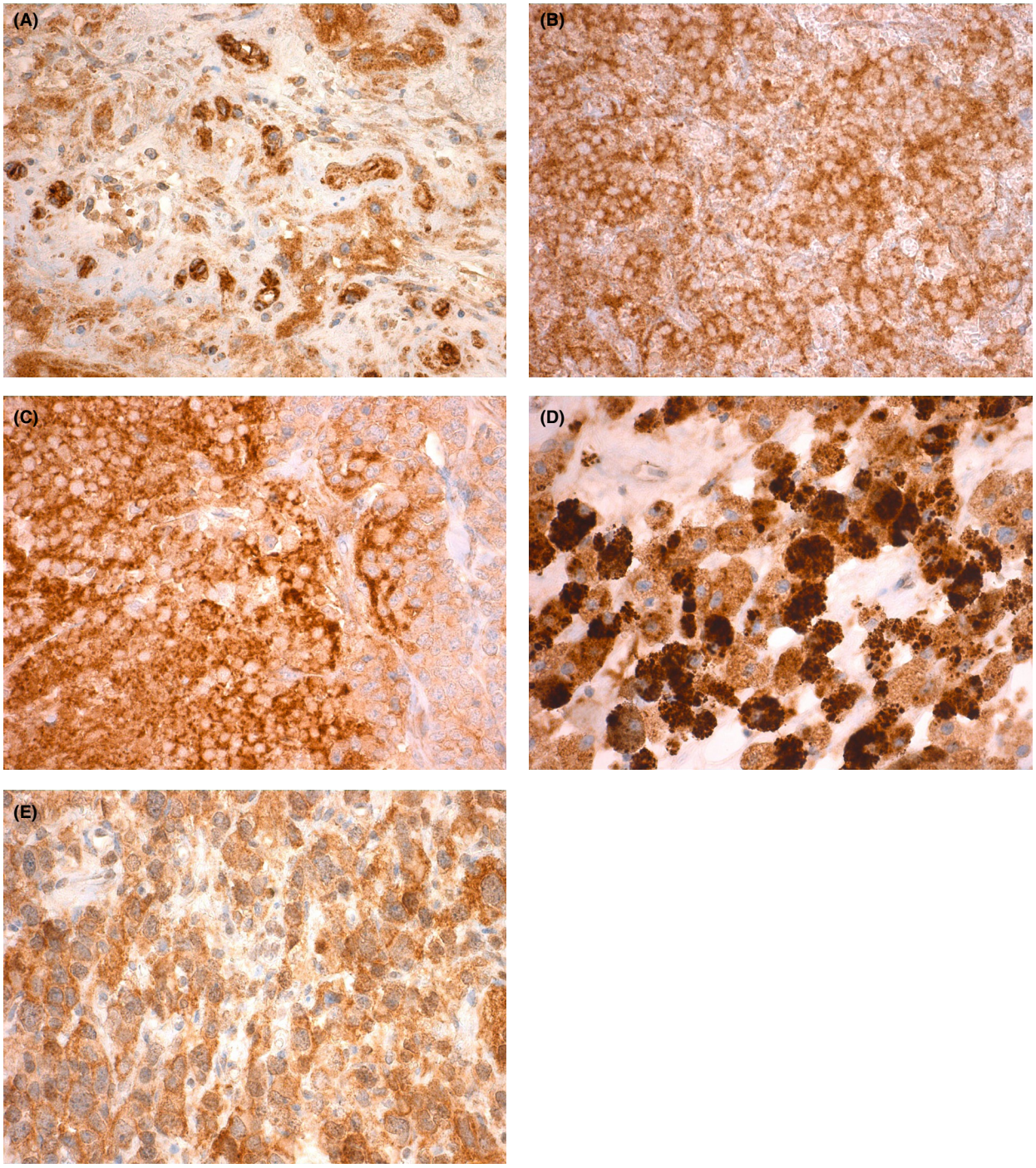


FIGURE 1 Immunostaining of neuroblastoma tissue for CD276 expression. Formalin-fixed, paraffin-embedded neuroblastoma tissue sections of five patients with aggressive and/or recurrent disease were stained for CD276 expression using immunohistochemistry and evaluated by two independent investigators for positivity of staining and staining intensity using an immunohistochemical grading score [Color figure can be viewed at wileyonlinelibrary.com]

CD34 as marker gene (Figure 3A). VSV-G pseudotyped lentiviral particles were produced to transduce the human NK cell line NK-92. Transduction efficiency was determined by flow cytometry of CD34 marker gene surface expression. Cells were subsequently single-cell-sorted and eight NK-92 clones were screened for CAR expression and proliferation. The NK-92 cell clone with the highest CD276-CAR expression was chosen for further experiments.

To analyze whether transduction of NK-92 cells with the CD276-CAR construct would impair proliferation, cell number, and viability as well as CAR expression was monitored regularly. We were able to show that neither proliferation nor viability (>90% at all times; data not shown) of CD276-CAR NK-92 cells were decreased compared to parental NK-92 cells. Additionally, CAR expression levels were stable for more than 6 months (Figure 3B-D).

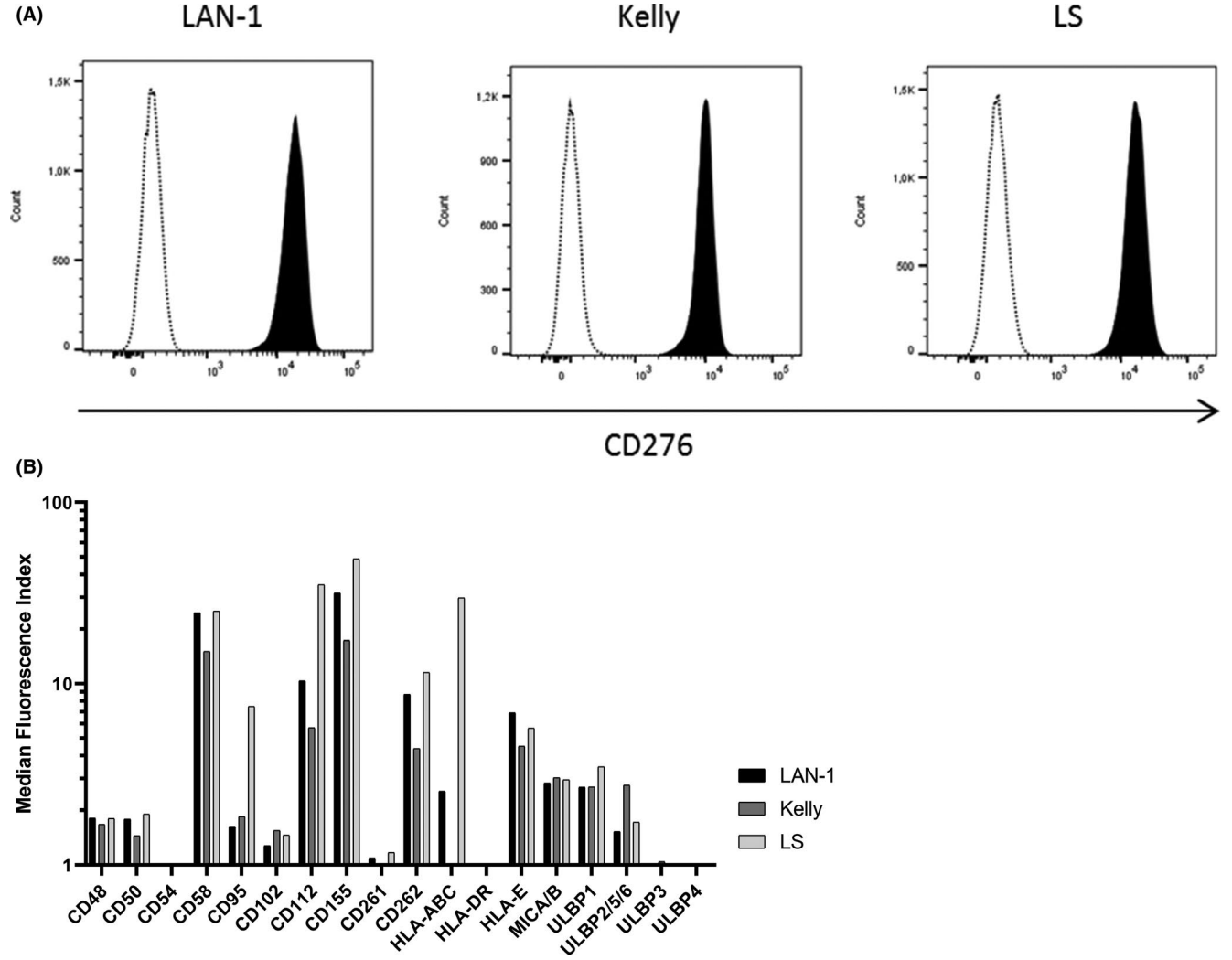


FIGURE 2 CD276 and NK ligand expression profile analysis of neuroblastoma (NB) cell lines. NB cell lines LAN-1, Kelly, and LS were analyzed for B7-H3 (CD276) surface expression (A) and characterized using a panel of known NK cell ligands (B) via flow cytometry

3.3 | CD276-CAR NK-92 cells express different receptor profile compared to primary NK cells

To examine whether CAR transduction had any effect on NK-92 receptor expression profile and how NK-92 cells compare to primary NK cells from healthy donors, cells were screened for the expression of a variety of activating and inhibitory NK cell receptors, as well as immune checkpoint molecules and chemokine receptors using flow cytometry.

Compared to primary NK cells, NK-92 and CD276-CAR NK-92 cells showed a slightly lower expression of DNAM-1 (CD226) while expression of NKG2D (CD314), two of the main activating NK receptors, was similar compared to isolated NK cells. Furthermore, parental and CD276-CAR-transduced NK-92 cells displayed high expression of CD94 and NKG2A (CD159a), two membrane receptors that form an immune checkpoint complex recognizing the human HLA-E peptide, and thus, inhibiting NK effector function. Interestingly, NKp80, a receptor expressed on all activated NK cells was only present on the isolated NK cells and not on NK-92 cells (Figure 4A).

NK-92 and isolated NK cells exhibited a similar pattern of immune checkpoint receptor expression (Figure 4B). PD-1, the receptor for PD-L1 and first- and second-line target for immune checkpoint inhibition-based cancer therapies, was not highly expressed. In contrast, NK-92 cells as well as isolated NK cells showed substantial expression of CD96 (TACTILE) and TIGIT, two recently emerging targets for immune checkpoint inhibition.

Flow cytometric analysis showed high expression of three of the most important chemokine receptors, CD183 (CXCR3), CD184 (CXCR4), and CD197 (CCR7), in both NK-92 as well as primary NK cells, while CD181 (CXCR1) expression was completely absent in the NK-92 cell line (Figure 4C).

3.4 | CD276-CAR NK-92 cells specifically lyse CD276$^+$ NB cell lines in vitro

To determine whether CD276-CAR NK-92 cells were able to specifically lyse NB cells through CAR signaling, they were co-incubated

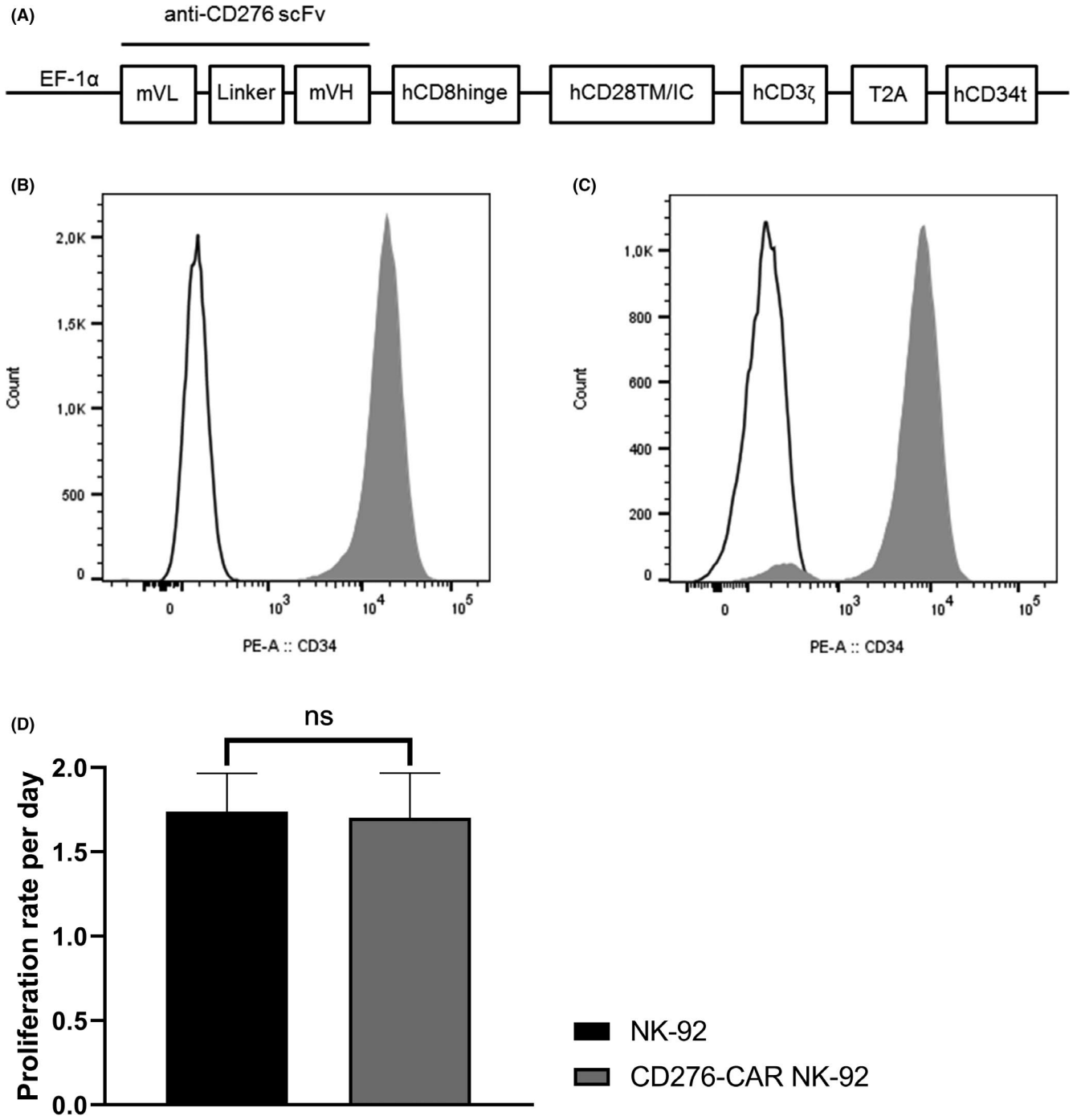


FIGURE 3 Schematic representation of CD276-CAR plasmid and generation of CD276-CAR NK-92 cells. Schematic representation of the lentiviral transfer plasmid encoding the CD276-CAR construct which was purchased from Creative Biolabs (A). The second generation CAR comprises a CD28 co-stimulatory domain and a CD34 tag sequence. CD34 marker gene expression was measured via flow cytometry on untransduced (grey, filled) and transduced NK-92 cells before (black line) and after (grey line) single-cell sort (B) and monitored for 6 mo (C). Average proliferation rate of parental NK-92 and sorted CD276-CAR NK-92 was measured over the same time frame and calculated as mean ± SD (D), n = 3, ns: $P = .6012$. CAR, chimeric antigen receptor.

with calcein-labeled NB cells at various effector-to-target (E:T) ratios. Compared to parental NK-92 cells, specific lysis of NB cells increased up to 80% after 2 hours even at low E:T ratios (Figure 5A). Importantly, CD276-CAR NK-92 cells did not show unwanted off-target effects since they did not induce any CAR-specific lysis of the CD276-negative control cell line KG1a. Since irradiation of NK-92 cells is required in all active clinical trials, we tested whether irradiation had an impact on cytotoxic efficacy. Parental NK-92 and CD276-CAR NK-92 cells were irradiated with 10 Gy and used as effector cells in a standard Calcein-release cytotoxicity assay (CRA) assay. Essentially, irradiation immediately prior to utilization did not significantly impact CAR-mediated lysis of the NB cell lines.

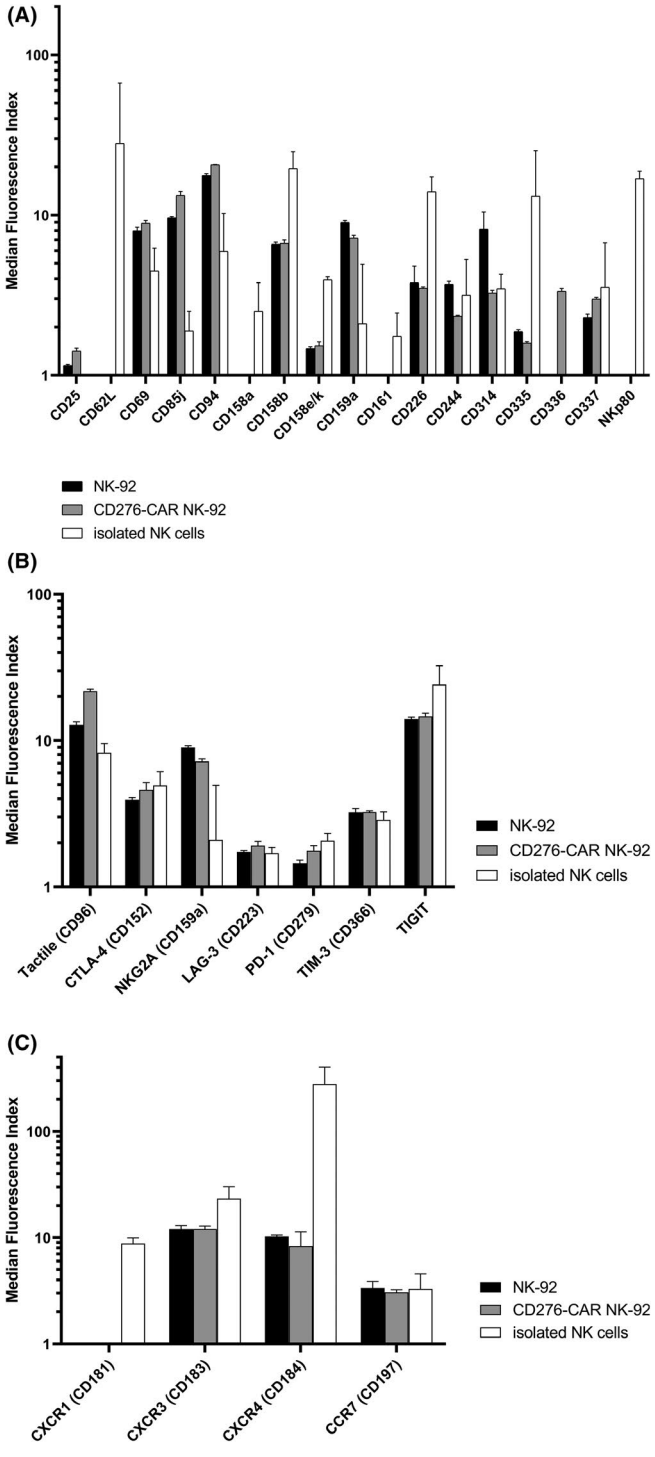


FIGURE 4 Characterization of CD276-CAR NK-92 cells. CD276-CAR-transduced and parental NK-92 cells were characterized for the expression of NK receptors (A), immune checkpoint molecules (B) and chemokine receptors (C) via flow cytometry. Results were compared to flow cytometric analysis of isolated NK cells from three healthy donors. Median fluorescence intensity ± SD was calculated using FlowJo software, n = 3. CAR, chimeric antigen receptor

However, CD276-CAR NK-92 cells gradually lost effector function and underwent apoptosis over time (Figure 5B).

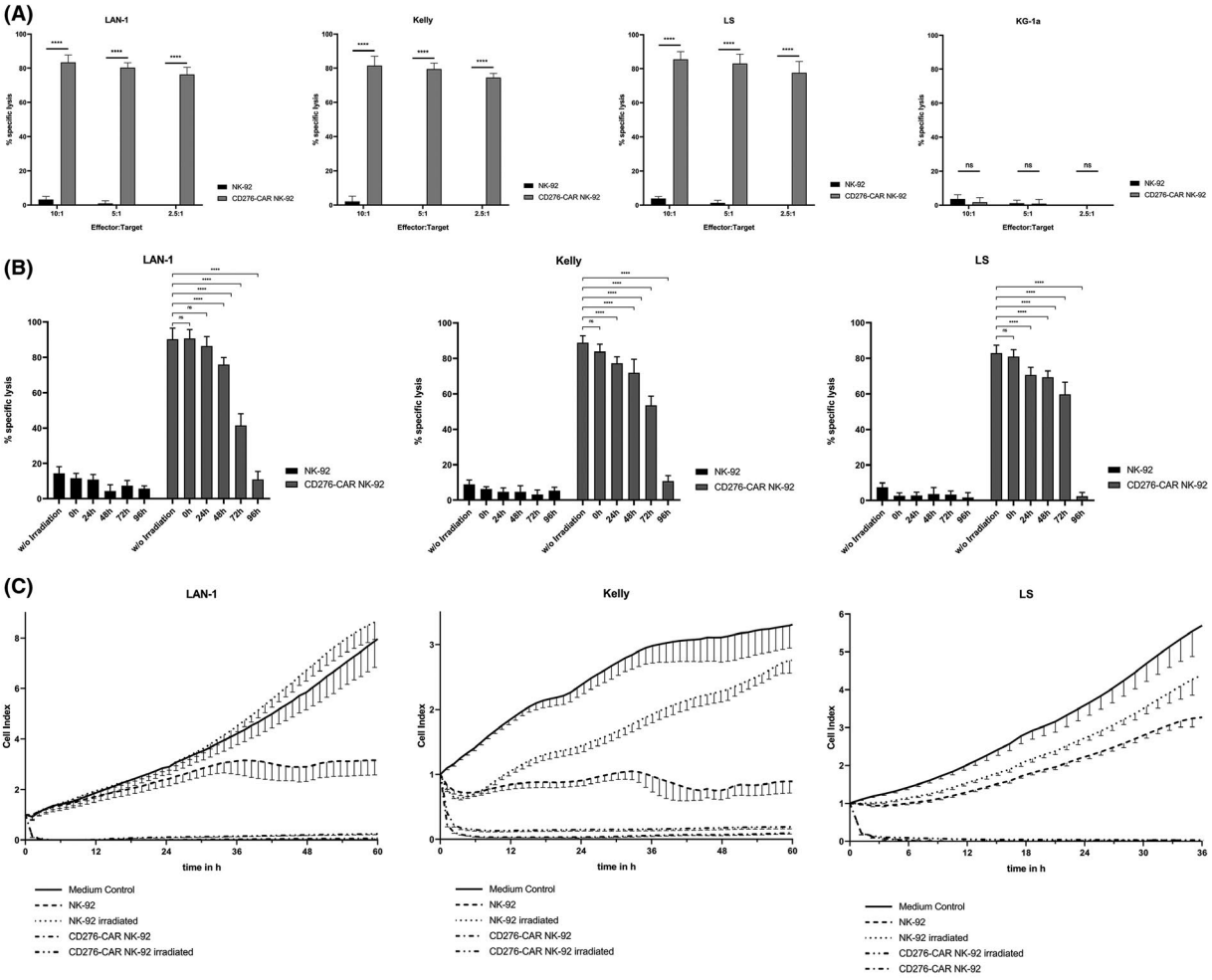
Next, we wanted to examine NK-92-mediated cytotoxicity of the NB cell lines over a longer time period. The xCELLigence RTCA system offers the opportunity to monitor label-free adherent cells in real-time and determine begin and end of cytotoxicity. NB cells were co-incubated with CD276-CAR NK-92 or parental NK-92 cells and electrical impedance was measured every 5 minutes for over 36 hours (Figure 5C). Electrical impedance is depicted as the dimension-less "cell index" which is directly proportional to the amount of adherent cells at certain time points compared to the beginning of the experiment. All three NB cell lines showed a significant decrease of their cell index not later than 2 hours after addition of CD276-CAR NK-92 cells. No lysis was detected when parental NK-92 cells were used. Importantly, CD276-CAR NK-92 cells were capable of eliminating a sufficient number of NB cells to fully prevent regrowth. Furthermore, utilization of irradiated CD276-CAR NK-92 cells did not impair cytotoxic ability and, likewise, did not allow for tumor regrowth.

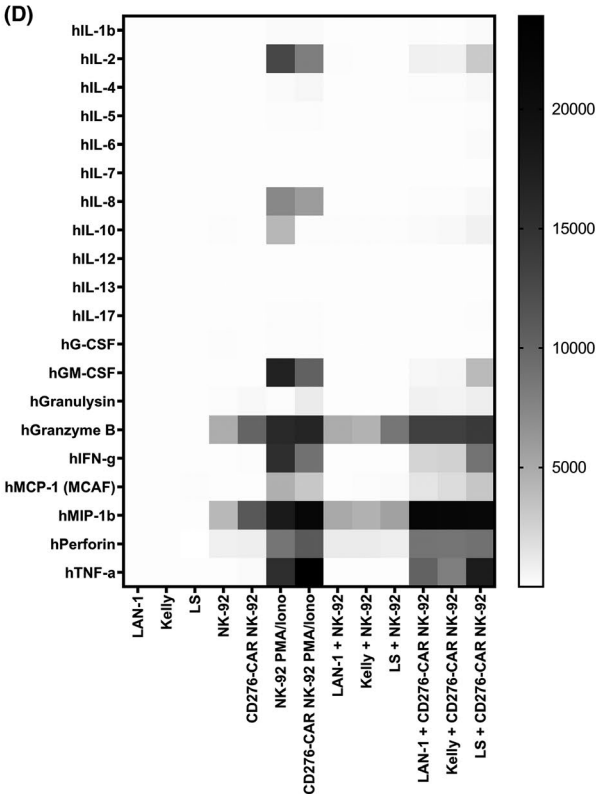
Next, the NK-92 cytokine secretion profile analyzed for a variety of cytokines, including NK cell effector molecules, using the Bio-Plex Pro human cytokine 17-plex assay (Figure 5D). After co-incubation with NB cells, CD276-CAR NK-92 cells drastically increased secretion of various cytokines such as IL-2 (10- to 36-fold) and IL-10 (5- to 19-fold). We also detected an increase in secretion of the pro-inflammatory molecules IFN-γ (21- to 82-fold) and TNF-α (38- to 85-fold). NK effector function was mainly based on perforin release (10-fold increase) and not granzyme B or granulysin secretion (1.3- and 2-fold increase respectively).

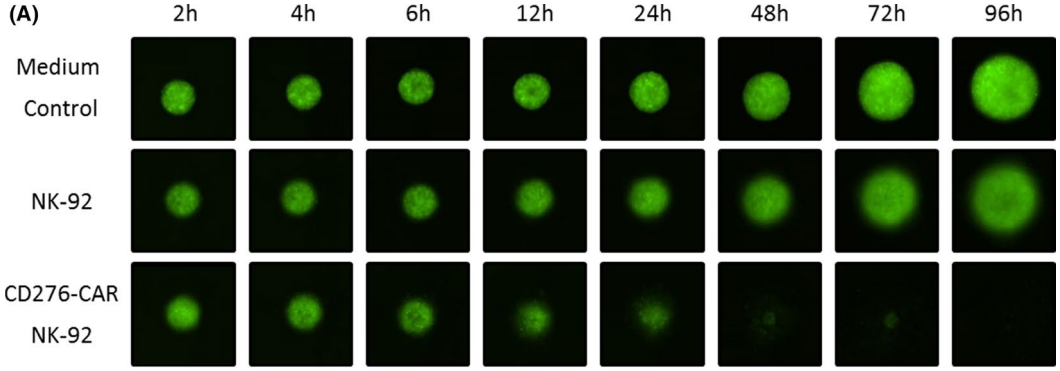
3.5 | CD276-CAR NK-92 cells successfully target NB spheroids

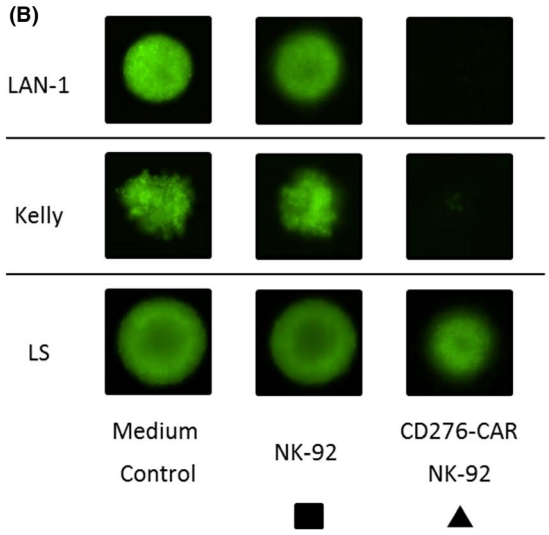
The majority of immunotherapy in vitro studies are based on tumor cell monolayer culture systems, neglecting the three-dimensional (3D)

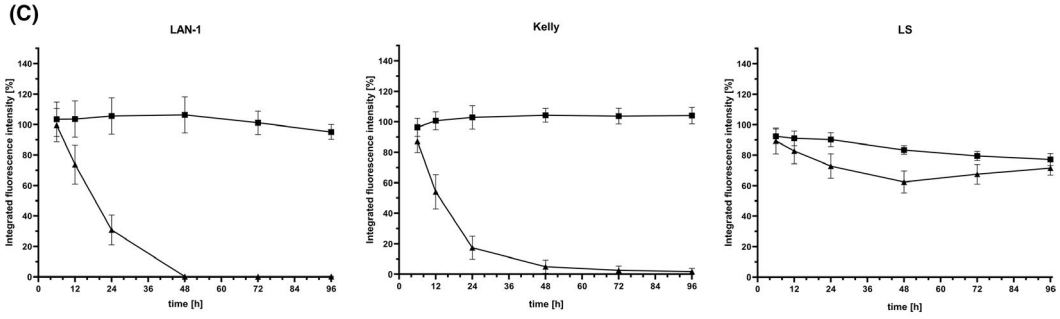
FIGURE 5 CD276-CAR NK-92-mediated tumor cell lysis. CD276-CAR NK-92 cells as well as untransduced control NK-92 cells were co-incubated with calcein-labeled neuroblastoma cell lines LAN-1, Kelly and LS as well as the CD276-negative acute myeloid leukemia cell line KG1a for 2 h. Specific tumor cell lysis is shown as mean ± SD, n = 3 (A). CD276-CAR and parental NK-92 cells were irradiated with 10 Gy and used as effector cells in a CRA with neuroblastoma (NB) cell lines LAN-1, Kelly, and LS at an E:T ratio of 5:1 after indicated time points. Specific tumor cell lysis is shown as mean ± SD, n = 3 (B). Irradiated and non-irradiated CD276-CAR NK-92 cells as well as untransduced NK-92 cells were co-incubated with unlabeled NB cell lines and constantly monitored over time using the xCELLigence RTCA system. NK-mediated tumor cell lysis is portrayed as decrease in the dimensionless "cell index", n = 3 (C). The release of cytokines by CD276-CAR NK-92 and parental NK-92 cells in the presence or absence of NB cells was measured using the Bio-Plex Pro human cytokine 17-plex assay and is shown as a heatmap (D). *$P < .1$; **$P < .01$; ***$P < .001$; ****$P < .0001$; ns = $P \geq .1$. CAR, chimeric antigen receptor

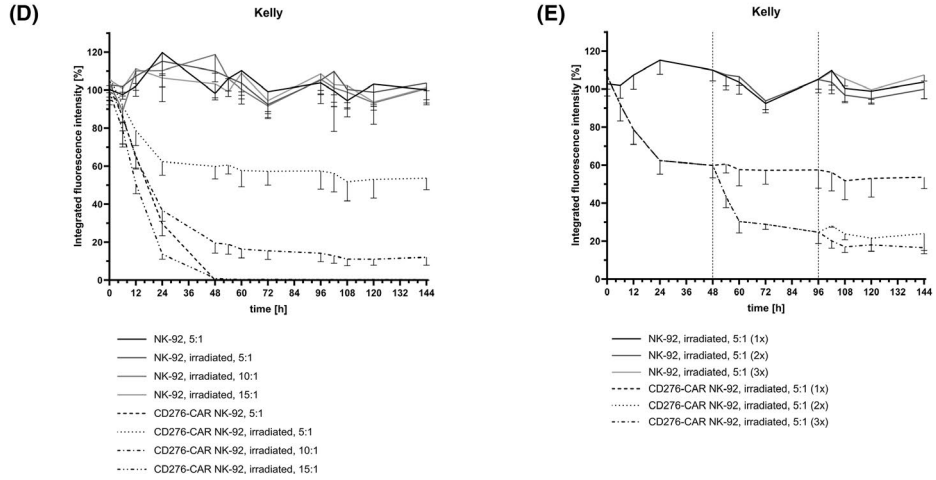

(A)
LAN-1
Kelly
LS
KG-1a
% specific lysis
Effector:Target
10:1
5:1
2.5:1
NK-92
CD276-CAR NK-92
ns
(B)
LAN-1
Kelly
LS
% specific lysis
w/o Irradiation
0h
24h
48h
72h
96h
NK-92
CD276-CAR NK-92
(C)
LAN-1
Kelly
LS
Cell Index
time in h
Medium Control
NK-92
NK-92 irradiated
CD276-CAR NK-92
CD276-CAR NK-92 irradiated


(D)
hIL-1b
hIL-2
hIL-4
hIL-5
hIL-6
hIL-7
hIL-8
hIL-10
hIL-12
hIL-13
hIL-17
hG-CSF
hGM-CSF
hGranulysin
hGranzyme B
hIFN-g
hMCP-1 (MCAF)
hMIP-1b
hPerforin
hTNF-a
20000
15000
10000
5000
LAN-1
Kelly
LS
NK-92
CD276-CAR NK-92
NK-92 PMA/Iono
CD276-CAR NK-92 PMA/Iono
LAN-1 + NK-92
Kelly + NK-92
LS + NK-92
LAN-1 + CD276-CAR NK-92
Kelly + CD276-CAR NK-92
LS + CD276-CAR NK-92


(A)
2h
4h
6h
12h
24h
48h
72h
96h
Medium Control
NK-92
CD276-CAR NK-92


(B)
LAN-1
Kelly
LS
Medium Control
NK-92
CD276-CAR NK-92


(C)
LAN-1
Kelly
LS
Integrated fluorescence intensity [%]
time [h]


(D)
Kelly
Integrated fluorescence intensity [%]
time [h]
NK-92, 5:1
NK-92, irradiated, 5:1
NK-92, irradiated, 10:1
NK-92, irradiated, 15:1
CD276-CAR NK-92, 5:1
CD276-CAR NK-92, irradiated, 5:1
CD276-CAR NK-92, irradiated, 10:1
CD276-CAR NK-92, irradiated, 15:1

(E)
Kelly
Integrated fluorescence intensity [%]
time [h]
NK-92, irradiated, 5:1 (1x)
NK-92, irradiated, 5:1 (2x)
NK-92, irradiated, 5:1 (3x)
CD276-CAR NK-92, irradiated, 5:1 (1x)
CD276-CAR NK-92, irradiated, 5:1 (2x)
CD276-CAR NK-92, irradiated, 5:1 (3x)

FIGURE 6 CD276-CAR NK-92-mediated lysis of 3D neuroblastoma spheroids. GFP-transduced neuroblastoma cell lines LAN-1, Kelly, and LS were grown as 3D spheroids and subsequently co-incubated with CD276-CAR NK-92 or parental NK-92 cells for 96 h in at least three individual experiments. Representative fluorescence images show LAN-1 spheroids (A). Integrated fluorescence intensity of neuroblastoma (NB) spheroids (LAN-1, Kelly, LS) was measured regularly using the Celigo S Imaging Cytometer (Nexcelom), representative fluorescence pictures of the NB spheroids are shown after co-incubation of 96 h (B, C). NK-92 and CD276-CAR NK-92 cells were irradiated prior to co-incubation and added to tumor spheroids (Kelly) at indicated E:T ratios and compared to non-irradiated effector cells (D). NB spheroids (Kelly) were treated with irradiated CD276-CAR as well as parental NK-92 cells at an E:T ratio of 5:1. Fresh, irradiated effector cells were added twice every 48 h (E). Integrated fluorescence intensity was compared to untreated control spheroids and is shown as mean ± SD, n = 3. CAR, chimeric antigen receptor [Color figure can be viewed at wileyonlinelibrary.com]

tumor structure, thereby allowing limited translation to the in vivo situation. Due to this fact, 3D multicellular NB spheroids were used to investigate infiltration and intratumoral cytotoxicity of CD276-CAR NK-92 cells. 3D cultures of the GFP-transduced NB cell lines Kelly, LAN-1 and LS were monitored for spheroid growth and fluorescence intensity using the Celigo S imaging cytometer. After 4 days of culture NB spheroids were co-incubated with either CD276-NK-92 or parental NK-92 cells and monitored for over 96 hours (Figure 6A).

While parental NK-92 cells did not have a substantial impact on spheroid growth, CD276-CAR NK-92 cells eradicated LAN-1 spheroids completely after less than 48 hours (Figure 6B,C). Similarly, spheroids established from the cell line Kelly were eliminated almost entirely after 72 hours. However, CAR-mediated cytotoxicity was not sufficient to completely lyse spheroids of the cell line LS over the given time period. CD276-CAR NK-92 cells demonstrated the ability to specifically target and eliminate NB cells in a 3D spheroid model.

In addidtion, we examined whether irradiation of CD276-CAR NK-92 cells had a negative impact on cytotoxic effect and we found that, by utilizing irradiated effector cells, the integrated fluorescence intensity of NB spheroids (Kelly cell line) only decreased to about 60% after 24 hours and remained stable for an additional 120 hours. Interestingly, by increasing effector to target ratio to 15:1, irradiated CD276-CAR NK-92 cells were able to completely lyse the NB spheroid in a time frame comparable to non-irradiated CD276-CAR NK-92 cells at an E:T ratio of 5:1 (Figure 6D). Thus, we tested whether sequential addition of irradiated CD276-CAR effector NK-92 cells at low E:T ratios could increase cytotoxic effector function. Neuroblastoma spheroids using the Kelly cell line were co-incubated with CD276-CAR and parental NK-92 that were irradiated prior to addition to the tumor cells. Every 48 hours effector cells were added. The introduction of fresh CD276-CAR NK-92 cells increased CAR-mediated cytotoxic effector function and expanded the therapeutic window (Figure 6E).

4 | CONCLUSIONS

The treatment of patients with refractory or relapsed NB still poses a big challenge for modern medicine. The latest standard therapy of high-risk NB, according to the Children's Oncology Group protocol, includes immunotherapy with mAb targeting disialoganglioside (GD2) in combination with immunostimulatory molecules and retinoic acid.[4,53-55] Furthermore, there are currently 12 active clinical trials for CAR T cell-based therapy of NB of which only one trial does not employ GD2 as its primary target antigen and GD2-CAR T cells show promising results in treatment of NB.[56,57] However, expression levels of GD2 vary greatly within NB and low expression of GD2 before immunotherapy correlated with higher relapse rate in patients under GD2-targeted therapy.[58] Furthermore, one of the main obstacles of CAR T cell therapies, besides cost-efficiency, is the occurrence of severe side effects due to on-target, off-tumor toxicity. Pre-clinical studies of NB mouse models showed the induction of fatal neurotoxic events after treatment with GD2-specific CAR T cells.[59,60] An ideal candidate for CAR T-based therapy needs to be abundantly expressed on the tumor surface and scarce on normal tissue to minimalize toxicity. Consequently, new options for targeted immunotherapy of NB are needed.

CD276 (B7-H3) is an important immune checkpoint molecule and member of the B7 superfamily. It recently emerged as a prognostic marker for various tumor types and a promising immunotherapeutic target structure.[40,61,62] CD276 is aberrantly expressed in a variety of solid cancers, including NB.[63,64] The high expression across multiple tumor types and restricted expression in normal tissue make CD276 an attractive pan-cancer target for immunotherapy. Currently, there are multiple active clinical trials evaluating the efficacy of omburtamab, a radioactive, 131 iodine-labeled anti-CD276 mAb that received FDA's breakthrough therapy designation in 2017 for the treatment of NB-associated CNS metastases. For relapsed or refractory high-risk NB the ongoing pivotal phase II trial is expected to enter the biologics license application process later in 2020.[65] As for CAR T cells, CD276 has been evaluated as a potential immunotherapy target for a variety of solid tumor entities in pre-clinical studies.[46] CD276-CAR T cell therapies show potent efficacy in preclinical models of a variety of malignancies, including pediatric solid tumors, brain tumors, pancreatic adenocarcinoma, liver cancer, colon cancer, breast cancer, cervical cancer, ovarian cancer, and melanoma.[26,45-48,66,67] To date, there are only a few clinical trials focusing in CD276 as target which are still in their early stages and there is not one active trial specifically for NB.

In the present study, we introduced a second generation CD276-targeting CAR to the well-established, FDA-approved NK cell line NK-92. Since NB cells generally have a low level of MHC class I expression, using NK cells is a very appealing option for immunotherapy.[68,69] Improved NK isolation and expansion protocols compensate for initially low cell quantities of autologous NK cells and allogeneic NK cells provide graft vs tumor effect while preventing graft vs host disease.[70-72] However, genetic modification of primary NK cells has

been and still is problematic with low transfection efficiencies and poor CAR functionality.[73-75]

Utilization of the NK-92 cell line, however, provides a flexible and consistent platform for CAR-engineered immune cell production. CD276-CAR expression as well as viability of NK-92 was stable for over 6 months and no loss of cytotoxic function could be observed. CD276-CAR NK-92 cells exhibited NK signaling independent of MHC expression as well as expression of other inhibitory ligands on NB cells.

The first in vivo studies as well as clinical trials with CAR-engineered NK-92 cells showed that they were less likely to induce severe side effects such as neurotoxicity and CRS.[76,77] Likewise, in the present study, IL-6, one of the driving forces of CRS was not produced by CD276-CAR NK-92 cells after co-incubation with NB cells. The secretion of pro-inflammatory cytokines such as IFN-γ and MIP-1b, as seen in our experiments, can further stimulate the endogenous immune system and, in turn, enhance anti-tumor activity.[17]

One of the key necessities of CAR-immune cell therapies to be effective in solid tumors and especially in the metastatic setting is their capability of homing and infiltration within tumors.[78,79] Our data clearly indicate that CD276-CAR NK-92 cells were able to efficiently infiltrate and lyse NB spheroids which mimic features of a solid tumor. Future testing of CD276-CAR NK-92 cells will include preclinical in vivo models for NB as well as evaluating strategies to protect CAR-engineered NK-92 cells from the immunosuppressive conditions of the tumor microenvironment.

As outlined, the use of an NK cell line such as NK-92 offers certain advantages over primary NK cells which can overcome obstacles like variable transduction efficiency and limited expansion potential, that can obstruct clinical translation of donor-derived NK cells.[80] In addition, the robust ex vivo expansion of NK-92 cells to high cell numbers under good manufacturing practice, an exquisite safety profile, as well as the ease of genetic modification make this cell line an ideal platform for targeting tumors with CARs.[81,82]

On the downside, due to their origin from a non-Hodgkin lymphoma patient, γ-irradiation of NK-92 cells has to be performed before transfusion into a recipient. Preclinical titration experiments demonstrated that an irradiation dose of 10 Gy is sufficient to inhibit proliferation of unmodified NK-92 cells as well as CAR-engineered NK-92 cells, without affecting their in vitro cytotoxicity for at least 48 hours or reducing in vivo antitumor activity in xenograft models.[77,83-85] Autologous CAR T-cell products can be frozen after transduction and will expand in vivo upon injection of relatively small amounts of the cells. In contrast, NK cells in general and especially NK cell lines need to be expanded to a therapeutic dose before infusion. Irradiation will prevent any in vivo expansion, which makes repeated therapeutic dosages necessary for continued control of cancer growth. Therefore, the manufacturing process has to be adjusted accordingly.

Adapting the strategy developed for unmodified NK-92 cells, Burger et al 2019 described the use of a qualified master cell bank of CAR-engineered NK-92 cells as a reliable source for subsequent production of patient doses for clinical trials.[77,86] To bridge the lag phase of cell proliferation after thawing and to have a therapeutic dose of CAR NK-92 cells readily available for a patient within <1 week, a process was established which relies on a maintenance culture of the CAR NK-92 cells. From this maintenance culture therapeutic dosages can be expanded in batch culture within 5-6 days upon seeding the cells at an initial density of 5×10^4 cells/mL in 2 L of medium in gas permeable cell culture bags. After approximately 3.5 doublings (doubling time of CAR NK 92 cells: 32-36 hours), the culture yields a total cell number of 1×10^9 which can be further expanded depending on the individual cell dose per patient or the number of patients treated simultaneously. In a recent phase I clinical trial with CD33-specific CAR NK-92 cells for the treatment of AML, no dose-limiting toxicities were observed upon repeated intravenous infusions of up to 5×10^9 irradiated cells per dose.[76]

In summary, we have generated CAR-engineered NK-92 cells which successfully eliminate NB cell lines in monolayer cultures as well as 3D models in vitro by targeting the pan-cancer antigen CD276, thus providing a potential new "off-the-shelf" treatment option for high-risk neuroblastoma and potentially other solid tumor entities.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study conformed to the US Federal Policy for the Protection of Human Subjects.

CLINICAL IMPLICATIONS

Adoptive immunotherapy with CAR-engineered immune cells has been extremely effective in hematologic malignancies and several clinical CAR NK-92 trials are ongoing. Here, we demonstrate the potential benefit of CAR-engineered NK-92 specifically targeting CD276 in neuroblastoma. CD276-CAR NK-92 cells can potentially also be used to target a broader spectrum of B7-H3-positive solid cancers. Our data encourage further research such as in vivo models and, subsequently, clinical trials.

AUTHOR'S CONTRIBUTION

SG, RH, and SS developed the idea. SG and SS designed the study, interpreted the results, and wrote the manuscript. SG, KCC, and CB carried out the experiments. ME provided the patient samples for IHC staining. HB, MS, and LF performed and evaluated the IHC staining. All authors have read and agreed to the published version of the manuscript.

ORCID

Stefan Grote https://orcid.org/0000-0001-8713-6709
Sabine Schleicher https://orcid.org/0000-0002-1847-2984

REFERENCES

1. Maris JM. Recent advances in neuroblastoma. *N Engl J Med*. 2010;362:2202-2211.
2. Matthay KK, Maris JM, Schleiermacher G, et al. Neuroblastoma. *Nat Rev Dis Prim*. 2016;2:16078.
3. Morgenstern DA, London WB, Stephens D, et al. Prognostic significance of pattern and burden of metastatic disease in patients with stage 4 neuroblastoma: a study from the International Neuroblastoma Risk Group database. *Eur J Cancer*. 2016;65:1-10.
4. Yu AL, Gilman AL, Ozkaynak MF, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med*. 2010;363:1324-1334.
5. Pinto NR, Applebaum MA, Volchenboum SL, et al. Advances in risk classification and treatment strategies for neuroblastoma. *J Clin Oncol*. 2015;33:3008-3017.
6. Cartellieri M, Bachmann M, Feldmann A, et al. Chimeric antigen receptor-engineered T cells for immunotherapy of cancer. *J Biomed Biotechnol*. 2010;2010:1-13.
7. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371:1507-1517.
8. Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet*. 2015;385:517-528.
9. Gardner RA, Finney O, Annesley C, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood*. 2017;129:3322-3331.
10. Srivastava S, Riddell SR. Chimeric antigen receptor T cell therapy: challenges to bench-to-bedside efficacy. *J Immunol*. 2018;200:459-468.
11. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia*. 1994;8:652-658.
12. Tam YK, Miyagawa B, Ho VC, Klingemann HG. Immunotherapy of malignant melanoma in a SCID mouse model using the highly cytotoxic natural killer cell line NK-92. *J Hematother*. 1999;8:281-290.
13. Swift BE, Williams BA, Kosaka Y, et al. Natural killer cell lines preferentially kill clonogenic multiple myeloma cells and decrease myeloma engraftment in a bioluminescent xenograft mouse model. *Haematologica*. 2012;97:1020-1028.
14. Tonn T, Schwabe D, Klingemann HG, et al. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy*. 2013;15:1563-1570.
15. Williams BA, Law AD, Routy B. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. *Oncotarget*. 2017;8:89256-89268.
16. Boyiadzis M, Agha M, Redner RL, et al. Phase 1 clinical trial of adoptive immunotherapy using "off-the-shelf" activated natural killer cells in patients with refractory and relapsed acute myeloid leukemia. *Cytotherapy*. 2017;19:1225-1232.
17. Zhang C, Oberoi P, Redner RL, et al. Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. *Front Immunol*. 2017;8:1-17.
18. Bin H, Yao T, Li W, Zeng Q, Chang D Efficiency of CAR-T therapy for treatment of solid tumor in clinical trials: a meta-analysis. *Dis Markers*. 2019;2019. https://doi.org/10.1155/2019/3425291.
19. Newick K, O'Brien S, Moon E, Albelda SM. CAR T cell therapy for solid tumors. *Annu Rev Med*. 2017;68:139-152.
20. Flem-Karlsen K, Fodstad O, Nunes-Xavier CE. B7–H3 immune checkpoint protein in human cancer. *Curr Med Chem*. 2019;26:1-20.
21. Hofmeyer KA, Ray A, Zang X, et al. The contrasting role of B7–H3. *Proc Natl Acad Sci USA*. 2008;105:10277-10278.
22. Luo L, Zhu G, Xu H, et al. B7–H3 promotes pathogenesis of autoimmune disease and inflammation by regulating the activity of different T cell subsets. *PLoS One*. 2015;10:e0130126.
23. Lee Y-H, Martin-Orozco N, Zheng P, et al. Inhibition of the B7–H3 immune checkpoint limits tumor growth by enhancing cytotoxic lymphocyte function. *Cell Res*. 2017;27:1034-1045.
24. Castellanos JR, Purvis IJ, Labak CM, et al. B7–H3 role in the immune landscape of cancer. *Am J Clin Exp Immunol*. 2017;6:66-75.
25. Zhang H, Zhang J, Li C, et al. Survival association and cell cycle effects of B7H3 in neuroblastoma. *J Korean Neurosurg Soc*. 2020;86:1-10.
26. Huang B, Luo L, Wang J, et al. B7–H3 specific T cells with chimeric antigen receptor and decoy PD-1 receptors eradicate established solid human tumors in mouse models. *Oncoimmunology*. 2020;9:1684127.
27. Seaman S, Zhu Z, Saha S, et al. Eradication of tumors through simultaneous ablation of CD276/B7-H3-positive tumor cells and tumor vasculature. *Cancer Cell*. 2017;31:501-515.e8.
28. Xu H, Cheung IY, Guo H-F, et al. MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7–H3: potential implications for immune based therapy of human solid tumors. *Cancer Res*. 2009;69:6275-6281.
29. Benzon B, Zhao SG, Haffner MC, et al. Correlation of B7–H3 with androgen receptor, immune pathways and poor outcome in prostate cancer: an expression-based analysis. *Prostate Cancer Prostatic Dis*. 2017;20:28-35.
30. Inamura K, Yokouchi Y, Kobayashi M, et al. Tumor B7–H3 (CD276) expression and smoking history in relation to lung adenocarcinoma prognosis. *Lung Cancer*. 2017;103:44-51.
31. Qin X, Zhang H, Ye D, et al. B7–H3 is a new cancer-specific endothelial marker in clear cell renal cell carcinoma. *Onco Targets Ther*. 2013;6:1667-1673.
32. Yamato I, Sho M, Nomi T, et al. Clinical importance of B7–H3 expression in human pancreatic cancer. *Br J Cancer*. 2009;101:1709-1716.
33. Zang X, Thompson RH, Al-Ahmadie HA, et al. B7–H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. *Proc Natl Acad Sci USA*. 2007;104:19458-19463.
34. Tekle C, Nygren MK, Chen YW, et al. B7–H3 contributes to the metastatic capacity of melanoma cells by modulation of known metastasis-associated genes. *Int J Cancer*. 2012;130:2282-2290.
35. Ye Z, Zheng Z, Li X, et al. B7–H3 Overexpression Predicts Poor Survival of Cancer Patients: A Meta-Analysis. *Cell. Physiol. Biochem*. 2016;39:1568-1580.
36. Dong P, Xiong Y, Yue J, et al. B7H3 As a promoter of metastasis and promising therapeutic target. *Front Oncol*. 2018;8:1-8.
37. Crispen PL, Sheinin Y, Roth TJ, et al. Tumor cell and tumor vasculature expression of B7–H3 predict survival in clear cell renal cell carcinoma. *Clin. Cancer Res*. 2008;14:5150-5157.
38. Liu Z, Zhang W, Phillips JB, et al. Immunoregulatory protein B7–H3 regulates cancer stem cell enrichment and drug resistance through MVP-mediated MEK activation. *Oncogene*. 2019;38:88-102.
39. MacGregor HL, Sayad A, Elia A, et al. High expression of B7–H3 on stromal cells defines tumor and stromal compartments in epithelial ovarian cancer and is associated with limited immune activation. *J Immunother Cancer*. 2019;7:1-17.
40. Picarda E, Ohaegbulam KC, Zang X, et al. Molecular pathways: targeting B7–H3 (CD276) for human cancer immunotherapy. *Clin. Cancer Res*. 2016;22:3425-3431.
41. Kramer K, Kushner BH, Modak S, et al. Compartmental intrathecal radioimmunotherapy: results for treatment for metastatic CNS neuroblastoma. *J Neurooncol*. 2010;97:409-418.
42. Loo D, Alderson RF, Chen FZ, et al. Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. *Clin Cancer Res*. 2012;18:3834-3845.

43. Powderly J, Cote G, Flaherty K, et al. Interim results of an ongoing Phase I, dose escalation study of MGA271 (Fc-optimized humanized anti-B7-H3 monoclonal antibody) in patients with refractory B7-H3-expressing neoplasms or neoplasms whose vasculature expresses B7–H3. *J Immunother Cancer*. 2015;3:O8.
44. Souweidane MM, Kramer K, Pandit-Taskar N, et al. Convection-enhanced delivery for diffuse intrinsic pontine glioma: a single-centre, dose-escalation, phase 1 trial. *Lancet Oncol*. 2018;19:1040-1050.
45. Du H, Hirabayashi K, Ahn S, et al. Antitumor responses in the absence of toxicity in solid tumors by targeting B7–H3 via chimeric antigen receptor T cells. *Cancer Cell*. 2019;35:221-237.e8.
46. Majzner RG, Theruvath JL, Nellan A, et al. CAR T cells targeting B7–H3, a pan-cancer antigen, demonstrate potent preclinical activity against pediatric solid tumors and brain tumors. *Clin Cancer Res*. 2019;25:2560-2574.
47. Nehama D, Di Ianni N, Musio S, et al. B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres. *EBioMedicine*. 2019;47:33-43.
48. Tang X, Zhao S, Zhang Y, et al. B7–H3 as a novel CAR-T therapeutic target for glioblastoma. *Mol Ther Oncolytics*. 2019;14:279-287.
49. Zhang C, Hu Y, Shi C, et al. Targeting natural killer cells for tumor immunotherapy. *Front Immunol*. 2020;11:60.
50. Zheng M, Yu L, Hu J, et al. Efficacy of B7-H3-redirected BiTE and CAR-T immunotherapies against extranodal nasal natural killer/T cell lymphoma. *Transl Oncol*. 2020;13:100770.
51. Tang X, Liu F, Liu Z, et al. Bioactivity and safety of B7-H3-targeted chimeric antigen receptor T cells against anaplastic meningioma. *Clin Transl Immunol*. 2020;9. https://doi.org/10.1002/cti2.1137.
52. Rudolph G, Schilbach-Stiickle K, Handgretinger R, et al. Cytogenetic and molecular characterization of a newly established neuroblastoma cell line LS. *Hum Genet*. 1991;86:562-566.
53. Voeller J, Sondel PM. Advances in anti-GD2 immunotherapy for treatment of high-risk neuroblastoma. *J Pediatr Hematol Oncol*. 2019;41:163-169.
54. Ahmed M, Cheung NKV. Engineering anti-GD2 monoclonal antibodies for cancer immunotherapy. *FEBS Lett*. 2014;588:288-297.
55. Gholamin S, Mirzaei H, Razavi S-M, et al. GD2-targeted immunotherapy and potential value of circulating microRNAs in neuroblastoma. *J Cell Physiol*. 2018;233:866-879.
56. Louis CU, Savoldo B, Dotti G, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood*. 2011;118:6050-6056.
57. Sait S, Modak S. Anti-GD2 immunotherapy for neuroblastoma. *Expert Rev Anticancer Ther*. 2017;17:889-904.
58. Terzic T, Cordeau M, Herblot S, et al. Expression of disialoganglioside (GD2) in neuroblastic tumors: a prognostic value for patients treated with anti-GD2 immunotherapy. *Pediatr Dev Pathol*. 2018;21:355-362.
59. Richman SA, Nunez-Cruz S, Moghimi B, et al. High-affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. *Cancer Immunol Res*. 2018;6:36-46.
60. Majzner RG, Weber EW, Lynn RC, Xu P, Mackall CL. Neurotoxicity associated with a high-affinity GD2 CAR. *Cancer Immunol Res*. 2018;6:494-495.
61. Zhou Z, Luther N, Ibrahim GM, et al. B7–H3, a potential therapeutic target, is expressed in diffuse intrinsic pontine glioma. *J Neurooncol*. 2013;111:257-264.
62. Heubach F, Schlegel P, Zekri L, et al. Abstract A131: targeting B7–H3 (CD276) in neuroblastoma: in vitro evaluation of Fc-optimized antibodies and immunocytokines. In: *Cancer Immunology Research*. Vol. 7. American Association for Cancer Research (AACR); 2019:A131. https://doi.org/10.1158/2326-6074.CRICIMTEATIAACR18-A131.
63. Gregorio A, Corrias MV, Castriconi R, et al. Small round blue cell tumours: diagnostic and prognostic usefulness of the expression of B7–H3 surface molecule. *Histopathology*. 2008;53:73-80.
64. Castriconi R, Dondero A, Augugliaro R, et al. Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis. *Proc Natl Acad Sci USA*. 2004;101:12640-12645.
65. Kramer K, Khan O, Haque S. Central nervous system neuroblastoma metastases pseudoprogression following intraventricular anti-B7-H3 radioimmunotherapy. *J Neurooncol*. 2019;144:227-229.
66. Theruvath J, Sotillo E, Mount CW, et al. Locoregionally administered B7-H3-targeted CAR T cells for treatment of atypical teratoid/rhabdoid tumors. *Nat Med*. 2020;26:712-719.
67. Yang M, Tang X, Zhang Z, et al. Tandem CAR-T cells targeting CD70 and B7–H3 exhibit potent preclinical activity against multiple solid tumors. *Theranostics*. 2020;10:7622-7634.
68. Wölfl M, Jungbluth AA, Garrido F, et al. Expression of MHC class I, MHC class II, and cancer germline antigens in neuroblastoma. *Cancer Immunol Immunother*. 2005;54:400-406.
69. Raffaghello L, Prigione I, Airoldi I, et al. Mechanisms of immune evasion of human neuroblastoma. *Cancer Lett*. 2005;228:155-161.
70. Cho D, Campana D. Expansion and activation of natural killer cells for cancer immunotherapy. *Korean J Lab Med*. 2009;29:89-96.
71. Benjamin JE, Gill S, Negrin RS. Biology and clinical effects of natural killer cells in allogeneic transplantation. *Curr Opin Oncol*. 2010;22(2):130-137.
72. Farag SS, Fehniger TA, Ruggeri L, et al. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*. 2002;100:1935-1947.
73. Xiao L, Cen D, Gan H, et al. Adoptive transfer of NKG2D CAR mRNA-engineered natural killer cells in colorectal cancer patients. *Mol Ther*. 2019;27:1114-1125.
74. Shimasaki N, Fujisaki H, Cho D, et al. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy*. 2012;14:830-840.
75. Parkhurst MR, Riley JP, Dudley ME, et al. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin Cancer Res*. 2011;17:6287-6297.
76. Tang X, Yang L, Li Z, et al. First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. *Am J Cancer Res*. 2018;8:1899.
77. Nowakowska P, Romanski A, Miller N, et al. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. *Cancer Immunol Immunother*. 2018;67:25-38.
78. Schürch CM. CARving up colorectal cancer organoids in vitro. *Genes Immun*. 2020;21:1-3.
79. Wallstabe L, Göttlich C, Nelke LC, et al. ROR1-CAR T cells are effective against lung and breast cancer in advanced microphysiologic 3D tumor models. *JCI Insight*. 2019;4:e126345. https://doi.org/10.1172/jci.insight.126345.
80. Zhang C, Oberoi P, Oelsner S, et al. Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. *Front Immunol*. 2017;8. https://doi.org/10.3389/fimmu.2017.00533.
81. Klingemann H. Are natural killer cells superior CAR drivers? *Oncoimmunology*. 2014;3:e28147.
82. Klingemann H, Boissel L, Toneguzzo F. Natural killer cells for immunotherapy—advantages of the NK-92 cell line over blood NK cells. *Front Immunol*. 2016;7:1-7.

83. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. *J Hematother Stem Cell Res*. 2001;10:535-544.
84. Schönfeld K, Sahm C, Zhang C, et al. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. *Mol Ther*. 2015;23:330-338.
85. Oelsner S, Friede ME, Zhang C, et al. Continuously expanding CAR NK-92 cells display selective cytotoxicity against B-cell leukemia and lymphoma. *Cytotherapy*. 2017;19:235-249.
86. Burger MC, Zhang C, Harter PN, et al. CAR-engineered NK cells for the treatment of glioblastoma: turning innate effectors into precision tools for cancer immunotherapy. *Front Immunol*. 2019;10:1-16.

How to cite this article: Grote S, Chan KC-H, Baden C, et al. CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma. *Adv Cell Gene Ther*. 2021;4:e105. https://doi.org/10.1002/acg2.105

cells

MDPI

Article

In Vitro Evaluation of CD276-CAR NK-92 Functionality, Migration and Invasion Potential in the Presence of Immune Inhibitory Factors of the Tumor Microenvironment

Stefan Grote *, Guillermo Ureña-Bailén, Kenneth Chun-Ho Chan, Caroline Baden, Markus Mezger, Rupert Handgretinger and Sabine Schleicher

Department of Hematology and Oncology, Children's Hospital, University Hospital Tuebingen, 72076 Tuebingen, Germany; guillermo.urena@med.uni-tuebingen.de (G.U.-B.); kc220@outlook.com (K.C.-H.C.); caroline.baden@student.uni-tuebingen.de (C.B.); markus.mezger@med.uni-tuebingen.de (M.M.); rupert.handgretinger@med.uni-tuebingen.de (R.H.); sabine.schleicher@med.uni-tuebingen.de (S.S.)
* Correspondence: stefan.grote@med.uni-tuebingen.de

Abstract: Background: Melanoma is the most lethal of all skin-related cancers with incidences continuously rising. Novel therapeutic approaches are urgently needed, especially for the treatment of metastasizing or therapy-resistant melanoma. CAR-modified immune cells have shown excellent results in treating hematological malignancies and might represent a new treatment strategy for refractory melanoma. However, solid tumors pose some obstacles for cellular immunotherapy, including the identification of tumor-specific target antigens, insufficient homing and infiltration of immune cells as well as immune cell dysfunction in the immunosuppressive tumor microenvironment (TME). Methods: In order to investigate whether CAR NK cell-based immunotherapy can overcome the obstacles posed by the TME in melanoma, we generated CAR NK-92 cells targeting CD276 (B7-H3) which is abundantly expressed in solid tumors, including melanoma, and tested their effectivity in vitro in the presence of low pH, hypoxia and other known factors of the TME influencing anti-tumor responses. Moreover, the CRISPR/Cas9-induced disruption of the inhibitory receptor NKG2A was assessed for its potential enhancement of NK-92-mediated anti-tumor activity. Results: CD276-CAR NK-92 cells induced specific cytolysis of melanoma cell lines while being able to overcome a variety of the immunosuppressive effects normally exerted by the TME. NKG2A knock-out did not further improve CAR NK-92 cell-mediated cytotoxicity. Conclusions: The strong cytotoxic effect of a CD276-specific CAR in combination with an "off-the-shelf" NK-92 cell line not being impaired by some of the most prominent negative factors of the TME make CD276-CAR NK-92 cells a promising cellular product for the treatment of melanoma and beyond.

Keywords: CD276; B7-H3; chimeric antigen receptor; NK-92; immune therapy; melanoma; CRISPR/Cas9

Citation: Grote, S.; Ureña-Bailén, G.; Chan, K.C.-H.; Baden, C.; Mezger, M.; Handgretinger, R.; Schleicher, S. In Vitro Evaluation of CD276-CAR NK-92 Functionality, Migration and Invasion Potential in the Presence of Immune Inhibitory Factors of the Tumor Microenvironment. *Cells* **2021**, *10*, 1020. https://doi.org/10.3390/cells10051020

Academic Editor: Ali R. Jazirehi

Received: 4 March 2021
Accepted: 22 April 2021
Published: 26 April 2021

1. Introduction

Malignant cutaneous melanoma, which develops from the pigment forming cells of the skin, is a highly aggressive tumor characterized by an increasing worldwide incidence [1]. Although melanoma accounts for only 4% of all skin cancers, it causes the highest number of skin cancer-related deaths [2]. The majority of patients diagnosed with melanoma have early-stage disease, and the prognosis is generally favorable although heterogeneous. In contrast, outcome for patients with metastatic disease is poor, with a five-year survival rate of approximately 10% [3].

Besides targeted therapy with BRAF and MEK inhibitors in BRAF V600 mutated melanomas, immune checkpoint inhibitors have revolutionized the treatment of patients with advanced melanoma and other cancers. Blockade of the CTLA-4 or/and PD-1 signaling axis enhances T-cell-mediated antitumor immune responses, leading to improved

survival and durable responses in patients [4]. However, a significant proportion of patients either does not respond or show progression after the initial response to checkpoint inhibitor treatment. Multiple mechanisms, such as downregulation of the immune checkpoint ligands by the tumor, activation of alternative cancer signaling pathways, mutations in genes involved in IFN-γ signaling, immuno-editing and changes in the tumor microenvironment (TME) have been shown to contribute to both primary and acquired resistance to immune checkpoint inhibitors [5,6]. A further challenge is posed by their mode of action, as immune checkpoint inhibitors can also induce immune-related adverse effects that require careful monitoring and immediate treatment [7–9]. Therefore, new treatment strategies for therapy-resistant progressed melanoma are still of great significance.

Novel immunotherapeutic approaches aim at utilizing the innate cytotoxic ability of lymphocytes by specifically redirecting immune cells to target tumor cells using chimeric antigen receptors (CAR), synthetic recombinant receptors that combine target antigen binding with immune cell activation [10]. Most prominently, CD19-targeting autologous T cells were recently approved by the US Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA) for the treatment of relapsed or refractory B-cell malignancies. CD19-CAR T cells have demonstrated tremendous clinical responses in treating acute lymphoblastic leukemia and certain types of relapsed or refractory large B cell lymphoma resulting in preponed approval after evaluation of phase 2 clinical studies [11,12]. The occurrence of, partially, severe side effects such as cytokine release syndrome (CRS) or neurotoxic events as well as the extensive manufacturing process of a custom, autologous T cell product, have yet hindered the inclusion of CAR T cells as a general treatment option [13].

NK cells have become an increasingly attractive alternative effector cell source for CAR-modification because of their potent innate anti-tumor activity. Phase 1 and 2 trials with CD19-CAR-engineered NK cells have shown impressive responses in patients with relapsed or refractory non-Hodgkin's lymphoma (NHL) or chronic lymphocytic leukemia (CLL). In addition, associated side effects such as graft-versus-host disease (GvHD), CRS or severe neurotoxicity were not reported [14]. However, the majority of studies including CAR-modified NK cells so far have been performed with NK-92 cells. NK-92 is an IL-2-dependent, continuously expanding human NK cell line which exhibits phenotypic and functional characteristics of activated, primary NK cells that has been approved by the FDA for patient treatment [15]. NK-92 cells were shown to be easily expandable in a GMP-compliant manner to sufficient cell numbers for clinical use [16]. Moreover, irradiated NK-92 cells have demonstrated clinical safety and persistent anti-tumor activity against hematological malignancies as well as solid cancers [17–21]. Compared to primary NK cells, they only express few inhibitory receptors such as NKG2A [22]. CAR-modified NK-92 cells could be manufactured as an "off-the-shelf"-available CAR immune cell product, reducing production complexity and, subsequently, financial expenditure [23]. Several early phase clinical trials with CAR-engineered NK-92 cells are currently being carried out in Europe, China and the US (clinicaltrials.gov; NCT 02742727, 02839954, 02892695, 03656705, 03940833).

Along with the tremendous success CAR therapies have had in treating hematological malignancies, so far, they have only demonstrated limited effectivity against solid tumors [24]. The biggest challenges are the lack of tumor-specific antigens, tumor antigen heterogeneity, immune cell homing and infiltration as well as the immunosuppressive TME.

CD276 (B7-H3) is a member of the B7 superfamily of immune checkpoints and recently emerged as an important prognostic tumor marker as well as a promising target structure for immunotherapy [25–27]. Although CD276 mRNA is reportedly ubiquitously expressed in various human tissue types, assessment of CD276 translational status using immunohistochemistry demonstrated only weak or absent protein expression on normal tissue [28,29]. Importantly, CD276 is overexpressed in a variety of solid tumors, including melanoma [27]. Such overexpression was reported to play a pivotal role in tumor vascularization, metastasis and overall poor clinical prognosis [30,31]. Since CD276 is expressed

on different cell types within the tumor, like differentiated as well as stem-like cells or even tumor vasculature, immunotherapy targeting B7-H3 may have great potential for complete tumor eradication [32,33]. So far, different anti-CD276 blocking antibodies have been examined in pre-clinical as well as phase 1 clinical studies and have demonstrated a good safety and powerful anti-tumor profile [34,35]. Additionally, CD276-specific chimeric antigen receptors have been developed and tested as CAR-engineered T cells in multiple pre-clinical studies targeting various solid tumors including melanoma [36–40].

The tumor microenvironment consists of a variety of cells including tumor-associated fibroblasts, macrophages, dendritic cells, neutrophils, regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC) as well as extracellular matrix and multiple secreted or cell membrane-presented molecules. Due to the immunosuppressive properties of the TME, tumor cells are able to escape immune-surveillance by impairing immune cell infiltration and cytotoxic functions, thus supporting tumor growth and metastasis [41].

To elicit a strong anti-tumor immune response in solid tumors, CAR-engineered NK cells need to overcome major challenges, such as insufficient homing and infiltration due to the lack of cognate chemokine signals and physiological barriers, immunosuppressive cells and cytokines, low pH, oxidative stress and lack of immune-stimulating cytokines [42,43]. Soluble factors such as TGFβ, which are highly secreted within the TME, have been shown to facilitate metastatic development as well as tumor escape mechanisms during treatment with primary immune cells [44,45]. TGFβ directly impairs NK cell immune effector function through inhibition of the T-bet transcription factor (SMAD3) which reduces cytotoxicity and IFN-γ secretion [42,46]. Furthermore, TGFβ upregulates expression of NK cell inhibitory receptors while at the same time downregulating activating receptors such as NKp30 and NKG2D or their respective ligands, such as MHC class I polypeptide-related sequence A (MICA), on cancer cells [47,48]. Moreover, metabolic dysregulation by the induction of nutrient-catabolizing enzymes such as indoleamine 2,3-dioxygenase (IDO) and the secretion of large amounts of lactate promote an acidic tumor environment and is linked to growth arrest of tumor-infiltrating NK cells [49,50]. Lactate accumulation in the TME is associated with limited NK cell-mediated cytotoxicity through downregulation of activating NK receptors [51]. Furthermore, melanoma cells exposed to an acidic pH demonstrate elevated levels of invasiveness and metastatic development [52,53]. Another factor impairing effectivity of tumor-infiltrating NK cells is the hypoxic environment within solid tumors. Hypoxia has been shown to downregulate activating NK cell receptors as well as the secretion of NK effector molecules such as perforin and granzyme B [54,55].

Contrary to primary NK cells, there is very few data investigating effects of the immunosuppressive TME for NK-92 cell-based immunotherapy. In the present study, we analyzed effectivity of CAR NK-92 cells targeting CD276 in vitro in the presence of low pH, hypoxia and other known factors of the TME.

2. Materials and Methods

2.1. Cell Lines and Culturing Conditions

Cutaneous melanoma cell lines FM-3 and WM115 were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, UK), the cell line Mel-Juso was purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany), the cell lines M14, SK-MEL-2 and UACC62 were acquired from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA) and the cell lines SK-MEL-5 and SK-MEL-28 were purchased from Cell Lines Services (CLS, Eppelheim, Germany). All tumor cell lines were maintained in RPMI 1640 GlutaMAX™ medium containing 2 mM stable L-glutamine and supplemented with 10% heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), referred to as RPMI complete medium.

Dermal fibroblasts were purchase from PromoCell (Heidelberg, Germany) and cultivated in RPMI complete medium. Cancer-associated fibroblasts were established as a

by-product from biopsy tumor samples provided by the Department of Orthopedic Surgery, University Hospital Tuebingen (UKT) and also cultivated in RPMI complete medium.

Lenti-X 293T cells (Takara Bio, Kyoto, Japan) were cultivated in DMEM high glucose (4.5 g/L) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing GlutaMAX supplemented with 10% FBS and 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA, USA). NK-92 cells were purchased from the American Type Culture Collection (ATCC) and maintained at a concentration of 10^5 cells/mL in MEM Alpha Medium containing GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% FBS and 100 U/mL IL-2 (Proleukin, Aldesleukin, Chiron, Emeryville, CA, USA) referred to as NK-92 complete medium.

All media contained 1× antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA) consisting of 100 U/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL amphotericin B. All cells and cell lines were maintained at 37 °C in a humidified 5% CO_2 atmosphere and were regularly tested for mycoplasma contamination.

2.2. Flow Cytometry

Cells were stained at 4 °C using the indicated antibodies in flow cytometry buffer made of PBS (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% FBS and 0.5 M EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Using a BD FACSCanto II flow cytometer, live cells were gated based on forward and side scatter. CD276-CAR expression on NK-92 cells was determined by CD34 marker gene expression.

2.3. Generation of Lentiviral Vectors

A second generation lentiviral vector plasmid encoding the CD276-specific chimeric antigen receptor (CD276-CAR) construct was acquired from Creative Biolabs, Shirley, NY, USA. Exact constitution of the CD276-CAR construct was previously described [22]. The transfer plasmid encoding a GFP-luciferase construct was obtained from Dr. Irmela Jeremias, Helmholtz Center, Munich, Germany.

Lentiviral particles (LVP) were produced in Lenti-XTM 293T (Takara Bio, Kyoto, Japan) after lipofection (Lipofectamine 3000, Thermo Fisher Scientific, Waltham, MA, USA) of a second generation packaging plasmid, a VSV-G envelope plasmid and the indicated transfer plasmid. LVP containing supernatants were concentrated using Lenti-X concentrator (Takara Bio, Kyoto, Japan) and stored at −80 °C until further use.

2.4. Lentiviral Transduction

Exact transduction process of CD276-CAR-engineered NK-92 cells was described previously [22]. Melanoma cells were seeded at 5.0×10^4 cells/mL or 1.25×10^5 cells/mL in RPMI medium without supplements and subsequently transduced with GFP-luciferase LVP for 16 h. Cells were maintained in RPMI complete medium. Transduction efficiency was determined by flow cytometry.

2.5. Calcein-Release Cytotoxicity Assay (CRA)

Target cell staining with Calcein AM (Thermo Fisher Scientific, Waltham, MA, USA) as well as the protocol for the calcein release-based cytotoxicity assay (CRA) was described previously [22,56].

2.6. Real-Time Impedance-Based Live Cell Analysis

Melanoma cell lines were adjusted to a concentration of 10^5 cells/mL in RPMI complete medium and seeded in E-Plate 96 VIEW (OLS, Bremen, Germany) micro-well plates. Effector CAR NK-92 cells were adjusted to an E:T ratio of 5:1 in NK-92 complete medium without IL-2 and co-incubated with the target cells. Utilizing the xCELLigence real-time cell analysis (RTCA) system, cells were monitored for at least 72 h. Melanoma cell viability was calculated using the RTCA 2.0 software and CAR mediated cytotoxicity was subsequently determined.

2.7. Cytokine Secretion Analysis

Cytokine release of CD276-CAR NK-92 cells was determined using the Bio-Plex Pro human cytokine 17-plex assay (Bio Rad, Hercules, CA, USA). The respective protocol was described previously [56].

2.8. Analysis of CD276-CAR NK-92 Cell-Mediated Cytotoxicity under Hypoxic Conditions

For the assessment of the influence of hypoxic conditions on cytotoxic efficacy of NK-92 cells, CD276-CAR-engineered as well as parental NK-92 cells were cultivated in a specialized incubator at 37 °C in a humidified 5% CO_2, 1% O_2 atmosphere for 24 or 168 h. Subsequently, they were utilized as effector cells in standard calcein release assays as described above under hypoxic conditions.

2.9. Testing the Influence of TGFβ on CD276-CAR NK-92 Cells

NK-92 and CD276-CAR NK-92 cells were cultivated in NK-92 complete medium with TGFβ at indicated concentrations for 48 h and subsequently used as effector cells in standard calcein release assays as described above.

2.10. Analysis of the Influence of Lactate on CD276-CAR NK-92 Cells

NK-92 and CD276-CAR NK-92 cells were cultivated in NK-92 complete medium with sodium L-lactate (Merck, Darmstadt, Germany) at indicated concentrations for 72 h. pH-values were measured using the LAQUAtwin pH-22 pH-meter (Horiba, Kyoto, Japan) and cells were subsequently used as effector cells in standard calcein release assays as previously described.

2.11. Generation of Melanoma Supernatants

FM-3, Mel-Juso and WM115 cells, human skin fibroblasts as well as cancer-associated fibroblasts (CAF) were seeded in 6-well plates in RPMI complete medium at a concentration of 6.25×10^4 cells/mL. They were cultivated at 37 °C for 72 h. Cells were harvested, centrifuged and the medium supernatant was collected carefully. pH values were measured using the LAQUAtwin pH-22 pH-meter (Horiba, Kyoto, Japan).

Next, CD276-CAR NK-92 cells as well as parental NK-92 cells were cultivated in melanoma or fibroblast supernatants at a concentration of 10^5 cells/mL for 48 h and subsequently used as effector cells in standard calcein release assays as described above.

2.12. Generation of Melanoma 3D Spheroids for the Assessment of CD276-CAR-Mediated Cytotoxicity of NK-92 Cells

GFP-transduced melanoma cells were adjusted to a concentration of 5.0×10^3 cells/mL and seeded in a 96-well low attachment U-bottom plate (Nexcelom, Lawrence, MA, USA). Spheroids were grown for 72 h and were subsequently co-incubated with CD276-CAR NK-92 cells. Fluorescence was measured using the Celigo S imaging cytometer (Nexcelom, Lawrence, MA, USA) at indicated time points over a period of 96 h. CAR-mediated cytotoxicity was calculated using the average integrated fluorescence intensity of melanoma spheroids.

2.13. Tracking of NK-92-Mediated Tumor Cell Invasion

NK-92 as well as CD276-CAR NK-92 cells were assessed for their invasion potential. (CD276-CAR) NK-92 cells were stained using CellTracker™ Deep Red dye (Thermo Fisher Scientific, Waltham, MA, USA) according to the staining protocol provided by the manufacturer. Melanoma 3D spheroids were co-incubated with NK-92 cells at an E:T ratio of 5:1 and monitored using the Celigo S imaging cytometer (Nexcelom, Lawrence, MA, USA) at indicated time points over a period of 120 h.

2.14. Assessment of NK-92 Migration

For the assessment of NK-92 migration potential, GFP-transduced WM115 melanoma cells were grown as 3D spheroids according to protocol. Melanoma spheroids were then incubated with or without CXCL12 and/or prostaglandin E_2 (PGE2) at indicated concentrations for the remaining experiment according to the publication by Berahovic at al. 2006 [57]. NK-92 cells as well as CD276-CAR NK-92 cells were seeded in a HTS Transwell-96 Well permeable supports with barcode, tissue culture treated, 5.0 µm pore size plate (Corning, Corning, NY, USA) at an E:T ratio of 20:1 in NK-92 complete medium without IL-2. The trans-well plate was put on top off the spheroid low-attachment plate to allow for NK-92 cell migration. After 24 h, the trans-well plate was removed and fluorescence of the spheroid was regularly measured using the Celigo S imaging cytometer (Nexcelom, Lawrence, MA, USA) over a period of 72 h. NK-92-mediated cytotoxicity was calculated using the average integrated fluorescence intensity of melanoma spheroids.

2.15. CRISPR/Cas9-Mediated Knock-Out of NKG2A in CD276-CAR NK-92 Cells

2.15.1. SgRNA Design

Five different sgRNAs (Table 1) were employed to disrupt the NKG2A gene. NKG2A-1, NKG2A-2 and NKG2A-3 were designed using the CHOPCHOP software version 3 [58]. NKG2A-4 and NKG2A-5 sequences were obtained from the NKG2A Human Gene Knock-out Kit (KN203062, OriGene, Rockville, MD, USA).

Table 1. Nucleotide sequences for NKG2A sgRNAs

Name	Nucleotide Sequence
NKG2A-1	GAAGCTCATTGTTGGGATCC
NKG2A-2	TTGAAGGTTTAATTCCGCAT
NKG2A-3	ACTGGAGTTCTTCGAAGTAC
NKG2A-4	AGGCAGCAACGAAAACCTAA
NKG2A-5	GGTCTGAGTAGATTACTCCT

2.15.2. Assessment of CRISPR/Cas9 In Vitro Cutting of NKG2A Gene

The cutting potential of the designed sgRNAs was determined by the in vitro cleavage of target DNA with ribonucleoprotein (RNP) complex protocol (IDT).

2.15.3. Generation of CRISPR-Mediated Knock-Out of NKG2A Gene in CD276-CAR NK-92 Cells

V3 Cas9 RNP and chemically modified sgRNAs were incubated at a molar ratio of 1:2 (45 pmol to 90 pmol) at room temperature for 15 minutes [59]. After complex formation, 10^5 NK-92 cells were transfected using the 10 µL Neon Transfection kit (ThermoFisher Scientific, Waltham, MA, USA) with the following electroporation settings: 1.200 V, 20 ms, 1 pulse (Neon System, ThermoFisher Scientific, Waltham, MA, USA). Finally, cells were transferred to a 48 well-plate in fresh NK-92 complete medium.

2.15.4. Assessment of CRISPR-Mediated Modification of NKG2A at Genomic Level in CAR-NK-92 Cells

On day 5 post-electroporation, the CRISPR-modified cells were harvested for DNA isolation. DNA was extracted with the NucleoSpin Tissue kit following the manufacturer's instructions (Macherey-Nagel, Düren, Germany) and was subsequently employed in a PCR reaction (100 ng). The genomic region including the CRISPR target site was specifically amplified with GoTaq Green DNA polymerase master mix (Promega, Madison, WI, USA) using following protocol: initial denaturation of 95 °C for 2 min followed by 40 cycles of 40 s at 95 °C, 40 s at 55 °C and 1 min at 68 °C. The PCR reaction was cleaned up from unincorporated nucleotides and other reagents with QIAquick PCR purification kit following the commercial protocol (Qiagen, Hilden, Germany) and then Sanger-sequenced

(Table 2) by Eurofins Genomics (Konstanz, Germany). The results were further analyzed with ICE software (Synthego, Redwood City, CA, USA).

Table 2. Nucleotide sequences for NKG2A PCR primers

Primer	Nucleotide Sequence
Forward 1	TACTCGTTCTCCACCTCACC
Reverse 1	TAACGTGAAAATTCCCCTTGTAATC
Forward 2	ATTTACCAGCCCATGAAGATGT
Reverse 2	TCCATGAAAAGCAAAAACTGAA

2.15.5. Measurements of CRISPR-Mediated Knock-Out Score of NKG2A at Protein Level in NK-92 Cells

Cells were harvested for FACS analysis 5 days after electroporation. NKG2A-FITC antibody (130-114-091, Miltenyi Biotec, Bergisch Gladbach, Germany) was employed for flow cytometry analysis (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA) following the commercial protocol. The gating strategy was set up using FlowJo software according to the positive population observed in the control sample. The knock-out score was calculated as the NKG2A negative population presented in the CRISPR-modified cells.

2.16. Data Analysis

All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Flow cytometry data were analyzed using FlowJo software V10.0.8 (FlowJo LLC., BD Biosciences, Franklin Lakes, NJ, USA).

3. Results

B7-H3 was shown to be a promising option of targeted immunotherapy. We previously showed that CD276-CAR NK-92 cells were effective in targeting neuroblastoma cells in vitro [22]. To examine whether CD276-CAR NK-92 cells can also successfully target melanoma, commercially available melanoma cell lines, among others FM-3, Mel-Juso and WM115, were screened for their CD276 surface expression using flow cytometry (Figure 1).

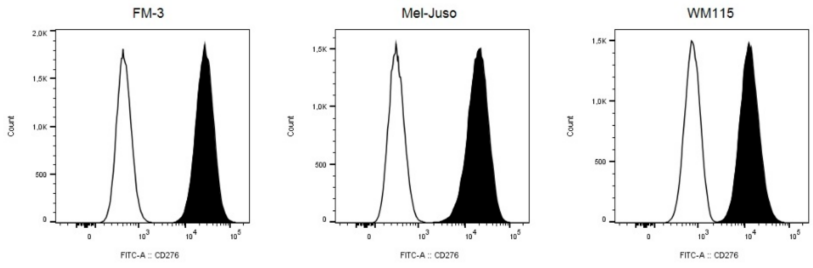


Figure 1. The commercially available melanoma cell lines FM-3 (**a**), Mel-Juso (**b**) and WM115 (**c**) were screened for their B7-H3 (CD276) surface expression using flow cytometry. CD276 antigen expression (black) was determined using a FITC-labeled anti-CD276 antibody and compared to the respective isotype antibody (white).

All melanoma cell lines that were screened during this study showed uniform abundance of CD276 expression. As a proof of principle, the three cell lines FM-3, Mel-Juso and WM115 were chosen for subsequent experiments.

We characterized the cell lines for surface expression of important NK cell ligands using flow cytometry (Table 3). All cell lines showed abundant expression of various types of human leukocyte antigens, including HLA-E, a major ligand for the inhibitory receptor complex CD94/NKG2A on NK cells. Furthermore, high expression levels of the major histocompatibility complex (MHC) class I chain-related protein A and B (MICA/B),

a protein that, upon binding to the natural killer group 2, member D receptor NKG2D (CD314), induces an activating signaling cascade in NK cells, could be demonstrated for the FM-3 and Mel-Juso cell lines. Moreover, all three cell lines expressed Nectin 2 (CD112), PVR (CD155) as well as death receptor 5 (CD262). By binding to the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), CD262 can induce NK-mediated apoptosis. CD112 and CD155 are pivotal ligands for the DNAX Accessory Molecule-1 DNAM-1 (CD226) triggering NK cell activation. However, CD112 as well as CD155 can also induce an inhibitory cascade by activation of the immune checkpoint receptor TIGIT on NK cells.

Table 3. Characterization of melanoma cell lines for NK ligand expression. Tumor cells were co-incubated with fluorescently labeled antibodies and analyzed using flow cytometry. MFI values were calculated using staining with the respective isotype control antibody.

	CD48	CD50	CD54	CD58	CD95	CD102	CD112	CD155	CD261	CD262
FM-3	1.50	1.49	22.71	34.67	3.08	1.28	9.17	56.28	0.97	9.65
Mel-Juso	1.38	1.48	9.40	55.23	0.93	1.26	15.70	80.10	2.79	6.23
WM115	1.49	1.65	1.98	144.49	3.45	1.21	5.46	22.80	1.63	9.40
	HLA-ABC	**HLA-DR**	**HLA-E**	**HLA-G**	**MICA/B**	**ULBP1**	**ULBP2/5/6**	**ULBP3**	**ULBP4**	
FM-3	34.24	14.53	4.89	20.68	11.37	0.49	2.52	0.48	1.20	
Mel-Juso	30.86	33.69	7.11	22.73	21.45	0.58	7.03	0.78	0.20	
WM115	87.21	44.80	8.07	21.88	3.12	0.66	2.43	0.52	0.42	

Next, the melanoma cell lines were used as target cells in standard calcein release assays (CRA) to assess the specific cytotoxic potential of CD276-CAR NK-92 cells (Figure 2a–c). CD276-CAR NK-92 cells as well as parental NK-92 cells were co-incubated with calcein-labeled melanoma cells. After two hours, a significant increase in specific CAR-mediated tumor lysis could be demonstrated for all three cell lines. Even at low E:T ratios, specific cytotoxicity increased up to 70%. Previously, we have shown that CD276-CAR NK-92 cells showed no undesirable off-target effects since CAR-mediated cytotoxicity only occurred in the presence of CD276 surface expression on target tumor cells [22]. In order to examine the kinetics of CAR-mediated cytolysis, CD276-CAR NK-92 as well as untransduced NK-92 cells were co-incubated with unlabeled melanoma cells and monitored using the xCELLigence real-time cell analysis (RTCA) system (Figure 2d–f). Electrical impedance is measured and interpreted as the dimensionless cell index. Increase in cell index describes tumor cell growth while cell index decrease is proportional to tumor cell death. A significant decrease in tumor cell viability could be shown within two hours upon addition of CD276-CAR NK-92 cells and no tumor regrowth was detectable over a longer period of time. Furthermore, we tested whether γ-irradiation of NK-92 cells with 10 Gy, as required in all NK-92 clinical trials, would decrease CAR-mediated cytotoxic potential. We found that the difference between irradiated and non-irradiated cytotoxicity was only marginal and did not reach statistical significance.

Next, a cytokine secretion profile for CD276-CAR and parental NK-92 cells upon co-incubation with melanoma cells was established to screen for the release of NK effector molecules (Figure 2g). Compared to parental NK-92 cells, co-incubation of CD276-CAR NK-92 cells with melanoma cells significantly increased secretion of various interleukins, among others, IL-6 (98- to 317-fold) and IL-10 (6- to 15-fold). Furthermore, a drastic increase of pro-inflammatory cytokines such as IFN-γ (44- to 81-fold) and TNF-α (101- to 225-fold) could be detected. Increase of NK effector molecule release, granulysin (4-fold), granzyme B (3- to 4-fold) and perforin (5- to 7-fold), was evenly distributed. Additionally, flow cytometric analysis showed no increase in surface expression of two important pathways in NK-mediated apoptosis induction, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), in CD276-CAR NK-92 cells upon co-incubation with melanoma cells (Figure 2h).

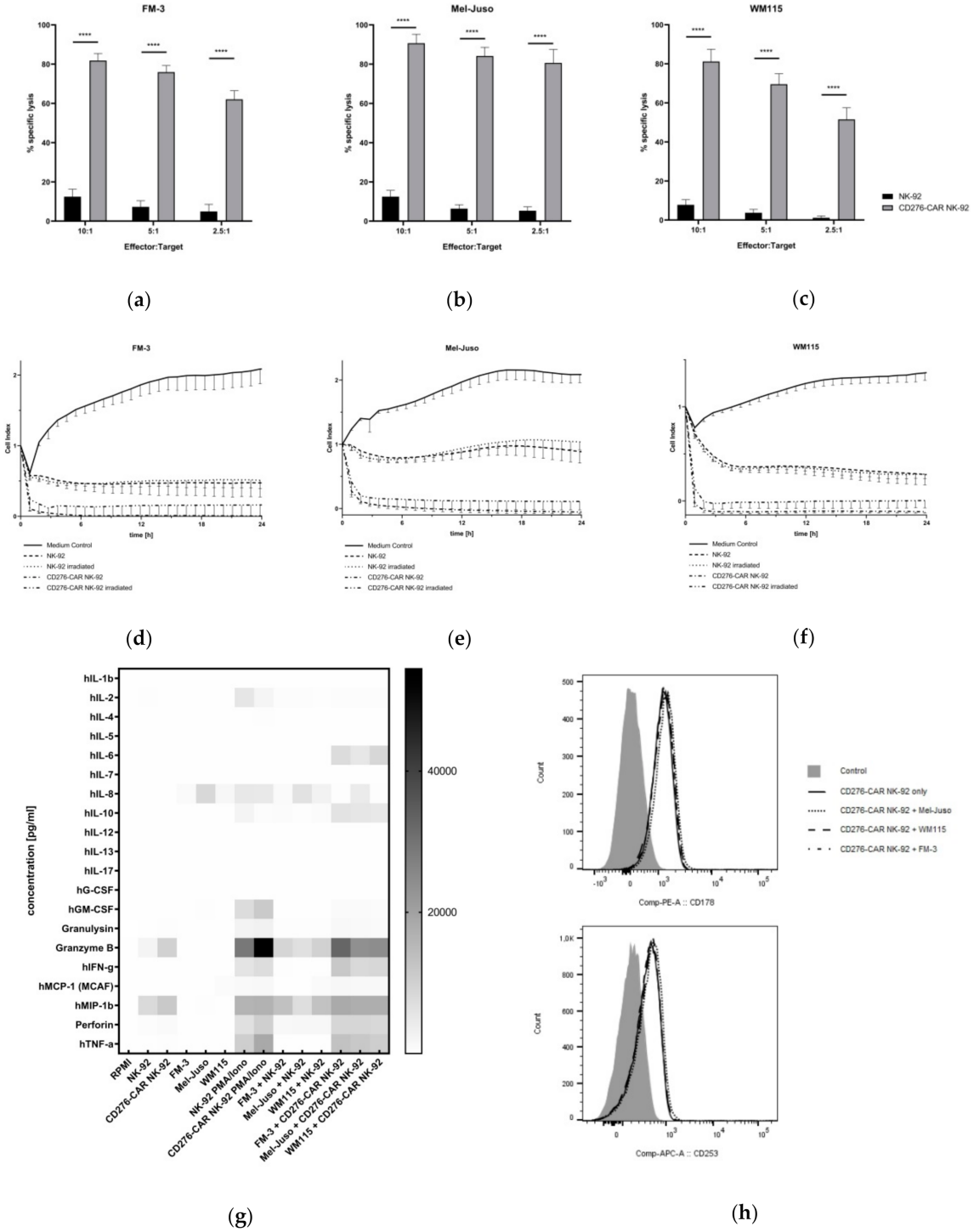


Figure 2. CD276-CAR NK-92 cells were co-incubated with calcein-labeled melanoma cell lines at indicated effector-to-target (E:T) ratios. Specific cytotoxicity was measured after 2 h and is shown as mean ± SD, $n = 3$ (**a**–**c**). To assess the kinetics of CD276-CAR-mediated tumor lysis, irradiated and non-irradiated CD276-CAR NK-92 as well as parental NK-92 cells were co-incubated with unlabeled melanoma cells at an E:T ratio of 5:1. Tumor cell viability was monitored using the real-time live cell analysis (RTCA) xCELLigence system, $n = 3$ (**d**–**f**). NK-92 cytokine secretion in the presence or absence of melanoma target cells was determined using the Bio-Plex Pro human cytokine 17-plex assay and is shown as a heatmap (**g**). CD276-CAR NK-92 cell surface expression levels of FasL (CD178) and TRAIL (CD253) were screened using flow cytometry before and after co-incubation with melanoma cell lines (**h**); **** $p < 0.0001$.

The immunosuppressive microenvironment surrounding solid tumors has been shown to negatively affect cytotoxic potential of NK cells. We examined how hypoxic or acidic culture conditions and soluble factors secreted by cancer cells or cancer-associated, such as transforming growth factor beta (TGFβ), influence CAR-mediated effector function of NK-92 cells.

First, NK-92 and CAR-transduced NK-92 cells were cultivated under hypoxic conditions (5% CO_2, 1% O_2) for 24 and 168 h. In a standard CRA, compared to cells cultivated in standard conditions, CD276-CAR NK-92 cells showed slightly decreased cytotoxic potential after hypoxic cultivation for 24 h but after cultivation for 168 h, specific CAR-mediated cytotoxicity drastically decreased to circa 20% (Figure 3a–c). Importantly, NK-92 cell viability was also impaired by 30% and 80%, respectively. Next, we investigated the effect of the immunosuppressive TGFβ. CD276-CAR NK-92 and NK-92 cells were cultivated with soluble TGFβ at concentrations of 10 ng/mL and 50 ng/mL for 48 h. Cytotoxic efficacy was assessed in a CRA and compared to untreated NK-92 and CD276-CAR NK-92 cells (Figure 3d–f). We found no significant decrease in cytotoxicity against all three melanoma cell lines at any E:T ratio, which suggests that TGFβ seems to have no negative effect on CD276-CAR NK-92-mediated tumor lysis.

Acidosis, as a fundamental feature of the TME, can impact functionality and tumor cell invasion capacity of primary immune cells such as NK cells. Here, we assessed whether an acidic environment during cell culture affects NK-mediated cytotoxicity of NK-92 cells. CD276-CAR and parental NK-92 cells were cultivated in NK-92 complete medium with different concentrations of sodium L-lactate for 48 h (Figure 3g–i). Cell supernatant pH values decreased from 8.0 in an untreated control to 7.5 and 7.2 in medium treated with 15 or 30 mM sodium L-lactate, respectively. Cytotoxic effector function of NK-92 as well as CAR-transduced NK-92 cells was not significantly impaired by acidity of culture medium. Notably, NK-92 cell proliferation and viability were not impaired either.

Lastly, we wanted to determine if incubation of NK-92 cells in the presence of soluble factors secreted by melanoma cells, such as TGFβ, IL-10, IDO, nitric oxide (NO) and prostaglandins, molecules that have been shown to be regularly secreted by tumor cells and to induce an immunosuppressive phenotype in primary immune cells, could also weaken immune effector function of NK-92 cells [60–63]. The melanoma cell lines FM-3, Mel-Juso and WM115 as well as human fibroblasts and cancer-associated fibroblasts were cultivated for 72 h. Supernatant was harvested and pH value was assessed. Compared to the control medium, pH values in the supernatant decreased from 8.0 to 7.4 $\pm$ 0.1. CD276-CAR and NK-92 cells were subsequently incubated in tumor cell supernatant for 48 h and used as effector cells in a CRA with all three melanoma cell lines (Figure 3j–o). Incubation of CD276-CAR NK-92 cells in supernatant from FM-3 culture did not impact cytotoxicity while CAR NK-92 cells incubated with Mel-Juso as well as WM115 cell culture supernatant did show slightly decreased CAR-mediated cytolysis of all three melanoma cell lines. Interestingly, cultivation in medium collected from fibroblasts and CAF did not impact CAR-mediated NK effector function.

All in all, no negative effect on CD276-CAR NK-92 cell-mediated cytotoxicity could be demonstrated with variances in acidity, immunosuppressive molecules like TGFβ or co-incubation with cancer-associated fibroblasts. Only long-lasting hypoxic culture conditions as well as incubation with tumor cell supernatants slightly affected CD276-CAR NK-92 cell function.

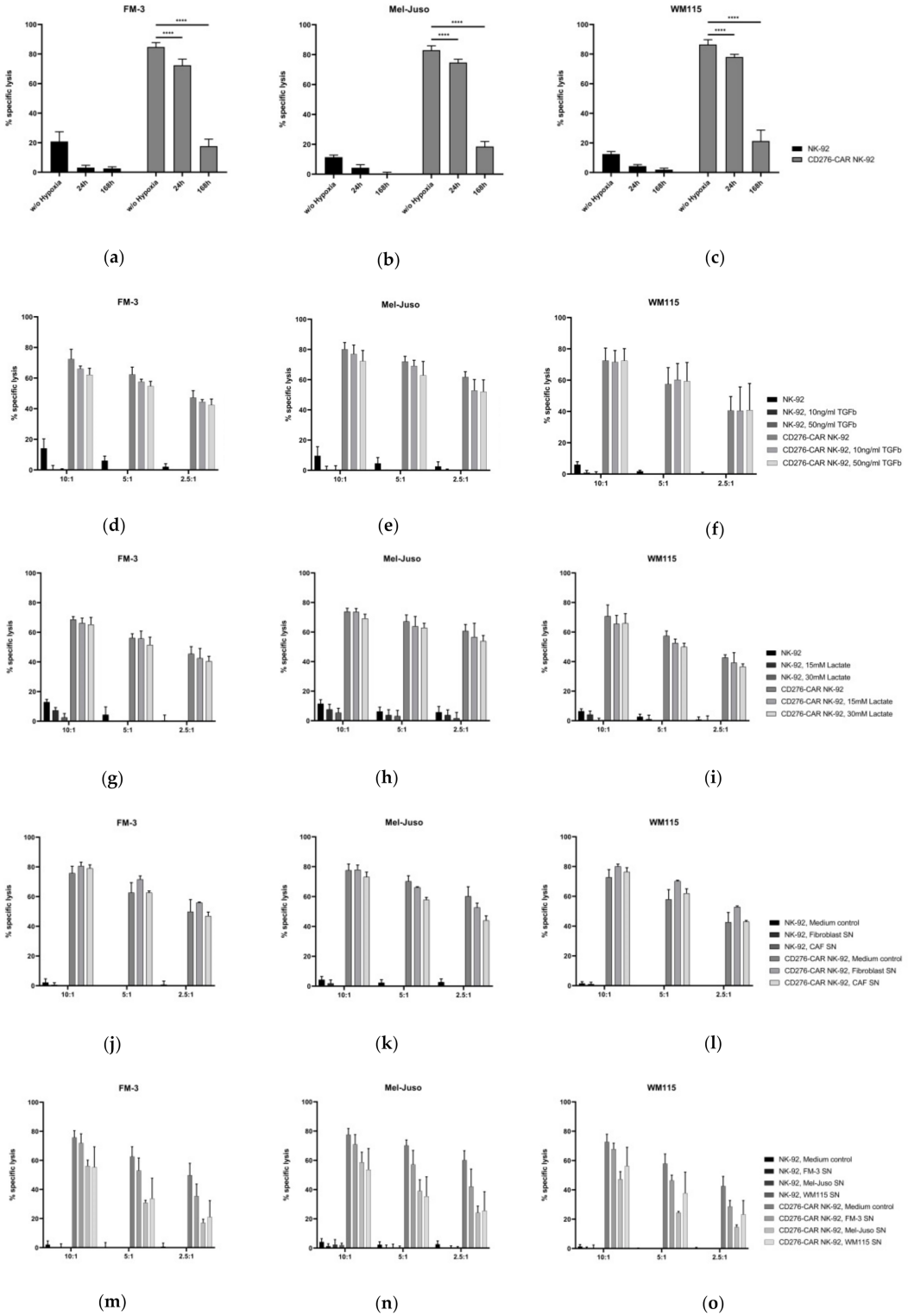


Figure 3. CD276-CAR NK-92 cells as well as parental NK-92 cells were co-incubated with calcein-labeled melanoma cell lines at indicated E:T ratios for 2 h. Specific cytotoxicity was assessed using a calcein release assay and is shown as mean ± SD, $n = 3$. Prior to the experiments, NK-92 and CD276-CAR NK-92 cells were either cultivated under hypoxic conditions (5% CO_2, 1% O_2) for 24 and 168 h (**a**–**c**), pre-treated with indicated concentrations of TGFβ (**d**–**f**), sodium L-lactate (**g**–**i**) or supernatants, previously collected from human fibroblast and cancer-associated fibroblast (CAF) culture (**j**–**l**) as well as melanoma cell line culture (**m**–**o**), for 48 h; **** $p < 0.0001$.

Evaluation of immune effector function in in vitro assays is usually restricted to two-dimensional tumor cell culture. The 3D cell cultures, better mimicking the in vivo situation, are physiologically more relevant and predictive than standard 2D assays. Therefore, three-dimensional melanoma spheroid models were established to examine whether CD276-CAR NK-92 prove to be as effective in tumor cell lysis as in a standard CRA. Melanoma cell lines were grown as spheroids for 72 h according to the protocol published by Vinci et al. 2012 and subsequently co-incubated with CD276-CAR NK-92 cells. Representative fluorescence pictures of tumor spheroids were taken regularly (Figure 4a–b) and compared to co-incubation with untransduced NK-92 cells. CAR-mediated tumor cell lysis of each of the three tumor cell lines was drastically increased after no later than 24 h after addition of NK effector cells (Figure 4c–e). Notably, WM115 spheroids were lysed fully after 48 h and tumor cell regrowth could not be observed at a later time point.

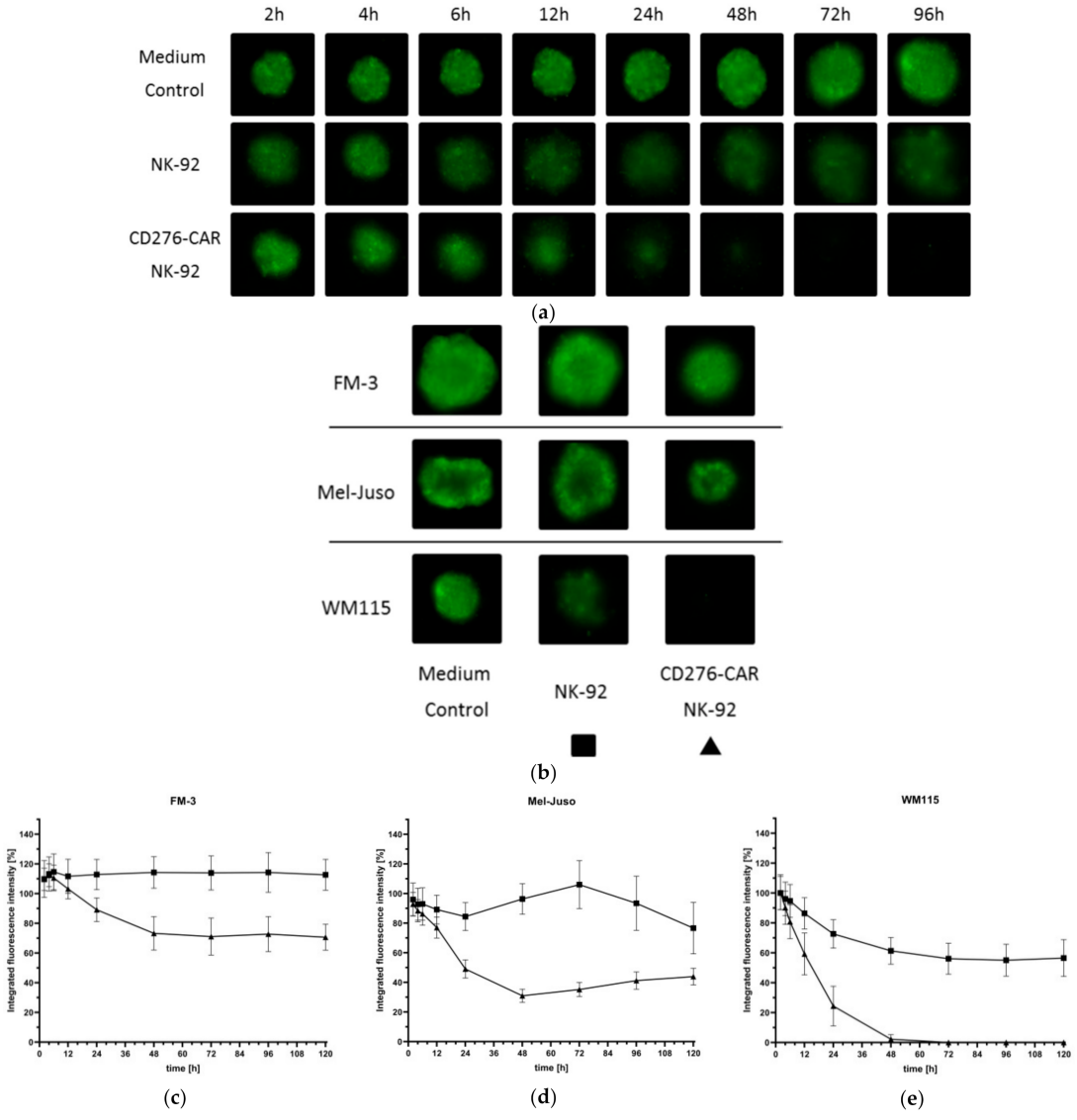


Figure 4. GFP-transduced cell lines FM-3, Mel-Juso and WM115 were grown as three-dimensional melanoma spheroids for 72 h before being co-incubated with CD276-CAR NK-92 or parental NK-92 cells for 96 h. Integrated fluorescence intensity of melanoma spheroids (FM-3, Mel-Juso and WM115) was measured regularly using the Celigo S imaging cytometer. Representative fluorescence images of WM115 spheroids from indicated time points (**a**) as well as all melanoma spheroids after 96 h (**b**) are shown. Integrated fluorescence intensity was compared to untreated control spheroids and is shown as mean ± SD, $n = 3$ (**c**–**e**).

We then utilized melanoma spheroids to assess the potential impairment of CAR-mediated cytotoxicity by the TME factors, thus, mimicking a more realistic in vivo scenario. Therefore, CD276-CAR NK-92 and parental NK-92 cells were cultivated under hypoxic conditions, under low pH, with WM115 cell culture SN and various combinations thereof for 48 h and then co-incubated with WM115 spheroids for 96 h. Fluorescence images of the melanoma spheroids were taken regularly (Figure 5a–b). Integrated fluorescence intensity of spheroids co-incubated with NK-92 and CD276-CAR NK-92 cells were compared to untreated control spheroids (Figure 5c–d). All melanoma spheroids that were co-incubated with CD276-CAR NK-92 cells were completely eradicated after 48 to 72 h. Only pre-cultivation of CAR NK-92 cells in medium with lactate-induced low pH slightly decreased the time course of tumor cell lysis compared to untreated cells. However, it did not impair overall cytotoxic function. Interestingly, parental NK-92 cells that had prior been cultivated in low pH showed significantly lower cytolytic effects compared to untreated NK-92 cells.

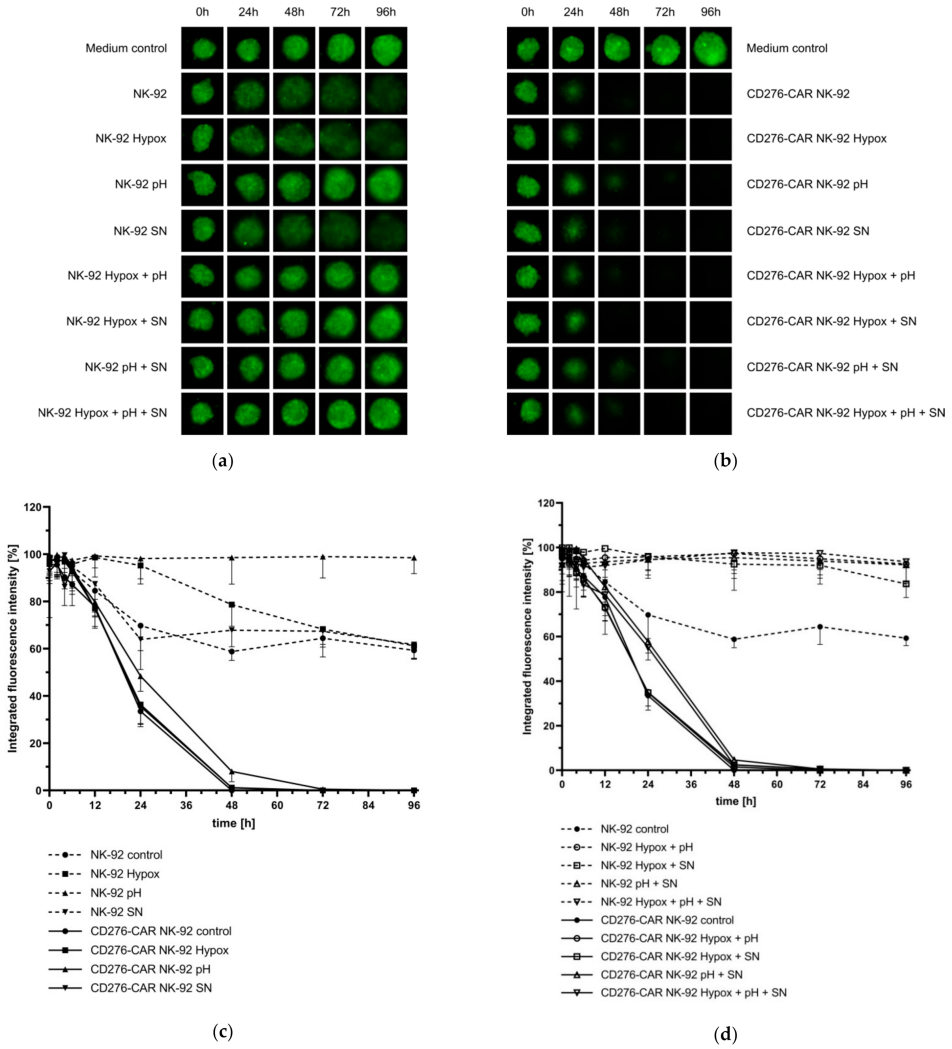
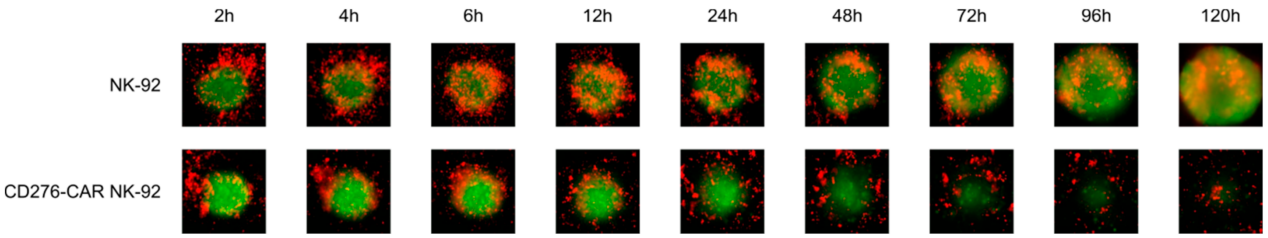


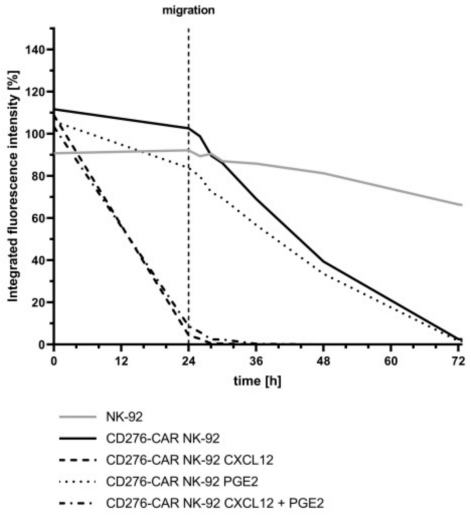
Figure 5. The GFP-transduced cell line WM115 was grown as 3D spheroid for 72 h. CD276-CAR NK-92 and parental NK-92 cells had been cultivated under hypoxic conditions, under low pH, with WM115 cell culture SN or various combinations thereof for 48 h and were added to the melanoma spheroids. Integrated fluorescence intensity of spheroids was measured regularly over 96 h using the Celigo S imaging cytometer. Representative fluorescence images of WM115 spheroids co-incubated with NK-92 (**a**) and CD276-CAR NK-92 (**b**) from indicated time points are shown. Average integrated fluorescence intensity was compared to untreated control spheroids and is shown as mean ± SD, $n = 3$ (**c**–**d**).

Overall, CAR-mediated cytotoxicity of melanoma cells did not drastically decrease after incubation of effector cells with various combinations of the immunosuppressive factors of the TME in either 2D or 3D cell culture systems.

For robust immunotherapy of solid tumors, primary immune cells need to be able to deeply infiltrate tumor tissue. To assess NK-92 tumor infiltration potential, NK-92 as well as CD276-CAR NK-92 cells were labeled with a red fluorescent dye and co-incubated with GFP-transduced WM115 spheroids for 120 h. Fluorescent images of the cells were taken regularly at indicated time points (Figure 6a). Interestingly, parental NK-92 cells seem to infiltrate tumor spheroids faster than their CAR-transduced counterparts but without increased tumor cell lysis. On the contrary, CD276-CAR NK-92 cells show high cytotoxic potential lysing tumor spheroids from the outside, indicating that lysis of the spheroids is not dependent on complete infiltration.



(**a**)



(**b**)

Figure 6. GFP-transduced WM115 melanoma cells were grown as 3D spheroids and co-incubated with CellTracker™ Deep Red-labeled NK-92 or CD276-CAR NK-92 cells for 120 h. Representative fluorescence pictures of spheroids at indicated time points are shown (**a**). WM115 spheroids were incubated with or without CXCL12 at a concentration of 100 nM. NK-92 cells (grey line) as well as CD276-CAR NK-92 cells (black lines) were pre-treated with or without prostaglandin E2 (PGE2) at a concentration of 5.7 µM and seeded in a HTS trans-well plate at an E:T ratio of 20:1. Cells were allowed to migrate for 24 h. Spheroid fluorescence was regularly measured using the Celigo S imaging cytometer over a period of 72 h. NK-92-mediated cytotoxicity was calculated using the average integrated fluorescence intensity of melanoma spheroids and is shown as mean (**b**); $n \geq 3$.

Migration potential is another important factor for the treatment efficacy of solid tumors using immune cells. Therefore, NK-92 migration potential was assessed using permeable trans-well plates. GFP-transduced WM115 tumor spheroids were grown according to protocol and CD276-CAR NK-92 cells were subsequently added in tissue culture-treated wells with 5.0 µm pore size. Untransduced NK-92 cells were used as controls. A small sample of tumor culture medium was supplemented with soluble CXCL12 at a concentration of 100 nM to enhance immune cell migration and some CD276-CAR NK-92 cells were

pre-treated with prostaglandin E2 (PGE2) at a concentration of 5.7 µM in order to block migration [64,65]. CXCL12 is a highly effective chemoattractant that is expressed in various cell types, including melanoma [66]. It binds to, among others, the C-X-C chemokine receptor type 4 (CXCR-4), which is expressed in NK-92 cells [22]. Cells were allowed to migrate for 24 h and integrated fluorescence intensity of tumor spheroids was measured regularly (Figure 6b). CD276-CAR NK-92 cells that were added to tumor spheroids in the presence of CXCL12 were able to migrate quickly and show high cytotoxic potential within 24 h. In the absence of supplemented CXCL12, cells seem to migrate in lower numbers, so a delayed tumor lysis was observed. Notably, the pre-treatment of NK-92 cells with PGE2 did not impair migration or cytotoxic potential independent of CXCL12 supplementation of tumor medium. To sum up, CD276-CAR NK-92 cells demonstrated the ability to effectively migrate to tumor sites and infiltrate and lyse melanoma cells in a 3D spheroid model system.

In the context of TME-mediated immune escape strategies, HLA-E expression is often elevated in melanoma [67]. We found abundant HLA-E expression in all melanoma cell lines that were used in this study. Its respective NK receptor, NKG2A (CD159a), is one of the few inhibitory receptors expressed by NK-92 cells. In order to assess the influence of HLA-E binding to NKG2A for NK-92-mediated cytotoxicity and to potentially further enhance the efficacy of the generated CD276-CAR NK-92 cells, the disruption of NKG2A (CD159a) was studied.

The predominance of NKG2A$^+$ NK cells in the tumor microenvironment reveals its importance in the suppressive axis between tumor and NK cells [68], leading to important clinical studies using anti-NKG2A inhibitory antibodies in the treatment of cancer [69,70]. As a matter of fact, the disruption of NKG2A in NK cells was proven to overcome tumor resistance [71]. Therefore, we hypothesized that the disruption of *KLRC1* (encoding for NKG2A protein) would boost the antitumor activity of CD276-CAR NK-92 as a result of bypassing this immune scape mechanism. To corroborate this idea, five different sgRNAs aiming for the coding sequence of the gene were considered. Three of them (NKG2A-2, NKG2A-4 and NKG2A-5) target the cytoplasmic domain, one (NKG2A-1) the intermembrane domain and one (NKG2A-3) the intracellular domain (Figure 7a). The obtained results revealed that NKG2A-5 induced the highest percentage of indels (82.5% and 71.5% in NK-92 and CD276-CAR NK-92 cells, respectively), and so achieved the highest level of disruption (77.8% and 67.2%) at protein level (Figure 7b–c).

The knock-out cells obtained by NKG2A-5 editing were subsequently sorted and expanded to generate pure NKG2A-KO NK-92 and CD276-CAR NK-92 cell lines. Next, the cells were co-cultured with the different melanoma cell lines in standard CRA. Slightly increased tumor cell lysis of melanoma cells was observed for NKG2A-KO NK-92 cells in comparison to their unmodified counterparts. However, this improvement was not demonstrated in NKG2A-KO CD276-CAR NK-92 cells, where the reported cytotoxic performance was similar to unmodified CD276-CAR NK-92 cells (Figure 7d). This was further underscored by pre-treatment of CD276-CAR NK-92 cells with a blocking antibody targeting NKG2A which, similarly, did not enhance CAR-mediated cytotoxicity suggesting that cytotoxic potential of CD276-CAR NK-92 cells is either not influenced by inhibitory ligands or the CAR-induced activation signal is strong enough to override inhibitory signaling.

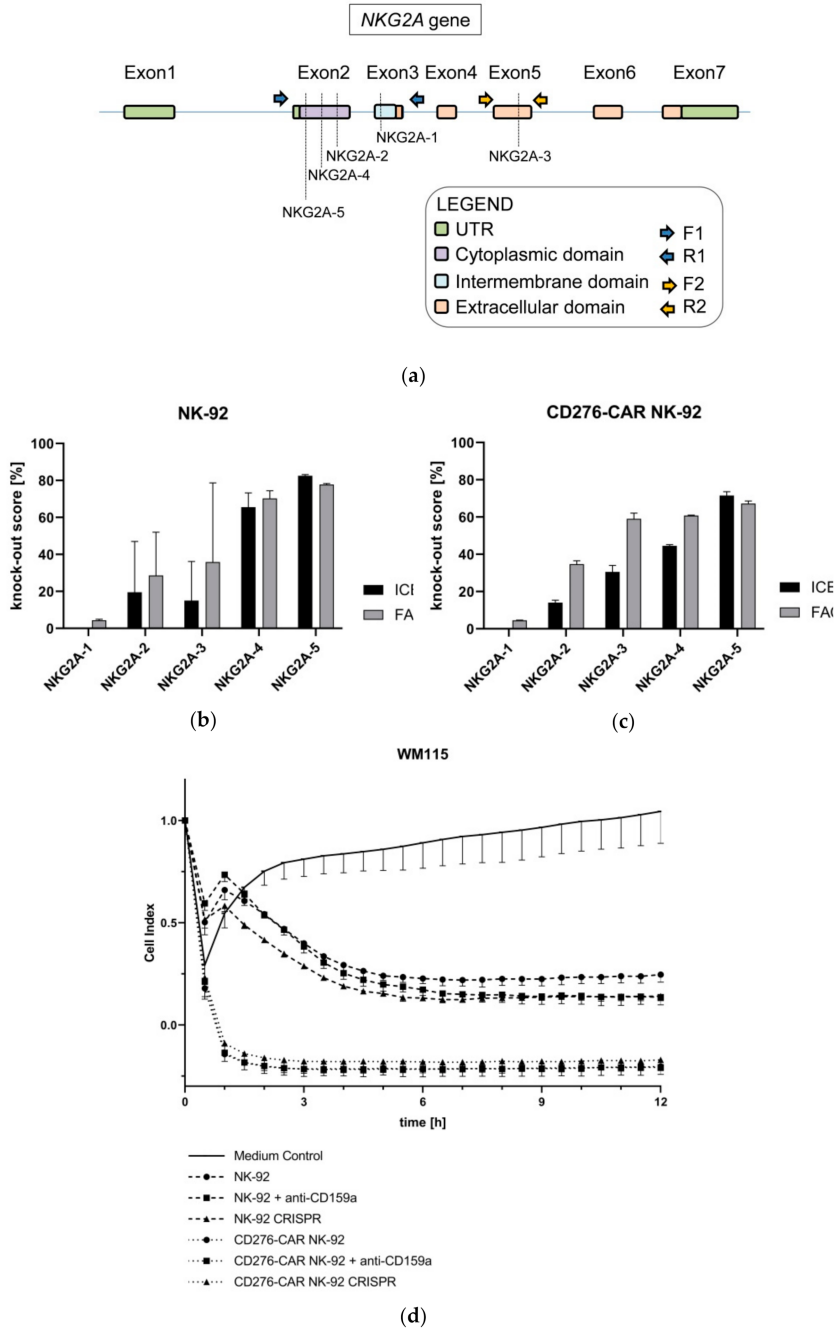


Figure 7. The schematic illustration shows the NKG2A (CD159a) gene and five potential sgRNA cutting sites in exons 2, 3 and 5 (**a**). Knock-out scores for NK-92 and CD276-CAR NK-92 cell NKG2A knock-out were calculated using Sanger sequencing (black) and flow cytometric analysis (grey) (**b**,**c**). Cytotoxic potential of knock-out variant 5 (NKG2A-5) was assessed using the xCELLigence real-time cytotoxicity assay and compared to unmodified controls as well as wildtype CD276-CAR and parental NK-92 cells that had been pre-treated with an inhibitory anti-NKG2A antibody (**d**); $n = 3$.

4. Discussion

Melanoma is one of the most common cancers worldwide with its incidence continuing to rise each year [72]. Even though immune checkpoint blockade drastically increased overall outcome for some patients, new treatment strategies for therapy-resistant melanomas are in dire need.

Chimeric antigen receptor-engineered T cells provide a therapeutic approach that involves the use of modified primary lymphocytes to specifically target cancer cells. Currently, only seven clinical trials examining the usage of CAR T cells for the treatment of melanoma, targeting among others disialoganglioside GD2 or vascular endothelial growth factor receptor 2 (VEGFR2), are on-going, while no clinical trials have yet been conducted with other CAR-engineered immune cells such as primary NK cells. Major obstacles for CAR efficiency in solid tumors include the identification of tumor-specific antigens as well as tumor antigen heterogeneity [24]. Additionally, due to the presence of the highly immunosuppressive microenvironment in this context, clinical results so far have been underwhelming [73].

One very promising clinical study, conducted at the Seattle Children's Hospital, is targeting the immune checkpoint molecule CD276 [74]. CD276 recently emerged as an important prognostic marker for various solid tumor entities and, thus, seems to be a promising immunotherapeutic target structure [25,26]. It was already shown in multiple studies that CD276 is aberrantly expressed in malign melanoma and the present study demonstrated CD276 abundance in all examined melanoma cell lines [75–77].

However, CAR T and CAR NK cells, regardless of their specific target structure, face complex obstacles when used in solid tumors. They need to successfully migrate through tissue to reach the tumor site, efficiently invade the tumor and also survive the suppressive TME that decreases anti-tumor response using various factors [78–80]. In the present study, we evaluated whether the recently established NK cell line CD276-CAR NK-92, that proved to be effective in targeting neuroblastoma, could also be an effective cellular product for the treatment of melanoma [22]. Furthermore, it was evaluated whether CAR-modified NK-92 cells are able to overcome negative factors of the TME, thus, making NK-92 cells a superior CAR vehicle for the treatment of melanoma and solid tumors in general.

Dynamic alterations during melanoma progression include the increase of growth factor secretion and, subsequently, growth of additional fibrous tissue surrounding the tumor site which in turn impairs effector cell migration [81]. Here, we showed that CD276-CAR NK-92 cells demonstrated successful eradication of various melanoma cell lines in vitro in 2D as well as 3D experiments. They were able to successfully migrate through small pores to the tumor site and penetrate melanoma spheroids while retaining their cytotoxic efficacy.

After successful extravasation, CAR-modified immune cells need to survive the unfavorable TME. The predominantly hypoxic and acidic conditions in the TME in combination with an anti-inflammatory cytokine milieu consisting of, among others, TGFβ can significantly impair CAR-mediated effector function [82–86]. TGFβ was demonstrated to directly impair NK cell functionality by downregulating activating NK receptors such as NKG2D and NKp30 or by suppressing the mTOR pathway [48,87]. Contrary to primary NK cells, CD276-CAR NK-92 cells showed significant resistance to soluble immunomodulatory factors. The cells retained their cytotoxic potential even after prolonged co-incubation with high concentrations of TGFβ.

Acidosis in the TME, caused by tumor cell secretion of substantial amounts of lactate, is associated with impaired cytotoxicity of primary NK cells as well as downregulation of activating NK cell receptors [88,89]. Conversely, effector function of CD276-CAR NK-92 cells was not impaired after cultivation with high concentrations of supplemented lactate. Moreover, cultivation in cell culture supernatant that was harvested from fibroblasts and cancer-associated fibroblasts, that play a pivotal role in tumor neo-vascularization, had also no detectable impact on CAR-mediated tumor lysis while melanoma supernatant only slightly affected CD276-CAR NK-92 cell function [90,91].

Hypoxic culture conditions for longer periods of time were drastically decreasing CAR-mediated target cell lysis of primary NK cells and NK-92 cells [54,55,92]. Since NK-92 cells have to be irradiated for clinical use to prevent uncontrolled in vivo proliferation due to being derived from a non-Hodgkin lymphoma, cell viability and effective cytotoxicity is restricted to a 48–72 h time window after irradiation. We previously demonstrated

that application of CD276-CAR NK-92 cells immediately after irradiation did not impair CAR-mediated cytotoxicity [22]. Therefore, decreased immune effector function induced by long-term exposure to hypoxia (>72 h) is not a critical issue.

NKG2A is, contrary to primary NK cells, one of the few inhibitory receptors expressed by CD276-CAR NK-92 cells and their respective tumor cell ligands, HLA-E and HLA-G, are commonly overexpressed in cutaneous melanoma [22,56,71]. Furthermore, HLA-E and HLA-G expression levels are often elevated in the course of typical melanoma immune escape strategies [67]. A CRISPR-mediated NKG2A knock-out as well as blocking the NKG2A receptor with an inhibitory antibody did not significantly boost CAR-mediated cytotoxic potential of CD276-CAR NK-92 cells, suggesting that CAR effectivity in CD276-CAR NK-92 cells is independent of inhibitory ligands. Since the CAR-mediated cytotoxicity exerted by parental CD276-CAR NK-92 itself is already very strong, we hypothesize that the implementation of the described CRISPR protocol for other NK-92-based immunotherapies could improve the outcome of the respective treatment.

The accumulated data for CD276-CAR NK-92 cells and their resistance to the tumor microenvironment is very promising. Other factors, including the influence of immunosuppressive cell such as myeloid-derived suppressor cells (MDSC) or CAR NK-92 cell trafficking to the tumor site, need to be investigated further. In the near future, we are planning on establishing various in vivo models, including PDX and humanized mouse models, to validate these preliminary results.

Combining the aforementioned positive features of a CD276-CAR, targeting a tumor-specific antigen, with the NK-92 cell line and its strong resilience to influences exerted by the TME generates a powerful cellular product that can be easily manufactured under GMP guidelines as a readily available, "off-the-shelf" treatment option for melanoma and other solid tumors.

Author Contributions: Conceptualization, S.G. and S.S.; methodology, S.G. and S.S.; software, S.G.; validation, S.G. and S.S.; formal analysis, S.G.; investigation, S.G., G.U.-B., K.C.-H.C., C.B. and M.M.; resources, R.H.; data curation, S.G.; writing—original draft preparation, S.G., G.U.-B. and S.S.; writing—review and editing, S.G. and S.S.; visualization, S.G.; supervision, S.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Clinician Scientist Program Tuebingen (N°. 440-0-0), Stiftung Förderverein für krebskranke Kinder Tübingen e.V., Stefan Morsch Stiftung and the University Children's Hospital of Tübingen.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by ethics committee at the Medical Faculty of the Eberhard Karls University and the University Hospital Tuebingen (reference number 008/2014BO2).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gershenwald, J.E.; Guy, G.P. Stemming the Rising Incidence of Melanoma: Calling Prevention to Action. *J. Natl. Cancer Inst.* **2016**, *108*, 381. [CrossRef] [PubMed]
2. Miller, K.D.; Siegel, R.L.; Khan, R.; Jemal, A. Cancer Statistics. *Cancer Rehabil.* **2018**, *70*, 7–30. [CrossRef]
3. Sandru, A.; Voinea, S.; Panaitescu, E.; Blidaru, A. Survival rates of patients with metastatic malignant melanoma. *J. Med. Life* **2015**, *7*, 572–576.
4. Ott, P.A.; Hodi, F.S.; Robert, C. CTLA-4 and PD-1/PD-L1 Blockade: New Immunotherapeutic Modalities with Durable Clinical Benefit in Melanoma Patients. *Clin. Cancer Res.* **2013**, *19*, 5300–5309. [CrossRef]
5. Koyama, S.; Akbay, E.A.; Li, Y.Y.; Herter-Sprie, G.S.; Buczkowski, K.A.; Richards, W.G.; Gandhi, L.; Redig, A.J.; Rodig, S.J.; Asahina, H.; et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat. Commun.* **2016**, *7*, 10501. [CrossRef]

6. Pitt, J.M.; Vétizou, M.; Daillère, R.; Roberti, M.P.; Yamazaki, T.; Routy, B.; Lepage, P.; Boneca, I.G.; Chamaillard, M.; Kroemer, G.; et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. *Immunity* **2016**, *44*, 1255–1269. [CrossRef]
7. Kourie, H.R.; Klastersky, J. Immune checkpoint inhibitors side effects and management. *Immunotherapy* **2016**, *8*, 799–807. [CrossRef]
8. Spain, L.; Diem, S.; Larkin, J. Management of toxicities of immune checkpoint inhibitors. *Cancer Treat. Rev.* **2016**, *44*, 51–60. [CrossRef]
9. Lyon, A.R.; Yousaf, N.; Battisti, N.M.L.; Moslehi, J.; Larkin, J. Immune checkpoint inhibitors and cardiovascular toxicity. *Lancet Oncol.* **2018**, *19*, e447–e458. [CrossRef]
10. Sadelain, M.; Brentjens, R.; Rivière, I. The Basic Principles of Chimeric Antigen Receptor Design. *Cancer Discov.* **2013**, *3*, 388–398. [CrossRef]
11. Neelapu, S.S.; Locke, F.L.; Bartlett, N.L.; Lekakis, L.J.; Miklos, D.B.; Jacobson, C.A.; Braunschweig, I.; Oluwole, O.O.; Siddiqi, T.; Lin, Y.; et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N. Engl. J. Med.* **2017**, *377*, 2531–2544. [CrossRef]
12. Maude, S.L.; Laetsch, T.W.; Buechner, J.; Rives, S.; Boyer, M.; Bittencourt, H.; Bader, P.; Verneris, M.R.; Stefanski, H.E.; Myers, G.D.; et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N. Engl. J. Med.* **2018**, *378*, 439–448. [CrossRef]
13. Yáñez, L.; Sánchez-Escamilla, M.; Perales, M.-A. CAR T Cell Toxicity: Current Management and Future Directions. *HemaSphere* **2019**, *3*, e186. [CrossRef]
14. Liu, E.; Marin, D.; Banerjee, P.; Macapinlac, H.A.; Thompson, P.; Basar, R.; Kerbauy, L.N.; Overman, B.; Thall, P.; Kaplan, M.; et al. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. *N. Engl. J. Med.* **2020**, *382*, 545–553. [CrossRef]
15. Gong, J.H.; Maki, G.; Klingemann, H.G. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* **1994**, *8*, 652–658.
16. Burger, M.C.; Zhang, C.; Harter, P.N.; Romanski, A.; Strassheimer, F.; Senft, C.; Tonn, T.; Steinbach, J.P.; Wels, W.S. CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy. *Front. Immunol.* **2019**, *10*, 2683. [CrossRef]
17. Tam, Y.K.; Martinson, J.A.; Doligosa, K.; Klingemann, H.G. Ex vivo expansion of the highly cytotoxic human natural killer cell line NK-92 under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. *Cytotherapy* **2003**, *5*, 259–272.
18. Swift, B.E.; Williams, B.A.; Kosaka, Y.; Wang, X.-H.; Medin, J.A.; Viswanathan, S.; Martinez-Lopez, J.; Keating, A. Natural killer cell lines preferentially kill clonogenic multiple myeloma cells and decrease myeloma engraftment in a bioluminescent xenograft mouse model. *Haematologica* **2012**, *97*, 1020–1028. [CrossRef]
19. Tonn, T.; Schwabe, D.; Klingemann, H.G.; Becker, S.; Esser, R.; Koehl, U.; Suttorp, M.; Seifried, E.; Ottmann, O.G.; Bug, G. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* **2013**, *15*, 1563–1570. [CrossRef]
20. Boyiadzis, M.; Agha, M.; Redner, R.L.; Sehgal, A.; Im, A.; Hou, J.-Z.; Farah, R.; Dorritie, K.A.; Raptis, A.; Lim, S.H.; et al. Phase 1 clinical trial of adoptive immunotherapy using "off-the-shelf" activated natural killer cells in patients with refractory and relapsed acute myeloid leukemia. *Cytotherapy* **2017**, *19*, 1225–1232. [CrossRef]
21. Williams, B.A.; Law, A.D.; Routy, B.; Denhollander, N.; Gupta, V.; Wang, X.-H.; Chaboureau, A.; Viswanathan, S.; Keating, A. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. *Oncotarget* **2017**, *8*, 89256–89268. [CrossRef] [PubMed]
22. Grote, S.; Chan, K.C.; Baden, C.; Bösmüller, H.; Sulyok, M.; Frauenfeld, L.; Ebinger, M.; Handgretinger, R.; Schleicher, S. CD276 as a novel CAR NK-92 therapeutic target for neuroblastoma. *Adv. Cell Gene Ther.* **2021**, *4*, e105. [CrossRef]
23. Zhang, C.; Oberoi, P.; Oelsner, S.; Waldmann, A.; Lindner, A.; Tonn, T.; Wels, W.S. Chimeric Antigen Receptor-Engineered NK-92 Cells: An Off-the-Shelf Cellular Therapeutic for Targeted Elimination of Cancer Cells and Induction of Protective Antitumor Immunity. *Front. Immunol.* **2017**, *8*, 533. [CrossRef] [PubMed]
24. Hou, B.; Tang, Y.; Li, W.; Zeng, Q.; Chang, D. Efficiency of CAR-T Therapy for Treatment of Solid Tumor in Clinical Trials: A Meta-Analysis. *Dis. Markers* **2019**, *2019*, 1–11. [CrossRef] [PubMed]
25. Zhou, Z.; Luther, N.; Ibrahim, G.M.; Hawkins, C.; Vibhakar, R.; Handler, M.H.; Souweidane, M.M. B7-H3, a potential therapeutic target, is expressed in diffuse intrinsic pontine glioma. *J. Neuro-Oncol.* **2013**, *111*, 257–264. [CrossRef]
26. Picarda, E.; Ohaegbulam, K.C.; Zang, X. Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy. *Clin. Cancer Res.* **2016**, *22*, 3425–3431. [CrossRef]
27. Flem-Karlsen, K.; Tekle, C.; Andersson, Y.; Flatmark, K.; Fodstad, Ø.; Nunes-Xavier, C.E. Immunoregulatory protein B7-H3 promotes growth and decreases sensitivity to therapy in metastatic melanoma cells. *Pigment. Cell Melanoma Res.* **2017**, *30*, 467–476. [CrossRef]
28. Huang, B.; Luo, L.; Wang, J.; He, B.; Feng, R.; Xian, N.; Zhang, Q.; Chen, L.; Huang, G. B7-H3 specific T cells with chimeric antigen receptor and decoy PD-1 receptors eradicate established solid human tumors in mouse models. *OncoImmunology* **2020**, *9*, 1684127. [CrossRef]
29. Zhang, H.; Zhang, J.; Li, C.; Xu, H.; Dong, R.; Chen, C.C.; Hua, W. Survival Association and Cell Cycle Effects of B7H3 in Neuroblastoma. *J. Korean Neurosurg. Soc.* **2020**, *63*, 707–716. [CrossRef]

30. Benzon, B.; Zhao, S.G.; Haffner, M.C.; Takhar, M.; Erho, N.; Yousefi, K.; Hurley, P.; Bishop, J.L.; Tosoian, J.; Ghabili, K.; et al. Correlation of B7-H3 with androgen receptor, immune pathways and poor outcome in prostate cancer: An expression-based analysis. *Prostate Cancer Prostatic Dis.* **2017**, *20*, 28–35. [CrossRef]
31. Zang, X.; Thompson, R.H.; Al-Ahmadie, H.A.; Serio, A.M.; Reuter, V.E.; Eastham, J.A.; Scardino, P.T.; Sharma, P.; Allison, J.P. B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 19458–19463. [CrossRef]
32. Crispen, P.L.; Sheinin, Y.; Roth, T.J.; Lohse, C.M.; Kuntz, S.M.; Frigola, X.; Thompson, R.H.; Boorjian, S.A.; Dong, H.; Leibovich, B.C.; et al. Tumor Cell and Tumor Vasculature Expression of B7-H3 Predict Survival in Clear Cell Renal Cell Carcinoma. *Clin. Cancer Res.* **2008**, *14*, 5150–5157. [CrossRef]
33. Seaman, S.; Zhu, Z.; Saha, S.; Zhang, X.M.; Yang, M.Y.; Hilton, M.B.; Morris, K.; Szot, C.; Morris, H.; Swing, D.A.; et al. Eradication of Tumors through Simultaneous Ablation of CD276/B7-H3-Positive Tumor Cells and Tumor Vasculature. *Cancer Cell* **2017**, *31*, 501–515. [CrossRef]
34. Loo, D.; Alderson, R.F.; Chen, F.Z.; Huang, L.; Zhang, W.; Gorlatov, S.; Burke, S.; Ciccarone, V.; Li, H.; Yang, Y.; et al. Development of an Fc-Enhanced Anti–B7-H3 Monoclonal Antibody with Potent Antitumor Activity. *Clin. Cancer Res.* **2012**, *18*, 3834–3845. [CrossRef]
35. Powderly, J.; Cote, G.M.; Flaherty, K.T.; Szmulewitz, R.Z.; Ribas, A.; Weber, J.S.; Loo, D.; Baughman, J.; Chen, F.; A Moore, P.; et al. Interim results of an ongoing Phase I, dose escalation study of MGA271 (Fc-optimized humanized anti-B7-H3 monoclonal antibody) in patients with refractory B7-H3-expressing neoplasms or neoplasms whose vasculature expresses B7-H3. *J. Immunother. Cancer* **2015**, *3*, O8. [CrossRef]
36. Tang, X.; Zhao, S.; Zhang, Y.; Wang, Y.; Zhang, Z.; Yang, M.; Zhu, Y.; Zhang, G.; Guo, G.; Tong, A.; et al. B7-H3 as a Novel CAR-T Therapeutic Target for Glioblastoma. *Mol. Ther. Oncolytics* **2019**, *14*, 279–287. [CrossRef]
37. Majzner, R.G.; Theruvath, J.L.; Nellan, A.; Heitzeneder, S.; Cui, Y.; Mount, C.W.; Rietberg, S.P.; Linde, M.H.; Xu, P.; Rota, C.; et al. CAR T Cells Targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity against Pediatric Solid Tumors and Brain Tumors. *Clin. Cancer Res.* **2019**, *25*, 2560–2574. [CrossRef]
38. Du, H.; Hirabayashi, K.; Ahn, S.; Kren, N.P.; Montgomery, S.A.; Wang, X.; Tiruthani, K.; Mirlekar, R.; Michaud, D.; Greene, K.; et al. Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 Via Chimeric Antigen Receptor T Cells. *SSRN Electron. J.* **2018**, *35*, 221–237.e8. [CrossRef]
39. Tang, X.; Liu, F.; Liu, Z.; Cao, Y.; Zhang, Z.; Wang, Y.; Huang, J.; Fan, S.; Zhao, S.; Chen, Y.; et al. Bioactivity and safety of B7-H3-targeted chimeric antigen receptor T cells against anaplastic meningioma. *Clin. Transl. Immunol.* **2020**, *9*, e1137. [CrossRef]
40. Zhang, Z.; Jiang, C.; Liu, Z.; Yang, M.; Tang, X.; Wang, Y.; Zheng, M.; Huang, J.; Zhong, K.; Zhao, S.; et al. B7-H3-Targeted CAR-T Cells Exhibit Potent Antitumor Effects on Hematologic and Solid Tumors. *Mol. Ther. Oncolytics* **2020**, *17*, 180–189. [CrossRef]
41. Quail, D.F.; A Joyce, J. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **2013**, *19*, 1423–1437. [CrossRef]
42. Melaiu, O.; Lucarini, V.; Cifaldi, L.; Fruci, D. Influence of the Tumor Microenvironment on NK Cell Function in Solid Tumors. *Front. Immunol.* **2020**, *10*, 3038. [CrossRef]
43. Nayyar, G.; Chu, Y.; Cairo, M.S. Overcoming Resistance to Natural Killer Cell Based Immunotherapies for Solid Tumors. *Front. Oncol.* **2019**, *9*, 51. [CrossRef]
44. Xie, F.; Ling, L.; van Dam, H.; Zhou, F.; Zhang, L. TGF-β signaling in cancer metastasis. *Acta Biochim. Biophys. Sin.* **2018**, *50*, 121–132. [CrossRef]
45. Wu, Y.; Kuang, D.M.; Pan, W.D.; Wan, Y.L.; Lao, X.M.; Wang, D.; Li, X.F.; Zheng, L. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology* **2013**, *57*, 1107–1116. [CrossRef]
46. Trotta, R.; Col, J.D.; Yu, J.; Ciarlariello, D.; Thomas, B.; Zhang, X.; Allard, J.; Wei, M.; Mao, H.; Byrd, J.C.; et al. TGF-β Utilizes SMAD3 to Inhibit CD16-Mediated IFN-γ Production and Antibody-Dependent Cellular Cytotoxicity in Human NK Cells1. *J. Immunol.* **2008**, *181*, 3784–3792. [CrossRef]
47. Pasero, C.; Gravis, G.; Guerin, M.; Granjeaud, S.; Thomassin-Piana, J.; Rocchi, P.; Paciencia-Gros, M.; Poizat, F.; Bentobji, M.; Azario-Cheillan, F.; et al. Inherent and Tumor-Driven Immune Tolerance in the Prostate Microenvironment Impairs Natural Killer Cell Antitumor Activity. *Cancer Res.* **2016**, *76*, 2153–2165. [CrossRef] [PubMed]
48. Castriconi, R.; Cantoni, C.; Della Chiesa, M.; Vitale, M.; Marcenaro, E.; Conte, R.; Biassoni, R.; Bottino, C.; Moretta, L.; Moretta, A. Transforming growth factor β1 inhibits expression of NKP30 and NKG2d receptors: Consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4120–4125. [CrossRef]
49. Uyttenhove, C.; Pilotte, L.; Théate, I.; Stroobant, V.; Colau, D.; Parmentier, N.; Boon, T.; van den Eynde, B.J. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med.* **2003**, *9*, 1269–1274. [CrossRef]
50. Frumento, G.; Rotondo, R.; Tonetti, M.; Damonte, G.; Benatti, U.; Ferrara, G.B. Tryptophan-derived Catabolites Are Responsible for Inhibition of T and Natural Killer Cell Proliferation Induced by Indoleamine 2,3-Dioxygenase. *J. Exp. Med.* **2002**, *196*, 459–468. [CrossRef]
51. Xie, H.; Hanai, J.-I.; Ren, J.-G.; Kats, L.; Burgess, K.; Bhargava, P.; Signoretti, S.; Billiard, J.; Duffy, K.J.; Grant, A.; et al. Targeting Lactate Dehydrogenase-A Inhibits Tumorigenesis and Tumor Progression in Mouse Models of Lung Cancer and Impacts Tumor-Initiating Cells. *Cell Metab.* **2014**, *19*, 795–809. [CrossRef] [PubMed]

52. Martínez-Zaguilán, R.; Seftor, E.A.; Seftor, R.E.; Chu, Y.W.; Gillies, R.J.; Hendrix, M.J. Acidic pH enhances the invasive behavior of human melanoma cells. *Clin. Exp. Metastasis* **1996**, *14*, 176–186. [CrossRef] [PubMed]
53. Rofstad, E.K.; Mathiesen, B.; Kindem, K.; Galappathi, K. Acidic Extracellular pH Promotes Experimental Metastasis of Human Melanoma Cells in Athymic Nude Mice. *Cancer Res.* **2006**, *66*, 6699–6707. [CrossRef] [PubMed]
54. Petrova, V.; Annicchiarico-Petruzzelli, M.; Melino, G.; Amelio, I. The hypoxic tumour microenvironment. *Oncogenesis* **2018**, *7*, 10. [CrossRef]
55. Sceneay, J.; Chow, M.T.; Chen, A.; Halse, H.M.; Wong, C.S.; Andrews, D.M.; Sloan, E.K.; Parker, B.S.; Bowtell, D.D.; Smyth, M.J.; et al. Primary tumor hypoxia recruits CD11b^{+}/Ly6C^{med}/Ly6G^{+} immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche. *Cancer Res.* **2012**, *72*, 3906–3911. [CrossRef]
56. Grote, S.; Mittelstaet, J.; Baden, C.; Chan, K.C.H.; Seitz, C.; Schlegel, P.; Kaiser, A.; Handgretinger, R.; Schleicher, S. Adapter chimeric antigen receptor (AdCAR)-engineered NK-92 cells: An off-the-shelf cellular therapeutic for universal tumor targeting. *Oncoimmunology* **2020**, *9*, 1825177. [CrossRef]
57. Berahovich, R.D.; Lai, N.L.; Wei, Z.; Lanier, L.L.; Schall, T.J. Evidence for NK Cell Subsets Based on Chemokine Receptor Expression. *J. Immunol.* **2006**, *177*, 7833–7840. [CrossRef]
58. Labun, K.; Montague, T.G.; Krause, M.; Cleuren, Y.N.T.; Tjeldnes, H.; Valen, E. CHOPCHOP v3: Expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* **2019**, *47*, W171–W174. [CrossRef]
59. Lamsfus-Calle, A.; Daniel-Moreno, A.; Antony, J.S.; Epting, T.; Heumos, L.; Baskaran, P.; Admard, J.; Casadei, N.; Latifi, N.; Siegmund, D.M.; et al. Comparative targeting analysis of KLF1, BCL11A, and HBG1/2 in CD34^{+} HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin. *Sci. Rep.* **2020**, *10*, 10133. [CrossRef]
60. Enk, A. Dendritic cells in tolerance induction. *Immunol. Lett.* **2005**, *99*, 8–11. [CrossRef]
61. A Clark, D.; Coker, R. Molecules in focus Transforming growth factor-beta (TGF-β). *Int. J. Biochem. Cell Biol.* **1998**, *30*, 293–298. [CrossRef]
62. Umansky, V.; Sevko, A. Melanoma-induced immunosuppression and its neutralization. *Semin. Cancer Biol.* **2012**, *22*, 319–326. [CrossRef]
63. Kusmartsev, S.; Gabrilovich, D.I. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis Rev.* **2006**, *25*, 323–331. [CrossRef]
64. Dillenburg-Pilla, P.; Patel, V.; Mikelis, C.M.; Zárate-Bladés, C.R.; Doçi, C.L.; Amornphimoltham, P.; Wang, Z.; Martin, D.; Leelahavanichkul, K.; Dorsam, R.T.; et al. SDF-1/CXCL12 induces directional cell migration and spontaneous metastasis via a CXCR4/Gαi/mTORC1 axis. *FASEB J.* **2015**, *29*, 1056–1068. [CrossRef]
65. Singh, T.; Katiyar, S.K. Honokiol Inhibits Non-Small Cell Lung Cancer Cell Migration by Targeting PGE2-Mediated Activation of β-Catenin Signaling. *PLoS ONE* **2013**, *8*, e60749. [CrossRef]
66. Bleul, C.C.; Fuhlbrigge, R.C.; Casasnovas, J.M.; Aiuti, A.; A Springer, T. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* **1996**, *184*, 1101–1109. [CrossRef]
67. Borst, L.; van der Burg, S.H.; van Hall, T. The NKG2A–HLA-E axis as a novel checkpoint in the tumor microenvironment. Clin. *Cancer Res.* **2020**, *26*, 5549–5556.
68. Platonova, S.; Cherfils-Vicini, J.; Damotte, D.; Crozet, L.; Vieillard, V.; Validire, P.; André, P.; Dieu-Nosjean, M.-C.; Alifano, M.; Régnard, J.-F.; et al. Profound Coordinated Alterations of Intratumoral NK Cell Phenotype and Function in Lung Carcinoma. *Cancer Res.* **2011**, *71*, 5412–5422. [CrossRef]
69. Ruggeri, L.; Urbani, E.; André, P.; Mancusi, A.; Tosti, A.; Topini, F.; Bléry, M.; Animobono, L.; Romagné, F.; Wagtmann, N.; et al. Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. *Haematologica* **2015**, *101*, 626–633. [CrossRef]
70. Muntasell, A.; Ochoa, M.C.; Cordeiro, L.; Berraondo, P.; de Cerio, A.L.-D.; Cabo, M.; López-Botet, M.; Melero, I. Targeting NK-cell checkpoints for cancer immunotherapy. *Curr. Opin. Immunol.* **2017**, *45*, 73–81. [CrossRef]
71. Kamiya, T.; Seow, S.V.; Wong, D.; Robinson, M.; Campana, D. Blocking expression of inhibitory receptor NKG2A overcomes tumor resistance to NK cells. *J. Clin. Investig.* **2019**, *129*, 2094–2106. [CrossRef]
72. Swetter, S.M.; Tsao, H.; Bichakjian, C.K.; Curiel-Lewandrowski, C.; Elder, D.E.; Gershenwald, J.E.; Guild, V.; Grant-Kels, J.M.; Halpern, A.C.; Johnson, T.M.; et al. Guidelines of care for the management of primary cutaneous melanoma. *J. Am. Acad. Dermatol.* **2019**, *80*, 208–250. [CrossRef]
73. Simon, B.; Uslu, U. CAR-T cell therapy in melanoma: A future success story? *Exp. Dermatol.* **2018**, *27*, 1315–1321. [CrossRef]
74. B7H3 CAR T Cell Immunotherapy for Recurrent/Refractory Solid Tumors in Children and Young Adults. Available online: https://clinicaltrials.gov/ct2/show/NCT04483778 (accessed on 25 January 2021).
75. Tekle, C.; Nygren, M.K.; Chen, Y.-W.; Dybsjord, I.; Nesland, J.M.; Maelandsmo, G.M.; Fodstad, Ø. B7-H3 contributes to the metastatic capacity of melanoma cells by modulation of known metastasis-associated genes. *Int. J. Cancer* **2011**, *130*, 2282–2290. [CrossRef]
76. Dong, P.; Xiong, Y.; Yue, J.; Hanley, S.J.B.; Watari, H. B7H3 as a Promoter of Metastasis and Promising Therapeutic Target. *Front. Oncol.* **2018**, *8*, 264. [CrossRef]
77. Flem-Karlsen, K.; Fodstad, Ø.; Nunes-Xavier, C.E. B7-H3 Immune Checkpoint Protein in Human Cancer. *Curr. Med. Chem.* **2020**, *27*, 4062–4086. [CrossRef]

78. Fridman, W.H.; Pagès, F.; Sautès-Fridman, C.; Galon, J. The immune contexture in human tumours: Impact on clinical outcome. *Nat. Rev. Cancer* **2012**, *12*, 298–306. [CrossRef]
79. Schlößer, H.A.; Theurich, S.; Shimabukuro-Vornhagen, A.; Holtick, U.; Stippel, D.L.; Von Bergwelt-Baildon, M. Overcoming tumor-mediated immunosuppression. *Immunotherapy* **2014**, *6*, 973–988. [CrossRef]
80. Kosti, P.; Maher, J.; Arnold, J.N. Perspectives on Chimeric Antigen Receptor T-Cell Immunotherapy for Solid Tumors. *Front. Immunol.* **2018**, *9*, 1104. [CrossRef]
81. Plaza, J.A.; Bonneau, P.; Prieto, V.; Sangueza, M.; MacKinnon, A.; Suster, D.; Bacchi, C.; Estrozi, B.; Kazakov, D.; Kacerovska, D.; et al. Desmoplastic melanoma: An updated immunohistochemical analysis of 40 cases with a proposal for an additional panel of stains for diagnosis. *J. Cutan. Pathol.* **2016**, *43*, 313–323. [CrossRef]
82. Ivey, J.W.; Bonakdar, M.; Kanitkar, A.; Davalos, R.V.; Verbridge, S.S. Improving cancer therapies by targeting the physical and chemical hallmarks of the tumor microenvironment. *Cancer Lett.* **2016**, *380*, 330–339. [CrossRef] [PubMed]
83. Kato, Y.; Ozawa, S.; Miyamoto, C.; Maehata, Y.; Suzuki, A.; Maeda, T.; Baba, Y. Acidic extracellular microenvironment and cancer. *Cancer Cell Int.* **2013**, *13*, 89. [CrossRef] [PubMed]
84. Renner, K.; Singer, K.; Koehl, G.E.; Geissler, E.K.; Peter, K.; Siska, P.J.; Kreutz, M. Metabolic Hallmarks of Tumor and Immune Cells in the Tumor Microenvironment. *Front. Immunol.* **2017**, *8*, 248. [CrossRef] [PubMed]
85. Almeida, F.V.; Douglass, S.M.; Fane, M.E.; Weeraratna, A.T. Bad company: Microenvironmentally mediated resistance to targeted therapy in melanoma. *Pigment. Cell Melanoma Res.* **2019**, *32*, 237–247. [CrossRef]
86. Jarnicki, A.G.; Lysaght, J.; Todryk, S.; Mills, K.H.G. Suppression of Antitumor Immunity by IL-10 and TGF-β-Producing T Cells Infiltrating the Growing Tumor: Influence of Tumor Environment on the Induction of $CD4^+$ and $CD8^+$ Regulatory T Cells. *J. Immunol.* **2006**, *177*, 896–904. [CrossRef]
87. Viel, S.; Marçais, A.; Guimaraes, F.S.-F.; Loftus, R.; Rabilloud, J.; Grau, M.; Degouve, S.; Djebali, S.; Sanlaville, A.; Charrier, E.; et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci. Signal.* **2016**, *9*, ra19. [CrossRef]
88. Husain, Z.; Huang, Y.; Seth, P.; Sukhatme, V.P. Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells. *J. Immunol.* **2013**, *191*, 1486–1495. [CrossRef]
89. Harmon, C.; Robinson, M.W.; Hand, F.; AlMuaili, D.; Mentor, K.; Houlihan, D.D.; Hoti, E.; Lynch, L.; Geoghegan, J.; O'Farrelly, C. Lactate-Mediated Acidification of Tumor Microenvironment Induces Apoptosis of Liver-Resident NK Cells in Colorectal Liver Metastasis. *Cancer Immunol. Res.* **2018**, *7*, 335–346. [CrossRef]
90. Shiga, K.; Hara, M.; Nagasaki, T.; Sato, T.; Takahashi, H.; Takeyama, H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers* **2015**, *7*, 2443–2458. [CrossRef]
91. Hutchenreuther, J.; Vincent, K.; Norley, C.; Racanelli, M.; Gruber, S.B.; Johnson, T.M.; Fullen, D.R.; Raskin, L.; Perbal, B.; Holdsworth, D.W.; et al. Activation of cancer-associated fibroblasts is required for tumor neovascularization in a murine model of melanoma. *Matrix Biol.* **2018**, *74*, 52–61. [CrossRef]
92. Solocinski, K.; Padget, M.R.; Fabian, K.P.; Wolfson, B.; Cecchi, F.; Hembrough, T.; Benz, S.C.; Rabizadeh, S.; Soon-Shiong, P.; Schlom, J.; et al. Overcoming hypoxia-induced functional suppression of NK cells. *J. Immunother. Cancer* **2019**, *8*, e000246. [CrossRef]