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STRATEGIES TO OPTIMIZE NATURAL KILLER CELL FUNCTIONS IN THE TUMOR MICROENVIRONMENT

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Strategies to optimize natural killer cell functions in the tumor microenvironment

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Popular science summary of the thesis

It is estimated that the risk of being diagnosed with cancer during one's lifetime is around 40% in high-income countries. According to statistics from the World Health Organization (WHO) the number of new cases has risen in the last decades while the number of patients that die because of cancer has decreased. Lifestyle changes, better and earlier diagnostics and improved treatment options are some of the reasons behind this trend. While surgery, chemo- and radiotherapy were the cornerstones of cancer treatment for several decades, immunotherapy has entered clinical practice with great success in the 21st century. The idea behind immunotherapy is to enhance the body's own ability to fight cancer instead of killing the tumor cells directly as it has been done for several years with the classical treatment options. There are many different forms of immunotherapy. One of them is called adoptive cellular immunotherapy (ACT). Here, different immune cells such as T cells or natural killer (NK) cells are grown outside the body in a laboratory and given to the patient in the form of an infusion. The immune cells can be genetically modified and/ or combined with other treatments. The work presented in this thesis focuses on NK cell based ACT to treat multiple myeloma (MM), the second most common blood cancer. Although the median length of survival has increased during the last decades, MM still remains incurable.

NK cells are immune cells that constitute approximately 5-20% of our white blood cells. They express different sensors, so called receptors, on their cell surface which enable them to detect tumor cells or virally infected cells. If this happens, different signals are triggered inside the NK cell that lead to its activation. An activated NK cell can directly kill other cells and release toxic substances to the environment in order to stop cancer growth or the spread of the virus. However, when cancer grows, it constructs an environment that reduces the ability of NK cells to sense or kill tumor cells which results in NK cell dysfunction and tumor growth but also inefficacy of ACT. To prevent this, the work presented in this thesis focuses on improving the sensing and killing ability of NK cells by genetic modification (**study I and II**) or by combination with an oncolytic virus (OV) (**study III**).

NK cells express many receptors on their cell surface that can either lead to activation and killing of the target cell or inhibition to prevent killing of normal cells. Whether activation or inhibition occurs depends on which receptors are stimulated on the NK cell. As mentioned before, cancer often reduces signals to activating receptors. It can, however, also increase signals to inhibitory receptors. One of them is called programmed cell death protein 1 (PD1). The function of NK cells can be diminished if the tumor cells activate PD1 on the NK cell. PD1 usually transmits negative signals and stops the killing ability of NK cells. To circumvent this, we designed several artificial receptors – so-called chimeric switch receptors (CSR) – in which the inhibitory signal of PD1 is replaced by an

activating signal to increase the function of NK cells upon engagement of PD1. We show in **study I** that different PD1-based CSR can be efficiently expressed on the surface of NK cells from healthy donors or patients with MM. The CSR$^+$ NK cells show a higher killing ability and release of toxic substances against different cancer cells compared to unmodified NK cells.

A similar concept is used in **study II** where NK cells are designed to express a so-called chimeric antigen receptor (CAR). This CAR enables NK cells to better sense tumor cells that express the receptor CD38 which is abundantly found on the surface of MM cells. However, NK cells can also express CD38. Thus, CD38-CAR$^+$ NK cells have the potential to kill each other. Our research shows that a specific culture technique leads to high numbers of NK cells with reduced surface expression of CD38. These CD38^{dim} NK cells can be efficiently modified to express a CD38-CAR. CD38^{dim} CD38-CAR$^+$ have a higher sensing and killing ability against MM cells compared to unmodified NK cells.

Lastly, we explore the interaction between an OV that is based on herpes-simplex virus I (HSV-1) and NK cells in **study III**. Oncolytic virotherapy is a new form of immunotherapy. OV are designed to specifically infect tumor cells and cause tumor cell killing as well as an increased immune response. However, the mechanisms on how OV and NK cells interact are not yet fully understood. In this study, we show that an HSV-1 based OV (HSV-1 OV) infects different tumor cells and causes a downregulation of several important ligands for NK cell sensing. Despite this, NK cells increase their ability to release toxic substances, suggesting other mechanisms of activation than through surface receptor modulation. Specifically, we show that the HSV-1 OV HSV1716 can be transferred from the tumor cell to the NK cell and thus increase the activation status of NK cells.

In summary, this thesis explores different strategies to improve the efficacy of adoptive NK cell immunotherapy against cancer, with a special focus on MM. Although the studies presented here are of early nature and more research is needed before clinical implementation can begin, the findings are promising and show a high feasibility for translation into a new therapy.

Populärwissenschaftliche Zusammenfassung

Das Risiko während seines Lebens eine Krebsdiagnose zu erhalten liegt bei ca. 40% in der westlichen Welt. Laut einer Statistik der Weltgesundheitsbehörde (WHO) ist die Anzahl der Krebsneuerkrankungen in den letzten Jahren gestiegen. Gleichzeitig ist aber die Anzahl der Patienten, die an ihrer Krebserkrankungen starben, gesunken. Dies beruht sowohl auf Lebensstilveränderungen, einer präziseren und früheren Diagnostik als auch verbesserten Therapiemöglichkeiten. Während Chirurgie, Chemotherapie und Strahlentherapie für Jahrzehnte die Grundpfeiler der Krebsbehandlung waren, hat Immuntherapie die Krebstherapie im 21. Jahrhundert revolutioniert und ist nun ein fester Bestandteil moderner Behandlungskonzepte. Es gibt verschiedene Formen der Immuntherapie. Eine davon nennt sich adoptive zelluläre Immuntherapie (ACT). Dabei werden Immunzellen wie z.B. T-Zellen oder Natürliche Killer (NK) Zellen außerhalb des Körpers im Labor kultiviert und Patienten in Form einer Infusion injiziert. Die Immunzellen können genetisch modifiziert sein und/ oder mit anderen Therapien kombiniert werden. In dieser Doktorarbeit wird eine NK-Zell-basierte zelluläre Immuntherapie für die Behandlung des Multiplen Myeloms (MM), der zweithäufigsten Blutkrebserkrankung, erforscht. Obwohl sich die Lebenserwartung von Erkrankten in den letzten Jahren deutlich verbessert hat, verbleibt MM unheilbar.

NK Zellen sind Immunzellen, die circa 5-20% unserer weißen Blutzellen ausmachen. Sie exprimieren verschiedene Sensoren, die man Rezeptoren nennt, auf Ihrer Zelloberfläche. Diese Rezeptoren helfen der NK Zelle, Tumorzellen oder virusinfizierte Zellen zu erkennen. Wenn dies klappt, werden Signale in der NK Zelle aktiviert, die zur Aktivierung führen. Eine aktivierte NK Zelle kann direkt andere Zellen töten oder toxische Substanzen in die Umgebung ausschütten, die das Tumorwachstum oder die Vermehrung des Virus stoppen. Wenn der Tumor jedoch wächst, schafft er eine Umgebung, die die Funktion von NK Zellen herabsetzt und zu Tumorwachstum führt sowie die Effektivität der ACT mindert. Diese Arbeit legt dar, wie durch genetische Modifikation (**Studie I und II**) oder der Kombination mit einem onkolytischen Virus (OV) (**Studie III**) die Funktionalität von NK Zellen erhalten bleibt.

NK Zellen exprimieren verschiedene Rezeptoren auf ihrer Zelloberfläche, die entweder zur Aktivierung und Tötung von bösartigen Zellen führen oder inhibierend, also bremsend wirken, um ein Abtöten normaler Zellen zu verhindern. Ob eine Aktivierung oder Inhibierung erfolgt, beruht darauf, welche Rezeptoren stimuliert werden. Wie schon erwähnt kann Krebs zu verminderten Signalen an aktivierende Rezeptoren führen. Es können aber auch vermehrt Signale an inhibierende Rezeptoren gesendet werden. Einen davon nennt man programmed cell death protein 1 (PD1). PD1 vermittelt negative Signale und stoppt NK Zellen davon, Tumorzellen abzutöten. Um dies zu verhindern, haben wir

künstliche Rezeptoren entwickelt – so genannte chimeric switch receptors (CRS) – die die negativen Signale von PD1 in positive Signale umwandeln, um die Funktion von NK Zellen nach Bindung von PD1 zu erhöhen. In **Studie I** zeigen wir, dass PD1-basierte CSR auf der Oberfläche von NK Zellen von gesunden Menschen und Patienten mit MM exprimiert werden können. Die CSR$^+$ NK Zellen haben im Vergleich zu unmodifizierten NK Zellen ein höheres Potential, verschiedene Tumorzellen zu töten und toxische Substanzen auszuschütten.

Eine ähnliche Herangehensweise wird in **Studie II** angewendet, in der NK Zellen hergestellt werden, die einen so genannten chimeric antigen receptor (CAR) auf ihrer Oberfläche exprimieren. Dieser CAR erhöht das Vermögen von NK Zellen, Tumorzellen zu erkennen, die den Rezeptor CD38 auf ihrer Zelloberfläche tragen. Im Vergleich zu gesunden Zellen ist die Expression von CD38 auf MM-Zellen um ein Vielfaches erhöht. Allerdings können auch NK Zellen CD38 auf ihrer Zelloberfläche tragen und somit sich selbst mit einem CD38-CAR töten. Unsere Forschung zeigt, dass eine bestimmte Zellkulturweise die Expression von CD38 auf NK Zellen mindert. Diese CD38^{dim} NK Zellen können erfolgreich einen CD38-CAR exprimieren. CD38^{dim} CD38-CAR$^+$ NK Zellen können im Vergleich zu unmodifizierten NK Zellen,Tumorzellen besser detektieren und aböten.

Schließlich untersuchen wir in **Studie III** das Zusammenspiel zwischen einem auf Herpes-simplex Virus Typ I (HSV-1) basierten onkolytischen Virus (OV) und NK-Zellen. Onkolytische Virustherapie ist eine neue Form der Immuntherapie. OV können Tumorzellen infizieren, um diese zu töten, und das Immunsystem aktivieren. Allerdings sind die Mechanismen wie OV und NK-Zellen miteinander interagieren noch nicht komplett verstanden. In unserer Studie zeigen wir, dass der HSV-1 basierte OV HSV1716 verschiedene Tumorzellen infizieren kann und zu einer Herabsetzung wichtiger Liganden für die Erkennung durch NK Zellen führt. Dennoch werden NK Zellen aktiviert, was durch das Vorliegen anderer Mechanismen erklärt werden muss. In dieser Studie zeigen wir, dass HSV1716 direkt auf NK Zellen übertragen werden kann und Ihren Aktivierungszustand erhöht.

Zusammenfassend werden in dieser Doktorarbeit Strategien entwickelt, die die Effektivität von NK Zell basierter AZT gegen Tumorzellen erhöhen. Ein spezielles Augenmerk wird dabei auf MM gelegt. Die hier präsentierten Studien befinden sich noch im Anfangsstadium und weitere Forschung ist notwendig, um die Ergebnisse im klinischen Alltag anwenden zu können. Die Umsetzung dieser vielversprechenden Ergebnisse ist jedoch zeitnah möglich.

Abstract

Immune cell–based therapies are currently changing the oncological treatment landscape worldwide. While genetically modified T cells have already been approved for clinical use in few hematological cancers, natural killer (NK) cell–based therapies are employed in early phase clinical trials. NK cells are generally regarded as an alternative to T cells as they possess the advantage of intrinsic killing capacity of malignant cells, cause less side effects post transplantation and can be used in an allogeneic setting. In the context of multiple myeloma (MM), the second most common blood cancer, two chimeric antigen receptor (CAR) T cell products are clinically approved and NK cell–based therapies show promising results in early phase clinical studies. However, relapses occur after CAR–T cell therapy and although NK cells show a good safety profile, it is evident that genetic modifications and combination therapies are needed to improve their efficacy. The research provided in this thesis aims to elaborate strategies to improve NK cell functionality in the tumor microenvironment (TME) through genetic modification and combination with an oncolytic virus, with a special focus on MM.

It is well established that immune cell dysfunctionality can occur through chronic stimulation via the programmed death protein 1 (PD1) pathway. Blocking the interaction of PD1 with its ligands PD–L1 and PD–L2 restores immune cell effector functions and is successfully applied in several hematological and solid malignancies. However, in MM, immune checkpoint inhibition has failed due to lack of efficacy in monotherapies and severe side effects in combination therapies. Therefore, **paper I** explores the feasibility of novel NK cell specific chimeric switch receptors (CSR) that re–direct inhibitory signaling into activating signaling in NK cells upon binding of PD1 ligands. The designed CSR are based on the extracellular domain of PD1 that is linked to the transmembrane and/or intracellular domains of DAP10, DAP12 and CD3ζ which are known to provide NK cell activation. In paper I, we show that primary PD1–CSR$^+$ NK cells exert superior cytotoxicity and cytokine release against PD–L1$^+$ cancer cell lines and primary autologous MM samples, establishing them as a promising strategy towards PD–L1$^+$ cancers.

Targeting CD38 with the monoclonal antibody (mAb) daratumumab has improved treatment outcomes for patients with both newly diagnosed and relapsed or refractory MM, but is also met with resistance to therapy. Moreover, the majority of peripheral blood NK cells express CD38 and are depleted during daratumumab treatment. In **paper II,** we have introduced an affinity–optimized CD38–CAR construct into CD38^{dim} primary NK cells that naturally occur during a long–term cytokine–based feeder cell–free expansion protocol. This approach leads to a functional NK cell product with superior cytotoxicity against CD38$^+$ MM cell lines and autologous MM samples with minimal risk for fratricide. The data provided here pave the way for advancing this approach as an alternative treatment option for MM .

Oncolytic viruses (OV) present an attractive immunotherapeutic platform for combination therapies and are currently tested in pre-clinical and early phase clinical trials for several malignancies. Herpes simplex virus 1 (HSV-1) based OV belong to the most utilized viruses and were shown to effectively infect and lyse malignant MM cells. However, their clinical applicability is met with several challenges, one being a rapid clearance by the immune system. NK cells naturally recognize virally infected cells and have been implicated in both promoting and hampering OV therapy. However, the exact molecular mechanism remain elusive. In **paper III** we have studied the interaction of primary NK cells with target cells that have been infected with the HSV-1 based OV HSV1716. The data provided in paper III show that primary NK cells increase their degranulation ability against HSV1716-infected target cells, despite the downregulation of important ligands for activating NK cell receptors. Primary NK cells become infected with HSV1716 through direct contact with target cells and increase their activation status. A better understanding of the molecular mechanisms behind NK cell recognition of HSV-1 OV infected target cells might improve immunotherapeutic combination approaches.

All in all, the constituting studies provide unique and novel data on how to improve NK cell-based immunotherapies for cancer. Although the main focus is laid on MM, the presented approaches can be expanded to other malignancies in order to improve NK cell therapies and increase NK cell fitness in an immunosuppressive TME.

List of scientific papers

I. **Susek KH,** Schwietzer YA, Karvouni M, Gilljam M, Keszei M, Hussain A, Lund J, Kashif M, Lundqvist A, Ljunggren HG, Nahi H, Wagner AK, Alici E.
    Generation of NK cells with chimeric switch receptors to overcome PD1-mediated inhibition in cancer immunotherapy.
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II. Karvouni M, Vidal-Manrique M, **Susek KH,** Hussain A, Gilljam M, Zhang Y, Gray JD, Lund J, Kaufmann G, Ljunggren HG, Ji H, Lundqvist A, Wagner AK, Guo W, Alici E.
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III. **Susek KH**, Albert J, Flore V, Gilljam M, Karvouni M, Meinke S, Wagner AK, Nahi H, Bresson D, Conner J, Powers C, Allen R, Alici E.
    The oncolytic virus HSV1716 infects and activates natural killer cells via direct cell-to-cell contact.
    Manuscript

Relevant scientific papers not included in this thesis

I.   Schwietzer YA*, **Susek KH** *, Chen Z, Alici E, Wagner AK
      A tractable microscopy- and flow cytometry-based method to measure natural killer cell-mediated killing and infiltration of tumor spheroids.
      Methods in Cell Biology, Academic Press, 2022

II.   Afram G, **Susek KH,** Uttervall K, Wersäll JD, Wagner AK, Luong V, Lund J, Gahrton G, Alici E, Nahi H.
      Improved survival in multiple Myeloma patients undergoing autologous stem cell transplantation is entirely in the standard cytogenetic risk groups.
      European journal of haematology 2021 106;4 546-554

III. **Susek KH** *, Gran C *, Ljunggren HG, Alici E, Nahi H.
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      European journal of haematology 2020 105;6 751-754

IV.   **Susek KH**, Karvouni M, Alici E, Lundqvist A.
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      Frontiers in immunology 2018 9; 2159-

* denotes equal contributions

Contents

List of abbreviations

aa	amino acid
ABB	antibody bipolar bridging
ACT	adoptive cellular immunotherapy
ADC	antibody-drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ADO	adenosine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	antigen presenting cell
Allo-SCT	allogeneic stem cell transplantation
Auto-SCT	autologous stem cell transplantation
BCMA	B-cell maturation antigen
BM	bone marrow
BCR	B cell receptor
cADPR	cyclic adenosine diphosphate-ribose
CAR	chimeric antigen receptor
CB	cord blood
CB-NK	cord-blood derived NK cells
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
cGAS-STING	cyclic GMP-AMP synthase stimulator of interferon genes
CIS	cytokine-inducible SH2-containing protein
CMV	cytomegalovirus
CNS	central nervous system
CR	complete remission
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
CSR	chimeric switch receptor
CTLA-4	cytotoxic T-lymphocyte–associated antigen 4

CXCL	C–X–C chemokine ligand
CXCR	C–X–C chemokine receptor
DAP	DNAX–activating protein
DC	dendritic cell
DLBCL	diffuse large B cell lymphoma
DNAM–1	DNAX Accessory Molecule–1
dsDNA	double-stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EC	extracellular
eIF–2α	eukaryotic initiation factor 2α
FACS	fluorescence-activated cell sorting
FAS ligand/ FasL	tumor necrosis factor ligand superfamily member 6
FDA	U.S. Food and Drug Administration
FHL	familial hemophagocytic lymphohistiocytosis
GvHD	graft-versus-host disease
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
HLA–I	human leukocyte antigen class 1
HPC	hematopoietic progenitor cell
HSCT	hematopoietic stem cell transplantation
HSV–1	herpes simplex virus type I
HSV–1 OV	herpes simplex virus type I oncolytic virus
HSV1716	herpes simplex virus 1716
HVEM	herpesvirus entry mediator
IC	intracellular
ICANS	immune effector cell-associated neurotoxicity syndrome
ICI	immune checkpoint inhibition
ICOS	inducible T-cell co-stimulator
ICR	Immune checkpoint receptor
IE	immediate early
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMiDs	immunomodulatory drugs

iPSC	induced pluripotent stem cell
iPSC-NK	induced pluripotent stem cell derived NK cells
IRF	interferon regulatory factor
IMiDs	immunomodulatory drugs
ICP	infected cell protein
ISG	IFN-stimulated genes
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
Kb	kilobases
KIR	killer immunoglobulin-like receptor
KO	knock-out
LAG3	lymphocyte activation gene-3
LAMP-1	lysosomal-associated membrane protein-1
LFA-1	lymphocyte function-associated antigen 1
mAb	monoclonal antibody
MDSC	myeloid-derived suppressor cell
MFI	mean fluorescence intensity
MHC-I	major histocompatibility complex class 1
MICA/B	MHC class I polypeptide-related sequence A and B
MNC	mononuclear cell
MM	multiple myeloma
MMAF	monomethyl auristatin-F
MRD	minimal residual disease
MTOC	microtubule-organizing center
NA	nicotinic acid
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD	nicotinamide dinucleotide
NADP	NAD phosphatase
NCR	natural cytotoxicity triggering receptor
NDMM	newly diagnosed MM
NK cell	natural killer cell
NKT cell	natural killer T cell
NKG2C	natural killer group 2 member C

NKG2D	natural killer group 2 member D
OS	overall survival
ORR	overall response rate
OV	oncolytic virus
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PB-NK	peripheral-blood derived NK cells
PD1	programmed cell death protein 1
PD-L1	programmed cell death protein 1 ligand 1
PD-L2	programmed cell death protein 1 ligand 2
PFS	progression-free survival
PI	proteasome inhibitor
pNK	primary NK cells
PNS	peripheral nervous system
PRR	pattern recognition receptors
PVR	poliovirus receptor
PVRL	poliovirus receptor ligand
ROS	reactive-oxygen species
RPMI	Roswell Park Memorial Institute
RRMM	relapsed/ refractory multiple myeloma
scFv	single-chain variable fragment
SHIP	SH2 domain-containing inositol-5-phosphatase
SHP-1	Src homology 2-domain-containing tyrosine phosphatase 1
SHP-2	Src homology 2-domain-containing tyrosine phosphatase 2
SLAMF7	signaling lymphocyte activation molecule F7
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF	transforming growth factor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM3	T cell immunoglobulin and mucin domain 3
TM	transmembrane
TME	tumor microenvironment
TNF	tumor necrosis factor
TLR	Toll-like receptor

TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
T-VEC	talimogene laherparepvec
UCB	umbilical cord blood
ULBP	UL16-binding proteins
VP	viral protein
VZV	varizella zoster virus
WHO	World Health Organization

1   Introduction

1.1   Cancer immunotherapy

Cancer is one of the leading causes of death worldwide according to data from the World Health Organization (WHO). While the incidence of cancer has gradually risen in the last decades, mortality rates generally decreased in Sweden and other high-income countries due to improvements in diagnostic and therapeutic options. During the 20th century, cancer treatment was mainly based on surgery, chemo- and radiotherapy. The introduction of cancer immunotherapy in the 21st century has drastically changed the therapeutic landscape and is experiencing an unprecedented growth, especially since the 2010's. Today, cancer immunotherapy comprises different approaches including antibody-based therapies, cancer vaccines, cytokine therapies, oncolytic viruses and cell-based therapies.

1.2   Multiple myeloma

Multiple myeloma (MM) is a hematological malignancy that is classified by the clonal proliferation of terminally differentiated B cells, also known as plasma cells. Due to the uncontrolled growth of malignant plasma cells within the bone marrow (BM) during disease progression, patients regularly present with anemia, immune system dysregulations, hypercalcemia, kidney injuries and destructive bone lesions. In Sweden, the annual incidence is approximately 7 per 100.000 inhabitants, making MM the second most common hematological malignancy [1]. The median overall survival (OS) is highly dependent on age at diagnosis and ranges from 7.8 years in patients 60 years and younger to 1.5 years in patients older than 80 years [1]. However, OS has significantly improved in recent years due to the availability of new treatment options (**Figure 1**).

Historically, melphalan was the first chemotherapeutic drug that showed a consistent therapeutic success in the treatment of MM, and the addition of prednisone even enhanced its effect [2, 3]. High-dose melphalan therapy resulted in the best anti-myeloma effect, but due to a prolonged myelosuppression it needed to be combined with stem cell transplantation [4-6]. Although allogeneic stem cell transplantation (allo-SCT) has shown long-term remissions and potential cure in some MM patients, it is not an available treatment option for the majority of patients to date due to severe side effects [7]. In direct comparison, median survival was better after autologous stem cell transplantation (auto-SCT) compared to allo-SCT, given a high procedure-related mortality in the latter [8, 9]. Although auto-SCT is often not curative, it remains the standard of care for eligible patients. Research efforts have therefore focused on improving induction and maintenance regimes before, and respectively after auto-SCT, to reduce the risk of minimal residual disease (MRD) and relapses. These regimes were early on based on drugs that modify and target the immune system. The treatment

landscape quickly evolved from the use of immunomodulatory drugs (IMIDs) and proteasome inhibitors (PIs) to monoclonal antibodies (mAb), bispecific antibodies and cell-based therapies. To date, immunotherapy is an integral part of MM treatment both as first-line therapy and in recurrent disease, either in combination with auto-SCT or alone.
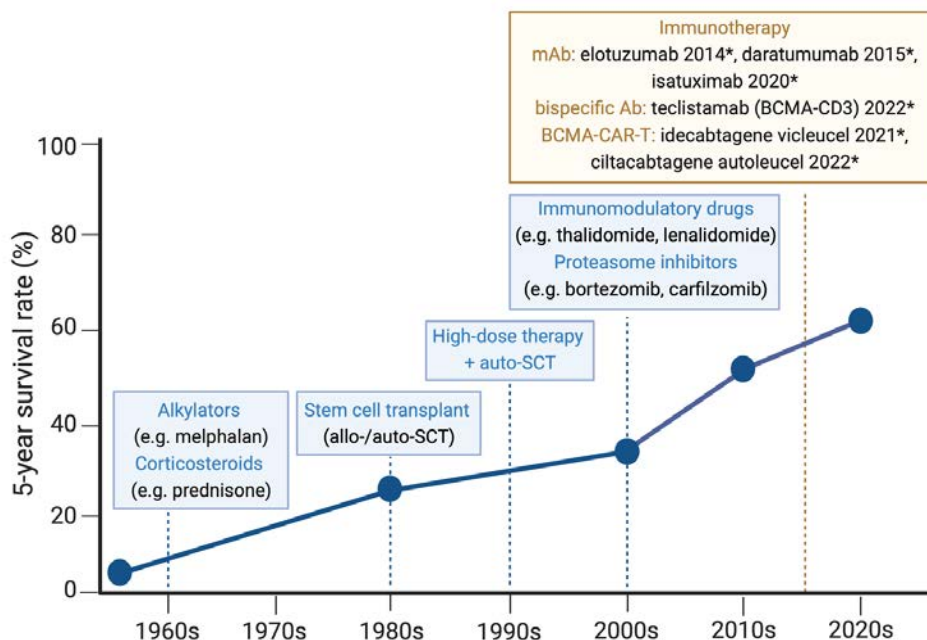

FIGURE 1 Milestones in MM treatment. The 5-year-survival rate has increased for patients with MM over the last decades, partially due to improvements in therapeutic options. To date, immunotherapy is an integral part of MM treatment regimes. * denotes year of FDA-approval. Image created with Biorender by the author.

Antibody-based therapies targeting the type II transmembrane (TM) glycoprotein CD38 belong to the most successfully applied immunotherapies in the context of MM [10]. Under physiologic conditions, CD38 is expressed at low levels on the surface of immune cells from both myeloid and lymphoid lineage such as T cells, NK cells, monocytes, granulocytes and dendritic cells as well as other non-hematopoietic cells [11]. CD38 serves as an adhesion molecule and receptor that can bind to CD31 in *trans* and to the T cell receptor (TCR), B cell receptor (BCR) or CD16 in *cis* and thus favors adhesion, migration, activation and proliferation [12, 13]. Moreover, CD38 has enzymatic activity and functions as a nicotinamide adenine dinucleotide (NAD+) glycohydrolase, leading to calcium mobilization and regulation [14]. In MM, CD38 is highly overexpressed on malignant plasma cells and can be targeted with the mAb daratumumab and isatuximab [15]. Daratumumb

is a fully humanized immunoglobulin G1κ (IgG1κ) mAb and was approved by the U.S. Food and Drug Administration (FDA) in 2015. It induces MM killing by several mechanisms, including direct cytotoxicity, antibody–dependent cellular cytotoxicity (ADCC), antibody–dependent cellular phagocytosis (ADCP) and complement–dependent cytotoxicity (CDC) [16, 17]. Several clinical trials have shown the efficacy of daratumumab monotherapy and combination therapy with IMiDs and PIs for both relapsed refractory MM (RRMM) and newly–diagnosed MM (NDMM) [18, 19]. Isatuximab, on the other hand, is a chimeric (human/murine) IgG1κ mAb that was approved by the FDA in 2020 for patients with RRMM in combination with pomalidomide and dexamethasone [20]. Isatuximab's predominant mode of action is the induction of ADCC [21].

Another mAb approved for the use in MM is the humanized IgG1κ mAb elotuzumab that targets the cell–surface glycoprotein signaling lymphocyte activation molecule F7 (SLAMF7). It was FDA–approved in 2014 and is currently approved for patients with RRMM in combination with lenalidomide and dexamethasone [22].

B–cell maturation antigen (BCMA) is a successfully targeted surface receptor in MM. Due to its nearly exclusive expression on plasma cells, several therapeutic approaches such as mAb, bispecific antibodies, trispecific antibodies, antibody–drug conjugates (ADC) and cell–based therapies have been tested and some even approved for clinical application [23]. One of the first FDA–approved BCMA-targeted therapies was belantamab-mafodotin (Blenrep), an ADC consisting of a humanized IgG1κ mAb and monomethyl auristatin–F (MMAF). After receiving accelerated approval in 2020 as a monotherapy in patients with RRMM, it was withdrawn by the FDA in November 2022 due to results from the phase III DREAMM–3 trial showing that Blenrep did not meet the criteria for progression–free survival (PFS) in comparison with pomalidomide and dexamethasone [24]. Currently, two BCMA-targeting chimeric antigen receptor (CAR) T cell therapies, namely idecabtagene vicleucel (Abecma) and ciltacabtagene autoleucel (Carvykti), as well as the BCMA–CD3 bispecific antibody teclistamab (Tecvayli) are FDA–approved for the treatment of RRMM patients [25-27].

Given the general success of immune checkpoint inhibition (ICI) in cancer with mAb that target the programmed death receptor 1 (PD1) pathway, this approach was also tested for the treatment of MM. Importantly, expression of the PD1 ligand PD-L1 was induced on malignant plasma cells as well as on other cells in the BM microenvironment of myeloma and, additionally, PD1 surface expression increased on T cells and NK cells [28-30]. Indeed, the expression of PD1 and its ligands correlated with disease stage with higher values observed in patients with MRD or at relapse; confirming the relevance of PD1 targeting approaches [30]. However, two large phase III clinical trials that investigated the combination of PD1/PD-L1 mAb, IMiDs and corticosteroids had to be halted due to severe side effects and increased mortality rates in the experimental arm [31, 32]. At the same time, monotherapy with mAb did not yield good clinical efficacy [33]. Therefore, PD1/ PD-

L1 checkpoint inhibition is currently not an approved treatment option for MM and efforts are ongoing to retarget the PD1 axis in MM [34].

The recent advances in immunotherapy for MM have gradually increased 5-year survival rates in the general MM population from approximately 30% in the late 20th century to currently 50-60%, making MM a chronic disease for some subgroups and even raising hope for a cure [35]. Research efforts are focused on improving combination therapies and selection of patients as well as discovering new molecular targets and therapeutic approaches. This thesis addresses and explores the possibility of NK cell-based therapies for MM. Specifically, genetically modified NK cells that express a chimeric switch receptor (CSR) targeting programmed death protein 1 (PD1) (paper I), a CD38-targeting chimeric antigen receptor (CAR) (paper II) and a combination with a herpes-simplex virus type I based oncolytic virus (HSV-1 OV) (paper III) are explored.

1.3 Molecular targets for the immunosuppressive tumor microenvironment (TME)

As mentioned above, immunotherapy can be successfully applied in MM and other malignancies. In order to prolong treatment responses and reduce the risk of relapse, however, multimodal treatment approaches need to be designed that target both tumor cells and the cancer-promoting microenvironment. Currently, several approaches and new molecular targets are under investigation for the treatment of MM with a plethora of exciting pre-clinical and clinical findings. This thesis focuses on the receptors PD1 and CD38 which are further described below.

1.3.1 PD1

PD1 is a type I transmembrane glycoprotein that was first described in 1992 as an apoptosis promoting receptor [36]. However, later studies found PD1 primarily expressed on activated T and B cells and suggested its involvement in regulating cell activation rather than inducing programmed cell death [37]. This was confirmed by murine knock-out (KO) studies which showed that loss of PD1 leads to an autoreactive T cell phenotype and the development of auto-immune disease [38]. To date, it is established that PD1 is upregulated on activated T cells and counterbalances T cell activation following T cell receptor (TCR) engagement in order to maintain self-tolerance and reduce auto-reactivity. Although the function of PD1 is best described in T cells, its expression was also found on other immune cells such as NK cells, natural killer T cells (NKT) as well as some myeloid and antigen-presenting cells (APC). The role of PD1 on these cell types is currently under investigation.

PD1 and its ligands belong to the CD28 family of proteins. Structurally PD1 is composed of an IgV-type extracellular domain that shares 21-33% sequence homology to cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), CD28 and inducible T-cell co-stimulator

(ICOS). Compared to other members of the CD28 family, however, PD1 is a monomer [39]. The intracellular (IC) domain contains a membrane proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a distant immunoreceptor tyrosine-based switch motif (ITSM). Mutational studies showed that phosphorylation of the ITSM is a key mediator of T cell inhibition while the ITIM only has a minor influence on the ability of PD1 ligation to inhibit T cell activation [40, 41]. In primary T cells, PD1 recruits both Src homology 2-domain-containing tyrosine phosphatase 1 (SHP-1) and 2 (SHP-2) [40, 42]. However, several studies showed that recruitment of SHP-2 to the ITSM plays a dominant role in inhibition of key signaling transducers downstream of both the TCR and CD28 pathways in T cells and the BCR pathway in B cells [40-44]. Mechanistically, phosphorylation of the ITSM is essential for the recruitment and binding of SHP-2 while phosphorylation of the ITIM leads to the conformational change and release of its auto-inhibited state and thus SHP-2 activation [45]. However, SHP-2 recruitment and activation can also occur through binding of the ITSM domains from two different PD1 molecules, resulting in dimerization [46]. Ultimately, PD1 signaling in T cells leads to reduced effector cytokine production, cell expansion and diminished cytotoxicity as well as metabolic alterations [47-50].

The ligands of PD1 are PD-L1 and PD-L2 [51, 52]. PD-L1 is ubiquitously expressed on non-hematopoietic cells such as vascular endothelial cells, heart, lung, pancreas, skeletal muscle and liver cells as well as APC and many different cancer cells. It can bind to both PD1 and the immune co-signaling molecule CD80 in *cis* or *trans* to prevent T cell co-stimulation and activation [53-56]. Structurally, PD-L1 is a type I transmembrane protein with similarities to the Ig light chain with at least five different isoforms described [57]. Although generally regarded as a surface protein, PD-L1 can also be found in intracellular compartments such as the cytosol and nucleus [57, 58]. Moreover, in certain cancer types an extracellular (EC) soluble PD-L1 has been described [59]. Although PD-L1 lacks a conventional signaling domain it can promote cancer cell-intrinsic signaling, leading to altered cancer cell metabolism, proliferation and survival, chemo-resistance and metastatic spread [57]. The expression of PD-L2, on the other hand, is more restricted and generally found on immune cells such as dendritic cells, macrophages and B cells as well as some cancer cells from esophageal, lung and kidney origin. Although structurally very similar to PD-L1, PD-L2 shows a higher binding affinity to PD1 and is involved in regulation of T cell activation [60]. It's function during tumorigenesis is less understood and currently under investigation.

The study of PD1 and other immune checkpoint receptors (ICR) in regulating T cell responses has led to major advancements in cancer therapy as ligands for ICR are often upregulated in the TME. Chronic signaling via the PD-1 axis leads to a less reactive, exhausted T-cell phenotype and consequently immune escape and tumor growth [61]. Inhibiting this interaction with mAb can restore T cell functionality [62]. To date, mAb that target PD1 and PD-L1 are an integral part of the treatment options for several cancer types,

with good clinical responses observed. However, resistances and treatment failures occur with some patients showing no responses at all (innate resistance) and some patients developing progressive disease under therapy (acquired resistance). Several mechanisms have been described for both innate and acquired resistance to PD1 therapy that can – among other things – affect the generation, activation and memory formation of tumor-reactive T cells. For instance, CD38 has been implied in sustaining T cell dysfunctionality following immune checkpoint therapy [63].

1.3.2 CD38

CD38 is a multifunctional protein that can act both as adhesion molecule, ectoenzyme and co-receptor [11]. It is expressed by several hematopoietic and non-hematopoietic cell types and is over-expressed by malignant cells and other cells within the immunosuppressive TME. Therefore, CD38 is depicted as a two-sided protein with an important role in immune cell proliferation, signaling and effector cell functionality and – on the other hand – also involved in tumor progression and other pathophysiological conditions such as aging, neurodegeneration and obesity [11].

The diverse functions of CD38 are attributed to its topological conformation, dimerization and localization within the cell and are context-dependent. Often, CD38 expression is found at the cell surface but it can be localized in intracellular compartments such as the endoplasmic reticulum, nuclear membrane and mitochondria [64, 65]. Structurally, CD38 is a 300 amino acid (aa) long type II transmembrane glycoprotein with a long extracellular domain and a short cytoplasmic tail that forms homodimers. However, CD38 can also be present as a type III transmembrane protein where the extracellular domain faces the cytosol [66]. CD38 is primarily regarded as an ectoenzyme since the extracellular domain has catalytic function and synthetizes two structurally distinct calcium messengers, namely cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) from the substrates nicotinamide adenine dinucleotide (NAD) and NAD phosphatase (NADP) [14, 67]. In immune cells, this enzymatic activity leads to the activation of calcium-permeable membrane channels as well as calcium mobilization from intracellular stores and cell activation [68, 69]. Moreover, as a type II glycoprotein CD38 can bind to its ligand CD31 that is expressed on endothelial cells [12, 70]. The interaction between CD38 and CD31 promotes immune cell migration and homing but also provides growth signals to promote cell proliferation, survival and differentiation [70, 71]. Lastly, CD38 is found at immune synapses in close association to the TCR, BCR and CD16 in T cells, B cells and NK cells, respectively, where it is regarded as a co-receptor, promoting cytotoxicity and effector cell functions [72-75].

Although CD38 was initially identified as a lymphocyte activation marker, it is currently being investigated based for its role as a pro-tumorigenic protein and immune checkpoint molecule that supports the immunosuppressive TME in hematological and solid

malignancies [76, 77]. For instance, the expression of CD38 on malignant plasma cells and its interplay with CD39 and CD73 in the BM niche leads to an accumulation of the immunosuppressive metabolite adenosine (ADO) and is correlated with MM progression [78]. ADO has been shown to impair the effector functions and metabolic fitness of tumor-infiltrating T cells in several studies [79, 80]. Similarly, ADO signaling through the A2A adenosine receptor leads to dysfunctional NK cells with reduced cytokine production ability and cytotoxicity and thus diminished anti-cancer activity [81-83]. Moreover, CD38 upregulation was observed in patients with melanoma and non-small cell lung cancer cells after exposure to PD1/ PD-L1 blockade and was suggested as a resistance mechanism to immune checkpoint blockade where a sequential T cell suppression occurs through increased ADO expression in the TME [63, 84].

All in all, targeting the immunosuppressive microenvironment seems to be a promising therapeutic approach. However, it is evident that different pathways and strategies need to be employed synergistically in order to achieve long-lasting clinical responses. This thesis explores the possibility of targeting PD1 and CD38 with genetically modified NK cells as well as NK cells in combination with oncolytic virotherapy to overcome resistances in the TME.

1.4   Natural killer cells

NK cells are innate lymphoid cells that can sense and rapidly kill virally infected or malignantly transformed cells without prior antigen exposure. Their activation depends on the balance of several germline-encoded inhibitory and activating receptors (**Figure 2**). Phenotypically, NK cells are characterised by the expression of the neural cell adhesion molecule (NCAM, also known as CD56) and the absence of surface TCR [85]. NK cells are a heterogenous cell population that can be found in the BM, peripheral blood (PB) and some organs such as the liver [86]. Classically, they are divided into CD56^{bright}CD16^{dim} cells that mainly produce chemokines and cytokines and CD56^{dim}CD16^{bright} subsets that are considered primarily cytotoxic [87].
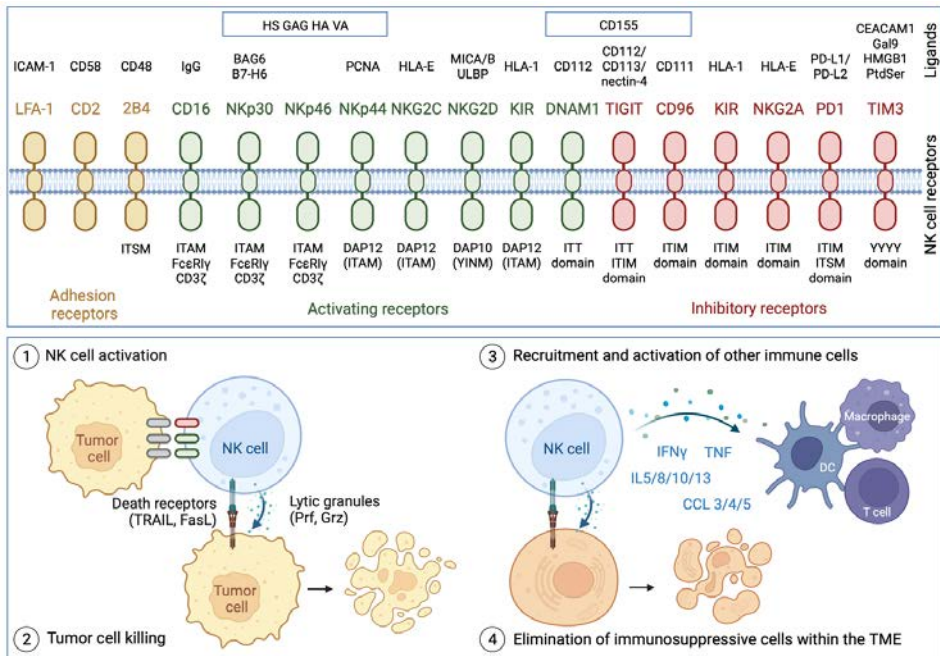
FIGURE 2 Aspects of NK cell biology. The upper panel visualizes the main activating and inhibitory NK cell receptors, their signaling domains and cognate ligands. The lower panel summarizes important NK cell effector mechanisms. Abbreviations: CCL: CC motif chemokine ligand; CD: cluster of differentiation; CEACAM1: Carcinoembryonic antigen-related cell adhesion molecule 1; DAP: DNAX-activating protein 10; DC: dendritic cells; FasL: Fas ligand; FGL1: Fibrinogen-like protein 1; GAG: glycosamino-glycans; Gal9: Galectin 9; Grz: granzyme; HA: hemagglutinin; HLA: Human leukocyte antigen; HMGB1: High mobility group box 1 protein; HS: heparane sulfate; IgG: immunoglobulin G; IFN: interferon; IL: interleukin; ITAM: Immunoreceptor tyrosine-based activation motif; ITIM: Immunoreceptor tyrosine-based inhibitory motif; ITT: immunoglobulin tail tyrosin; ITSM: Immunoreceptor tyrosine-based switch motif; MICA/B: MHC class I chain-related protein A and B; MHC: Major histocompatibility complex; PCNA: Proliferating cell nuclear antigen; PD-L1/L2: programmed death ligand 1/ ligand 2; Prf: perforin; PtdSer: phosphatidylserine; TME: tumor microenvironment; TNF: tumor necrosis factor; TRAIL: Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand; ULBP: UL16 binding protein; VA: viral antigens. Image created with Biorender by the author.

1.4.1 Activating NK cell receptors

NK cells express several activating receptors on their cell surface that can together recognize a plethora of different ligands. Among the best characterized activating NK cell receptors are the NCR NKp30, NKp44 and NKp46 as well as killer immunoglobulin like receptors (KIR) such as KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1 [88, 89]. Generally, KIR receptors with a short intracellular tail act as activating receptors while KIR receptors with a long intracellular domain exert an inhibitory function [90]. Other main activating receptors are CD226 (DNAX Accessory Molecule-1, DNAM-1), natural killer group 2 member C and D (NKG2C and NKG2D) and CD16 (FcγRIIIA) [91–94]. Ligands for activating NK cell receptors are often overexpressed in cells affected by cellular stress,

infection or oncogenic mutations. Importantly, most activating NK cell receptors recognize several different ligands and although many of them have already been identified, new receptor-ligand interactions are regularly described. NCR, for instance, recognize a vast array of host- and pathogen- associated ligands such as hemagglutinin of influenza virus [88]. However, not all NCR binding partners have yet been discovered and soluble ligands might exist. Cancer cells often upregulate ligands for NKG2D and DNAM-1, which include MHC class I polypeptide-related sequence A and B (MICA/B), UL16-binding proteins (ULBP) for NKG2D and CD112 (also known as Nectin-2) as well as CD155 (also known as PVR, poliovirus receptor) for DNAM-1 [95, 96]. One of the strongest activating receptors on NK cells is CD16 which binds the constant region (Fc) of immunoglobulins and triggers ADCC. In this way, target cells that are opsonized by antibodies can be effectively eliminated [97].

Moreover, adhesion molecules play an important role in the cytotoxic process by enabling adhesion and conjugate formation with target cells, initiation of the immunological synapse and polarization for granule release. Integrins, the CD2 family of the immunoglobulin superfamily of molecules and the related signaling lymphocyte activation molecule (SLAM) family of immune cell receptors are examples of receptors that are involved in these processes. Especially, the integrin lymphocyte function-associated antigen 1 (LFA-1, also known as CD11a/CD18) as well as CD2 and the SLAM receptor 2B4 (SLAMF4, CD244) play key roles in adhesion, synapse formation and initiation of cytotoxicity in NK cells [98-105]. A more detailed description of NK cell effector mechanisms is given further below.

1.4.2   Inhibitory NK cell receptors

The engagement of inhibitory receptors on NK cells generally leads to a shut-down of signaling pathways downstream of activating receptors and thus mediates NK cell inhibition in order to prevent self-recognition and auto-immunity. A large group of inhibitory receptors on NK cells belong to the KIR family. Both activating and inhibitory receptors engage with major histocompatibility complex class 1 (MHC-I) molecules [106]. Ligation of self-MHC-I with inhibitory KIR suppresses NK cell activation while alterations or loss of self-MHC-I molecules, which often occur in tumor cells, release the "break" and trigger NK cell cytotoxicity [107]. This is also referred to as *missing-self* recognition [107, 108]. Another important inhibitory receptor on NK cells is the heterodimer NKG2A/CD94 that binds human leukocyte antigen E (HLA-E). HLA-E is a non-classical MHC-I molecule and compared to other classical HLA-I molecules expressed at low levels on healthy cells and upregulated in cancer [109]. Since NKG2A is expressed on the majority of NK cells and its engagement leads to NK cell inhibition it is also regarded as an NK cell specific ICR [100, 110, 111]. Indeed, blocking NKG2A with the mAb monalizumab was shown to increase the anti-tumor activity of NK cells and T cells [112-114]. Currently, several clinical trials are testing monalizumab in combination with other immune checkpoint inhibitors such as PD1

axis blockade against different cancer types (NCT02671435) [115]. Finally, CD161 (also known as KLRB1; Killer cell lectin-like receptor subfamily B, member 1) that binds lectin-like transcript 1 (LLT1) and killer cell lectin like receptor G1 (KLRG1) that binds to cadherins are other inhibitory NK cell receptors. Their role in the TME and potential for targeted therapy approaches is currently being evaluated [110, 116–120].

1.4.3 Immune checkpoint receptors and molecules in NK cells

ICR are inhibitory receptors which recognize ligands expressed on healthy cells and thus prevent auto-immunity and a prolonged inflammatory response. ICR are therefore often upregulated upon activation. This system is hijacked by tumor cells which induce an inflammatory milieu, leading to chronic stimulation and finally immune cell exhaustion. The success of immune checkpoint blockade has generally sparked interest in inhibitory receptors that can be targeted in order to restore immune cell activation within the TME. Although initially studied in T cells, the role of ICR on other immune cells such as NK cells is increasingly recognized. To date, clinically relevant ICR are NKG2A, PD1, T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) and lymphocyte activation gene 3 (LAG3). Therapies targeting these axes are currently in clinical trials or already approved for cancer therapy [121, 122].

The ICR PD1, TIGIT and TIM3 were shown to be expressed on NK cells from cancer patients, resulting in NK cell dysfunctionality that can be restored by immune checkpoint blockade [28, 123–126]. However, the role of PD1 in regulating NK cell functionality under healthy conditions is not fully elucidated and thus PD1 is not recognized as a canonical ICR in NK cells. For instance, it was shown that different splicing variants of PD1 mRNA and a pool of cytoplasmic PD1 protein are expressed in several NK cell subsets, especially CD56^{dim} NK cells [127]. The signals that govern its surface expression, however, are still not known and most NK cells from healthy individuals do not express PD1 on the cell surface at high levels [127]. Indeed, PD1 expression is primarily found on NK cells from patients with cancer, especially on tumor-infiltrating NK cells [128]. Another exception appears to be individuals who are serologically positive for cytomegalovirus (CMV) infection. PD1 expression is increased in a subset of NK cells phenotypically classified as CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ as well as CD56^{dim}CD57$^+$NKG2C$^+$ that corresponds to an adaptive NK cell subset [129, 130]. Similar to CD8$^+$ T cells, adaptive NK cells are memory-like cells that display faster and stronger effector functions upon re-exposure and are implied in better control of acute lymphoblastic leukemia (ALL) cells [131]. Both PD1 and LAG3 were reported to be upregulated on adaptive NK cells upon chronic stimulation, leading to reduced NK cell effector functions against tumor targets [130, 132]. Likewise, the expression of PD1 on some tumor-infiltrating NK cells is associated with reduced effector cell functions which can be restored by blocking the PD1 pathway [133, 134]. Importantly, NK cells contribute to the overall success of anti-PD1/PD-L1 therapy by exerting direct anti-tumor cytotoxicity

and/or by re-shaping the TME [135-137]. Compared to PD1, TIGIT expression was found on NK cells from healthy donors and inversely correlated with their effector cell functionality [138]. TIGIT+ NK cells displayed less cytokine production and degranulation compared to TIGIT- NK cells. Importantly, TIGIT binds the same ligands as the activating receptor DNAM-1, namely CD112 (nectin-2 / PVRL2, poliovirus receptor ligand 2) and CD155 (PVR, poliovirus receptor), and has been implicated in preventing NK cells from killing of normal cells [139, 140]. TIGIT has a stronger affinity to CD155 and recognizes additionally two other ligands, namely nectin-4 (PVRL4, poliovirus receptor ligand 4) and nectin-3 (CD113/ PVRL3, poliovirus receptor ligand 3) [141, 142]. Moreover, CD96 has been described as an inhibitory receptor within the DNAM-1/TIGIT/PVR axis which competes with TIGIT and DNAM-1 for CD155 binding [143]. Currently, efforts are ongoing to target this immune checkpoint axis to alleviate T cell and NK cell functions for improved cancer immunotherapy [122, 144]. Although the inhibitory role of TIGIT and TIM-3 on NK cells from cancer patients has been described in numerous studies, the contribution of NK cells to the overall efficacy of blocking approaches has to be determined in clinical trials.

Besides receptor-ligand based immune checkpoints, metabolic checkpoint molecules have been described for NK cells. One of them is the cytokine-inducible SH2-containing protein (CIS) which modulates IL-15 mediated signaling in NK cells [145]. IL-15 is an important cytokine for NK cell activation, proliferation and survival [146]. Upon IL-15 receptor ligation, CIS becomes upregulated and dampens IL15 mediated signaling. Thus, CIS deletion results in improved proliferation, cytokine production and cytotoxicity [145]. Similarly interleukin-1 receptor 8 (IL-1R8) has been described to control NK cell anti-tumor and anti-viral functions [147]. IL-1R8 mediated signaling inhibits IL18 induced NK cell activation [147]. Recently it was shown that IL-1R8 silencing in NK cells improves NK cell cytokine release and cytotoxicity against several different cancer cells *in vitro* [148]. Other molecular checkpoints that negatively regulate NK cell function and are thus currently under investigation for targeting approaches are the E3 ubiquitin ligases Cbl-b and TRIM29 [149, 150]. More examples of pathways that are explored in order to unleash NK cell cytotoxicity and effector cell functionality in the TME are the adenosine pathway as discussed earlier, targeting of the phosphatase SHP-1 as well as mitochondrial polarization and apoptosis [81-83, 151-154]. Lastly, the ability of NK cells to recognize target cells, form an immunological synapse and induce killing can be impaired in the TME. The engagement of the receptor NgR1, for instance, was shown to destabilize the NK cell immunological synapse and thus impair NK cell mediated tumor control [155]. Taken together, several new inhibitory immune checkpoint receptors and molecules have been identified that not only inhibit signaling pathways downstream of activating receptors but also interfere with other aspects of NK cell effector mechanisms such as their metabolic fitness or capacity for target cell recognition, synapse formation and ultimately killing.

1.4.4   NK cell effector mechanisms

Upon NK cell activation, several effector mechanisms are induced. NK cell cytotoxicity leads to target cell killing, while the secretion of cytokines, chemokines and growth factors enhances the recruitment and activation of other immune cell types.

NK cell cytotoxicity is exerted via two main mechanisms: The secretion of cytotoxic proteins is generally regarded as a fast process, occurring within few minutes after target cell recognition [156, 157]. A slower mode of killing occurs via direct receptor-ligand interaction (so called death-receptor-mediated killing) [156]. Upon activation, NK cells form an immunological synapse with the target cell that requires integrin-mediated adhesion [99, 158]. This is followed by the polarization of the microtubule-organizing center (MTOC) and the reorganization of the actin cytoskeleton. Lytic granules are then transported via microtubules towards the synapse where they merge with the plasma membrane and release cytotoxic proteins [159]. This process is termed *degranulation*. The granules are lysosome-related organelles and contain perforin and granzymes. Perforin inserts itself into the membrane of the target cell where it leads to pore formation and osmotic lysis [160]. This enables granzymes to enter the cytosol of the target cell. There are five different granzymes described, with granzyme B being the most studied in cytotoxic cells. Granzyme B induces a caspase-dependent cell death, while the other granzymes can induce a caspase-independent apoptosis, leading to reactive-oxygen species (ROS) production, disruption of mitochondrial function and chromatin condensation [156]. Upon fusion with the plasma membrane, the lysosomal-associated membrane protein 1 (LAMP-1, also known as CD107a) becomes exposed on the cell surface and functions as a marker of degranulation in functional assays [161].

Receptor-mediated cytotoxicity can occur via three different receptor-ligand systems: tumor necrosis factor (TNF), tumor necrosis factor ligand superfamily member 6 (FAS ligand/FasL) and TNF-related apoptosis-inducing ligand (TRAIL) [162, 163]. Their corresponding ligands are TNF receptor 1 and 2 for TNF and CD95 (also known as APO-1/Fas) for FasL. TRAIL can bind 4 membrane-bound ligands and one soluble ligand, however only TRAIL receptor 1 (also known as DR4) and TRAIL receptor 2 (also known as DR5) can induce apoptosis while TRAIL receptor 3 and 4 induce NFkB signaling [164–166]. As type II transmembrane proteins FasL and TRAIL are also known as *death receptors*. In unstimulated NK cells, both FasL and TRAIL are stored in secretory granules and only become expressed on the surface of NK cells upon activation [167, 168]. These granules are distinct from the cytotoxic granules that contain perforin and granzymes [169]. When death receptor ligands are engaged on the target cell, an intracellular signaling program is initiated which leads to the formation of the death-inducing signaling complex. This leads to the activation of caspase 8 and 10 and ultimately a caspase-dependent initiation of apoptosis [170, 171].

NK cells secrete many proinflammatory cytokines, chemokines and growth factors which in turn enhance immune responses. These include for instance interferon γ (IFNγ), TNF, interleukins (IL) 5, 10 and 13, the growth factor granulocyte–macrophage colony-stimulating factor (GM-CSF) as well as chemokines such as C–C motif chemokine ligand (CCL) 3, 4, 5 and IL8 [87]. IFNγ and TNF are stored in granules and are released upon activation. Compared to perforin and granzymes, this secretion seems to be non-directional [172]. Secretion of IFNγ by NK cells, for instance, promotes maturation, activation and recruitment of dendritic cells, macrophages and T cells [173, 174]. TNF can either induce direct apoptosis of tumor cells, block pro-tumorigenic regulatory T (Treg) cells and favor effector T cell functionality as well as recruit pro-inflammatory immune cell subsets [175]. NK cells secrete several other T cell recruiting chemotactic factors such as IL8, macrophage inflammatory protein 1α (MIP-1α) or CCL5 [176]. Moreover, NK cells led to the accumulation of conventional type I dendritic cells which are important for T cell effector functions [177]. However, NK cells do not only promote other immune cells but can also eliminate them as a way of regulating immune cell homeostasis. For instance, activated T cells were shown to upregulate ligands for NKG2D and DNAM-1 receptors and became more susceptible to NK cell-mediated cytotoxicity [178]. This was implicated in controlling overt T cell responses and prevent autoimmunity [178]. Similarly, NK cells eliminated immature dendritic cells via the TRAIL receptor ligand pathway [179]. The regulatory function of NK cells is exemplified in patients with familial hemophagocytic lymphohistiocytosis (FHL) that lack NK cell cytotoxic functions and present with systemic inflammation [180-182].

In summary, NK cells are not only effector immune cells of the innate immune system but also serve as a bridge between innate and adaptive immunity. They are implicated in the surveillance of transformed cells but also in the regulation of other immune cell effector mechanisms.

1.4.5   NK cell signaling pathways of activating receptors

NK cells express several activating receptors which integrated signaling leads to activation and cytotoxicity as described above. However, many activating receptors do not have a signaling function on their own but rather depend on adaptor proteins. The most prominent of these are the immunoreceptor tyrosine-based activating motif (ITAM)–bearing adaptor proteins CD3ζ, FcεRIγ and DNAX–activating protein (DAP) 12 as well as DAP10 which signals via a YINM motif. The association of the adaptor proteins occurs through charged amino acids in the TM regions of the proteins. Generally, the adaptor proteins possess a negatively charged aspartate residue which associates with a positively charged lysine or arginine within the TM region of their corresponding receptors [183, 184]. When an activating receptor binds to its ligand on the target cell, phosphorylation of the signaling subunit initiates downstream signaling and NK cell activation.

FcεRIγ and CD3ζ associate with CD16 and the NCR NKp30 and NKp46 by either formation of homodimers or heterodimers, whereas DAP12 associates as a homodimer with NKp44 and several activating KIR [185]. FcεRIγ and DAP12 have one ITAM whereas CD3ζ has three ITAM motifs [185]. The downstream signaling of the ITAM is very similar to the one described for B and T cells [186, 187]. Upon activation, Src family tyrosine kinases such as Lck, Fyn, Src, Yes, Fgr and Lyn phosphorylate the two tyrosine residues within the ITAM motif. This leads to recruitment and activation of the tyrosine kinases Syk and Zap70 [188]. Syk has been implicated in downstream signaling of the BCR and Zap70 in downstream signaling of the TCR [189–191]. It has been shown that NK cells can recruit both signaling molecules, which can likely fine-tune NK cell activation thresholds and effector responses [192]. For instance, differential expression of Zap70 and Syk is associated with adaptive NK cells [192]. Further downstream signaling involves phosphorylation of the TM protein LAT (linker for activation of T cells) and SLP-76 which form a three-protein complex with other adaptor proteins such as Gads [193]. The signaling then branches into several modules leading to activation of the PLCγ, PI3K-Akt, Vav1, MAPK and ERK pathways [193]. This results in cytoskeleton reorganization and calcium mobilization which is important for cell polarization and the release of cytotoxic granules. Moreover, the transcription of several genes encoding cytokines and chemokines is enhanced. Loss of Syk and ZAP70 in murine models was implied in impaired cytokine production but intact cytotoxicity towards several tumor cell lines [194–196]. However, silencing of DAP12 signaling impaired human NK cell lysis of tumor target cells, suggesting that ITAM are involved in triggering direct cytotoxicity [197]. Dissecting each signaling pathway in specific enhancement of direct cytotoxicity or cytokine production is difficult due to the plasticity of NK cell signaling pathways and the potential role of other activating receptors such as NKG2D-DAP10 signaling in ITAM deficient NK cells [198].

In human NK cells, the activating receptor NKG2D forms a homodimer that can recruit two homodimers of DAP10, leading to a hexameric protein complex [199]. Murine NK cells have two NKG2D isoforms, named NKG2D-L(ong) and NKG2D-S(hort). While the first isoform associates with DAP10, as seen in human NK cells, the latter can also recruit DAP12 [200]. Compared to ITAM, DAP10 has a very short signaling subunit with the sequence YINM. The phosphorylation of this motif by Src family kinases leads to the recruitment of the p85 subunit of PI3K and activation of the PI3K pathway [201]. Additionally, the Grb2 adaptor protein is recruited which induces phosphorylation of Vav1, PLCγ2 and SLP-76 and leads to PI3K pathway activation [202]. For full cytotoxicity to occur both subunits need to be bound [202]. Compared to ITAM-mediated signaling, LAT recruitment is not involved in DAP10 signaling [202]. Generally, DAP10 signaling triggers direct cytotoxicity of NK cells towards target cells [198, 203, 204]. In mice, DAP10 has also been implicated in NK cell proliferation upon activation [205].

1.4.6 NK cell signaling pathways of inhibitory receptors

Many inhibitory NK cell receptors signal through an ITIM. Upon ligand engagement, ITIM are phosphorylated and recruit phosphatases such as SHP-1, SHP-2 and lipid phosphatase SH2 domain-containing inositol-5-phosphatase (SHIP) [206-210]. Among these phosphatases, SHP-1 has been described as a central negative regulator of NK cell cytotoxicity and important for NK cell responsiveness [154, 211, 212]. SHP-1 leads to the dephosphorylation of the activating signaling cascade at a proximal step and thus modifies downstream signaling [213]. For instance, SHP-1 was shown to dephosphorylate Vav1 and thus block actin-dependent activation signals in NK cells [214]. Moreover, dephosphorylation of LAT and PLCγ by SHP-1 was shown to dampen NK cell cytotoxicity [215, 216]. However, ITIM-bearing NK cell receptors do not only block activation signals triggered by activating NK cell receptors but can also signal independently. This has been shown for NKG2A/CD94 signaling that induces Crk phosphorylation and thus inhibits CD16-mediated NK cell activation [217, 218]. All in all, inhibition of NK cell cytotoxicity by inhibitory NK cell receptors is a tightly regulated process, where not all mechanisms are yet fully elucidated.

1.5 Natural Killer cell immunotherapy

1.5.1 NK cells in the immunosuppressive TME

Immune cell functions in the TME can be impaired due to several mechanisms, including:

- o Upregulation of ligands for inhibitory receptors
- o Downregulation of ligands for activating receptors
- o Reduced chemokine expression and immune cell recruitment
- o Increased expression of inhibitory cytokines and immunosuppressive metabolites (e.g. transforming growth factor (TGF) ß, adenosine, ROS)
- o Reduced availability of nutrients and oxygen
- o Increased stiffness due to alterations in extracellular matrix (ECM) composition and angiogenesis
- o Conversion of anti-tumorigenic immune cell subsets to pro-tumorigenic immune cells subsets (e.g. myeloid-derived suppressor cell (MDSC), tumor-associated macrophage (TAM), Treg cells).

All of the above-mentioned mechanisms have been described for NK cell inhibition in the TME of hematological and solid malignancies. Discussing each mechanism in detail would, however, go beyond the scope of this thesis. As an example it can be stated that inhibitory receptors such as CD96, NKG2A, TIGIT and PD1 were shown to be upregulated on NK cells from patients with MM, colon and liver cancer and associated with a dysfunctional, exhausted NK cell phenotype that could be reverted by blocking the respective inhibitory axis [28, 123, 219-221]. Moreover, downregulation of surface expression of the activating

NK cell receptors DNAM-1, NKG2D and some NCR was described for several cancers, resulting in impaired NK cell cytotoxicity [222, 223]. Chronic signaling in the TME additionally lead to lower expression of death receptors, perforin and IFNγ on tumor-infiltrating NK cells upon target cell recognition [224–226]. Metabolic impairments were associated with an overall decreased effector cell function [227]. Among the chemokines, C-X-C chemokine receptor (CXCR) 3 and its ligands C-X-C chemokine ligand type (CXCL) 9/10/11 were implicated in recruitment of NK cells to solid malignancies [228-230]. The role of ADO has also been investigated in NK cells as discussed earlier and was associated with reduced cytotoxicity and cytokine release ability while blocking ADO receptors on NK cells could restore these functions [81, 231]. Lastly, the TME is often hypoxic due to increased oxygen demands by the tumor and defects in angiogenesis. Hypoxia on the other hand has multiple effects on NK cells, spanning from alterations in the surface receptor repertoire to metabolic reprogramming [232–235]. Furthermore, alterations in the TME can give rise to other immunosuppressive cell types such as MDSC, TAM and Treg cells that additionally hamper NK cell functions and lead to a vicious cycle of immunosuppression [236]. Due to the multifaceted roles of NK cells, boosting NK cell responses within the TME can have a profound impact on treatment outcomes [237]. Therefore, this work focuses on increasing NK cell functionality by adoptive NK cell transfer in combination with genetic modifications or oncolytic virotherapy.

1.5.2 Adoptive NK cell therapy

Adoptive cellular immunotherapy (ACT) describes the infusion of functionally competent effector cells, such as T cells and NK cells. CAR-T cell therapy was the first ACT that reached clinical translation and commercialization and is currently available outside clinical studies. Although CAR-T cell therapy has produced long-term sustained remissions in many cancer cases, it has several drawbacks [238, 239]. Due to the high risk of alloreactivity and graft-versus-host-disease (GvHD) observed in T cell therapy, an autologous approach needs to be used that is both time- and resource-consuming. Employing the patient's own cells limits its eligibility as many cancer patients may not have sufficient cells to yield a viable product due to pre-treatment and lymphopenia. Moreover, the majority of patients treated with CAR-T cells experience severe side effects such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Studies are ongoing to improve CAR-T cell therapy and generate easily available off-the-shelf products with reduced toxicity. However, these approaches often require further genetic editing such as TCR removal [240]. NK cells, on the other hand, have been associated with good clinical tolerance and very low risk of GvHD even in an haploidentical/allogeneic setting and are therefore explored as off-the-shelf cellular sources for ACT [241-243]. Research efforts are ongoing in order to translate adoptive NK cell therapy to the clinic. The selection of a reliable and effective NK cell source, a good manufacturing practice (GMP)-compliant and efficient expansion protocol

as well as possibilities to enhance NK cell fitness and antitumor functions *in vivo* are main aspects under investigation.

Currently, several sources for adoptive NK cell therapy are explored such as peripheral-blood derived NK cells (PB-NK), cord-blood derived NK cells (CB-NK), induced pluripotent stem cell derived NK cells (iPSC-NK) and the NK cell line NK92. Each of these cell sources poses advantages and limitations. For instance, PB-NK show a mature phenotype with expression of activating NK cell receptors such as CD16 for ADCC and high *in vitro* cytotoxicity. Indeed, autologous PB-NK cell infusions were the first form of adoptive NK cell therapy tested in clinical trials [242]. Despite their good tolerance and expansion *in vivo,* initial studies showed a lack of clinical activity against hematological and solid cancers [244]. The field thus moved towards allogeneic NK cells, especially, after studies were conducted that showed a good clinical response of alloreactive NK cells with unleashed cytotoxicity against AML in T cell depleted HLA-mismatched BM grafts [243]. Complete remissions of AML were even seen outside the setting of hematopoietic stem cell transplantation (HSCT) although larger studies did not confirm this correlation and suggested the need for adequate pre-conditioning regimes prior to NK cell transfer [242, 245]. Thus PB-NK cells were established as a potential source for adoptive NK cell therapies, however, it became evident that they needed to be combined with adequate conditioning regimes and other therapies such as mAb, bispecific antibodies or genetic modifications to unfold their full potential. PB-NK cannot be manufactured as a homogenous cell product, since inter-donor variability occurs and the possible yield from one donor is limited.

Another robust source for pNK cells is umbilical cord blood (UCB) that generally contains a higher percentage of NK cells than peripheral blood and is also a rich source for hematopoietic progenitor cells (HPC) that could potentially be expanded to mature NK cells [246]. CB-NK cells are readily available as frozen products, stored worldwide in blood banks and do not require apheresis from patients or healthy donors. Compared to PB-NK they show a lower cytotoxicity and cytokine release against the NK-sensitive cell line K562 and other stimuli which could be alleviated by IL2 and IL15 stimulation [246, 247]. Although CB-NK cells possess all features of primary NK (pNK) cells, they generally express lower levels of adhesion molecules and activating receptors such as CD2 and CD16 as well as higher levels of certain inhibitory receptors such as NKG2A and thus are regarded as more immature compared to PB-NK [248]. These limitations can be overcome by genetic modifications and, indeed, CD19-CAR CB-NK cells were the basis for the first published phase I/II clinical study with genetically modified NK cells that reported clinical efficacy [249, 250]. A total of 11 patients with different lymphoid tumors were treated of which 7 showed complete remission (CR) and persistence of NK cells could be observed up to 12 months after infusion. Similar to PB-NK cells, CB-NK cells, are not an off-the-shelf product as donor-dependent variabilities may occur.

Stem-cell derived NK cells may overcome current limitations of PB-NK and CB-NK cells regarding homogeneity and reproducibility. iPSC-NK cells are thus increasingly employed in pre-clinical and clinical trials. The generation of iPSC-NK cells requires the establishment of a reliable producer cell line, extensive pre-clinical testing and a more complex differentiation protocol but was shown to be clinical feasible [251]. Indeed, iPSC-NK cells displayed a high anticancer activity in several murine models of hematological and solid cancers [252, 253]. Moreover, genetic modification of iPSC is feasible and shows promising results in early phase clinical trials [254-258].

Lastly, the NK cell line NK92 has been extensively studied as an off-the-shelf product for adoptive NK cell therapy due to the possibility of large GMP-compliant expansions as well as high susceptibility to genetic modifications [259]. Due to safety reasons, however, NK92 cells need to be irradiated prior to patient infusions which limits *in vivo* persistence and durable clinical efficacy [259]. Moreover, NK92 do not express CD16 and other activating NK cell receptors, limiting their use in combination with ADCC. Therefore, an NK92 cell line has been generated that expresses a high-affinity variant of CD16 as well as endogenous IL2 (haNK) and is currently tested in few clinical trials (NCT04050709, NCT04390399, NCT04052061) [260].

All in all, the field of adoptive NK cell therapy is booming and different NK cell sources are investigated in numerous clinical trials worldwide. Lessons learned so far emphasize the need for clinical scale GMP-compliant expansion, transduction and combination therapy protocols to unleash NK cells' full clinical potential, translation and commercialization. These important steps are extensively reviewed elsewhere [261-264]. Shortly, for the large-scale expansion of NK cells different systems and platforms exist that usually require an *ex vivo* culture of 2-3 weeks after harvest but may take longer depending on additional differentiation and activation steps [262]. On average, therapeutic doses of $5x10^6$ to $1x10^8$ NK cells per kilogram body weight are needed and this expansion can be achieved either in a so-called feeder-cell free expansion system, relying on the supplementation of cytokines, antibodies or small molecules or a feeder-cell based expansion system [265]. The choice of expansion system depends on several factors such as the source of NK cell as well as local and regulatory restrictions. As an example, feeder-cell free soluble cytokine supplements generally contain IL2 with the addition of IL12, IL15, IL18 or IL21 [262]. Commonly used feeder cells are autologous peripheral blood mononuclear cells (PBMC) or tumor cells such as the erythroleukemic cell line K562 that is genetically modified to express membrane bound IL15, IL21, MICA and/ or 4-1BB ligands [262]. Regarding the genetic modification of NK cells commonly used systems are either based on viral transductions (lentivirus, retrovirus) or non-viral, for instance, through electroporation [263, 265]. In the studies presented in this thesis, we employed a PB-NK cell expansion protocol based on autologous PBMC and IL2 supplementation combined

with either lentiviral (**study I**) or retroviral (**study II**) transduction of CSR or CAR constructs, respectively [266, 267].

1.5.3 Chimeric antigen receptor (CAR) immune cell therapy

A CAR is a synthetic fusion protein expressed on the cell surface that consists of an extracellular domain, targeting a specific antigen via a single-chain variable fragment (scFv) of an antibody, a TM region as well as intracellular signaling domains and co-stimulatory domains [268, 269]. Depending on the number of intracellular domains, different generations of CAR constructs can be distinguished. The initial CAR designs were inspired by T cell signaling moieties and usually employ CD3ζ signaling which contains three ITAMs (1st generation). The second and third generations added one or two co-stimulatory domains such as CD28 and 4-1BB while the fourth and fifth generations additionally contain chemokine or cytokine receptor signaling units. The first studies on chimeric receptors to augment T cell functionality were published by a group at the Weizmann Institute in Israel in the early 1990s and several adaptations followed that finally led to the FDA-approval of the first CAR-T cell product Kymriah (CTL-019) in 2017 [270, 271]. To date, modifications and improvements of CAR designs are ongoing that aim to improve efficacy and minimize toxicity. Moreover, research efforts are ongoing to adapt CAR designs for NK cells and their unique signaling properties in order to obtain their full potential. For instance, it was shown that NK-specific CARs that employ the TM region from NKG2D instead of CD28 as well as CD3ζ intracellularly in combination with 2B4 or DAP10 co-stimulatory domains confer a higher cell killing and degranulation capacity than NK cells engineered to express a classical third generation CAR construct with CD3ζ/CD137/CD28 signaling [254]. Importantly, mutation studies showed that the NKG2D and 2B4 domains led to improved cytotoxicity upon target recognition. In the currently published NK-CAR clinical trial, a classical T cell specific CAR with CD28/CD3ζ domains was employed [250]. To evaluate the efficacy of NK-specific CAR, the results from ongoing and future studies are eagerly awaited. In **study II** of this thesis a CD38-targeting CAR with CD28/CD3ζ signaling is employed that shows improved target cell killing of both MM cell lines and autologous BM mononuclear cells (BM MNC).

1.5.4 Chimeric switch receptor (CSR) immune cell therapy

CSR are synthetic surface receptors in which the intracellular domain of an inhibitory receptor is replaced by one or several activating signaling domains, aim being to prevent immune cell suppression upon target cell recognition. The need for this approach was formulated due to difficulties in translating CAR-T cell therapy for the treatment of solid malignancies. Although highly effective against hematological cancers, CAR-T cell therapy did not show similar efficacy for solid cancers, partially due to poor trafficking, limited persistence and infiltration as well as T cell inhibition within the TME. The latter encouraged the development of adoptive T cell products with cell-intrinsic disruption of

inhibitory receptors – especially PD1 – by gene modification. These PD1[disrupted]/PD1-KO T cells were deemed to have a better safety profile and higher efficacy than the combination of immune checkpoint blockade with mAb and adoptive T cell therapy. Indeed, several clinical trials with clustered regularly interspaced short palindromic repeats (CRISPR) engineered PD1[disrupted]/PD1-KO T cells were initiated [272, 273]. Although this approach was successful in several murine models, new data indicates that PD1-KO might also impair T cell functionality [274]. Specifically, PD1-KO CAR-T cells were shown to have an impaired proliferative capacity and cytotoxicity [275]. In a recent phase I trial with CAR-T cells that harbored additional disruptions of PD1 and TCR, lack of clinical efficacy and diminished T cell persistence were observed [276]. In contrast, a CSR consisting of PD1/CD28 was shown to augment the efficacy of CAR-T cells in murine cancer models [277, 278]. Specifically, PD1-CD28^{+} CAR-T cells had a higher killing capacity, cytokine production ability as well as infiltration and proliferation rate compared to CAR-T cells alone in murine prostate and gastric cancer models. Similarly, a TIGIT/CD28 based CSR enhanced T cell functionality upon repetitive target cell exposure and enhanced T cell-mediated eradication of murine melanoma [279]. Currently, there are few clinical trials available testing PD1-based CSR in a clinical setting; one for relapsed and refractory B cell lymphoma (NCT03258047) and one for solid cancers with pleural or peritoneal metastasis (NCT04684459) [280]. In the first-in-human study, PD1-CD28^{+} CD19-CAR-T cells showed good clinical activity in patients with diffuse large B cell lymphoma (DLBCL) with CR in 7 out of 17 cases as well as no severe CRS or ICANS [281]. As discussed previously, PD1 is not expressed on the surface of PB-NK cells under physiologic conditions but can be upregulated in cancer due to chronic stimulation, leading to NK cell dysfunctionality. PD1-based CSR consisting of PD1 linked to the TM and intracellular domains of NKG2D, DAP10 and/ or 4-1BB were designed and tested in the NK92 cell line [282-284]. These studies confirmed the feasibility of the approach in improving NK92 cytotoxicity towards different tumor cell lines and in murine cancer models. The work presented in this thesis (**study I**) further evaluated the feasibility of PD1-based CSR in NK cells. Compared to previous studies, the work mainly focuses on pNK cells from both healthy donors and patients with MM against autologous BM MNC. Moreover, different NK cell specific signaling domains were compared side-by-side. All in all, this helps to further pave the way for clinical implementation of NK cell specific CSR.

1.6 Oncolytic virotherapy

Oncolytic virotherapy is a form of immunotherapy that utilizes genetically modified viruses which preferentially infect and replicate within tumor cells. Their potential lies in the ability to both induce immunogenic cell death in cancer cells and enhance anti-tumor immune functions [285]. As of October 2023 at least four oncolytic viruses and one non-oncolytic virus have been approved for clinical use [286]. These include H101 (Oncorine), an adenovirus-based OV approved in China for the treatment of head and neck tumors;

talimogene laherparepvec (T-VEC), an HSV-1 based OV approved for the treatment of malignant melanoma in the United States, Europe and Australia; ECHO-7 (Rigivir), an echovirus-based OV approved in Armenia, Georgia and Latvia for the treatment of malignant melanoma as well as teserpaturev, an HSV-1 based OV for the treatment of glioblastoma in Japan. However, Rigivir has been discontinued in 2019 due to manufacturing issues. The adenovirus-based non-oncolytic virus nadofaragene firadenovec (Adstiladrin) that encodes IFNα-2b received FDA-approval in December 2022 for the treatment of bladder cancer [287]. Multiple other OV platforms are currently under development and in early clinical trials [288]. However, to date, OV monotherapy only shows modest clinical activity. Several reasons can be identified, among which a substantial anti-viral immune response is an important factor that limits, for instance, systemic delivery or viral propagation and efficacy in the TME. This might explain why T-VEC has not been developed beyond the local treatment of melanoma since its first approval in 2015 [289]. Nevertheless, the field is moving forward due to a great potential of OV in combinatorial treatment modalities with other immunomodulatory drugs, chemotherapeutic agents or cellular therapies. Research performed for this doctoral thesis (**study III**) focuses on the interplay between HSV-1 based OV and NK cells.

1.6.1   Human herpesviruses

Herpesviruses are a large group of double-stranded DNA viruses (dsDNA) with more than 100 known viral species infecting amphibians, fish, mammals and humans. Currently, eight human herpesviruses have been described that belong to the three different subfamilies of α-, β- and γ-herpesviruses (**Table 1**). A hallmark of herpesviruses is their ability to establish a lifelong, latent or quiescent infection which can reactivate, spread to other hosts via direct contact or aerosol and cause one or several cycles of disease.

TABLE 1 Humanotropic herpesviruses

Subfamily	Colloquial name	Numerical designation	Disease
α	Herpes simplex type 1 (HSV-1)	HHV-1	acute gingivostomatitis, eczema herpeticum, herpetic keratoconjunctivitis, encephalitis
	Herpes simplex type 2 (HSV-2)	HHV-2	genital ulcers, meningitis
	Varizella zoster virus (VZV)	HHV-3	chickenpox, shingles
β	Cytomegalovirus (CMV)	HHV-5	congenital defects, transplant complications
	Human herpesvirus 6A	HHV-6A	unknown
	Human herpesvirus 6B	HHV-6B	exanthema subitum, convulsions, mononucleosis-like disease
	Human herpesvirus 7	HHV-7	exanthema subitum
γ	Epstein-Barr virus (EBV)	HHV-4	infectious mononucleosis, Burkitt's lymphoma, B cell lymphoma
	Kaposi's sarcoma associated herpesvirus (KSHV)	HHV-8	Kaposi's sarcoma

Structurally, herpesviruses are composed of a complex virion, comprising a capsid, tegument and a trilaminar envelope (**Figure 2A**). The linear dsDNA is approximately 125–290 kilobases (kb) large, encodes approximately 80-180 viral proteins and is located within the capsid. The tegument is an amorphous mass containing both viral and cellular proteins as well as viral mRNA. The envelope consists of viral glycoproteins and other proteins derived from cell membranes and is important for cell attachment and viral entry.
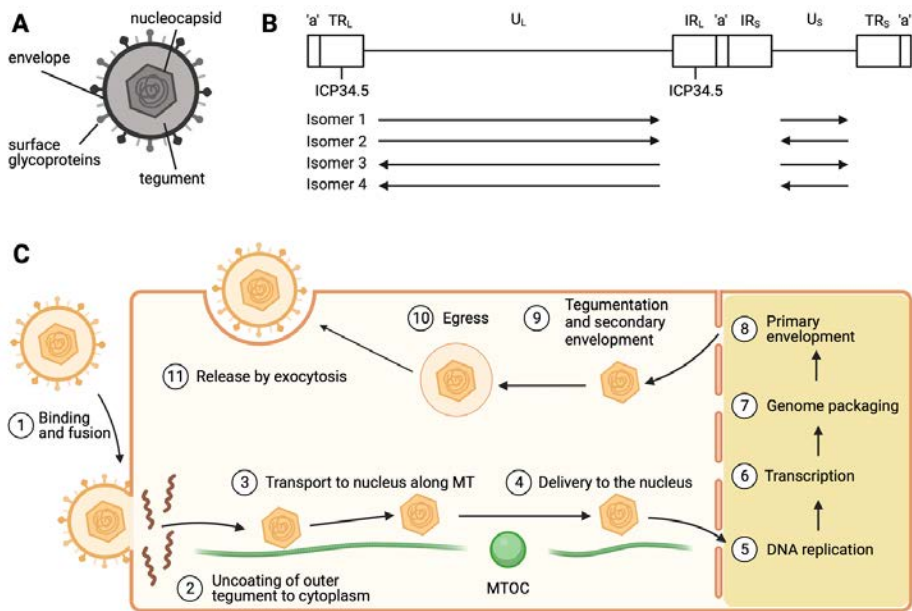
FIGURE 3 Aspects of HSV–1 biology. A) HSV–1 is composed of a complex virion structure where the linear dsDNA is contained within the nucleocapsid that is surrounded by an amorphous mass, called the tegument, and an envelope which contains surface glycoproteins and other proteins for cell attachment and viral entry. **B)** Illustration of the HSV–1 genome with its different regions designated as 'a' sequence, terminal repeat long (TR$_L$), unique long (U$_L$), internal repeat long (IR$_L$), internal repeat short (IR$_S$), unique short (U$_S$) and terminal repeat short (TR$_S$) sequences. The 'a' sequences allow the inversion of the unique fragment orientation, leading to different reading directions and creating four different isomers that are present in equal ratios. **C)** Illustration of HSV-1 life cycle. After attachment and fusion of the envelope to the cell surface, tegument proteins are released into the cytoplasm and the nucleocapsid transported to the nucleus where viral DNA replication, transcription and genome packaging occurs. The nucleocapsid is then released into the cytosol where it acquires its tegument and envelope before being released by exocytosis. Image created with Biorender by the author; inspired by [290].

1.6.2 Herpes simplex virus type 1 (HSV-1)

HSV-1 is one of the most prevalent viruses worldwide with an estimate of 65-70% of humans being infected, leading to a high research interest in HSV-1 biology, epidemiology and therapy [291]. During primary infection, HSV-1 infects epithelial cells of the mucosa or skin, causing lytic replication and production of viral particles that then infect neurons of the peripheral nervous system (PNS) and less frequently central nervous system (CNS). Importantly, HSV-1 can stay dormant in neurons for several years (latent infection) and reactivate (active infection) upon certain stimuli such as cellular stress due to environmental stressors (e.g. temperature, toxins, mechanical damage). The switch between active and latent infection is not yet fully understood but requires a complex genetic regulation and interplay with the immune system. In the following, a brief introduction of the HSV-1 genome, the infectious cycle in cultured cells as well as immune responses to HSV-1 is given.

HSV-1 genome and nomenclature

Depending on the laboratory strain and clinical isolate, the genome of HSV-1 is a dsDNA of approximately 152 kb and divided into different segments (**Figure 2B**). Two major unique fragments can be distinguished that are referred to as unique long (U_L) and unique short (U_S). These are framed by so called inverted repeats denoted as repeat long (R_L) or repeat short (R_S) which are located both at the end (terminal) of the genome and then named TR_L or TR_S or internally and then named IR_L or IR_S, respectively. The repeated segments are bound by shorter so called "a" sequences in one or more copies at both ends and between the IR_L/IR_S junction. These "a" sequences enable the inversion of the unique fragment orientation and thus formation of four genomic isomers that are formed at equal ratios [292]. The current nomenclature of the approximately 80 identified genes is straightforward since they are numbered based on their position left of the conventional genome isomer orientation, e.g. R_L1, R_L2, U_L1-56, R_S1 and U_S1-12. However, HSV-1 genes can also be named based on their function as immediate-early (IE), early or late genes and other historic nomenclatures exist. Thus, gene R_L2 is also known as IE1 or $\alpha0$. The nomenclature of viral proteins is yet more complex and can refer to the gene encoding the protein or follow a different nomenclature. Generally, two protein families are distinguished and referred to as infected cell protein (ICP) or viral protein (VP) and named in ascending order based on their gel mobility, a technique that separates proteins based on their mass and physicochemical properties. Older papers, however, classified the ICPs based on their apparent molecular size and used the prefix Vmv (e.g. Vmv110 for ICP0). Viral proteins also have names based on their function such as thymidine kinase or glycoproteins. It is important to understand that the systemic gene numbering system is used for all herpesviruses, but, due to genomic differences the encoded proteins might differ. This leads to the fact that although the gB proteins of HSV-1 and HCMV are related, gB is the gene product of UL27 in HSV-1 and UL55 in HCMV, respectively.

HSV-1 infectious cycle

Although HSV-1 primarily infects epithelial cells in humans and establishes latency in neuronal cells, it can infect and replicate within a large variety of different cell types *in vitro*, often mimicking active infection. The infectious cycle comprises different steps from viral entry to viral gene expression as well as assembly and egress of viral particles (**Figure 2C**) [290, 293, 294]. Multiple entry pathways for HSV-1 have been described, mainly a pH-independent fusion of the viral envelope and the plasma membrane and a pH-dependent endocytic process. Although the details and differences of these pathways are not yet fully elucidated and probably depend on the properties of the studied cell, it is generally accepted that they require a multi-step process involving both viral envelope glycoproteins and their cognate cellular surface receptors. A protein complex of gD, gH/L and gB is generally regarded as the viral "core fusion machinery". The initial binding of gD to its cognate receptors induces a conformational change that enables gH/L to interact and activate gB that in turn undergoes a conformational change, leading to membrane fusion. Cognate receptors of gD include herpesvirus entry mediator (HVEM), nectin-1 and nectin-2 (CD112) as well as 3-O-sulfated heparan sulfate proteoglycan (3-O HSPG). Known gB receptors are paired immunoglobulin-like Type 2 receptor-α (PILRα), myelin-associated glycoprotein (MAG) and non-muscle myosin heavy chain (NMHC)-IIA. Upon membrane fusion, the tegument proteins are released into the cytoplasm and the capsid is transported in a dynein-dependent manner along microtubules to the MTOC and thereafter to the nuclear membrane where the viral dsDNA is released into the nucleoplasm. The transcription of the IE genes is stimulated by the viral tegument protein VP16 and relies on the host transcriptional apparatus. IE proteins then enable the transcription of early genes that occur independently of viral DNA replication while late genes are transcribed only after viral DNA replication has been initiated. Viral DNA replication as well as capsid formation and DNA packaging occur in the nucleus. Once this process is complete, the capsid is released through the nucleus into the cytoplasm where it is assembled to a viral particle containing a tegument and envelope. The mature viruses are then transported to the cytoplasmic membrane and released through exocytosis into the extracellular space. However, HSV-1 can also spread via direct cell-to-cell contact, utilizing the cell's transmembrane adhesion proteins, in order to escape immune response. Unfortunately, the molecular mechanisms regulating this process are to date only poorly understood. Mutational studies have implied a role of the gE/gI heterodimer in contributing to syncytia formation, transport of viral particles to cell junctions and ultimately viral spread via lateral cell junctions [295-297]. All in all, the infectious cycle is a rapid process in cultured cells. It is estimated that in the most commonly used cell types that are infected with HSV-1 at a multiplicity of infection (MOI) sufficient to infect all cells, all groups of viral genes are expressed simultaneously and maximal progeny viral yields are reached by 24 hours post infection (p.i.).

As mentioned before, human herpesviruses are able to switch between an active and a latent phase, suggesting that they have developed mechanisms to efficiently evade recognition by the immune system. Indeed, several mechanisms are described as to how HSV-1 interferes with intracellular immune responses, the release of proinflammatory cytokines and chemokines and the recognition of infected cells by the innate and adaptive immune systems [298, 299]. In brief, cells have the ability to sense foreign material by several pattern recognition receptors (PRR) and RNA/DNA sensing receptors in order to initiate cellular pathways to shut down protein synthesis, initiate programmed cell death, prevent viral replication and spread as well as alarm neighboring cells and the immune system. The most studied DNA sensing pathways involved in HSV-1 recognition are the toll-like receptor (TLR) signaling pathway and the cyclic GMP-AMP synthase stimulator of interferon genes (cGAS–STING) pathway. Activation of these pathways leads to activation of type I interferons which in turn activates IFN-stimulated genes (ISG) that create an antiviral state in the infected cell and lead to activation of the immune system. ICP0 and ICP27 have, for instance, been shown to limit the induction of type I IFN and ISG activation by interfering with downstream TLR and cGAS–STING signaling respectively [300–302]. Moreover, HSV-1 has been shown to evade immune cell recognition by both T cells and NK cells through downregulation of MHC class I molecules as well as ligands for NKG2D and DNAM-1 [303-305]. Despite this, numerous other strategies are described on how HSV-1 circumvents immune recognition, for instance, by interfering with the complement system or by modulating dendritic cell functions. All in all, herpesviruses have co-evolved with humankind and other vertebrate hosts for millions of years and thus established a complex system to control host immune responses.

1.6.3   HSV-1-based OV

HSV-1 has been investigated and translated as an oncolytic agent due to several reasons [306]. Firstly, HSV-1 infects and replicates in several cells due to the abundant expression of HSV-1 cellular receptors. This also means that HSV-1 is comparably easy to propagate in cell cultures to reach high viral titers needed for therapies. Secondly, HSV-1 has a large genome which facilitates its genetic modification and the insertion of multiple transgenes. Moreover, HSV-1 is not known to integrate into the host genome. Lastly, antiviral treatments, e.g. acyclovir, are available that could potentially be used in case of side effects. A drawback of HSV-1 as an OV, on the other hand, is the high prevalence and existence of antiviral-immunity that inhibits systemic delivery, repeated dosing and mounting of an adequate immune response.

Currently, several different HSV-1 mutants are investigated in pre-clinical research or early phase clinical trials [307]. In **study III** of this thesis, the interaction between NK cells and the HSV-1 based OV HSV1716, also known as seprehvir (1st generation) or seprehvec

(2nd generation), is studied. HSV1716 differs from the wildtype HSV-1 strain by a deletion in both copies of the R_L1 gene, leading to the loss of ICP34.5. HSV1716 does not contain any further transgenes [308]. ICP34.5 is a multi-functional protein that plays a role in establishing anti-viral immunity, nuclear egress of the nucleocapsid and control of viral replication versus latency in neuronal cells. The latter also led to the description of ICP34.5 as a neurovirulence factor. Specifically, ICP34.5 mutant HSV-1 strains were shown to infect different cells of the PNS and CNS in murine models, but were highly attenuated in their replicative function and rather promoted establishment of latency [309]. This lead to the exploration of ICP34.5 mutant HSV-1 strains for gene therapy and OV treatment of CNS malignancies such as glioblastoma [310]. Mechanistically, ICP34.5 leads to dephosphorylation of the eukaryotic initiation factor 2α (eIF-2α) and thus counteracts the protein kinase R (PKR)-mediated phosphorylation of eIF-2α and shut-off of host protein synthesis [311]. Moreover, ICP34.5 can bind to Beclin-1 and inhibit autophagy [312]. Thus, ICP34.5 deletions highly attenuate the viral replicative function in healthy cells but are compensated for in cancer cells due to their aberrant signaling pathways. Additionally, ICP34.5 controls anti-viral immune responses by inhibiting STING signaling and ISG activation as well as MHC class II surface presentation [313, 314].

Seprehvir has been tested in several phase I/II trials for high-grade glioma, melanoma, sarcoma and mesothelioma [315-320]. All of these studies demonstrated an excellent safety profile with no serious side effects, but unfortunately lack robust anti-tumor efficacy. One explanation can be the high attenuation of HSV1716 which prompted the development of other HSV-1-based ICP34.5 deleted OV that harbor additional modifications to boost anti-tumor functions and stimulate immune responses [307, 321]. Alternatively, HSV1716 can be combined with other forms of cancer treatment such as chemotherapy, radiotherapy or immunotherapy.

1.6.4   NK cells and HSV-1 based OV

Patients who lack NK cells are highly susceptible to severe and recurrent herpesvirus (HSV) infections, which establishes the role of NK cells in HSV-1 recognition and infection control [322, 323]. NK cells were also shown to target dorsal root ganglia, where HSV-1 primarily resides during latency, in an NKG2D dependent manner [324]. Moreover, NK cells have been implicated in the recognition and elimination of HSV-1 OV infected tumor cells, emphasizing a potential enhancement of NK cell-based immunotherapy with the help of HSV-1 OV. For instance, glioblastoma-bearing mice showed prolonged survival with a combination of HSV-1 OV and NK cells compared to HSV-1 OV without NK cells which was further enhanced by the addition of bortezomib [325]. Also, the function of NK cells, expressing a CAR that targets epidermal growth factor (EGFR) against breast cancer brain metastases was potentiated by the addition of an HSV-1 OV in murine models [326]. Another interesting mechanism how NK cell functionality could be boosted with HSV-1 OV is the concept of antibody bipolar bridging (ABB) [327]. Viral gE that is expressed on

infected target cells can bind to the Fc receptor of immunoglobulins at another epitope than CD16. Although initially described as a viral evasion mechanism from host immune responses, it was shown that ABB can mediate enhanced NK cell effector functions similar to ADCC, which is interesting in combinatorial treatment approaches with mAb [328].

In summary, HSV-1 OV can potentially boost the function of NK cells within the TME, however, it is increasingly becoming evident that combination therapy is only successful in a certain *window of opportunity*, taking e.g. the timing of the treatment and the number of NK cells into account [329, 330]. Early clearance of virally infected cells by NK cells can impede the oncolytic effect [331]. Therefore, more studies are needed to elucidate the exact mechanism of recognition of HSV-1 OV infected target cells by NK cells to design and improve combinatorial treatment approaches.

2 Research aims

This thesis investigates new immunotherapeutic approaches to increase the effector functionality of NK cells in the TME. A special focus is set on improving treatment options for MM. While **paper I** and **paper II** focus on genetic modifications of NK cells, **paper III** elaborates the combination of NK cells with the oncolytic virus HSV1716.

The specific aims are:

Paper I. **To re-direct PD1-mediated inhibitory signaling in NK cells and re-target immune checkpoint inhibition**. ICI with mAb failed in MM despite the expression of PD1 on immune cells and PD1 ligands in the myeloma BM microenvironment. In this study, several NK cell specific PD1-CSR were designed and tested in order to switch the inhibitory signal into an activating signal in NK cells upon PD1 engagement and thus overcome NK cell dysfunctionality in the TME and, additionally, target the PD1 pathway in MM.

Paper II. **To increase CD38-targeted NK cell cytotoxicity**. Targeting CD38 with the mAb daratumumab showed great clinical efficacy in MM but is also met with resistance to therapy, relapse and increased risk of infection due to fratricide and NK cell depletion. This study explores the pre-clinical feasibility of fratricide resistant $CD38^{dim}$ CD38-CAR-NK cells as an alternative CD38-targeted therapy for MM.

Paper III. **To explore the synergy between HSV-1 OV and NK cells**. HSV-1-based OV showed promising pre-clinical and clinical results for melanoma and glioblastoma and are currently explored for MM. NK cells were implicated in both improving and hampering the result of OV treatment. This study investigates mechanisms of NK cell activation upon HSV-1 OV infection in order to improve and design combinatorial treatment approaches.

3 Ethical considerations

Medical research should focus on improving life and well-being of the society. This requires that it is performed in a way that is transparent, meticulous, fact-based and well documented. Reports of scientific misconduct have shed a negative light on the scientific community which endangers the trust of the society and funding opportunities. While performing research, it is therefore necessary to reflect upon good laboratory practices and ethical considerations on a regular basis.

The research presented here has several ethical implications. These can arise from work with sensitive data, patient-derived material as well as practical application of medical research in a clinical setting or as a commercial product. Most of the experiments are based on cell lines or samples from anonymized healthy donors. By institutional guidelines, this does not require any ethical permits. However, it is important to note that some commercially available cell lines were not taken with legal consent from the donors. The most prominent case is *Henrietta Lacks* whose cells are used as HeLa cells by many laboratories. To date, healthy individuals that donate blood give their informed consent whether their material can be used for research purposes. Many donors will not be informed about the exact intention and experiment their blood cells are used for. As a researcher it is important to be aware of this and use those resources carefully. In a recent study, participants raised concerns regarding the potential of unacceptable future research, especially in the field of human cloning, or a fear of financial profiteering from their samples by pharmaceutical companies [332]. Another concern was sample storage with a potential of donor re-identification and misuse of collected data. Here, we applied strict rules to not store any data that could potentially lead to the identification of the donor when freezing isolated PBMC for future use. In the case of patient material, however, donor identification is often necessary and we have to follow all rules regarding biobanking [333]. In **paper I** and **paper II**, patient material was used which requires an ethical permit that was granted to us (ethical permit number: 2019-04973 and 2020-02119). Many ethical questions can be raised regarding biobanking, such as issues of private propriety, handling of results but also patient safety during sample collection. In the studies presented here, the patients donated blood via venous puncture and bone marrow via a biopsy which are both invasive procedures. Therefore, the research samples were collected when the patient was admitted to hospital for regular follow-up or if sample collection was medically advised for treatment purposes. In conjunction with this, extra material was taken for research purposes from patients that gave their formal consent. The personal data were stored according to General Data Protection Regulation (GDPR) rules and were pseudonymized and only available for researches involved in the project. The data were not disclosed to third parties or to group members not involved in the projects. Finally, the performed research aimed at improving cancer therapy for future patients with MM and did not impede current patient treatment.

4 Results and discussion

4.1 Paper I

Generation of NK cells with chimeric switch receptors to overcome PD1–mediated inhibition in cancer immunotherapy

Background

Targeting the immune checkpoint receptor PD1 and its ligands with mAb marked a milestone in cancer therapy and led to the attribution of the 2018 Nobel Prize. Although ICI with mAb is a successful integral part of treatment concepts for several malignancies to date, it does not show efficacy in all cancers. Specifically in MM, ICI with mAb has failed due to inefficacy of monotherapy and severe side effects in combination treatments [31, 32, 334]. Different approaches are currently under evaluation since PD1 and PD–L1/L2 upregulation occurs in MM patients, confirming the relevance of this axis [29, 335]. As discussed earlier, ACT with both T cells and NK cells is already in clinical practice or in clinical trials for MM. Our laboratory focuses on the development of autologous NK cell–based immunotherapies for the treatment of MM [266]. In a phase I study it was shown that autologous NK cells can be safely infused into patients with MM and persist for up to four weeks [336]. Further studies are currently ongoing to improve the efficacy of autologous NK cells. Although the majority of PB–NK cells from healthy individuals does not express PD1 on their surface, an upregulation of PD1 on NK cells is observed in MM and associated with reduced effector cell functionality, possibly reducing the efficacy of ACT [28, 337].

Aim of the study

Based on the above, it is clear that PD1 and its ligands are targetable in MM, but not with the currently available approaches. Moreover, there is a risk of reduced efficacy of ACT due to immunosuppression in the TME. We have therefore designed PD1–based CSR in order to re-target the PD1 checkpoint axis in MM and provide an additional mode of action. Additionally, PD1-CSR shall improve the efficacy of NK cells in the TME by promoting a positive signal upon engagement with PD1 ligands and prevent inhibition.
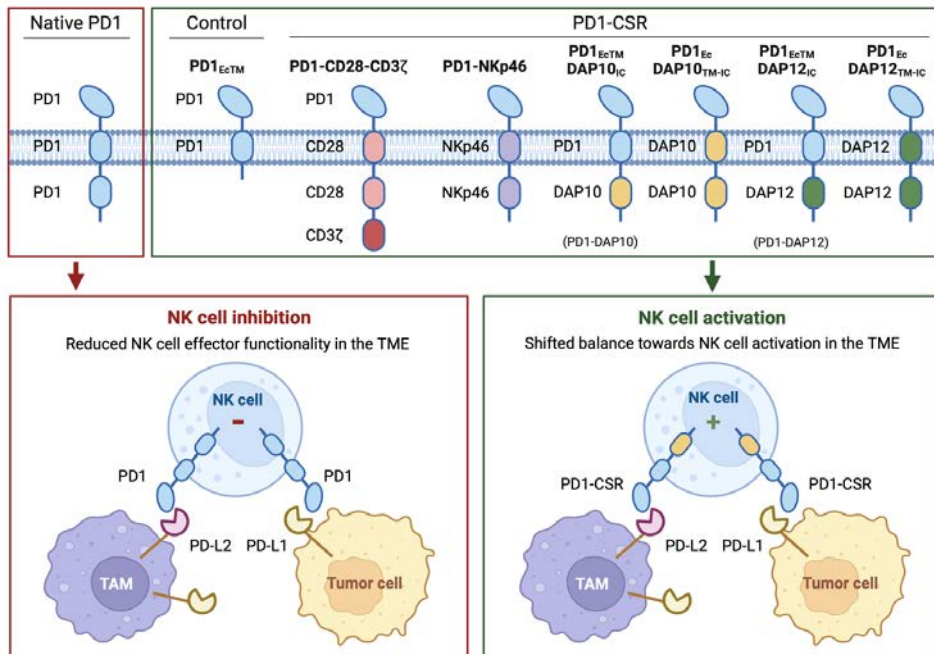
FIGURE 4 Graphical abstract for study I. Six different PD1–CSR constructs were generated by replacing the transmembrane and/ or intracellular domains of PD1 with different domains from known activating NK cell receptors or adaptor proteins. As a control, a truncated PD1 construct that lacks an intracellular signaling motif was designed. The PD1–CSR with the intracellular domains of DAP10 (PD1$_{EcTM}$DAP10$_{IC}$) or DAP12 (PD1$_{EcTM}$DAP12$_{IC}$) only are referred to as PD1–DAP10 and PD1–DAP12 in the main text to facilitate reading. PD1–CSR increase NK cell effector functions upon ligand binding. Abbreviations: CSR: Chimeric switch receptor; Ec: extracellular; IC: intracellular PD1: programmed death protein 1; PD–L1/L2: programmed death ligand 1/ ligand 2; TAM: tumor associated macrophage; TM: transmembrane; TME: tumor microenvironment. Image created with Biorender by the author.

Results

In **study I**, we designed six different PD1–based CSR for optimal signaling in NK cells and tested their functionality in both NK92 and pNK cells from healthy donors and patients with MM against both PD–L1$^+$ tumor cell lines and autologous primary BM MNC from MM patients.

The aim was to find a signaling domain that would increase NK cell degranulation and killing ability upon PD1 engagement to a level, sufficient to tip the balance towards activation and work in conjunction with other immunotherapies such as CAR constructs or mAb. Therefore, we focused on single signaling domains and not chimeras of different signaling domains and were particularly interested in the YINM–bearing DAP10 and ITAM–bearing DAP12 adaptor proteins, known to associate with activating NK cell receptors. For those, two different types of CSR were designed by employing either the full length DAP10 and

DAP12 molecules or the signaling domains only (**Paper I, Fig. 1A–C**). Moreover, a CSR based on the activating receptor NKp46 which recruits ITAM-bearing CD3ζ homodimers or CD3ζ/ FcεRIγ heterodimers was designed (**Paper I, Fig. 1A–C**). As controls, a signaling deficient truncated PD1-CSR as well as a PD1-CSR based on CD28/CD3ζ expressed in second-generation CAR constructs were generated (**Paper I, Fig. 1A–C**). Our study confirms that the signaling domain of DAP10 or DAP12 alone confers an activation signal in both NK92 cells and pNK cells. Both NK92 cell lines with either the signaling domain of DAP10 (PD1$_{EcTM}$DAP10$_{IC}$) or DAP12 (PD1$_{EcTM}$DAP10$_{IC}$) increased degranulation measured by CD107a expression as well as IFNγ and TNF secretion against PD-L1$^+$ cancer cells (**Paper I, Fig. 2A, Fig. S2A–D**). Moreover, both CSR increased NK cell-mediated killing of tumor cells in regular co-cultures as well as against tumor cell spheroids (**Paper I, Fig. 2B, Fig. 3A–D**). PD1$_{EcTM}$DAP10$_{IC}$ and PD1$_{EcTM}$DAP12$_{IC}$ CSR were stably expressed under culture conditions or during stimulation (**Paper I, Fig. S3D,E**). In contrast, the PD1-NKp46 and PD1-CSR with the full length DAP12 did not show a stable expression over time in NK92 cells and displayed a receptor downregulation after activation (**Paper I, Fig. S3D,E**). In the following, the constructs named PD1$_{EcTM}$DAP10$_{IC}$ and PD1$_{EcTM}$DAP12$_{IC}$ will be referred to as PD1-DAP10 and PD1-DAP12 to facilitate reading.

With the positive results obtained with the PD1-DAP10$^+$ and PD1-DAP12$^+$ NK92 cell lines, we decided to transduce pNK cells from PBMC of healthy donors. Our results show that PD1 surface expression is only detected on a small fraction of PB-NK cells from healthy donors and does not increase under IL2 stimulation *in vitro* (**Paper I, Fig. 4A**). Unmodified PD1$^+$ NK cells showed a reduced degranulation and cytokine secretion ability when co-cultured with PD-L1$^+$ target cells (**Paper I, Fig. 4C–E, Fig. S7**). Abrogating the interaction between native PD1 and PD-L1 with the introduction of a truncated signaling-deficient PD1 construct on NK cells prevented the decreased degranulation (**Paper I, Fig. 4C–E, Fig. S7**). On the other hand, both PD1-DAP10 and PD1-DAP12 pNK cells increased degranulation and cytokine secretion against PD-L1$^+$ target cells (**Paper I, Fig. 4C–E, Fig. S7, Fig. S8D–F**). Most importantly, this increase could be further augmented by adding the CD20 targeting antibody rituximab (**Paper I, Fig. 4F–H, Fig. S7**). One of rituximab's way of interaction is the induction of ADCC via CD16 on NK cells [338]. This highlights the potential for combining PD1-CSR with other NK cell targeting therapies such as mAb capable of ADCC, bispecific antibodies or CAR constructs. In contrast to NK92, there was no clear increase in target cell killing by either PD1-DAP10$^+$ or PD1-DAP12$^+$ pNK cells which might be explained by the fact that we have not sorted the transduced pNK cells for PD1 positivity (**Paper I, Fig. 4I, Fig. S9A,B**). On average, the transduction efficiency reached 40–50 %, including pNK cells with both high surface expression of PD1 (PD1^{bright}) and lower surface expression of PD1 (PD1^{dim}) (**Paper I, Fig. 4A,B**). PD1 surface expression did not increase on untransduced pNK cells under culture and expansion conditions, allowing the assumption that both PD1^{bright} and PD1^{dim} pNK cells are PD1-CSR$^+$ cells. In the following, they will be referred to as PD1-CSR^{bright} and PD1-CSR^{dim} cells. While the PD1-CSR^{dim} cells abrogated PD1 mediated NK cell

inhibition, the PD1–CSR^bright cells increased degranulation and cytokine secretion (**Paper I, Fig. 4C–E, Fig. S8A–F**). Unfortunately, it was beyond the scope of this study to investigate the differences between PD1–CSR^dim and PD1–CSR^bright pNK cells in detail. Thus, it remains unclear whether an NK cell product with low levels of PD1–CSR^bright and higher levels of PD1–CSR^dim pNK cells would be sufficient to re-model the TME and improve overall tumor control in combination therapies *in vivo*. Moreover, future research needs to elucidate whether differences in surface expression levels of PD1–CSR are, for instance, due to the transduction method and transduction efficacy or reflect different functional states of pNK cell subsets.

Finally, we transduced primary expanded NK cells, obtained from PBMC of MM patients with both PD1-DAP10 and PD1-DAP12 constructs. We tested their ability to increase degranulation and cytokine secretion against autologous BMNC. Of the three donors tested, only donor one showed a detectable level of PD-L1 surface expression by flow cytometry on live CD138+ malignant plasma cells (**Paper I, Fig. 5A, Fig. S10A**). Degranulation and cytokine secretion by PD1-DAP10+ and PD1-DAP12+ autologous pNK cells from donor one increased by three to four-fold against the autologous BM MNC (**Paper I, Fig. 5B-E**). Degranulation by PD1–CSR+ pNK cells was not increased against autologous BM MNC from the other two donors where no significant PD-L1 expression was detected. However, the expression of IFNγ and TNF was increased in donor two (**Paper I, Fig. 5F–I**) and decreased in donor three (**Paper I, Fig. 5J–M**). This might be due to the expression of both PD-L1 and PD-L2 on other cells within the TME. However, due to limitations of clinical material we could only analyze PD-L1 and PD-L2 expression in donors one and two and not in donor three (**Paper I, Fig. S10**). While both PD-L1 and PD-L2 could be detected on BM MNC from donor one, only low levels of PD-L2 were detected on BM MNC from donor two (**Paper I, Fig. S10B**).

In summary, our study confirms that PD1-based CSR with NK cell–specific signaling domains are stably expressed in NK92 cells and pNK cells from both healthy donors and patients with MM and increase NK cell functionality against PD-L1+ target cells and autologous BM MNC. Most importantly, this is the first published study on NK cell specific PD1-CSR in pNK cells from healthy donors and patients with MM which demonstrates feasibility of clinical implementation.

Discussion and significance

The immune checkpoint receptor PD1 and its ligands PD-L1 and PD-L2 were discovered in the 1990s [36, 339]. PD1's function as an inhibitory receptor has been widely studied in T cells where PD1 is overexpressed during T cell activation and prevents autoimmunity and a prolonged inflammatory response [38, 340]. Targeting the PD1 and PD-L1/L2 axis with mAb marked a breakthrough in cancer immunotherapy [341]. Since then, PD1 expression has also been studied in other cell types such as NK cells. Several studies showed that

PD1 surface expression on PB-NK cells from healthy individuals is low and only expressed by certain subsets of PB-NK cells such as CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ and CD56^{dim}CD57$^+$NKG2C$^+$ NK cells from CMV$^+$ individuals [129, 130, 337]. However, PD1 mRNA and protein expression were detected intracellularly in the majority of PB-NK cells from both healthy donors and cancer patients independent of surface PD1 expression [127]. The factors governing its surface expression have not yet been fully elucidated and remain controversial throughout different studies [127]. It seems that chronic stimulation and certain cytokines such as IL12, IL15 and IL18 play a role in PD1 surface expression on NK cells [130, 134, 342]. More recently, it was suggested that pre-formed PD1 that is stored in granzyme B$^+$ granules was rapidly mobilized to the cell surface after target cell contact and degranulation, but not due to IL15 stimulation alone [343]. PD1$^+$ NK cells exhibited reduced effector functions against PD-L1$^+$ cancers which could be reverted by adding pembrolizumab, confirming that PD1 has an inhibitory function in NK cells [343]. Furthermore, it was shown that murine NK cells acquired PD1 from PD1 expressing leukemia cells by trogocytosis in a SLAM receptor dependent manner [344]. In line with previous findings, we found that only a small fraction of PB-NK cells expressed PD1 on their cell surface. Moreover, PD1$^+$ NK cells expressed lower levels of CD107a and the cytokines IFNγ and TNF after contact with PD-L1$^+$ target cells which is in accordance with an inhibitory function of PD1 in NK cells. In contrast, we did not observe an increase in PD1 expression after degranulation or contact with target cells during short-term co-culture. Also, in our study, pNK cells from patients with MM did not show a higher expression of PD1. Although some studies found that NK cells from certain patients with cancer, such as MM and renal cell carcinoma do increase PD1 surface expression, data on this varies [28, 345]. In line with our observation, another study did not confirm an increased expression of PD1 on NK cells from patients with MM [346]. A possible explanation for varying results could be different previous lines of MM treatment and disease duration affecting both the NK cell and the malignant plasma cell phenotype. For instance, glucocorticoids that are often used in the treatment of patients with MM induced PD1 expression on MM cells *in vitro* [342]. Additionally, the IMiD lenalidomide was shown to increase PD-L1 surface expression on MM cells while BCMA-targeting drugs reduced the expression of surface PD-L1 on malignant plasma cells [347].

It is conceivable that CSR could be generated based on other inhibitory receptors or employed in combination to target multiple inhibitory pathways in the TME. We have, for instance, observed that PD1-CSR^{high} cells from one of the MM patients expressed less IFNγ against autologous BM MNC but we were not able to elucidate the reasons behind this inhibition. Other immunosuppressive soluble or receptor-mediated pathways might play a role. NK cells do express a plethora of targetable inhibitory receptors such as NKG2A, TIGIT, and TIM3 [139, 348-350]. NKG2A recognizes the non-classical HLA-I molecule HLA-E and has become of interest for cancer immunotherapy. For instance, monalizumab, an NKG2A-targeting mAb is currently being investigated in several clinical cancer trials [114,

351]. Moreover, CRISPR–based editing of NKG2A improved the anti–myeloma activity of NK cells in a pre–clinical murine cancer model [352]. Among the immune checkpoint inhibitors, the role of TIGIT in NK cells is so far the best characterized as it shares the same ligands as the activating receptor DNAM–1, namely CD112 and CD155. Engagement of TIGIT on NK cells leads to NK cell dysfunction which can be restored with blocking antibodies against TIGIT [123]. Recently, it was reported that NK cells from patients with NDMM overexpressed TIGIT due to chronic stimulation via CD155 expressing BM derived mesenchymal stem cells and showed a lower degranulation and cytokine secretion capacity [353]. However, so far, published studies on CSR for NK cell–based immunotherapies only focused on the PD1 and PD–L1/L2 pathway [282–284]. Further studies to evaluate the benefit of CSR based on other inhibitory receptors on NK cells and possible combinations are needed.

Previous studies on PD1–based CSR in NK cells have focused merely on DAP10 signaling, either via NKG2D mediated recruitment of DAP10 adaptor proteins or the full–length DAP10 signaling domain. These studies have additionally employed 4–1BB as a co–stimulatory domain [282–284]. In contrast, our study is the first one to compare DAP10, DAP12 or CD3ζ signaling, using not only primary tumor samples as opposed to cell lines, but also healthy donor and patient–derived NK cells as effector cells. It was shown that PD1–NKG2D–41BB NK92 cells increased lysis of PD–L1$^+$ target cells but did not induce cytokine secretion upon PD–L1 engagement [282, 283]. In contrast, PD1–DAP10–41BB CSR NK92 cells induced higher killing of PD–L1$^+$ target cells and showed an increased secretion of IFNγ but not TNFα [284]. It can only be speculated upon why DAP10 but not NKG2D based CSR increased cytokine secretion in NK92 cells, given that one NKG2D dimer can recruit two DAP10 adaptor proteins [199]. Besides differences in experimental setup, the availability of DAP10 adaptor molecules to associate with NKG2D might play a role. For instance, NK92 cells only express low levels of NKG2D and might therefore not have enough preformed DAP10 adaptor proteins available for sufficient signaling via the transduced CSR [354]. We observed that PD1–DAP10$^+$ NK92 cells increase killing of PD–L1$^+$ 786–O cells, but not the secretion of IFNγ and TNFα, while they do increase cytokine secretion against PD–L1$^+$ Raji cells. On the other hand, the PD1$_{Ec}$DAP10$_{TM–IC}$$^+$ NK92 cells showed an opposite increased cytokine secretion against 786–O cells but not Raji cells. Both DAP12–based CSR, namely PD1–DAP12$^+$ and PD1$_{Ec}$DAP12$_{TM–IC}$$^+$ NK92 cell lines had a robust and strong increase of cytokine secretion and target cell killing upon PD–L1 engagement. In the context of pNK cells, both PD1–DAP10$^+$ and PD1–DAP12$^+$ NK cells showed a consistently high increase in cytokine secretion against PD–L1$^+$ target cells, with a tendency of higher IFNγ and TNFα expression by PD1–DAP12$^+$ NK cells. It is therefore conceivable, that the optimal signaling domain for CSR or CAR constructs does depend on the NK cell source and differs between PB–NK cells, NK cell lines or other NK cells. Further studies to evaluate optimal signaling domains for NK cell–based CSR or CAR constructs are needed. To date, the majority of CAR–NK cell clinical trials uses CD28, CD3ζ

and 4-1BB as signaling domains [355]. However, pre-clinical studies showed promising results with CAR-NK cell products, employing DAP10 or DAP12 as co-stimulatory domains [254, 356].

While CAR-T cell therapies have shown breakthrough success in the clinical treatment landscape of hematological malignancies, their applicability for the treatment of solid tumors remains poor [238, 239, 357]. Therefore, PD1-based CSR have been developed in an attempt to prevent PD1-mediated T cell inhibition in the TME of solid tumors and enhance the efficacy of CAR-T cells [277]. Particularly, they pose the advantage of an additional activating signal that promotes T cell proliferation, while approaches using PD1-KO CAR-T cells showed impairment of T cell function [275]. Clinical trials comparing PD1-KO or PD1-CSR based CAR-T cell therapies are needed in the future. So far, CD19-CAR[+] PD1-CD28-CSR^{+} T cells have shown good clinical response with acceptable toxicity for 17 adult patients with PD-L1[+] B cell lymphoma [281]. Specifically, CD19-CAR[+] PD1-CD28 CSR^{+} T cells showed superior proliferation and effector cell functionality compared to CD19-CAR^{+} T cells, but did not lead to a higher degree of CRS or ICANS in human patients [281]. Whether PD1-based CSR or CAR would, however, increase systemic toxicity in patients, given the abundant expression of PD-L1 and PD-L2, is unclear. Preclinical studies with PD-L1 targeting high-affinity NK-92 cells (PD-L1-t-haNK) showed promising results in murine cancer models and are currently in early phase clinical trials (NCT04050709, NCT04847466, NCT04927884) [358]. Clinical data regarding the toxicity profile and efficacy from prospective trials are eagerly awaited.

Summary

We have genetically modified NK cells to increase effector cell functionality upon PD1 engagement. This study contributes to current knowledge by affirming that, although expressed at low levels in pNK cells, PD1 acts as an inhibitory receptor on NK cells. Abrogating native PD1 signaling restores NK cell degranulation and target cell killing. This can be done by introducing a CSR with a positive signaling as exemplified in this study. Compared to previous work, our study contributes to current knowledge by testing and comparing different signaling domains in two different NK cell sources, namely the NK-92 cell line and PB-NK cells from both healthy donors and patients with MM. This work is important in order to improve NK cell-based therapies with chimeric receptors that are optimized in their signaling domains for the specific cell type. However, our work has been performed *in vitro* and future work needs to focus on evaluating efficacy and toxicity *in vivo*.

4.2 Paper II

Challenges in αCD38–CAR–NK cell–based immunotherapy in multiple myeloma: Harnessing the CD38^{dim} phenotype of cytokine–stimulated NK cells as a strategy to prevent fratricide

Background

Targeting CD38 with the mAb daratumumab and isatuximab has become an integral part of MM treatment. Despite their overall clinical success, however, treatment failures and resistances occur. The overall response rate of daratumumab monotherapy for patients with RRMM ranges between 30% and 40% [359]. While a higher surface expression of CD38 on MM cells is associated with a better response, a reduction of CD38 surface expression on MM cells occurs in both responders and non–responders [360]. Thus, other resistance mechanisms must exist. One of them is the reduction and dysfunction of effector immune cells [361]. As previously mentioned, the effects of daratumumab highly depend on ADCC which is mainly mediated by NK cells [16]. Daratumumab monotherapy significantly reduces the number of NK cells due to fratricide; although no clear correlation to clinical efficacy could be drawn [362]. This was explained by the persistent cytotoxic functions of remaining CD38^{low}/CD38$^-$ NK cells capable of ADCC [362]. In line, MM patients who receive daratumumab have a higher risk for infectious complications, partially due to NK cell depletion [363]. Adoptive transfer of NK cells in combination with daratumumab was implied to yield better clinical efficacy [361, 364].

The overall clinical success of anti–CD38 mAb therapy underlies the development of CD38–based CAR constructs. Promising pre–clinical results with anti–CD38–CAR–T cells led to the initiation of early phase clinical studies in patients with different CD38$^+$ hematological malignancies (NCT03464916) [365, 366]. However, due to the ubiquitous expression of CD38, this approach harbors the risk of severe side effects. CAR–NK cells on the contrary pose an alternative cell source with reduced risk of severe toxicity and potential to exert ADCC. This approach is therefore tested in several pre–clinical and clinical trials with CD38-KO NK cells in combination with CD38-CAR constructs and/or daratumumab [367-370].
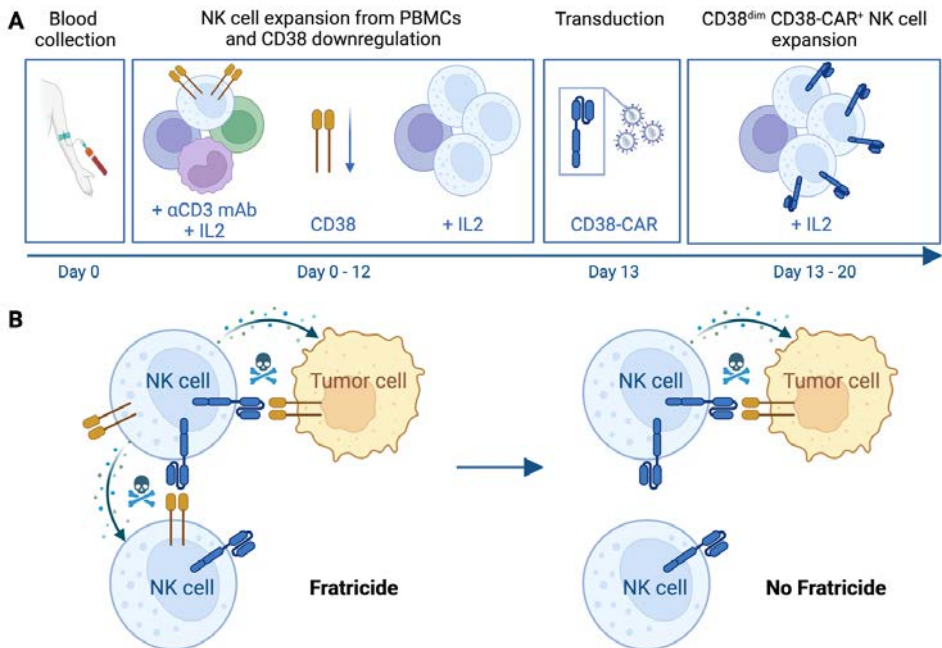
FIGURE 5 Graphical abstract for study II. A) Overview of pNK cell expansion and transduction protocol. PBMC were collected from healthy donors or patients with MM by venous puncture and isolated with a density gradient medium. Stimulation with an anti-CD3 mAb on the day of isolation and continuous supplementation of IL2 led to an expansion of pNK cells with low CD38 expression. Retroviral transduction of pNK cells with the CD38-CAR was performed on day 13. Cells were thereafter further expanded and employed for experimental assays. **B)** Donwregulation of CD38 on pNK cells reduces the risk of fratricide by CD38-CAR^{+} pNK cells. Image created with Biorender by the author.

Aim of the study

In order to develop an alternative CD38-targeting therapy with good efficacy and minimal risk of severe side effects, we explored the feasibility of NK cells expressing an affinity-optimized CD38-CAR construct that targets a distinct epitope than daratumumab for the treatment of MM. To minimize the risk of fratricide, CD38^{dim} NK cells, generated without genetic engineering in a feeder-free expansion protocol, were employed.

Results

In **study II**, we have employed a feeder-free NK cell expansion system together with an optimized retrovirus-based transduction protocol to obtain a fratricide resistant highly cytotoxic CD38^{dim} CD38-CAR^{+} NK cell product with good activity against CD38^{+} MM cells.

The CD38-CAR construct is an affinity optimized 2nd generation CAR which consists of a CD38-specific scFv, named CD38A2, the co-stimulatory domain CD28 and the

intracellular signaling domain CD3ζ that was provided to us by our collaborators (**Paper II, Fig. 1A,**). CD38A2 recognizes a different epitope than daratumumab. After successful transduction, CD38-CAR$^+$ NK92 cells significantly increased degranulation measured by CD107a expression, cytokine production and killing of the CD38$^+$ MM cell lines RPMI8226 and MM1S (**Paper II, Fig. 1B–G, Fig. S1**).

Next, we aimed to translate our findings to pNK cells and used a GMP-compliant feeder-cell-free NK cell expansion protocol that is based on long-term IL2 stimulation and has been already employed in a clinical trial with good efficacy and safety [336]. Phenotypic analyses revealed a gradual decrease of CD38 on NK cells during the course of expansion without impacting the ability of NK cells to degranulate or kill target cells (**Paper II, Fig. 2, Fig. S3**). Based on these results, we reasoned that a transduction of NK cells on day 13 of the expansion protocol provided a good timepoint with low CD38 expression, good viability and cytotoxicity as well as optimal transduction efficacy (**Paper II, Fig. 3A**). A retrovirus-based transduction system was employed to generate CD38-CAR$^+$ pNK cells. Functional testing revealed a high degranulation and killing ability with increased pNK cell proliferation against the CD38$^+$ target cell lines RPMI8226 and MM1S (**Paper II, Fig. 3B–E, Fig. S5**).

Due to the encouraging results obtained from healthy donor pNK cells, we moved on to test our expansion and transduction system in pNK cells from patients with MM against autologous BM MNC. These BM MNC comprised approximately 2–20% of malignant CD138$^+$ plasma cells with varying expression of CD38 (**Paper II, Fig. 4A**). Although CD38 downregulation did not follow the same kinetics observed with healthy donor pNK cells, transduction efficacy and NK cell expansion were sufficient (**Paper II, Fig. 5A–C**). Most importantly, CD38-CAR$^+$ pNK cells significantly increased degranulation and cytokine expression against BM MNC (**Paper II, Fig. 5D–E**).

All in all, our data confirm that CD38^{dim} CD38-CAR$^+$ NK cells confer a high cytotoxic activity against CD38$^+$ MM cells. Importantly, CD38^{dim} NK cells can be generated without additional gene editing through an optimized GMP-compliant feeder-cell free expansion system.

Discussion and significance

As mentioned before, CD38 targeting with daratumumab resulted in good clinical responses in patients with both RRMM and NDMM but is also met with resistance to therapy and relapses. Therefore, new CD38 targeting therapies are needed.

Given the overall success of genetically modified immune cells, CD38-CAR therapy might be an alternative treatment approach that can also be applied to other diseases with high CD38 expression such as acute myeloid leukemia (AML) or ALL [366, 371]. However, the ubiquitous expression of CD38 also results in higher risk for severe side effects. In a recent case report, a patient with relapsed B-ALL was given CD38-CAR$^+$ T cells and experienced

severe CRS [371]. In another trial performed on patients with RRMM a dual-targeting anti-BCMA anti-CD38 CAR-T cell was tested, and toxicities were reported to be mild and manageable [372]. Other clinical trials are currently ongoing and their results eagerly awaited (NCT03464916). Nevertheless, the general notion is that optimization of the CD38-CAR is needed to increase safety of CAR-T cell therapy [373].

CAR-NK cells pose an alternative to CAR-T cells with a generally better safety profile and are currently tested as immune cell sources for CD38-CAR therapy. For instance, primary CD38-KO CD38-CAR^{+} NK cells efficiently increased killing of BM MNC from AML patients [367]. Although data from clinical trials with CD38-CAR-NK cells are lacking, the combination of CAR-NK cells and daratumumab has recently been tested in a phase I clinical trial in patients with RRMM [374]. The presented interim results of the tested NK cell product FT576 suggest a good safety profile with no reported CRS or ICANS in a total of nine patients that were treated, encouraging the further exploration of this approach. FT576 and another NK cell product, named iDuo-MM CAR iPSC, currently tested for the application in MM, are derived from pluripotent stem cells and harbor several genetic modifications such as the insertion of a BCMA-CAR to target MM cells, a high-affinity non-cleavable CD16 variant for improved ADCC, a recombinant IL15 molecule and the genetic knock-out of native CD38 to prevent daratumumab mediated NK cell fratricide [256, 369]. These cells showed a high and specific tumor cytotoxicity and cytokine production in *in vitro* and *in vivo* tumor models. Given the important role of CD38 in signal transduction and proliferation, complete deletion of its expression might, however, cause unwanted NK cell impairments which can be masked with multiple gene-engineering approaches. For instance, CD38 was shown to play a role in immune synapse formation and conjugation between NK cells and target cells [375]. Blocking CD38 on NK cells thus resulted in a reduced cytotoxicity against virus-infected and malignantly transformed cells. In another study, CD38 KO NK cells showed resistance towards daratumumab mediated fratricide, a longer persistence in daratumumab treated *in vivo* murine models as well as metabolic changes which favored oxidative phosphorylation and cholesterol synthesis [364]. However, the direct effect of these metabolic changes on NK cell cytotoxicity and proliferation were not further investigated and the study did not provide data on CD38 KO NK cell cytotoxicity and degranulation capacity in the absence of daratumumab. Another study, however, showed that CD38 KO NK cells did not differ phenotypically from WT NK cells and showed the same level of proliferation, degranulation and cytokine production when in co-culture with target cells [376]. Thus, despite the known role of CD38 in different cellular and metabolic processes, its deletion or downregulation in genetically modified NK cells does not seem to impair functionality but instead augment cytotoxicity, especially in the context of MM and daratumumab. This is underlined by another study confirming that CD38^{-/low} NK cells from healthy donors or daratumumab treated MM patients displayed a higher cytotoxic potential against MM cells [377].

A direct comparison between CD38 KO and CD38^{dim} NK cells regarding their cytotoxic ability as well as proliferative and cytokine release capacity is currently lacking. Given the multi-faceted roles of CD38 and its function not only as a surface receptor but also an intracellular protein, might motivate the preference of CD38^{dim} cells over CD38 KO cells. Moreover, no CRISPR-based therapy has yet been approved for clinical use and remains in early phase clinical evaluation [378]. Although preliminary results with CRISPR-engineered adoptive immune cells look promising, their long-term safety and clinical risk are uncertain [273, 379]. However, the majority of NK cell engineering approaches for the treatment of MM employ a genetic KO of CD38. This is also based on the fact that feeder-cell based expansion systems for NK cells were used where a high upregulation of CD38 during culture is observed [367, 376]. Generally, feeder-cell based expansion protocols form the basis of most clinical and pre-clinical trials as they lead to robust and efficient NK cell expansions with a favorable *in vitro* phenotype and functionality. However, feeder-cell free expansion systems that primarily rely on cytokines are under development. They pose the advantage of easier regulatory approval and adaptability to GMP-compliant systems as the risk of infusing potentially viable feeder cells is omitted. In this study, we have employed a feeder-cell free expansion system that was utilized as a basis for a recently published clinical trial as mentioned before [336, 380]. The natural downregulation of CD38 during this expansion poses an advantage due to the reduced risk of fratricide after introduction of the CD38-CAR construct and the omission of additional genetic modifications. Most importantly, our clinical trial showed that CD38 remains downregulated shortly after infusion of the NK cells into the patient, emphasizing the clinical translation of this approach. Further clinical studies to investigate the feasibility and translation of CD38^{dim} CD38-CAR$^+$ NK cells are warranted.

Summary

Here, we have employed an NK cell expansion and transduction protocol to generate fratricide resistant CD38^{dim} CD38-CAR$^+$ NK cells for the treatment of MM. In line with previous studies, expanded NK cells do sustain their cytotoxicity and cytokine production capacity despite the downregulation of CD38. Importantly, CD38^{dim} CD38-CAR$^+$ NK cells were generated based on a novel CD38-CAR construct that targets a different epitope compared to daratumumab. Given the good *in vitro* efficacy and specificity, further evaluation of this approach is warranted. Future studies should focus on evaluating the *in vitro* and *in vivo* potential for daratumumab resistant or naïve MM patients. Together with the encouraging data from **study I**, a multiplex targeting approach is conceivable with CD38^{dim} CD38-CAR$^+$ PD1-CSR$^+$ NK cells with or without the addition of daratumumab for sustained and elevated NK cell responses against MM.

4.3 Paper III

The oncolytic virus HSV1716 infects and activates natural killer cells via direct cell-to-cell contact

Background

OV have entered the stage as immunotherapeutic tools since the FDA-approval of the HSV-1-based OV T-vec in 2015 [381]. Although the field of oncolytic virotherapy is developing fast with several launched clinical trials in recent years, T-vec therapy is still restricted to local injections in patients with malignant melanoma and only few new OV have been clinically approved. Nevertheless, the OV platform remains an attractive immunotherapeutic tool due to a wide range of possible genetic modifications and combination therapies. In order to improve clinical application of OV-based immunotherapies, a better understanding of the interaction with the immune system is necessary. NK cells have been, for instance, implicated in anti-HSV-1 mediated immunity [322, 323]. However, controversy exists whether they are favorable or not in combination with OV. As an example, NK cells increased killing of HSV-1 OV infected malignant plasma cells, resulting in improved tumor control in one study, while another implied NK cells in increased killing of HSV-1 OV infected glioma cells which limited the efficacy of OV therapy and tumor control [331, 382]. Elucidating the interplay between NK cells and HSV-1 OV might help to shed light on these opposing results and help to define potential combination therapy approaches.

Aim of the study

We sought to understand how a clinically applied HSV-1-based OV, namely HSV1716, stimulates the effector functions of pNK cells in order to enable clinical translation of HSV-1 OV and adoptive NK cell therapy approaches. To date, the combination of HSV-1 OV and NK cells in a clinical setting is challenging as not much is known about their interaction. Therefore, extensive phenotypic and functional characterizations of both infected target cells and co-cultured pNK cells at different time points were performed.
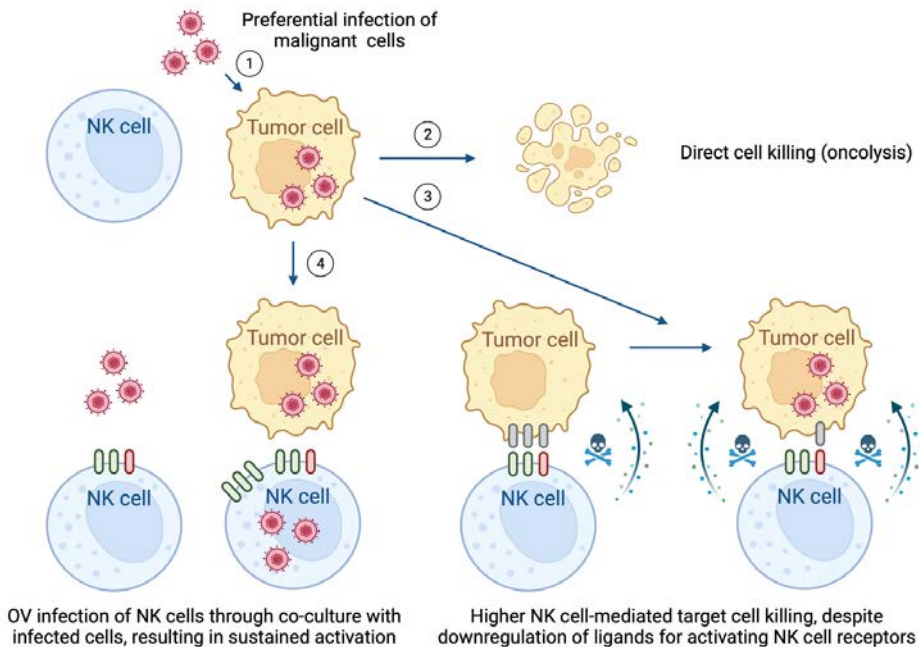
FIGURE 6 Graphical abstract for study III. HSV1716 preferentially infects malignantly transformed cells (1) and leads to oncolysis (2) and phenotypic changes such as downregulation of main ligands for activating NK cell receptors (3). Despite this, NK cells increase degranulation and cytokine release towards infected tumor cells (3). NK cells become infected with HSV1716 during direct co-culture with infected target cells, but not through viral inoculum alone, resulting in an increased and sustained expression of activation markers and DNAM-1 (4). Image created with Biorender by the author.

Results

In **study III**, we show that HSV1716 induces the downregulation of ligands for main activating NK cell receptors on several different human tumor cell lines. Despite this, pNK cells enhance their functional responses in terms of degranulation and cytokine release towards HSV1716-infected target cells. Most interestingly, pNK cells become infected with HSV1716 during co-culture with infected target cells and display a higher and sustained activation status.

First, the ability of HSV1716 to infect and lyse the tumor cell lines RPMI8226, MM1S, 786-O and SKOV3 was assessed. HSV1716-infected all human tumor cell lines and led to oncolysis and phenotypic changes, especially regarding the surface expression levels for known ligands of NKG2D and DNAM-1. The MM cell lines RPMI8226 and MM1S particularly showed a higher susceptibility to infection compared to 786-O and SKOV3 cells (**Paper III, Fig.1A-D, Fig. S1A**). While infected target cells generally remained HLA-A,B,C positive, a small but significant decrease in surface expression was observed in RPMI8226 and SKOV3 cells, but not MM1S or 786-O cells over a time period of 72 hours (**Paper III, Fig. 1E-**

H, Fig. S1B,C). The surface expression of the NKG2D ligand MICA/B decreased on all tested cell lines, while ULBP2,5,6 downregulation was observed in the adherent cell lines 786-O and SKOV3 (**Paper III, Fig. 1E–H**). Moreover, CD112 surface expression increased on RPMI8226 and MM1S and decreased on 786-O and SKOV3, while CD155 expression decreased on all infected target cells except SKOV3 (**Paper III, Fig. 1E–H, Fig. S1B,C**). It is important to mention that CD112 (nectin-2) is not only a ligand for DNAM-1 and TIGIT, but also an entry receptor for HSV-1, which might be reflected by differences in CD112 expression on infected versus uninfected cells.

Next, the degranulation ability of pNK cells against HSV1716-infected target cells was analyzed. pNK cells that were co-cultured for four hours with previously infected target cells increased degranulation and cytokine secretion by two- to three-fold compared to culture with non-infected target cells (**Paper III, Fig. 2A,B**). In accordance with their higher activation status, the expression of CD16 and NKp46 decreased while NKp44 surface expression increased on pNK cells degranulating against virally infected target cells (**Paper III, Fig. 2C–F**). Moreover, the expression of LAG3 showed a decrease in CD107a$^+$ pNK cells degranulating against HSV1716-infected target cells (**Paper III, Fig. 2C–F**). The surface expression of other main activating and inhibitory receptors remained largely unaltered between NK cells degranulating against mock-infected or HSV1716-infected target cells (**Paper III, Fig. 2C–F**).

Given the strong activation of pNK cells within a short-term co-culture with infected target cells and the decrease of several ligands for activating NK cell receptors, we were wondering whether other, for example soluble factors might play a role. To investigate this further, we co-cultured pNK cells in conditioned media alone or in combination with uninfected or infected target cells for 4, 24, 48 and 72 hours. An HSV1716 virus that encodes the red fluorescent protein dsRed was employed in these studies. Interestingly, we observed dsRed expression in pNK cells after 24 hours of co-culture with infected target cells, allowing the assumption that pNK cells got infected with HSV1716 (**Paper III, Fig. 3A,B**). Expression of dsRed in pNK cells was also observed when conditioned medium from virally infected target cells was added to a co-culture of fresh uninfected RPMI8226 or MM1S and pNK cells (**Paper III, Fig. 3B**). However, culture of pNK cells in the conditioned media of infected target cells alone did not lead to an increase of virally encoded dsRed with the exception of conditioned media from RPMI8226 (**Paper III, Fig. 3B**). Moreover, direct infection of pNK cells with HSV1716 at high MOIs did not result in dsRed expression, indicating that HSV1716 might infect pNK cells via direct cell-to-cell transfer (**Paper III, Fig. S3**) Infected dsRed$^+$ NK cells showed an increased and sustained expression of the activation markers CD69 and CD25 and a corresponding decrease of CD16, all signs of activation (**Paper III, Fig. 4, Fig.5C–F, Fig. S5A–D**). Interestingly, the expression of DNAM-1 was also increased on dsRed$^+$ pNK cells, while there were no changes in NKG2D expression levels (**Paper III, Fig. 4, Fig. 5C–F, Fig. S4, Fig. S5A–D**).

All in all, we demonstrate that pNK cells increase their degranulation and cytokine secretion capacity against HSV1716-infected target cells despite the downregulation of important ligands for activating NK cell receptors. After direct cell-to-cell contact with infected target cells, but not in the presence of viral inoculum, pNK cells start to express virally encoded dsRed, suggestive of a direct transfer of viral vectors between target and pNK cells. Importantly, HSV1716-infected pNK cells display a higher and sustained expression of activation markers and DNAM-1.

Discussion and significance

NK cells have been implicated in mediating immunity against herpesviruses since patients who lack NK cells have a higher risk for severe and recurrent herpesvirus infections [383]. However, herpesviruses are also characterized by their ability to establish latency and cause immunoevasion, a process aimed at suppressing viral detection and clearance by immune cells such as NK cells. This is often achieved by downregulating ligands for activating NK cell receptors, for instance, NKG2D and DNAM-1.

Nearly all herpesviruses have been shown to downregulate NKG2D ligand expression [384]. MICA/B and the ULBPs are often referred to as "stress-induced ligands" since they are rarely detected on healthy cells, but upregulated upon DNA damage response in malignantly transformed or virally infected cells. For instance, late viral gene products from HSV-1 were able to downregulate MICA and ULBP2 surface expression on HeLa cells through intracellular retention [304]. Another study performed on ARPE-19 cells confirmed a downregulation of both surface expression and total protein level of MICA, ULBP2 and ULBP3 upon HSV-1 infection [385]. Interestingly, although a decrease in surface expression of ULBP1 was also observed, total protein levels did not differ between infected and non-infected cells, suggesting that HSV-1 might use different mechanisms for NKG2D ligand downregulation [385]. However, the exact cellular mechanisms leading to a downregulation of MIC and ULBP receptors by HSV-1 are not yet fully elucidated. It has been shown that the viral microRNA miR-H8, encoded by HSV-1, interferes with the surface expression of ULBP2 and ULBP3, but not MICA/B or ULBP1 [386]. In the case of the beta-herpesvirus CMV, the expression of UL16 and UL142 have been implicated in surface retention of MICA/B and ULBP1,2,3,6 but a correlate protein has not yet been identified for HSV-1 [387-390]. Our study supports previous findings as we observed a downregulation of MICA/B and ULBP2,5,6 receptors in different cell lines upon HSV1716 infection.

The modulation of the DNAM-1 ligands CD112 and CD155 is differently regulated by different herpesvirus families and strains. The expression of UL141, encoded by CMV, leads to a downregulation of CD155 and CD112 surface expression and protection from NK cell-mediated lysis [391, 392]. In the case of the alpha-herpesviruses PRV and HSV-2, the expression of gD reduced the expression of CD112, but not CD155, in a process involving acidification and degradation of CD112 [305]. This resulted in reduced binding of DNAM-1

and reduced NK cell-mediated killing of virally-infected or gD-transfected target cells. Interestingly, UL141 and gD do not share sequence homology, suggesting that herpesviruses have indeed evolved different mechanisms of interfering with DNAM-1 ligands to circumvent NK cell effector functions, emphasizing that DNAM-1 plays an important role in NK cell-mediated targeting of virally infected cells. However, gD from HSV-1 does not display a similar affinity for CD112 as PRV or HSV2 [393]. In line with this, the oncolytic HSV-1 strain 34.5ENVE did not alter the surface expression of CD112 or CD155 in glioma cells [325]. Our study shows that HSV1716 infection leads to a higher expression of CD112 on the MM cell lines RPMI8226 and MM1S and a decrease of CD112 on the renal cell carcinoma cell line 786-O and the ovarian cancer cell line SKOV3. Importantly, CD112 is also known as nectin-2 and is not only a DNAM-1 ligand but also serves as a receptor for HSV-1 entry [394]. Thus, the upregulation of CD112 might reflect a population sensitive to HSV1716 infection. CD155 surface expression was downregulated on all the tested cell lines infected with HSV1716 over a period of 72 hours. Further studies investigating the impact of HSV-1 infection on the expression of CD112 and CD155 are unfortunately lacking. However, it can be speculated that HSV-1 uses different mechanisms to modulate CD112 and CD155 surface expression, probably dependent on the infectious cycle as well as the type and cellular state of the host cell. It should be emphasized that CD112 and CD155 are also ligands for the inhibitory receptor TIGIT. HSV-1 and other herpesviruses might have adapted different strategies to modulate the DNAM-1/TIGIT axis to favor NK cell inhibition and immunoevasion during active or latent infection. It was shown that CD112 and ULBP1 surface expression increased upon EBV reactivation and were important in augmenting NK cell-mediated lysis of productively infected EBV^{+} B cells [395].

Given the strong activation of NK cells towards HSV1716-infected target cells, we also investigated the expression of HLA-A,B,C receptors. Generally, MHC-I downregulation leads to increased NK cell cytotoxicity due to release of inhibitory NK cell signaling. It has been shown, that ICP47, encoded by HSV-1, interferes with the transport of MHC-I to the plasma membrane and thus leads to NK cell cytotoxicity of HSV-1 infected cells *in vitro* [303, 396, 397]. However, we did not observe a marked decrease of HLA-A,B,C receptors upon HSV1716 infection in the four cell lines tested. In accordance, other studies have not observed a marked HLA-I downregulation as a trigger of NK cell cytotoxicity against HSV-1 infected cells [398]. Earlier studies showed that HSV-1 infected HLA-I-deficient cell lines were killed at a higher level than non-infected HLA-I-deficient cells [399]. However, expression of HLA-I even increased killing of HSV-1 infected target cells, suggesting a role of HLA-I in regulating NK cell-mediated killing of virally infected cells [399]. Indeed, certain KIR haplotypes have been associated with the severity of HSV-1 infection and other viral diseases in humans [400, 401]. Thus, not the overall downregulation of HLA class I molecules but alterations in HLA-I and KIR phenotype seem to play a role in regulating NK cell responses to HSV-1.

Furthermore, we investigated the phenotype of NK cells degranulating against HSV1716-infected versus non-infected cells. NK cells that were co-cultured with virally infected target cells showed a higher expression of CD107a, IFNγ and TNF with a corresponding activation-induced decrease of CD16 and NKp46. In line with the activation status, a higher expression of NKp44 was observed on pNK cells, co-cultured with virally infected target cells. NCR, especially NKp44 and NKp46, have been previously implicated in recognition of viral infection [402, 403]. For instance, the immediate early protein ICP0, encoded by HSV-1, was shown to be important in activating NK cells towards HSV-1 infected target cells [398]. Specifically, it was suggested that ICP0 triggered expression of ligands for NCR that were identified as the main NK cell receptors involved in HSV-1 recognition. In the context of OV, it was demonstrated that NK cells were rapidly activated by HSV-1 OV infected target cells and led to a strong killing of tumor cells and thus impairment of viral spread within the tumor [331]. This was mediated through increased expression of ligands for NKp30 and NKp46 on infected target cells. Blocking of either NKp30 or NKp46, but not DNAM-1 or NKG2D, significantly decreased the killing of HSV-1 OV infected glioma cell lines. The role of NKp44 was not investigated in this study. It has to be added that the strong downregulation of CD16 that we have observed can – additionally to activation – also be mediated through engagement of CD16 by viral receptors such as the glycoproteins. Especially, the gE/gI complex has been shown to bind immunoglobulins at a distinct site than CD16, leading to antibody-bipolar bridging and NK cell activation [328] .

We questioned whether soluble factors could explain the increased NK cell activation towards HSV1716-infected target cells. However, conditioned media harvested from infected target cells – with the exception of RPMI8226 - did not increase the activation status of pNK cells; neither did viral inoculum. NK cells needed to be in direct contact with infected target cells to show virally encoded dsRed expression. This suggests that HSV1716 can be transferred to pNK cells via direct cell-to-cell contact. However, a direct infection of pNK cells due to very high viral titers present in conditioned media from e.g., RPMI8226 cells or due to an upregulation of HSV-1 entry receptors cannot be excluded. Based on our data, we assume that a direct cell-to-cell transfer is possible, but it remains unclear whether this is mediated through certain receptors or through other, for instance, endocytic processes such as macropinocytosis [404, 405]. Although HSV-1 entry through viral glycoproteins and HSV-1 entry receptors is the most and best studied pathway, both a direct cell-to-cell spread via adhesion proteins or through endocytosis have been described for HSV-1 [293]. Similarly, wildtype HSV-1 and VZV were shown to productively infect pNK cells in a contact-dependent manner [406]. Both VZV and HSV-1 infected pNK cells displayed a lower degranulation and cytokine secretion against uninfected target cells as well as a decrease in CD69 expression [406]. This was due to the interference with intracellular signaling events by VZV, but not modulation of surface expression of activating receptors. In contrast, HSV1716-infected or exposed pNK cells were highly

activated after 24, 48 and 72 hours of co-culture, determined by the increase of CD69 and CD25 expression and decrease of CD16. Interestingly, HSV1716-infected pNK cells displayed a higher expression of DNAM-1, but not NKG2D. Whether this activation status also translates to higher CD107a expression, cytokine secretion or target cell killing must be determined. The differences observed in the activation status of pNK cells with either HSV1716 or wildtype HSV-1 might be due to the lack of ICP34.5, a protein known to activate protein phosphatase 1A, in HSV1716. However, studies evaluating the impact of HSV-1 infection of pNK cells or other primary immune cells are lacking. It is conceivable that viral proteins can interfere with intracellular signaling pathways. For instance, VP11/12 of HSV-1 was able to infect the human T cell line Jurkat and activate the PI3K/Akt pathway, although the effect on the cytotoxic potential of Jurkat cells was not investigated in this study [407]. In NK cells, the PI3K/Akt pathway is complex and plays an important role in mediating NK cell effector functions [408]. Because of its multi-faceted roles, it is often hijacked by herpesviruses to facilitate viral entry and replication [409]. Interestingly, early clinical studies with systemic or local administration of HSV1716 reported no viral shedding or viremia, but confirmed the presence of viral genes in blood PCR samples in some patients [318, 319]. However, neither the exact cell type harboring the OV nor the clinical impact were further investigated. Recently, it was shown that macrophages are sensitive to HSV-1 infection and, therefore, these cells were tested as possible carrier cells for delivery of HSV1716 to the TME in a murine breast cancer metastasis model [410]. Several pre-clinical and early phase clinical studies are currently ongoing to explore the feasibility of immune cells or mesenchymal stem cells as delivery vehicles for OV [411, 412]. Given that the seroprevalence of HSV-1 is approximately 70% in the adult population, the development of systemic delivery approaches for HSV1716 and other HSV-1 based OV is crucial for their broader clinical application [291]. This study shows that pNK cells which are currently in clinical trials for ACT can be loaded with HSV1716 and maintain an activated phenotype. Future research needs to address the feasibility of this approach.

Summary

We describe that pNK cells become infected with HSV1716, likely through direct contact with infected target cells and consequently increase their activation status. In line with previous work, this study confirms that HSV-1 infection leads to downregulation of ligands for activating NK cell receptors on target cells. Nevertheless, pNK cells increase their degranulation activity and cytokine secretion towards infected cells. Previous work has mainly focused on elaborating changes in receptor and ligand surface expression to analyze the interaction between HSV-1 and NK cells. However, we build on current knowledge by providing data that HSV1716 is able to infect pNK cells *in vitro* and change their activation status. Unlike the infection with wildtype HSV-1 or VZV this does not lead to paralysis of pNK cells. Future work needs to focus on the entry mechanisms involved

in HSV1716 infection of pNK cells as well as the effects of HSV1716 infection on intracellular signaling events in pNK cells and their biological significance *in vivo*.

5 Conclusions

Cell-based immunotherapy is currently changing the treatment landscape of several malignant diseases. Further improvements in therapy outcomes are anticipated through combination therapies that also take the TME into account. This thesis provides evidence that genetic modification of NK cells improves their effector cell functionality upon engagement of PD1 (**paper I**) and targeting of CD38 (**paper II**) as well as in combination with an HSV-1-based OV (**paper III**). Overall, this thesis presents original and novel findings that can be utilized to improve treatment options for patients with MM. Specific conclusions from the constituting papers are presented below:

Study I

o PD1-based CSR with transmembrane and/or intracellular domains of CD28/CD3ζ, NKp46, DAP10 and DAP12 can be succesfully expressed and are functional in NK92 cells and pNK cells from healthy donors and patients with MM.

o PD1-DAP10$^+$ and PD1-DAP12$^+$ NK92 and pNK cells increase target cell killing, degranulation and cytokine release upon PD1 engagement *in vitro*, both when in co-culture with different target cell lines and against tumor spheroids.

o PD1-DAP10 and PD1-DAP12 CSR augment pNK cell degranulation and cytokine release in combination with ADCC.

o PD1-DAP10$^+$ and PD1-DAP12$^+$ pNK cells from patients with MM show a higher degranulation and cytokine release against autologous PD-L1/L2$^+$ BM MNC *in vitro*.

Study II

o CD38 downregulation occurs naturally on NK cells during a feeder-cell free GMP-compliant expansion protocol that relies on long-term IL2 stimulation. This can be exploited as an alternative approach to genetic KO of CD38 in order to generate fratricide-resistant NK cells.

o *Ex vivo* expanded healthy donor derived CD38^{dim} CD38-CAR$^+$ NK cells show improved cytotoxicity and cytokine release against CD38$^+$ MM cell lines *in vitro*.

o CD38^{dim} CD38-CAR$^+$ NK cells that were *ex vivo* expanded from patients with MM show improved degranulation and cytokine release against autologous BM MNC *in vitro*.

Study III

o HSV1716 infects different tumor cell types *in vitro*, induces oncolysis and leads to phenotypic changes in the surface expression of main activating NK cell ligands.

o NK cells increase degranulation and cytokine release against HSV1716-infected target cells, despite the downregulation of main ligands for activating NK cell receptors.

- NK cells from healthy donors become infected with HSV1716 via direct cell-to-cell contact with tumor cells but not through viral inoculum.
- HSV1716-infected pNK cells from healthy donors display an activated phenotype with increased expression of CD69, CD25 and DNAM-1.

6 Future outlook

The recent years have shown major advancements in cancer treatment and survival, leading to an overall drop in mortality among all cancer types by 32% compared to the early 1990s [413]. This is partially due to elimination of risk factors and cancer prevention programs as well as improved diagnostics and overall better treatment options. The prognosis and survival for patients with MM follows this trend and has gradually increased over the last decades [414]. To date, there are available treatment options for MM patients that have progressed on several lines of standard treatment; one of them being BCMA-targeted CAR-T cells. Although CAR-T cells show an overall efficacy of more than 80%, they come with their own limitations and challenges such as a resource and time-consuming manufacturing process, high rates of severe adverse events and the need for personalized customization of conventional CAR-T cell therapy which increases overall treatment costs [415]. Current research efforts are therefore focusing on "off-the-shelf" immunotherapies. Recently, interim results of the UNIVERSAL trial were published that investigated the safety and tolerability of allogeneic BCMA-CAR-T cells in patients with RRMM [416]. Of the 43 patients evaluated in the study approximately 56% developed CRS, 14% ICANS and 88% had an adverse event of grade $\geq$ 3 which is in line with data from conventional autologous T cells. In comparison, data from the only currently published CAR-NK cell trial with cord-blood derived HLA-mismatched CD19-CAR-NK cells reported no CRS or ICANS. In line, adoptive transfer of unmodified autologous or allogeneic NK cells did not result in severe side effects [336, 417, 418]. Clinical trials to assess the tolerability and efficacy of off-the-shelf BCMA-CAR-NK cells for the treatment of MM are ongoing [256, 369]. Current data support the notion that CAR-NK cells are a safe "off-the-shelf" adoptive immunotherapy; but genetic modifications and combination regimes are needed to increase their efficacy. This thesis provides data on how to improve NK cell based immunotherapy. Future research efforts should focus on validating the findings presented here before clinical testing and implementation is possible.

In **study I** we have evaluated the degranulation and killing ability of PD1-DAP10$^+$ and PD1-DAP12$^+$ pNK cells and observed that mainly PD1-CSR^{bright} expressing pNK cells increase these effector cell functions against PD-L1$^+$ cancer cells. However, a large proportion of transduced pNK cells expressed PD1-CSR at an intermediate level (PD1-CSR^{dim}) and, although they abrogated inhibition, they did not increase degranulation or cytokine secretion. Future studies should focus on the role of the PD1-CSR^{dim} versus PD1-CSR^{bright} cell populations and elaborate which ratio of PD1-CSR^{bright} pNK cells within the NK cell product is sufficient to tip the balance towards activation in the TME. Potentially, different plasmids or transduction protocols could be applied to increase the ratio of PD1-CSR^{bright} pNK cells. Additionally, more patients should be included in the study to further validate and confirm our data. It would be especially interesting to evaluate the role of native PD1

signaling in competition with PD1-CSR and also include combinations with CAR constructs or other mAb such as daratumumab. Lastly, CSR can be constructed and tested based on other more NK cell specific checkpoints such as TIGIT.

In **study II** we have proposed CD38^{dim} CD38-CAR$^+$ NK cells as an alternative to CD38-KO CD38-CAR$^+$ NK cells although we have not compared these two cell populations side by side in terms of metabolic fitness and effector cell functionality. Future research should focus on elaborating this, especially since our IL2-based expansion protocol seems to induce CD38 downregulation in healthy donor derived pNK cells and to a lesser extent in pNK cells from MM patients. Moreover, it is yet unclear whether this approach can be applied to other cell sources such as iPSC derived NK cells. Investigating the factors leading to CD38 downregulation would be beneficial in this context. Moreover, the CD38-CAR construct used in this study targets a different epitope on CD38 compared to daratumumab and it would be of interest to evaluate this approach on samples from daratumumab-resistant or refractory patients.

In **study III** we show that pNK cells increase their degranulation ability against HSV1716-infected target cells and start to express virally encoded dsRed. HSV1716-infected pNK cells display an activated phenotype. The relevance of these findings for ongoing clinical studies with HSV-1 based OV remains yet unclear. From a translational point of view it might be interesting to study the mechanisms behind HSV1716 infection of pNK cells. Modulation of this pathway could, for instance, be enabling towards an HSV-1 OV cell carrier system for systemic delivery. This might, however, also require additional modifications to the OV in order to specifically switch between active and latent state of the virus.

In summary, the field of cell-based cancer immunotherapy is rapidly evolving and entering the clinical stage with so far promising results. In the future, NK cells will be an integral part of this immunotherapeutic armamentarium. However, more research is needed to advance their translation for the treatment of both hematological and solid cancers. A special focus should lay on advancing cost-effective and GMP-compliant transduction and expansion methods that are accessible to a larger patient cohort. Moreover, genetic modifications and combination therapies are needed to advance NK effector cell functionality and overcome resistances to immunotherapy.

7 Acknowledgements

Working in science can be a very rewarding experience but at the same time demanding and frustrating. A famous quote by Bertrand Russell says "The time you enjoy wasting is not wasted time" and I believe it is true, but only if it is also filled with meaningful interactions. This PhD journey would not have been possible without the constant support, inspiration and encouragement from all the wonderful people I met throughout the years. Thank you!

"The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind."
(Khalil Gibran)

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