

DISSERTATION

Targeting CCR7 with CAR engineered NK cells for the
treatment of T cell non-Hodgkin's lymphoma (T-NHL)

CCR7 als Zielstruktur gentechnisch entwickelte CAR-NK Zellen
zur Behandlung von T Zellen-Non-Hodgkin-Lymphome (T-NHL)

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Table of Contents

I Tables	6
II Figures	6
III Abbreviation	8
IV Zusammenfassung	13
V Abstract	15
1 Introduction	17
1.1 Overview on T cell biology	17
1.1.1 T cell immunity.....	17
1.1.2 T cell development and maturation	18
1.2 T cell malignancies	20
1.2.1 Classification of T cell malignancies.....	20
1.2.2 Pathogenesis of T-NHL	21
1.2.3 Treatment landscape of T-NHL.....	23
1.3 Adoptive cell therapy	24
1.3.1 Chimeric antigen receptors (CAR)	26
1.3.2 CAR-T cell therapy for the treatment of hematological malignancies	29
1.3.3 CAR-T cell therapy for T cell malignancies: challenges and proposed solutions .	30
1.3.4 Preventing T cell aplasia with suicide genes, safety switches and short-lived CAR cells.....	32
1.4 NK cells as cellular vehicle for CAR-based therapy	33
1.4.1 Overview on NK Cell Biology	33
1.4.2 Unmodified NK cell therapy	35
1.4.3 CAR- NK cell therapy	36
1.5 Chemokines and their receptors as therapeutic targets in cancer	38
1.5.1 Chemokines and chemokine receptors	38
1.5.2 Biological roles of chemokines and chemokine receptors in homeostasis and diseases	39
1.5.3 The homeostatic chemokine receptor CCR7	40
1.5.4 CCR7 in blood cancer	41
2 Aim of the thesis	44

3 Material and Methods	45
3.1 Material	45
3.1.1 Oligonucleotides	45
3.1.2 Plasmids and retroviral vectors	46
3.1.3 Antibodies.....	49
3.1.5 Primary cells and culture media	50
3.1.6 Cell lines and culture media.....	50
3.1.7 Kits.....	52
3.1.8 Instruments.....	53
3.1.9 Software	53
3.1.10 Mice	54
3.2 Methods	54
3.2.1 Molecular biology	54
3.2.2 Cell culture	59
3.2.3 Immunological techniques	62
3.2.4 Animal experiments.....	64
3.2.5 Statistics.....	65
4 Results	66
4.1 Exploiting the T cell homing receptor CCR7 as a T-NHL selective target 66	
4.1.1 CCR7 is expressed on T-NHL cell lines.....	66
4.1.2 T cell neoplasms exhibit a wide range of CCR7 surface expression levels.....	68
4.1.3 A curated microarray database revealed CCR7 expression in various T-NHL malignancies	69
4.1.4 CCR7 is highly expressed on primary T-NHL samples.....	71
4.2 Construction of CCR7 CAR	73
4.2.1 <i>In silico</i> design of the CCR7 CAR construct.....	73
4.2.2 Cloning of the CCR7 CAR construct	74
4.2.3 The CCR7 CAR was efficiently expressed on the surface of Jurkat cells	75
4.2.4 Construction of humanized versions of the rat CCR7 scFv.....	76
4.2.5 Generation of stable retrovirus-producing packaging cell lines	79
4.2.6 Stable transduction of NK-92 and YTS effector cells with CCR7 CAR.....	79
4.3 CCR7 CAR enhances NK-92 effector functions against CCR7 positive cell lines <i>in vitro</i>.....	81

4.3.1 CCR7 CAR NK-92 cells mediated cellular cytotoxicity towards CCR7 expressing tumor entities.....	81
4.3.2 Cytolytic granules are secreted by the CCR7 CAR NK-92 cells in an antigen-dependent fashion	84
4.3.3 Effector cytokines are released upon coincubation of CCR7 CAR NK-92 cells with CCR7 positive cell lines	85
4.4 CCR7 CAR (PB)-NK cells exhibit specific cytolytic efficacy against T-NHL cell lines <i>in vitro</i>.....	87
4.4.1 CCR7 CAR can be successfully expressed in PB-NK cells	87
4.4.2 CCR7 CAR endows PB-NK cells with specific effector functions towards T-NHL cells lines <i>in vitro</i>	88
4.5 Patient-derived T-NHL samples are potently eradicated by CCR7 CAR-NK cells <i>in vitro</i>	91
4.5.1 CCR7 CAR-NK cells kill primary patient-derived T-NHL cells	91
4.6 Expression pattern for the target antigen CCR7	93
4.6.1 CCR7 is selectively expressed in the hematopoietic compartment	93
4.6.2 Autologous CCR7 CAR (PB)-derived NK cells react towards benign B and naïve T cells <i>in vitro</i>	95
4.6.4 CCR7 is absent on non-hematopoietic primary cells and cell lines	97
4.6.5 Coincubation of CCR7 CAR-NK cells with CCR7 negative primary cells and cell lines does not trigger the release of effector cytokines.....	99
4.8 Establishment of a T-NHL xenograft model.....	100
4.8.1 HuT-78 lymphoma cells can be efficiently engrafted in NSG mice	101
4.8.2 A single dose of CCR7 CAR (PB)-NK cells revealed a moderate anti-tumor efficacy in HuT-78 engrafted NSG mice.....	103
4.9 Pharmacological control of CCR7 CAR-NK cells using Dasatinib	105
4.9.1 100 nM Dasatinib ablates cytolytic activity and cytokine secretion	105
5 Discussion	107
5.1 The chemokine receptor CCR7 as a target	107
5.1.1 Physiological and pathophysiological expression of CCR7	107
5.1.2 Adoptive immunotherapy for T cell malignancies	109
5.1.3 Safety of anti-CCR7 therapies of T-NHL.....	111

5.2 CAR-NK cells for T-NHL immunotherapy	114
5.2.1 CAR-NK cells offer advantages over CAR T cells in the treatment of T-NHL....	115
5.2.2 CAR-NK cell therapies	118
5.2.3 CCR7 CAR-NK cell therapy	119
5.3 Next generation CARs	121
5.3.1 Improving safety through the integration of control switches.....	121
5.3.2 Implementing logic-gated CARs for combinatorial antigen targeting	122
5.4 Limitations of the study	123
5.5 Future directions	124
5.6 Conclusions	125
6 References	127
7 Statuary Declaration	150
8 Curriculum Vitae	151
9 Publications	151
10 Acknowledgments	152
11 Certificate of the accredited statistician	155

I Tables

Table: Classification of T- or NK cell neoplasms.....	21
Table 2: Oligonucleotides.....	45
Table 3: Plasmids and retroviral vectors	46
Table 4: List of antibodies for flow cytometry	49
Table 5: Primary cells and culture media	50
Table 6: Cell lines and culture media	50

II Figures

Figure 1: Schematic overview of adoptive T cell transfer (ATC) strategies for cancer treatment.....	24
Figure 2: Structural composition of different generation of chimeric antigen receptors (CARs).	29
Figure 3: A brief flow chart of CAR engineered-NK cell therapy.	37
Figure 4: Flow cytometric analysis of surface expression of CCR7 on T-NHL cell. ..	67
Figure 5: Quantification of CCR7 surface density on selected T-NHL cell lines.....	69
Figure 6: Cancer types with the highest CCR7 expression level in the Genevestigator database.	70
Figure 7: CCR7 surface expression on patient-derived primary T-NHL samples.....	73
Figure 8: Schematic representation of the CCR7 CAR construct.	74
Figure 9: Cloning of CCR7 CAR construct.	75
Figure 10: The CCR7 CAR construct was efficiently expressed on the surface of Jurkat cells.....	76
Figure 11: Workflow of humanization of the rat CCR7 scFv.....	78
Figure 12: CAR surface expression on stable retrovirus-producing packaging 293VecGalV cells.	79

Figure 13: Efficient retroviral vector-mediated gene transfer into NK-92 and YTS cell lines.....	81
Figure 14: The CCR7 CAR specifically retargets NK-92 cells against T-NHL cell lines <i>in vitro</i>	83
Figure 15: CCR7 CAR-engineered NK-92 cells lyse CCR7 positive target cell lines <i>in vitro</i>	85
Figure 16: CCR7 CAR NK-92 cells react against antigen positive T-NHL cell lines by secreting IFN- γ <i>in vitro</i>	86
Figure 17: The CCR7 CAR construct was successfully transduced into human NK cells.	88
Figure 18: CCR7 CAR (PB)-NK cells mediated cellular cytotoxicity against CCR7 expressing tumor entities.	90
Figure 19: CCR7 CAR-engineered NK-92 cells lyse primary T-NHL samples expressing CCR7.	92
Figure 20: Analysis of CCR7 expression on peripheral blood lymphocytes and myeloid cells.	95
Figure 21: Autologous CCR7 CAR (PB)-NK cells reactivity towards benign naïve T cells and mature B cells.	97
Figure 22: CCR7 is not expressed on unrelated primary cells and cell lines.....	98
Figure 23: CCR7 CAR-NK cells do not exhibit <i>in vitro</i> reactivity against CCR7 negative primary cells and cell lines.	100
Figure 24: Development of a mouse xenograft model for T-NHL.....	102
Figure 25: Antitumor activity of CCR7 CAR-(PB) NK cells against T-NHL xenograft <i>in vivo</i>	104
Figure 26: 100 nM Dasatinib-mediated inhibition of cytolytic activity and cytokine secretion.	106

III Abbreviation

7AAD	7-aminoactinomycin D
AB	A and B antigen
ACT	adoptive cell therapy
ADC	antibody-drug conjugate
ADCC	antibody-dependent cell-mediated cytotoxicity
ALCL	Anaplastic Large Cell Lymphoma
ALK	anaplastic lymphoma kinase
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APC	antigen-presenting cell
ATC	adoptive T cell transfer
ATLL	Adult T cell Leukemia/Lymphoma
BBB	blood-brain barrier
BCL	B cell lymphoma
BCMA	B cell maturation antigen
BL	Burkitt's Lymphoma
BLR	Burkitt's lymphoma receptor
BM	bone marrow
BSA	bovine serum albumin
BV	Brentuximab Vedotin
CAR	chimeric antigen receptor
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
CDR	complementary determining region
cHL	Classical Hodgkin's Lymphoma
CHOEP	CHOP combined with etoposide
CHOP	cyclophosphamide, doxorubicin, vincristine, and prednisolone
CLA	Cutaneous lymphocyte antigen
CLL	CLL
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
CTCL	Cutaneous T Cell Lymphoma
cTEC	cTEC

CTLA	cytotoxic T-lymphocyte-associated protein
DAP	DNAX-activating protein
DC	dendritic cell
DLBCL	Diffuse Large B cell Lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DN	double-negative
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DP	double-positive
EBV	Epstein-Barr virus
EC	endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGFR	EGFR
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FDA	Food and Drug Association
FDC	follicular dendritic cell
FL	Follicular Lymphoma
FMO	fluorescence minus one
FR	framework region
FRC	fibroblastic reticular cells
FT	Fate Therapeutics
FWD	forward
GDP	guanosine-5'-diphosphate
GFP	green fluorescent protein
GI	gastro-intestinal tract
GM-CSF	granulocyte-macrophage colony-stimulating factor
gMFI	geometric mean fluorescense intensity
GMP	good manufacturing practice
GPCR	G protein-coupled receptor
GTP	guanosine-5'-triphosphate
GvHD	graft-versus-host disease
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	high endothelial venule
HIV	human immunodeficiency virus
HL	Hodgkin's Lymphoma

HLA	human leukocyte antigen
HPV	human papillomavirus
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
HSV-TK	herpes simplex virus thymidine kinase
HTLV	human T-lymphotropic virus
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intracellular adhesion molecule
ICN	intracellular cleaved form of Notch1
IFN	interferon
IG	immunoglobulin
IL	interleukin
IMGT	international ImMunoGeneTics information system
iPSC	induced pluripotent stem cells
IS	immunological synapsis
ITAM	immunoreceptor tyrosine-based activation motif
IVIS	in vivo imagin system
KIR	killer cell immunoglobulin-like receptor
KO	knock out
LAMP	lysosomal-associated membrane protein
Lck	lymphocyte-specific protein tyrosine kinase
LFA	lymphocyte-function-associated protein
LGL	Large Granular Lymphocytic Leukemia
LH	light chains
LMP	latent membrane protein
LN	lymph nodes
LPC	lymphoid progenitor cells
LTR	long terminal repeat
LUC	luciferase
MACS	magnetic-activated cell sorting
MAGE	melanoma antigen gene
MART	melanoma differentiation antigen
MCL	Mantle Cell Lymphoma
MF	Mycosis Fungoides
MHC	MHC
MIC	MHC class I chain-related protein
MLNK	cytokine-induced memory-like NK
MLV	murine leukemia virus 10A1

MM	multiple myeloma
MTCL	Mature T cell Lymphomas
NF- κ B	nuclear factor kappa-light chain enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NHL	Non Hodgkin's Lymphoma
NHP	non-human primates
NK	natural killer
NOD	non-obese diabetic
NSG	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1 Wjl} /SzJ
NY-ESO	New York esophageal squamous cell carcinoma
ORR	overall response rate
OS	overall survival
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDX	patient-derived xenograft
PE	phycoerythrin
PFS	progression free survival
PMA	phorbol myristate acetate
PTCL	Peripheral T cell Lymphoma
PTLC-NOS	Peripheral T Cell Lymphoma Not Otherwise Specified
REV	reverse
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
SAP	SLAM-associated protein
SD	signaling domain
SEM	standard error of the mean
SLO	secondary lymphoid organ
SMASH	small molecule-assisted shutoff
SNP	single nucleotide polymorphism
SP	signal peptide
SS	Sézary Syndrome
T-NHL	T cell Non Hodgkin's Lymphoma
T-PLL	T Cell Prolymphocytic Leukemia
TAA	tumor-associated antigens
TCL	T cell lymphoma
TCM	central memory T cell

TCR	T cell receptor
TFH	follicular t helper cell
TIL	tumor infiltrating lymphocyte
TKI	tyrosine kinase inhibitor
TM	transmembrane domain
TME	tumor microenvironment
TNF	TNF
TRAC	T cell receptor alpha chain
TRAIL	TNF-related apoptosis-inducing ligand
TRBC	T cell receptor beta chain
Treg	regulatory T cell
TRUCK	universal cytokine-mediated killing
TSA	tumor-specific antigen
UCART	universal CAR T cell
UCB	umbilical cord blood
UV	ultraviolet
VH	variable heavy chain
VL	variable light chain
WHO	World Health Organization

IV Zusammenfassung

T-Zell-Non-Hodgkin-Lymphome (T-NHL) ist eine heterogene Gruppe lymphoider Neoplasien, die durch die Ausbreitung bösartiger T-Zellen gekennzeichnet sind. Verschiedene Subtypen weisen ein unterschiedliches klinisches Verhalten und Gesamtprognose auf. Für diese Patienten stehen nur begrenzt wirksame Behandlungen zur Verfügung, insbesondere bei der Erstbehandlung, was zu häufigen Rezidiven und hoher Morbidität führt. Die CAR-T-Zell-Therapie ist sehr erfolgreich bei der Behandlung fortgeschrittener B-Zellmalignitäten. Die Anwendung von CAR-T Zellen bei T Zellmalignitäten hat sich aufgrund der gemeinsamen Expression von Zielantigenen auf den therapeutischen, normalen und malignen T Zellen als herausfordernd erwiesen. Natürliche Killerzellen (NK Zellen) sind angeborene Immuneffektoren, die eine natürliche zytotoxische Aktivität gegen Tumoren zeigen, die durch CAR-Engineering weiter verbessert werden kann. Ihr biologisches Potenzial, ein sofort einsatzbereites allogenes Zellprodukt zu sein, machen sie zu einer attraktiven Plattform für die CAR Zell-Therapie, insbesondere für T Zellneoplasien. In dieser Arbeit wurde ein T Zell Homing Rezeptor (CCR7) als Zielstruktur für CAR-NK Behandlung von T-NHL Malignitäten vorgeschlagen. CCR7 ist ein homöostatischer Chemokinrezeptor mit Expression auf Subentitäten von Immunzellen, der das Homing dieser Zellen in sekundäre lymphoide Organe steuert. Bei hämatologischen Tumorerkrankungen, insbesondere bei T Zelleukämien und Lymphomen, legen Veröffentlichungen nahe, dass CCR7 die Zellmigration steuert und damit den Krankheitsverlauf entscheidend beeinflusst. Ein CCR7 CAR-NK Zellprodukt der zweiten Generation wurde entwickelt, wobei als allogene NK Zellquelle die NK-92 und die YTS Zelllinie sowie PBMCs von gesunden Spendern verwendet wurden. In dieser Arbeit verlieh der CCR7 CAR-NK-92 Zellen eine hohe Avidität, was es ihnen ermöglichte, i) T-NHL Zelllinien und primäre CCR7-positive T-NHL Zellen in verschiedenen *in vitro* Kokulturen i) zu erkennen und ii) zu töten. Diese Ergebnisse wurden mit primären CCR7 CAR-NK Zellen bestätigt. CCR7 wird hoch und häufig auf T-NHL exprimiert, während die Expression auf gesundem Gewebe auf Subpopulationen des hämatopoetischen Systems beschränkt ist. Die On-Target-/Off-Tumor-Analyse ergab i) eine bevorzugte Erkennung von T-Zellsubpopulationen, die CCR7 hoch exprimieren, eine verringerte Eliminierung von B-Zellen, die moderate

CCR7-Spiegel exprimieren, und ii) keine Off-Target-Effekte gegenüber CCR7-negativen, nicht hämatopoetischen Zellen. Weiterhin wurde ein Maus-Xenotransplantatmodell für eine T-NHL entwickelt. Ein erster Versuch zeigte, dass die Verabreichung einer Einzeldosis des CCR7 CAR-NK Zellprodukts das Tumorstadium mäßig kontrollierte, was darauf hindeutet, dass wiederholte Verabreichungen vermutlich wirksamer sind. Zusammen stellt die vorliegende Arbeit eine neuartige Therapiestrategie für Patienten mit T-NHL dar.

V Abstract

T cell non-Hodgkin's lymphoma (T-NHL) encompasses a heterogeneous group of lymphoid neoplasms characterized by the expansion of malignant T cells. Different subtypes exhibit varying clinical behaviour and overall prognosis. Limited effective treatments are available for these patients, especially in the frontline. This contributes to the frequent relapse rates and high morbidity, primarily due to the lack of effective targeted treatments. Chimeric antigen receptor (CAR) cellular therapy has been successful in treating refractory or relapsed B cell and plasma cell malignancies by redirecting T cells using CARs. Expanding the use of CAR T cells to treat T cell malignancies has proven challenging due to the shared expression of targetable antigens between the therapeutic, normal, and malignant T cells. Natural killer (NK) cells are innate immune effectors, showing natural cytotoxic activity against tumors which can be further improved by CAR engineering. Their unique biological features and the potential of being an off-the-shelf allogeneic cellular product render them an attractive platform for CAR cell therapy, foremost for T cell lineage neoplasms. Herein, a T cell homing receptor (CCR7) was proposed as a candidate for CAR-NK retargeting for T-NHL. CCR7 is a homeostatic chemokine receptor with restricted expression on subsets of immune cells that drives lymphocyte homing to secondary lymphoid organs. In hematological malignancies, particularly T cell lineage leukemias and lymphomas, evidence suggests that CCR7 orchestrates cell trafficking, critically impacting disease progression and treatment outcomes. A second generation CAR construct was generated and CCR7 CAR-NK cell products were developed, using as allogeneic NK cell sources the NK-92 and YTS cell lines, and PBMCs from healthy donors. In this study, the CCR7 CAR conferred NK-92 cells with high avidity, enabling them to i) recognize, and ii) kill T-NHL cell lines and patient-derived T-NHL cells with high, intermediate, or low CCR7 surface expression in various *in vitro* coculture assays. These findings were corroborated in PB-derived NK cells. CCR7 was highly and frequently expressed on T-NHL and its expression on healthy tissues was limited to the hematopoietic system. On-target/off-tumor-analysis revealed i) the preferential recognition of T cell subpopulations highly-expressing CCR7 and reduced killing of B cells expressing moderate CCR7 levels, and ii) no off-target effects towards CCR7-negative non-hematopoietic cells. A mouse xenograft model of T-NHL was

established. Preliminary *in vivo* testing demonstrated that administration of a single dose of the CCR7 CAR-NK cell product moderately controlled tumor growth, suggesting that repetitive administrations might be more effective. Together, the work herein presents a novel therapeutic strategy for patients suffering from T-NHL.

1 Introduction

The process of carcinogenesis is considered one of the most intricate phenomena in biology. Cancer can be viewed as a complex system that undergoes regulations across various spatial and temporal dimensions. More than 200 different diseases are labelled as "neoplasm" which differ in genetics, cell and tissue biology, pathology, and response to therapy. Weinberg and Hanahan enumerated a set of functional traits that cells acquire as they transit from benign to neoplastic states in an attempt to unite all types of human cancer at the phenotypical level. The core hallmarks of cancer include independence in proliferative signaling, resistance to growth inhibition, evasion of programmed cell death, unrestricted replicative capacity, sustained tissue invasion and angiogenesis, and metastasis [1]. The ability to evade immunological destruction was labelled as an "emerging hallmark" given that the postulated theory of immune surveillance required further validations. The existence of antitumor immunity as a defense barrier in controlling tumor growth is now recognized. Manipulation of some of these immunological circuits led to the groundbreaking approach of immune checkpoint blockade therapy. Cancer immunotherapy utilizes a diverse toolkit of approaches, namely antibodies, vaccines, cytokines, and cell-based therapies, to reactivate an averted pre-existing immune response or provoke a de novo immune response. Alongside radiation, surgery, and chemotherapy, cancer immunotherapy has firmly established itself as the fourth pillar of cancer care.

1.1 Overview on T cell biology

1.1.1 T cell immunity

T cells have a fundamental role in orchestrating the adaptive immune response, coordinating antigen-specific responses against various infections and malignancies. Their diverse reactivity is achieved through unique T cell receptors (TCR) on the cell surface, allowing recognition of foreign antigens while ignoring self-antigens. TCRs interact exclusively with antigenic peptides when bound to major histocompatibility complex (MHC) class I or class II molecules on antigen-presenting cells (APCs) a phenomenon known as MHC restriction of T cells [2]. MHC class I is expressed by most nucleated cells and presents processed antigens from intracellular sources.

MHC class II is primarily expressed by specialized APCs (dendritic cells, macrophages and B cells) that present exogenous antigens processed in lysosomes [3]. In addition to TCR signaling, T cell responses rely on the co-engagement of CD4 (for MHC class II) or CD8 (for MHC class I) co-receptors. These co-receptors define two main T cell lineages: cytotoxic T cells (CD8), which directly kill target cells upon antigen-MHC class I engagement, and helper T cells (CD4) that regulate immune responses by interacting with a network of other effector cells through cytokine and chemokine signaling [4].

1.1.2 T cell development and maturation

During embryonic development, T lymphocytes originate from hematopoietic stem cells (HSC) situated in the fetal liver. In later developmental stages, HSCs migrate to the bone marrow (BM) where they differentiate into lymphoid progenitor cells (LPC). LPCs exit the BM, enter the bloodstream, and gain access to the thymic cortex via the cortico-medullary junction. Within the thymus, they commit to the T cell lineage and lose their potential to differentiate into other cell types. This cell population, termed double-negative (DN1) thymocytes, lacks the TCR and does not express the co-receptors CD4 or CD8. The DN phase progresses through four differentiation stages (DN1-DN4) within the thymic cortex. During the DN3 stage, the TCR β -chain undergoes V(D)J recombination, catalyzed by recombination-activating gene (RAG1/2) recombinases which induce double-strand breaks at the TCR β locus and promote nonhomologous end joining. This β -chain later combines with a pre-TCR α -chain to form a functional pre-TCR on the thymocyte surface. Only thymocytes with actively signaling pre-TCR complexes proceed to mature during the β -selection process. Those failing this process remain arrested at the DN3 stage. Thymocytes that successfully undergo β -selection (late DN3 and DN4) proliferate and give rise to CD4+ and CD8+ double-positive (DP) thymocytes [5]. It is during this stage that the TCR α -chain is rearranged via V(D)J recombination, resulting in the formation of the mature $\alpha\beta$ -TCR. DP cells interact with peptide-major histocompatibility complex (MHC) complexes presented by cortical thymic epithelial cells (cTEC). Through positive selection, T cells with TCRs binding these complexes with low affinity receive vital survival factors and differentiate into either CD4 or CD8 single-positive (SP) cells

based on their affinity towards MHC I or MHC II. T cells with high or no affinity towards MHC complexes undergo apoptosis by neglect [6]. Concomitantly, DP and SP thymocytes that respond to self-MHC are subjected to negative selection. This process ensures the maintenance of central tolerance and deficiencies in it can result in severe autoimmune disorders [7]. SP naïve T cells that survive the selection exit the thymus, patrolling secondary lymphoid organs and peripheral tissues. Cytokines produced in their surroundings guide the lineage differentiation of naïve T cells. Interleukin 12 (IL-12) and interferon-gamma (IFN- γ) stimulate T_H1 differentiation, crucial for antiviral, antimicrobial, and cell-mediated immunity. Conversely, IL-4 gives rise to T_H2 cells protecting the host against extracellular parasites. Specific cytokine combinations drive the development of T_H17 lineage which contributes to immune responses at mucosal sites. The combination of IL-6, IL-21, and/or IL-27, along with IL12 stimulation, induces follicular helper T (T_{FH}) differentiation. This lineage has a fundamental role in the formation of germinal centers and B cell antibody responses [8]. Maintaining immunological tolerance toward self-tissues relies on the action of regulatory T (Treg) cells, a type of CD4⁺ T cell. They specialize in suppressing potentially harmful T cell responses and preventing autoimmune diseases [9]. In addition, T cells can differentiate into memory T cells, providing enduring protection against pathogens for decades following antigen exposure [10]. Activation of helper and cytotoxic T cells depends on two signals originating from APCs: i) signal 1 starts with the interaction between the peptide-MHC complex and the TCR, leading to downstream signaling; ii) signal 2 involves the costimulatory protein CD28 on T cells binding to B7 proteins (CD80 and CD86) on APCs upregulated during infections. T cells without signal 2 undergo programmed cell death or changes that render them unresponsive to further activation. Interaction between T cells and APCs induces lymphocyte-function-associated protein 1 (LFA-1) in T cells which interacts with intracellular adhesion molecule 1 (ICAM-1) on APCs. This binding extends cell engagement and promotes T cell activation. An inhibitory molecule called cytotoxic T-lymphocyte-associated protein 4 (CTLA4), expressed by T cells, delivers a negative feedback signal to regulate T cell activation. CTLA4 is similar to CD28 but exhibits a higher affinity for B7 binding, thereby suppressing T cell activation [11].

1.2 T cell malignancies

T cell malignancies comprise a rare and heterogeneous group of diseases characterized by abnormal clonal proliferation and dysfunctional T cell activity, encompassing T cell lymphomas (TCLs) and T cell leukemias, with mature and precursor forms. Compared with B cell malignancies, patients facing relapsed or refractory disease have limited therapeutic options with demonstrated clinical efficacy.

1.2.1 Classification of T cell malignancies

The World Health Organization (WHO) Classification of lymphoid malignancies was updated in 2022 and is based on the postulated cell of origin, differentiation stage, cytomorphology, and site of the disease. Over 100 entities are listed in the 5th edition of the WHO of Haematolymphoid Tumours (WHO-HAEM5) [12]. **Table 1** provides a recent histological classification of T cell and NK cell lymphoid proliferations and lymphomas subtypes adapted from the WHO-HAEM5. T- or NK cell neoplasms are not listed under two separated categories as the determination of T or NK- cell of origin may be challenging in some instances. In WHO-HAEM5, precursor T cell neoplasms include T lymphoblastic Leukemia/Lymphoma (T-ALL). Mature T cell Lymphomas (MTCLs), historically known as "peripheral" T cell Lymphomas (PTCLs), comprise the large majority of all T cell non-Hodgkin's Lymphoma cases and include 39 different subtypes. Broadly speaking, they can be grouped into 4 main categories: 1) nodal, 2) cutaneous, 3) extranodal, and 4) leukemic/disseminated diseases. Additionally, WHO-HAEM5 lists a new family of tumor-like lesions with T cell predominance, namely, Autoimmune Lymphoproliferative Syndrome (ALPS), Kikuchi-Fujimoto Disease (KFD), and Indolent T lymphoblastic Proliferation (ITLP). In this thesis, PTCL-NOS, Mycosis Fungoides (MF), Sézary Syndrome (SS), and Anaplastic Large Cell Lymphoma (ALCL) will be described.

<ul style="list-style-type: none"> • Precursor T cell neoplasms
<ul style="list-style-type: none"> • T lymphoblastic Leukemia/Lymphoma
<ul style="list-style-type: none"> • Mature T/NK cell Lymphomas
<p>1) Nodal</p> <ul style="list-style-type: none"> • Anaplastic Large Cell Lymphoma (ALK+, ALK-, Breast Implant-Associated) • Nodal T_{FH} Cell Lymphoma (Angioimmunoblastic-type, Follicular type, NOS) • EBV-positive Nodal T and NK cell Lymphoma
<ul style="list-style-type: none"> • Cutaneous • Mycosis Fungoides • Primary Cutaneous CD30+ T Cell Lymphoproliferative disorders • Primary Cutaneous CD4+ and CD8+ Lymphoproliferative disorders • Primary Cutaneous gamma/delta T Cell Lymphoma • Sézary Syndrome (SS) • Subcutaneous Panniculitis-like T Cell Lymphoma • Primary Cutaneous Peripheral T Cell Lymphoma, NOS
<p>2) Other extranodal</p> <ul style="list-style-type: none"> • Peripheral T Cell Lymphoma NOS (PTCL-NOS) • Extranodal NK/T Cell Lymphoma, nasal type • Enteropathy-type T Cell Lymphoma • Hepatosplenic T Cell Lymphoma
<p>3) Leukemic/disseminated</p> <ul style="list-style-type: none"> • T Cell Prolymphocytic Leukemia (T-PLL) • T Large Granular Lymphocytic Leukemia (T-LGL) • Adult T cell Leukemia/Lymphoma • NK Large Granular Lymphocytic Leukemia • Aggressive NK Cell Leukemia
<p>Tumor-like lesions with T cell predominance</p> <ul style="list-style-type: none"> • Kikuchi-Fujimoto Disease (KFD) • Indolent T Lymphoblastic Proliferation (ITLP) • Autoimmune Lymphoproliferative Syndrome (ALPS)

Table 1: Classification of T- or NK cell neoplasms. Data adapted from Salem, 2021 [13]. (ALK) anaplastic lymphoma kinase, (NOS) not otherwise specified, (T_{FH}) T-follicular helper, (EBV) Epstein–Barr virus, (GI) gastrointestinal.

1.2.2 Pathogenesis of T-NHL

Mature T cell Lymphomas (or PTCL) belong to the large group of NHLs and, therefore, are abbreviated as T-NHLs. In Western countries, T-NHL accounts for 5 to 10% of all NHLs with 3500-7000 new cases/year. The distribution of different subtypes of T-NHL shows marked geographic and racial variability. For instance, these diseases are more common in the Caribbean, Latin America and Asia, in part due to human T-lymphotropic virus type-1 (HTLV-1) and EBV viral infections [14]. The number of T-

NHL new cases in Germany is 1/100,000 inhabitants (Robert Koch Institute, 2019). T-NHL most commonly affects adults and older individuals, typically diagnosed at a median age of 60 years (men-to-women-ratio 3:1), except for some specific types that are common in younger patients, like ALCLs. The clinical presentation of T-NHL can differ widely, depending on the subtype and extent of disease involvement. Common symptoms include swollen lymph nodes, sometimes with spread to organs like the skin, bone, central nervous system, or gastrointestinal tract. In some cases, an enlarged liver and spleen can be observed too. PTCL often exhibits pan-T cell markers like CD2, CD3, and CD5 [15]. Due to a multitude of overlapping characteristics, the differential diagnosis of T cell lymphoma entities is difficult. This requires a comprehensive evaluation of clinical, laboratory, and immunohistology data and the expertise of specialists for appropriate categorization. Multiple T cell lymphoma subtypes with an undefined immunophenotype and an absence of specific clinical characteristics are grouped under the PTCL-NOS category, which is the most common form of PTCL in Western countries. A gene expression analysis identified two molecular subgroups based on the expression of TBX21 and GATA3 transcription factors that are master regulators of T_H1 and T_H2 lineage differentiation, correspondingly [16]. Within the PTCL-TBX21, mutations in DNMT3A correlated with a CD8+ T cell cytotoxic gene signature [17].

ALCL represents a distinct subgroup of PTCL originating from T_H17 cells and expressing T_H17-associated markers such as IL-17A, IL-17F, and CD30. ALCL can be divided into ALK+ and ALK- subtypes, each with distinct epidemiological, pathogenetic, and clinical differences. ALK+ ALCLs bear a genomic rearrangement involving the ALK gene and the NPM1 gene, leading to the generation of a protein with constitutive tyrosine kinase activity [18]. ALK+ cases show a significantly better prognosis and strong responsiveness to initial anthracycline-based chemotherapy compared to ALK- cases [19].

Cutaneous T cell Lymphomas (CTCL) originate from the malignant proliferation of skin-homing CD4+ T cells. Approximately 75% of all CTCL cases are constituted by Mycosis Fungoides (MF) and Sézary syndrome (SS) which are two major subtypes of CTCL [20]. MF cases with skin-limited manifestations show a favorable prognosis. In contrast, in the SS leukemic variant, tumor cells (Sézary cells) accumulate in the

visceral organ and peripheral blood with a subsequent poor prognosis [21]. MF and SS arise from distinct CD4+T cell populations. Flow cytometric analysis has revealed that MF skin lesions are characterized by a T cell effector memory (Tem) phenotype with the presence of CCR4 and CLA, whilst SS cells display a central memory T (Tcm) phenotype as indicated by the expression of CCR7+, CD27+, and L-selectin+ [22].

1.2.3 Treatment landscape of T-NHL

Frontline therapies for T cell lymphoma rely on established protocols for Diffuse Large B cell Lymphoma (DLBCL) and consist of cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP), or CHOP combined with etoposide (CHOEP), and subsequent consolidative autologous stem cell transplant in first remission. In contrast to DLBCL, the survival rates for PTCL patients are notably lower, ranging from 40-50%, and the overall prognosis remains unfavorable for most PTCL cases. Patients who experience relapsed or refractory (R/R) disease have a median progression-free survival (PFS) of about three months and a median overall survival (OS) of 5-11 months [23, 24]. In such cases, high-dose salvage chemotherapy is employed, especially for patients eligible for allogeneic stem cell transplantation. Given the disappointing results of conventional chemotherapy, there has been a significant effort in recent years to explore novel targeted approaches. These include epigenetic modulators, proteasome inhibitors, kinase inhibitors, ALK inhibitors, immunomodulatory agents, checkpoint inhibitors, and monoclonal antibodies (mAb). Aletuzumab is a humanized mAb targeting CD52 expressed on both healthy and cancerous B- and T lymphocytes. It demonstrated efficacy in PTCL Phase II trials when administered alongside CHOP or other agents. However, its standard use for PTCL is in question due to severe side effects and toxicity [25, 26]. Brentuximab Vedotin (BV) is a CD30- directed-drug-conjugated mAb that received FDA approval in 2018 for the first-line treatment of PTCLs expressing CD30 [27]. In the same year, a second mAb, Mogamulizumab, targeting the CC chemokine receptor (CCR4), was approved for R/R CTCLs [28]. While new approaches to conventional PTCL treatments have been proposed in recent years, their impact on response rates, particularly long-term outcomes such as PFS and OS, still needs improvement, and relapse rates continue to be exceedingly high.

1.3 Adoptive cell therapy

Cancer immunotherapies leverage the potential of the patient's immune system to battle cancer. They can be subdivided into "active" and "passive" based on their mechanisms of action. Checkpoint inhibitors and anticancer vaccines are examples of active immunotherapies and exert antitumor efficacy upon the engagement of the host immune system. Monoclonal antibodies and adoptive cell therapy (ACT) are endowed with intrinsic antineoplastic activity and are considered passive immunotherapies. ACT has been among the most significant breakthroughs in immune oncology over the past decade. Based on the idea that tumor-specific T cells could eradicate cancer, ACT relies on the reinfusion of T cells extracted from excised tumors [tumor infiltrating lymphocytes (TIL)] or genetically modified T cells targeting tumor antigens [T cell receptors (TCR) or chimeric antigen receptors (CAR)]

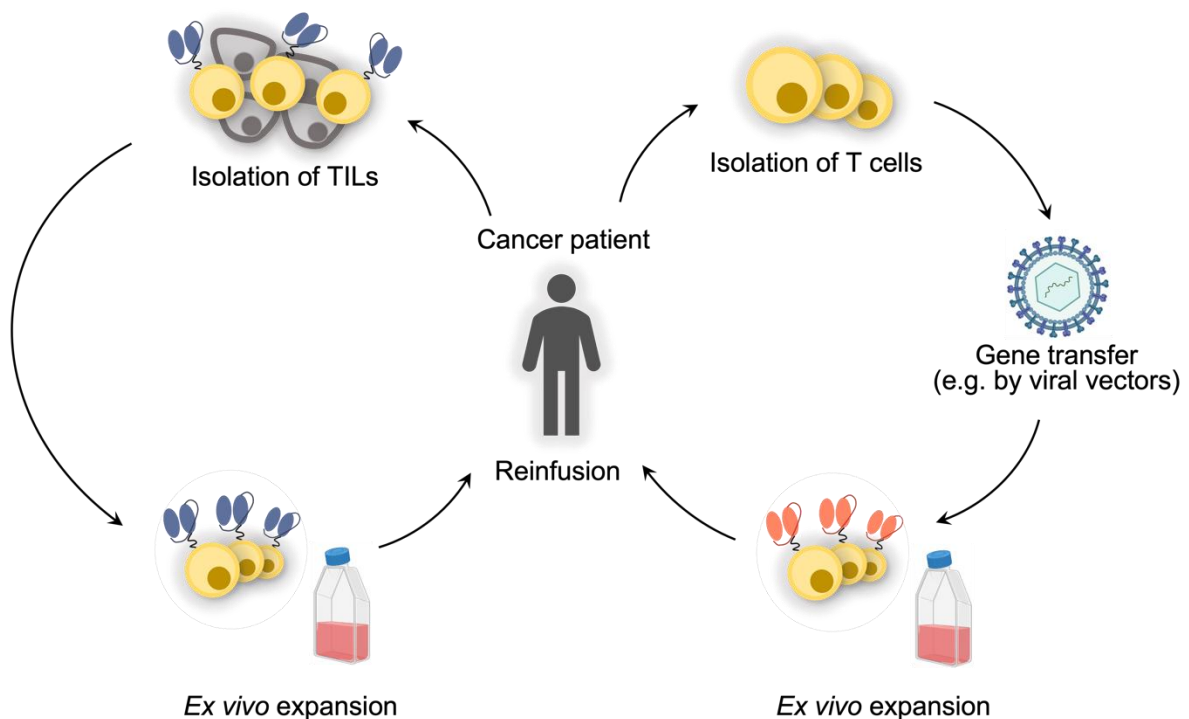


Figure 1: Schematic workflow of adoptive T cell transfer (ATC) strategies for cancer treatment. Tumor-infiltrating T cells are extracted from the patient's surgically resected tumor, expanded ex vivo with a high dose of IL-2, and then reintroduced into the patient (left panel). Alternatively, T cells can be derived from the patient's peripheral blood mononuclear cells (PBMCs). These cells undergo genetic modification using viral vectors for T cell receptor (TCR) or chimeric antigen receptor (CAR) gene transfer, enabling them to specifically

recognize and eliminate tumor cells upon re-infusion into the patient (right panel). The figure was created with Illustrator. The culture flask was obtained from BioRender.com.

The first clinical success of ACT occurred in the 1980s when Steven Rosenberg at the NIH/NCJ USA adoptively transferred heavily pre-treated patients affected by metastatic melanoma with TILs isolated from their tumors and expanded *ex vivo* under high IL-2 concentration [29, 30]. This approach induced objective cancer regression in 50% of patients [31]. Limitations of TIL-based immunotherapy is the restricted access to removable metastases or tumors, the systemic toxicity due to high-dose IL-2 required for TIL survival *in vivo*, and the exhaustion of the final TIL product driven by the immunosuppressive tumor microenvironment (TME). There are two distinct categories of antigens for targeted therapies: i) tumor-specific antigens (TSAs) or neoantigens, arising from nonsynonymous mutations and exclusively expressed on neoplastic cells; ii) tumor-associated antigens (TAAs) that are overexpressed on tumor cells but may also be present, albeit often at low levels, on benign tissues. Autologous T lymphocytes can be equipped with an antigen specific TCR. TCRs can be derived from TILs of patients or identified with screening platforms, such as transgenic mice harboring human MHC molecules and TCR $\alpha\beta$ loci ([32]; HuTCR). The gene encoding TCR α and β chains are genetically introduced into T cells, redirecting them against the antigen. The TCR chains lack intrinsic signaling capacity and require interaction with accessory signaling molecules. When a TCR binds its cognate peptide MHC complex, a non-covalent oligomeric complex forms with endogenous CD3 signaling molecules (CD3 ζ , CD3 $\delta\epsilon$, and CD3 $\gamma\epsilon$), leading to the phosphorylation of ITAMs in the intracellular portion of the CD3 subunits. This process results in T cell activation, initiating effector functions in an antigen-specific fashion [33]. Class I MHC complexes display cleaved peptides, mainly from intracellular proteins including i) tumor-specific mutated proteins (neoantigens) arising from tumor-specific somatic mutation (neoantigens) [(e.g. or KRAS G12D/G12V [34] and PIK3CA H1047L [35]] or from viral antigens [(e.g. human papillomavirus (HPV) viral oncogenes E6 and E7 [36, 37]; Epstein-Barr virus (EBV) latent membrane protein LMP1 and LMP2 viral oncogenes [38]]; ii) aberrantly transcribed cancer-associated differentiation antigens [(e.g., New York esophageal squamous cell carcinoma (NY-ESO), melanoma antigen gene

(MAGE) and melanoma differentiation antigen (MART-1)[39–41]]. In 2006, Morgan et al. pioneered the application of MART-1 specific TCR-T cell therapy to treat patients with melanoma. An objective response rate (ORR) of 12% was achieved [42]. In 2011, Robbins et al. treated patients with melanoma and synovial sarcoma with autologous T cells transduced with a TCR targeting NY-ESO-1 [43]. An ORR of 61% (11/18) was observed in the synovial sarcoma group, while the melanoma group showed an ORR of 55% (11/20), with no observed toxicity to normal tissues [44]. Since their introduction to clinical practice, clinical trials employing TCR-modified T cells have been scarce, and the first TCR T cell therapy was only approved in January 2022 by the US Food and Drug Association (FDA) for the treatment of uveal melanoma [45]. The MHC-/HLA-restriction pattern poses a significant obstacle by limiting the number of eligible patients for TCR therapy.

A third ACT approach, CAR-engineered T cell therapy, utilizes (unrestricted) variable antibody domains to enable antigen recognition in an HLA-independent fashion. The details of CAR-based approaches will be elucidated in the following chapters. T cells have been so far the best characterized and widely used immune effectors for ACT. However, the interest in utilizing innate immune cells as cellular vehicles in ACT, such as NK cells, has grown exponentially.

1.3.1 Chimeric antigen receptors (CAR)

CARs are synthetic receptors that combine antigen-specificity with T cell activating properties in a single fusion protein. Due to their ability to bind antigenic peptides expressed on the cell surface in an HLA-unrestricted fashion, CAR engineered T cells may prove more efficacious where TCR T cell therapy may fail, for example upon downregulation of MHC expression or defective antigen processing. More importantly, CAR-T cell therapy holds promise for targeting tumors for which MHC-presented peptides are yet to be identified [46]. The first generation of CARs, termed "T-bodies", was pioneered in 1989 by Eshhar and colleagues. These constructs contained an extracellular antibody-derived single-chain variable fragment (scFv) comprising heavy (VH) and light chains (LH) fused to cytoplasmic CD3 zeta (CD3 ζ) or Fc receptor gamma chain (γ) domains [47, 48]. This configuration endowed the engineered T cell with anti-tumor effector functions [49], however, it resulted in being ineffective in

clinical trials due to poor activation and, consequently, lack of persistence of infused cells [50]. Under physiological T cell responses, optimal T cell activation occurs upon engagement of the TCR with cognate peptide-loaded MHC (signal 1), followed by costimulation (signal 2) and subsequent cytokine-mediated differentiation and expansion (signal 3) [51]. The provision of signal 1 without CD28 costimulation was shown to lead to a limited T cell proliferation or induce anergy and apoptosis [52]. Hence, Maher and colleagues pioneered a new generation of CAR-T cells by incorporating costimulatory molecules such as CD28 into the CAR. This configuration ameliorated T cell expansion and preserved their function when challenged with repeated antigen exposure [53, 54]. The modular composition of a CAR generally consists of four major components: 1) a scFv comprising heavy (VH) and light chains (LH) linked via a flexible linker 2) a spacer (hinge) that anchors the scFv to the transmembrane domain (TM) 3) a hydrophilic alpha helix TM 4) intracellular signaling domain (SD).

CAR-T cell activation requires cell-cell interaction and "immunological synapse" (IS) formation between the CAR-T cell and the target cells. The majority of CARs utilized in clinical settings signal through the CD3z chain, activating downstream effectors associated with the TCR/CD3 complex. Ligand binding initiates CAR signaling through Lck and Fyn kinases belonging to the Src family. The CD3z contains three tyrosine rich ITAMs, each phosphorylated by Lck and Fyn at two tyrosine residues. These phosphorylated ITAMs recruit Zap-70, triggering three primary signaling pathways: calcium influx, nuclear factor kappa-light chain enhancer of activated B cells (NF- κ B), and Ras-MAPK [55]. Primarily, these activating pathways unleash effector mechanisms i) granzymes ii) cytokines iii) Fas iv) TRAIL v) TNF [56–58].

Second generation CARs combine the antigen binding domain with costimulatory signaling domains, such as CD28 and/or 4-1BB, and CD3z activation domain. Breakthroughs were achieved in the clinic with second generation CAR-armored T cells (see next chapter). Third generation CARs were defined by the introduction of multiple costimulatory components intended to overcome the limitations of each costimulatory domain and enhance the net antitumor efficacy of the cells, for example, OX40 and CD28/4-1BB [59], or 4-1BB and CD28 [60, 61]. More recently, Del Bufalo et al. treated 30 R/R neuroblastoma patients with a third generation anti-GD2 CAR T

cell product harboring CD28 and 4-1BB as costimulation, and iCasp9 as safety switch. An overall response of 63% was achieved [62]. A third generation CAR T cell therapy targeting CD19 (CD28 and 4-1BB) was administered to 13 R/R ALL patients. The one year follow-up revealed a progression-free survival (PFS) rate of 38% [63].

T cells engineered with fourth generation CARs or T cells redirected for universal cytokine-mediated killing (TRUCK) additionally incorporate a nuclear factor of activated T cells (NFAT) transcription factor-inducible or constitutive expression cassette encoding transgenic products, primarily cytokines but not limited to them. Some proinflammatory cytokines, namely IL-7, IL-12, IL-15 and IL-18 are being tested in different TRUCK approaches [64, 65]. Released cytokines can foster survival and proliferation of the cellular products in CIS or repolarize the TME by acting in TRANS. Fifth generation CARs include alternative accessory domains, namely truncated cytokine receptors e.g. (IL-2R β) and STAT3/5 transcription factor binding motif within the signaling module, that facilitate favorable cytokine signaling without the risk of inducing adverse effects by their secretion [66]. **Figure 2** illustrates the different CAR generations. Future developments will convert CAR-engineered cells into living micro-factories, being able to deposit therapeutically active products in CAR-targeted milieus.

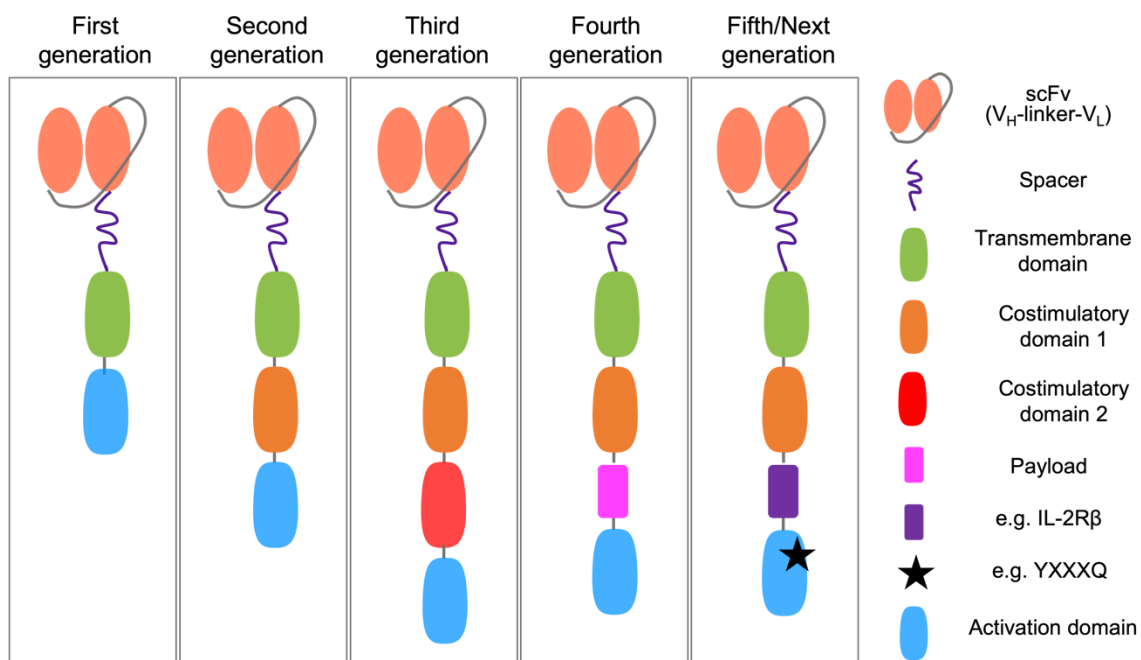


Figure 2: Structural composition of different generation of chimeric antigen receptors (CARs). CARs consist of an extracellular antigen-targeting moiety, the scFv, linked to a spacer, a transmembrane domain (TM) and intracellular signaling domain. First generation CARs feature only the CD3z. Subsequently, one (second generation) or two (third generation) costimulatory domains, such as CD28 and 4-1BB were added to the CARs. Fourth generation CARs or TRUCK (T cells redirected for universal cytokine-mediated killing) are additionally equipped with an inducible expression payload (e.g. IL-12 or IL-18) upon antigen stimulation. Fifth or next generation CARs include alternative accessory domains such as truncated cytokine receptors (e.g. IL-2R β) and STAT3/5 transcription factor binding motif (YXXXQ) within the signaling module. The figure was created with Illustrator.

1.3.2 CAR-T cell therapy for the treatment of hematological malignancies

Remarkable clinical responses led to the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approval of six autologous CAR-T cell products for treating patients with R/R B cell hematological malignancies. In CAR-T cell therapy, peripheral T cells are obtained from patient blood by leukapheresis, activated using antibodies or antibody-coated beads, and genetically modified, typically utilizing a lentivirus or retrovirus, to incorporate the CAR molecule. CAR-T cells are then expanded *in vitro* and infuse back into the patient. To enable efficient engraftment and robust expansion of adoptively transferred CAR-T cells, patients are pre-treated with lymphodepletion chemotherapy (cyclophosphamide and fludarabine) before T cell infusion. Reports have shown that myeloreduction results in the eradication of immunosuppressive factors in the microenvironment, such as removal of T-regs, and increase of homeostatic chemokines, e.g., IL-7 and IL-15, thereby boosting CAR T cell expansion [67, 68]. CAR T cell therapies targeting CD19 were the first approved CAR-based therapies for the treatment of B cell malignancies. CD19 is a pan-B cell marker ubiquitously expressed on B cell leukemias and lymphomas. Four CD19 targeting CAR-T cell products are currently available: 1) tisagenlecleucel (Kymriah, Novartis, NCT02445248, 2017) for the treatment of pediatric and young adults with B-ALL [69], adult patients with Diffuse Large B cell Lymphoma (DLBCL) [70]; 2) Axicabtagene Ciloleucel (Yescarta, Kite Pharma, NCT02348216, 2018) for adults with diffuse large B cell Lymphoma (DLBCL) and Non-Hodgkin's Lymphoma (NHL) [71] and more recently for Follicular Lymphoma (FL) (2022) [72]; 3) Brexucabtagene Autoleucel (Tecartus, Gilead, NCT02601313, 2020) for adults with Mantle Cell Lymphoma (MCL) [73] and R/R B-ALL [74]; 4) Lisocabtagene Maraleucel (Breyanzi, Juno,

NCT02631044, 2021) for DLBCL and FL (grade 3B, FL3B) [75]. Depending on the CAR-T cell product, overall response rates (ORR) and complete responses (CR) vary ranging from 52-85% and from 40-59%, depending on the specific product used. The two other FDA-and EMA-approved CARs are directed against B cell maturation antigen (BCMA) that is overexpressed on multiple myeloma (MM) cells (Yan, 2019). Two anti-BCMA CAR T cell products are available for MM indications, namely Idecabtagene Vicleucel (Abecma, Bristol Myers Squibb, NCT03361748, 2021) and Ciltacabtagene Autoleucel (Carvykti, Janssen Biotech, NCT03548207, 2022) with ORR of 73% and 98%, respectively [76, 77].

1.3.3 CAR-T cell therapy for T cell malignancies: challenges and proposed solutions

Successful translation of CAR-T cell therapy to tackle T cell malignancies has faced obstacles due to similarities between normal, malignant, and therapeutic T cells. Circulating tumor cells exist in the peripheral blood of T-ALL [78, 79] (Marks, 2009; Litzow, 2015) and TCL patients (World Health Organization classification of tumors. 4th edition, 2017) and are phenotypically similar to their normal counterparts; thus, they may be harvested and subsequently CAR-transduced, leading to co-infusion of "CAR tumor T cells". Given that T cell antigens are shared between therapeutic CAR-modified T cells and normal T cells, mutual killing of CAR T cells, also referred to as "fratricide", may occur, thereby, impeding the manufacturing and long-term persistence of the product in patients. Furthermore, targeting T lineage antigens widely expressed on normal T cells would cause a prolonged T cell aplasia after administration. To address the aforementioned challenges, numerous engineered cell-based approaches have been developed (**Table 2**).

Challenges	Solutions	Target Antigen	Cell Modification	References
Fratricide	Genetic deletion of the target antigen	CD7	CD7 KO/ base edited	[80]
	Blockade of target antigen expression	CD7	CD7 protein expression blocker	[81]
	Pharmacologic inhibitors (Ibrutinib and Dasatinib)	CD7	None	[82]
	Natural selection	CD7	None	[83]
	Reversible CAR expression	CD5	Tet-Off	[84]
T cell aplasia	Safety switch	CD4	Use of Alemtuzumab	[85]
Product contamination	Use allogeneic CAR-T cells	CD3, CD7	CD3/CD7 and TCR KO	[86, 87]
Fratricide/T cell aplasia	Targeting a restricted T cell antigen	CD1a, CD30, CD37, TRBC, CCR4	None	[88–92]
	Genetic deletion of the target antigen/ Use allogeneic CAR-T cells	CD2	CD2 and TCR KO	[93]

Table 2: Strategies to overcome challenges in CAR-T cell therapy for T cell lineage malignancies. CAR-T cells redirected against antigen shared with normal T cells recognize not only malignant cells but also normal and therapeutic T cells. This gives rise to three main problems i) fratricide, ii) T cell aplasia, iii) product contamination with malignant T cells. When targeting pan-T cell antigens (e.g. CD2, CD4, CD5, CD7), mutual killing (“fratricide”) of the CAR-T cells occurs. Abrogation of target expression (e.g. via gene editing or protein blockers) or pharmacological inhibition (e.g. via Ibrutinib and Dasatinib, see below) of the CAR signaling or reversing the CAR expression (e.g. via Tet-Off system) are the latest methods to prevent it. Naturally selected unmodified CAR-T cell products have been also developed. Alternatively, fratricide can be mitigated by selecting targets with restricted expression to T cell subsets (e.g.

CD1a, CD30, CD37, TRBC, CCR4) which also helps address extensive T cell aplasia. Another way to circumvent T cell aplasia is via the application of safety switches (e.g. alemtuzumab) which deplete the infused CAR-T cells. Distinguishing between normal and circulating tumor cells is difficult due to their similarities. To avoid product contamination, CAR-T cells can be generated using TCR-edited third-party donor T cells. Adapted from Kai-Lin Fang, Lee and Zhang, 2023, Cancers.

1.3.4 Preventing T cell aplasia with suicide genes, safety switches and short-lived CAR cells

In contrast to B cell aplasia, which is well tolerated, persistent T cell depletion predisposes patients to life-threatening infections. Hence, various approaches have been applied for the selective ablation of therapeutic CAR-T cells and reverse constitutive damage to the normal T cell compartment whenever necessary. To date, three classes of suicide gene technologies exist and are classified based on their ability to i) convert non-toxic drugs to toxic compounds ii) cause inducible caspase-9 (iCASP9) dimerization iii) induce antibody-dependent cellular cytotoxicity (ADCC) in gene-modified T cells. The first introduced suicide marker is the viral enzyme herpes simplex virus thymidine kinase (HSV-TK). Upon transferring the enzyme gene and subsequent treatment with ganciclovir, HSV-TK produces a toxic compound, ultimately aborting DNA chain elongation in proliferating cells [94]. Limitations of this strategy are the potential for immunogenicity of HSV-TK and its slow pharmacokinetics. A more recent approach consists in incorporating an inducible caspase-9 (iCasp9) human sequence into the CAR construct. Administration of the synthetic drug AP1903 results in fast clearance of CD19 CAR-engineered T cells [95]. A second ADCC-based safety switch is the truncated human epidermal growth factor receptor (huEGFRt). Genetically removal of the extracellular domains harboring the binding site for endogenous ligands as well as cytoplasmatic signaling domains rendered this protein functionally inert but still recognizable by the clinically approved anti-EGFR antibody Cetuximab. Application of Cetuximab results in ADCC-mediated depletion of CAR-T cells. A phase I clinical study is being conducted to treat patients with MUC16ecto+ solid tumors with an anti MUC-1 CAR T cell product additionally equipped with hEGFRt (NCT02498912). More recently, pharmacological control of CAR-T cell functions has been achieved via clinically approved tyrosine kinase inhibitors (TKI) as a novel alternative approach to conventional suicide genes. TKI

Dasatinib was shown to inhibit Lck phosphorylation and subsequent CAR signaling, thereby reversibly preventing CAR-T cell activation [96]. Interestingly, TKI Dasatinib and Ibrutinib were used to avert the self-targeting of CD7 CAR T cells during manufacturing, thus obviating the need for additional modifications of the CD7 gene [82]. Another strategy to prevent the prolonged depletion of normal T cells is to use innate cells with limited lifespans as immune effectors for CAR therapy, namely NK cells and $\gamma\delta$ -T cells. Importantly, utilizing NK cells to target a T cell antigen offers an additional way to circumvent fratricidal issues. Indeed, several groups have commenced preclinical investigations using NK-92 cells redirected against T cell antigens such as CD5, CD7, CD4, and CD3 [97–100].

1.4 NK cells as cellular vehicle for CAR-based therapy

Alternative cellular vehicles are being utilized for CAR engineering in addition to T cells. Among these, NK cells have garnered the most attention as they naturally possess anti-tumor cytotoxic functions and a safety profile. In the context of T cell malignancies, the absence of shared target T cell antigens and their short lifespan make NK cells ideal for CAR retargeting, circumventing the risk of fratricide during production and unwanted prolonged T cell aplasia in patients.

1.4.1 Overview on NK Cell Biology

NK cells are in the first line of defense against cancer and microbial infections [101]. They were first described in the 1970s as lymphocytes with the innate capacity to destroy tumors without prior antigen stimulation [102, 103]. NK cells, classified as group 1 innate lymphoid cells, are classically identified by surface expression of CD56 and lack of the clonotypic TCR and associated CD3 molecules. In human, NK cells can be found in the blood circulation, lymphoid tissues (bone marrow, spleen), and non-lymphoid tissues (uterus, liver, lung). Based on the relative density of CD56, NK cells can be further divided into two major subsets: CD56^{dim} and CD56^{bright}. The former display greater developmental maturity, enhanced cytolytic capacity, and expression of high levels FcRgIII (CD16), which make them potent effectors of antibody-dependent cell-mediated cytotoxicity (ADCC). The latter are less mature, less

cytotoxic, and produce abundant amounts of immunoregulatory cytokines. In healthy adults, NK cells account for 5-20% of circulating lymphocytes, and approximately 90% of them are CD56^{dim}. CD56^{brigh} NK cells are more predominant in tissues [104].

Some aspects of NK cell development remain unclear and under investigation. The consensus is that human NK cells originate from a multipotent CD34+ hematopoietic progenitor, which is in the BM. NK cell maturation takes place in the BM as well as in lymphoid organs and, in contrast to T cell, does not require the thymus [105, 106]. There is contradictory data on the lineage relationship between CD56^{brigh} and CD56^{dim} subsets. The debate centers around whether CD56^{brigh} is an immature precursor of CD56^{dim} or if they have distinct origins [107]. NK cells constantly interact with their surroundings using an array of innate activating and inhibitory receptors, enabling them to differentiate between healthy and potentially infected or transformed cells. NK cell stimulation and effector functions are controlled by the net balance of these signals. To ensure tolerance towards healthy cells, inhibitory signals typically prevail. Among the inhibitory receptors, the killer cell immunoglobulin-like receptor (KIR) family is of pivotal importance. KIRs bind to MHC class I molecules, also known as human leukocyte antigen (HLA) in humans. During viral infection or malignant transformation, MHC class I is frequently downregulated or absent. In accordance with “missing self-hypothesis”, NK cells sense this loss of inhibition, shifting the balance towards activation, ultimately leading to the destruction of the target cells [108]. The absence of MHC class I is, however, insufficient to evoke complete NK activation, which requires the recognition of cellular stress- and virus-associated molecules by NK cell activating receptors. For example, MHC class I polypeptide-related sequence A (MICA) and MICB serve as ligands for the activating receptor NKG2D, and are upregulated in tumorigenesis, infection, or DNA damage [109, 110].

Morphologically, NK cells in peripheral blood appear as large lymphoid cells with abundant cytoplasmic granules [111]. When NK cells recognize target cells, an immunological synapse (IS) forms at the contact point and direct release of the lytic granules occurs at the IS interface within minutes. These granules contain cytotoxic effectors, such as perforin and granzymes. Pores are created in the target plasma membrane by perforin, allowing granzymes to enter and activate caspases, ultimately leading to target cell death [112]. Detection of lysosomal-associated membrane

protein 1 (LAMP1, also referred to as CD107a) on the cell surface can serve as a marker for this process known as 'degranulation' [113]. NK cells can also eliminate tumor cells by engaging death receptors such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand in a granule-independent but caspase-dependent fashion [114]. In addition to their natural cytotoxicity, NK cells are major effectors of ADCC. They engage the Fc portion of antibody-coated targets via the activating receptor CD16 which triggers NK-mediated target cell killing and secretion of cytokines [115].

Upon activation, NK cells secrete multiple pro-inflammatory mediators, including interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines. For example, the secretion of IFN- γ promotes the polarization of Th1 cells, triggers MHC class II expression on APC, and stimulates macrophage activation [116, 117].

1.4.2 Unmodified NK cell therapy

NK cells for infusion can be isolated from multiple sources, including peripheral blood (PB) and umbilical cord blood [118, 119]. Alternatively, they can be differentiated from hematopoietic progenitor cells [120] and induced pluripotent stem cells (iPSCs) [121, 122]. NK cell lines have also been utilized. The NK-92 cell line was derived from a 50-year-old patient with NHL [123], and it is the only one that entered FDA-approved clinical trials. Infusion of NK-92 cells requires lethal irradiation to prevent proliferation and potential for tumor transfer to the patients. Most clinical trials have used PB-derived NK cells, and the starting material was typically leukapheresis. Freshly isolated NK cells are typically in a resting state; therefore, short-term (12-16 h) exposure to IL-2 is employed as a method to prime the cells prior to injection [124]. Obtaining therapeutically relevant cell numbers for adoptive NK cell transfer might be challenging as these cells represent a minor fraction of blood lymphocytes. Sustained proliferation responses in NK cells are achieved through cultivation with feeder cells such as autologous PBMC [125] and the K562 Chronic Myelogenous Leukemia cell line [126–128]. The absence of HLA class I coupled with enforced expression of additional NK stimulatory molecules such as 4-1BBL and membrane-bound cytokines (IL-15 or IL-21) on K562 provides NK cells with potent contact-dependent expansion

stimuli [126, 127]. Studies with autologous NK cells have produced unsatisfactory outcomes. For instance, the administration of NK cells derived from T cell-depleted leukapheresis after overnight incubation with IL-2 (1,000 U/ml) in R/R lymphoma patients was ineffective [129]. Similar results were observed in patients with metastatic melanoma or renal cell carcinoma who received autologous NK cells expanded with IL-2 OKT3 feeder cells and irradiated autologous PBMC for ten days following lymphodepletion [130]. The lack of success in adoptively transferring autologous NK cells can be attributed, in part, to the recognition of self-HLA molecules on the surface of tumor cells by NK cells, leading to a reduction in their cytotoxic activity. An early study demonstrated the antileukemic efficacy (graft-versus-leukemia, GvL) of alloreactive NK cells in a mouse model of Acute Myeloid Leukemia (AML), which was further supported by data from AML patients undergoing hematopoietic stem cell transplantation (HSCT) without causing graft-versus-host disease (GvHD). This finding revealed that NK cell alloreactivity resulting from KIR-mismatch played a major role in the GvL effect and correlated with a lower occurrence of relapse following transplantation [131]. The *in vivo* persistence of allogeneic NK cells can be hampered by immune rejection. To mitigate this, recipients receive lymphodepletion chemotherapy, including fludarabine and cyclophosphamide, before infusion of NK cells. In addition, to sustain the survival and proliferation of infused NK cells, cytokine support, typically IL-2 or IL-15, is provided to the recipients subcutaneously or intravenously [121, 132].

1.4.3 CAR- NK cell therapy

NK cells can be equipped with CARs to reprogram their specificity to a particular tumor target. So far, the majority of CARs employed in CAR-NK cell studies have consisted of CAR constructs optimized for T cell signaling and function. Although certain signaling and costimulatory domains, like CD3z and 4-1BB, are common to both NK cells and T cells, the function of other molecules, such as CD28, in NK cells remains not yet fully elucidated [133]. Given that NK cells have their distinct activating and inhibitory receptors governing their cytotoxicity, it was suggested to employ NK-specific intracellular signaling domains like DNAX-activating protein 10 (DAP10), or immunoreceptor tyrosine-based activation motifs (ITAM)-containing domains such as

DAP12 and 2B4 could boost cytotoxicity. DAP10 signals through the activating receptor NKG2D, whereas DAP12 transmits signals through NKG2C, NKp44, and KIRs. When the 2B4 activating receptor binds to its ligand CD48, the adapter molecules like SLAM-associated protein (SAP) is recruited through its immunoreceptor tyrosine-based switch motif (ITSM) for signal transduction [134]. Studies indicated that cytolytic functions were effectively elicited in CD19 CAR NK cells when utilizing either a DAP10 or CD3z signaling domains [126], but the most optimal response was observed upon integration of both domains into the CAR construct [135]. Li and colleagues conducted a comparative study to evaluate one CAR T cell construct alongside nine distinct CAR-NK cell variants directed against the same mesothelin antigen harboring four distinct transmembrane domains (TM) and various intracellular signaling domains (SD). Superior *in vitro* and *in vivo* activity was achieved when NKG2D (TM), 2B4 (costimulatory domain) and CD3z (SD) were combined [122].

The safety profile of NK cells in allogeneic settings renders NK cells a superior cellular vehicle for CAR engineering compared to T cells. This unique characteristic enables the use of donor-derived NK cells that can be specifically redirected towards the antigen of interest with a CAR, thus creating bank doses of 'off-the-shelf' cellular products that can potentially be administered to any cancer patient (**Figure 2**).

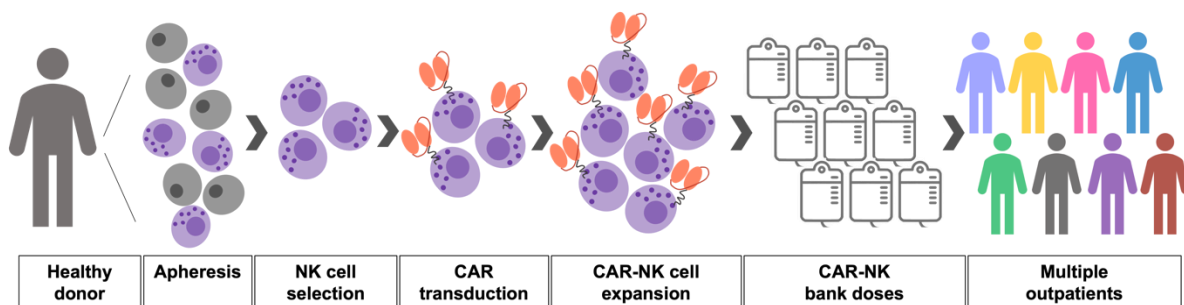


Figure 3: A brief flow chart of CAR engineered-NK cell therapy. Red star indicates that functional NK cells can be derived from various allogeneic sources: umbilical cord blood (UCB), peripheral blood (PB), hematopoietic stem cells (HSC) and cell lines such as NK-92. This figure was created with Illustrator. The blood bag was obtained from BioRender.com

Preclinical studies have extensively evaluated the efficacy of CAR-expressing NK-92 cells, targeting a range of cancers including B cell lymphoid malignancies [136–138], Multiple Myeloma [139], Acute Myeloid Leukemia (AML) [140], Breast Carcinoma [141, 142], Neuroblastoma [143], and Glioblastoma [144, 145]. In the case of T cell malignancies, four CAR-NK cell therapies have been developed, targeting CD3, CD5, and CD7. These therapies showed enhanced cytotoxicity against T-ALL and T-cell lymphoma *in vitro* and in NSG mouse models [97–100].

1.5 Chemokines and their receptors as therapeutic targets in cancer

Chemokines and their receptors are best known for their primary function in orchestrating the directional migration of various immune cells in the body. However, over the past decades, they have been recognized as key players in diverse physiological processes, including development, epithelial homeostasis, and angiogenesis. Notably, dysregulation in the chemokine system is implicated in chronic inflammatory disorders and in cancer biology, thus rendering chemokine receptors potential therapeutic targets.

1.5.1 Chemokines and chemokine receptors

Chemokines constitute a family of small soluble chemotactic cytokines of approximately 70 amino acid residues with more than 50 known members in humans [146]. They tightly regulate the trafficking and positioning of cells between and within tissues in a spatiotemporal fashion. Four major groups of chemokines exist, categorized as C, CC, CXC, and CX3C, based on the quantity and arrangements of conserved cysteine residues forming disulfide bonds on their N-terminus [147]. Chemokines exert their biological functions by binding to seven-transmembrane (7TM) domain G α i protein-coupled chemokine receptors (GPCR) [148] and are structurally categorized into four groups, (CX3CR, CXCR, CCR, and XCR), based on their ligands [149]. To date, a total of 24 different receptors have been characterized. They measure 350 amino acid residues in length and consist of an extracellular ligand-binding portion (the N-terminus), seven α -helical domains traversing the membrane with three intracellular and extracellular loops and an intracellular region (the C-terminus) [150,

151]. Chemokine receptors are bound to a heterotrimeric GTP-binding protein (G protein) consisting of α , β and γ subunits in their inactivated form. Upon chemokine binding, GDP/GTP exchange and dissociation of the $G\alpha$ and $G\beta\gamma$ subunits occur. The $G\alpha$ -GTP-bound subunit activates many downstream effectors, including Src and PI3 kinases, involved in cytoskeletal reorganization, focal adhesion formation and cell motility [152]. Additionally, activated GPCRs can trigger pro-proliferative and anti-apoptotic signaling circuits via G protein-independent β -Arrestin-mediated activation of ERK1/2 and Akt kinases [153, 154]. Resolution of the response is marked by β -arrestin recruitment, leading to receptor internalization and degradation/recycling [155]. In most instances, the interaction between chemokine receptors and their ligands is promiscuous as they bind to more than one chemokine and vice versa. This renders the chemokine system highly complex [156].

1.5.2 Biological roles of chemokines and chemokine receptors in homeostasis and diseases

Based on their expression patterns, chemokine receptors and their corresponding ligands fall within two functional categories: homeostatic and inflammatory. The homeostatic receptors CXCR4, CXCR5, and CCR7 define "cellular highways" that navigate cell homing, positioning, and trafficking to tissue-specific niches under basal conditions [157, 158]. CXCR4 and its exclusive ligand CXCL12 have a fundamental role in embryonic development [159, 160]. The primary source of CXCL12 is resident stromal cells, namely endothelial cells (ECs) in the bone marrow and secondary lymphoid organs (SLO), where it regulates the retention of hematopoietic stem and progenitor cells and controls the germinal center organization [161, 162]. CXCR5, together with CCR7, participates in the development and functional organization of SLO. Its ligand, CXCL13, is secreted by follicular dendritic cells (FDCs) and attracts CXCR5⁺ cells, forming the B cell zone of SLOs. Under homeostatic conditions, fibroblastic reticular cells (FRCs) of the T cell zone constitutively secrete CCR7 ligands, CCL19 and CCL21, which attract CCR7⁺ cells, including B cells, dendritic cells, naïve, regulatory, and central memory T cells. Inflammatory chemokines attract circulating leukocytes and other cell types in infection, inflammation, tissue injury, and tumor scenarios and their expression is induced or upregulated by pro-inflammatory

mediators. CCL1–5 and CXCL1–11 chemokines belong to this group. Specifically, CXCL9–11 regulate activated T cell trafficking, whereas CXCL1–8 regulate neutrophil trafficking. The navigation of T cells, macrophages and monocytes is under the guide of inflammatory CC chemokines. Dysregulation of chemokine/chemokine receptor axes is linked to an array of disease states, including infectious (malaria and HIV), autoimmune (rheumatoid arthritis, psoriasis, and multiple sclerosis) pulmonary (chronic obstructive pulmonary disease and asthma), vascular and metabolic (diabetes and obesity) disorders as well as cancer progression [163].

1.5.3 The homeostatic chemokine receptor CCR7

According to the classical paradigm, the homeostatic chemokine receptor CCR7 serves as a passport for the entry of naïve lymphocytes into secondary lymphoid tissues, such as lymph nodes and spleen, and their subsequent positioning within defined functional compartments. CCR7, also called CD197, was discovered in 1993 as orphan G protein-coupled receptors exclusively expressed in lymphocytes and termed Epstein–Barr virus-induced gene 1 (EBI1) [164]. The same gene was subsequently identified in a homology screen for chemokine receptors in Burkitt's lymphoma and named Burkitt's lymphoma receptor 2 [165]. After characterizing its chemokine ligand ELC, EBI1/BLR2 was renamed to CCR7 [166]. CCL19 (also known as ELC or MIP-3b) and CCL21 (also known as SLC or 6CK) are the sole binders for CCR7 [167]. Under homeostatic conditions, fibroblastic reticular cells (FRCs) of the T cell zone are the main source of both chemokines in mouse and human SLOs [168]. However, CCL21 can also be produced by high endothelial venules (HEVs) in mice [169]. In inflammation, additional sources of CCR7 ligands are activated dendritic cells (DC) being recruited into draining lymph nodes [170]. As a member of 7TM receptor families, CCR7 signals through heterotrimeric G proteins and their downstream effectors. Various immune cells constitutively express CCR7 on their surface, namely naïve (T_n), central memory (T_{cm}), and regulatory T cells (T_{reg}), naïve B cells, single positive (SP) and double negative (DN) thymocytes [171–174]. CCR7 can be also acquired by semi-mature and fully-mature DCs as well as CD56⁺CD16⁻ NK cells upon pathogen encounter [104, 175, 176]. T_n, T_{cm} and T_{reg} penetrate lymph nodes (LN) following a stepwise program regulated by CCR7 interacting with HEVs surface-

anchored CCL21. The subsequent intranodal migration of T cells on FRCs within the paracortical T cell-rich area occurs in a CCL19/CCL21-mediated fashion. On their way through the LN, T cells scan the antigen-presenting DCs that swarmed from the periphery via the CCR7-CCL19/CCL21 axis. CCR7 knockout (KO) mouse models elucidated the key role of this chemokine receptor in driving leukocyte migration to lymphoid tissues. These mice remained viable, fertile, and harbored structural aberration limited to lymphoid tissues. Interestingly, the overall microarchitecture of LNs and Peyer's patches were impaired as they were largely devoid of DCs, T cells, and to a lesser degree of B cells, however, CCR7-KO mice developed organized ectopic tertiary lymphoid structures in mucosal sites such as the stomach, intestine, and lungs. Adoptive transfer experiments demonstrated that CCR7-deficient i) T cells fail to home to the LNs but exclusively accumulated in the splenic red pulp, ii) B cells could migrate both to the splenic white pulp and the LNs, iii) DCs could not reach the draining LNs from the skin upon mobilization stimuli. In general, these animals displayed deleted, but intact, T cell and B cell responses and deficits in tolerance to self-antigen which rendered them more susceptible to develop generalized multi-organ autoimmunity despite the absence of clinically evident autoimmune disorders [177, 178].

1.5.4 CCR7 in blood cancer

Multiple lines of evidence suggest that cell trafficking coordinated by the chemokine receptor CCR7 has a central role in the pathophysiology of various hematological neoplasms. Compelling data report that various subtypes of leukemias and lymphomas exhibit CCR7 on their surface due to their lymphoid lineage and/or developmental stage. Among B lineage malignancies, CCR7 overexpression is consistently detected on the surface of virtually all PB, LN, or BM-sampled chronic lymphocytic leukemia (CLL) cells. In this context, it has been linked to nodal dissemination as well as intranodal positioning of malignant cells in close vicinity to pro-tumorigenic accessory cells, (stromal cells, follicular DC, and CD40L+CD4+ TH cells), which can trigger the constitutive secretion of CCL19 and CCL21, and, thus, creating a tolerogenic and protective microenvironment. Underlying genetic factors in the overexpression of CCR7 remain elusive but, interestingly, a single nucleotide

polymorphism (SNP) in the gene correlated with a higher risk of developing CLL (rs3136687) [179, 180]. Similar patterns were described in Burkitt's Lymphoma (BL). Albeit expression data of primary BL samples are scarce, studies in the syngeneic E μ -Myc mouse model of BL have demonstrated that CCR7 guides malignant clones to the T cell zone of SLOs and support the creation of protective niches via crosstalk with resident accessory and stromal compartments [181]. Interestingly, CCR7 axis had a prominent role in navigating not only tumor cells but also pro-tumorigenic activated T cells and CCR7+ Treg from the circulation in classical Hodgkin's Lymphoma [182, 183].

In B Cell Acute Lymphoblastic Leukemia (B-ALL), expression data reported variably low levels of this receptor, suggesting a limited impact in guiding this malignancy into lymphoid niches. However, in a previous study, CCR7 expression at the time of diagnosis was linked to cerebral presentations, which prompted the hypothesis that the receptor might bear additional pathogenic functions, namely CNS homing in the early phase of the disease [184]. As per B cell neoplasms, T cell lineage malignancies encompass multiple entities that are postulated to derive from the thymic or post-thymic stage of T cell development with immature or differentiated phenotypes. Accordingly, CCR7 expression would be highest in entities with a DN or SP thymocyte, Tn, Tcm or Treg pattern. In T-ALL relapsed patients, CNS manifestations are frequently present. CCR7 receptor was found significantly up-regulated in T-ALL samples with activating mutations in Notch1 gene [185]. Remarkably, pre-clinical T-ALL mouse models overexpressing the intracellular cleaved form of Notch1 (ICN1) substantiated the evidence that augmented CCR7 favored neurotropism of the malignant cells [184]. In the case of T Cell Prolymphocytic Leukemia (T-PLL), one study found that CCR7 was overexpressed in 86.5% of cases of the examined cohort at the time of diagnosis and, more importantly, the proportion of PB derived T-PLL cells expressing CCR7 at diagnosis and overall survival within a 8 year follow-up inversely correlated. In addition, the pathogenic role of CCR7 in fostering lymphotropism of malignant cells was confirmed in this key scenario [186]. Upregulated CCR7 transcript and protein were detected in treatment refractory Adult T cell Leukemia/Lymphoma (ATLL) patients with acute and progressive disease and associated with a poor prognosis and nodal involvement. [187].

In the case of Cutaneous T Cell Lymphoma (CTCLs), the expression of CCR7 has been identified as a marker of advanced Mycosis Fungoides (MF) and mediator of subcutaneous as well as lymphoid dissemination of cutaneous lesions [188]. Additional roles of CCR7 in epidermotropism and proliferation within the skin of SS leukemic cells exhibiting high mRNA and protein levels have been also acknowledged [189]. With regards to Anaplastic Large Cell Lymphoma (ALCL), two studies reported overexpression of CCR7 in the ALK-negative variant of ALCL compared to ALK-positive or primary cutaneous ALCL [190, 191]. Also, samples from Peripheral T Cell Lymphoma Not Otherwise Specified (PTCL-NOS) patients exhibited CCR7 expression as part of their Tcm signature [192].

Two clinical studies are underway to validate CCR7 as a therapeutic candidate for blood cancers. A phase-I clinical trial was announced by Novartis involving patients with R/R CLL and NHLs who are being treated with a humanized anti-CCR7 antibody-drug conjugate, named JBH492 (NCT042140704). A second phase-I clinical is being conducted by Catapult Therapeutics evaluating the safety and efficacy of CAP-100, a humanized anti-CCR7 monoclonal antibody with antagonist activity, in patients with R/R CLL or Small Lymphocytic Lymphoma (SLL) at Dana-Farber Cancer Institute (NCT04704323) [193].

2 Aim of the thesis

T cell non-Hodgkin's lymphoma (T-NHL) comprises a highly heterogeneous disease of the lymphoid compartment marked by the expansion of malignant T cells. Relapse rates are frequent due to the scarce availability of effective treatment options and clinical outcomes are generally poor. Chimeric antigen receptor (CAR) T cell therapy has produced remarkable responses in patients with relapsed and refractory patients B cell and plasma cell malignancies. A challenge in translating CAR T cell therapy into T cell malignancies is the shared target antigen expression between normal, therapeutic, and malignant cells, leading to i) self-destruction of CAR-T cells, ii) on-target toxicities against normal T cells iii) product contamination with cancerous T cells. The distinct biological features of Natural Killer (NK) cells make them a superior cellular vehicle for CAR retargeting in the treatment of T cell lineage cancer. In this thesis, the chemokine receptor CCR7, which orchestrates lymphocyte homing to secondary lymphoid organs, was identified as a candidate target for CAR-NK cell therapy. Various lines of evidence indicate that cell trafficking regulated by CCR7 has a fundamental role in the pathophysiology of numerous T cell leukemias and lymphomas.

The aims of this thesis were:

- 1) Characterization of CCR7 expression on T-NHL derived cell lines and primary tumor cells from T-NHL samples;
- 2) Generation of an anti-CCR7 CAR construct, development of CCR7 CAR NK-92 and YTS cellular products and conducting extensive *in vitro* functional studies to prove CAR-specific cytotoxic activity against tumors;
- 3) Establishing protocols for peripheral blood (PB)-derived NK cell generation and *in vitro* functional testing of CCR7 (PB)-CAR-NK cells;
- 4) Developing a T-NHL xenograft mouse model for T-NHL and preclinical *in vivo* testing of the anti-lymphoma efficacy of the herein established CCR7 CAR-NK cells;
- 5) Assessing CCR7 expression profile on benign tissues and addressing potential safety concerns surrounding CCR7 CAR-NK cell therapy.

3 Material and Methods

3.1 Material

3.1.1 Oligonucleotides

Table 2: Oligonucleotides

Primer ID	Sequence (5' - 3')	Experimental Purpose
A1-NotI_CCR scFv FWD	TTACAGGCGGCCGCCACC ATGGATTTTCAGGTGCAGA TCTTCAGC	Oligonucleotide for amplification of CCR7 scFv for cloning in the mp71 vector with Kozak consensus sequence and NotI restriction site
A2-CCR_scFv_REV	GGCACCAAGCTGGAATCA AGAGACCCGCCGAGCCCA AGAGCA	Oligonucleotide for amplification of CCR7 scFv for cloning in the mp71 vector
A3-CCR-H28 WFD	GGCACCAAGCTGGAAATC AAGAGACCCGCCGAGCCC AAGAGC	Oligonucleotide for amplification and cloning of a 2 nd generation CAR backbone in the mp71 vector
A4-H28_BsiWI_REV	TACGGCTTTCGTTTTCTCC T	Oligonucleotide for amplification and cloning of a 2 nd generation CAR backbone in the MP71 vector binding in the MP71-PRE region with BsiWI restriction site
A7-NotI_BspEI_P2A_FWD	ACAGGCGGCCGCTGGTCC TCCGGAGCCACCAACTTC AGCCTGCTG	Oligonucleotide for amplification and cloning of P2A_mTq in the mp71 vector with BspEI restriction site
A8-mp71_UniCAR_REV	CATGCTATTGCTTCCCGTA CGGCTT	Universal oligonucleotide for (i) amplification and cloning of P2A_mTq in the mp71 vector and (ii) sequencing of all mp71_CAR construct binding in the MP71-PRE region
A9-mp71_uniCAR-FWD	GCCCCTGGGAGACGTCCC AGCGGCC	Universal oligonucleotide for (i) amplification of the 2 nd generation SP6 CAR cassette and (ii) sequencing of all mp71_CAR constructs binding in the introns downstream the 5' LTR

A10- BspEI_Univ_mp71C AR_REV	CAGGCCCTGCCCCCTCGC GGCTCCGGATGGTCC	Universal oligonucleotide for amplification of the 2 nd generation SP6 CAR cassette binding in the CD3 ζ domain with BspEI restriction site
A12-Univ_mp71- P2A_FWD	CAGCAGGAGCGCAGACGC CCCCGCG	Universal oligonucleotide for sequencing of all mp71_CAR_P2A constructs binding in the P2A domain
A13-Univ_mp71- P2A_REV	TCTTGTAGTTGCCGTCGTC CTTGAA	Universal oligonucleotide for sequencing of all mp71_CAR_P2A constructs binding in the P2A domain
A17-Univ_8R1	TTTGCCAGAGCCGCTTGTA GAGCCGCTAGACACTGTG ACCAG	Universal oligonucleotide for amplification and cloning of the r/pH/fH VHs in pH/fH scFv variants
A18-Univ_8F2	GGC TCT ACA AGC GGC T CT GGC AAA CCT GGA TCT GGC GAG GGA	Universal oligonucleotide for amplification and cloning of the r/pH/fH VLs binding in the linker region in pH/fH scFv variants

3.1.2 Plasmids and retroviral vectors

Table 3: Plasmids and retroviral vectors

Plasmid ID	Description
20AALJLP_2764886 QAD_NK_human CCR7 ScFv	GeneArt plasmid containing rat anti-human CCR7 scFv clone 3D12 (rVH-linker-rVL)
H28-MP71_human CXCR5 CAR_IgG1_CD28_CD28_CD3z	Retroviral vector MP71 expressing anti-human CXCR5 CAR (VH-linker-VL-IgG1 Δ -CD28-CD3 ζ)
M5-MP71_3D12_human CCR7 CAR_IgG1_CD28_CD28_CD3z	Retroviral vector MP71 expressing rat anti-human CCR7 CAR (rVH-Linker-rVL-IgG1 Δ -CD28-CD3 ζ)
M7-MP71_empty_P2A_mTq	Retroviral vector containing P2A-mTq
M8+_fH_CCR7 CAR _NK	GeneArt plasmid containing fully humanized (fH) anti-human CCR7 CAR (fHVH-linker-fHVL-IgG1 Δ -CD28-CD3 ζ -P2A)

M9+_pH_CCR7 CAR_NK	GeneArt plasmid containing partially humanized (fH) anti-human CCR7 CAR (pHVH-linker-pHVL-IgG1Δ-CD28-CD3ζ-P2A)
M10-MP71_SP6_28z_P2A_mTq	Retroviral vector MP71 expressing anti-human SP6 CAR (VH-Linker-VL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M12-MP71_3D12_CCR7_28z_P2A_mTq	Retroviral vector MP71 expressing rat anti-human CCR7 CAR (rVH-Linker-rVL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M17- MP71 CCR7 (rVH-L-fHVL) 28z_P2A_mTq	Retroviral vector MP71 expressing anti human CCR7 (rVH-linker-fHVL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M18- MP71 CCR7 (fHVH-L-rVL) 28z_P2A_mTq_mp71	Retroviral vector MP71 expressing anti human CCR7 (fHVH-linker-rVL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M19- MP71 CCR7 (rVH-L-pHVL) 28z_P2A_mTq	Retroviral vector MP71 expressing anti human CCR7 (rVH-linker-pHVL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M20 - MP71 CCR7 (pHVH-L-rVL) 28z_P2A_mTq	Retroviral vector MP71 expressing anti human CCR7 (pHVH-linker-rVL-IgG1Δ-CD28-CD3ζ-P2A-mTq)
M28+_CCR7 scFv_[(rVH)-linker-pHVL(T14P) -IgG1Δ]	GeneArt plasmid containing partially humanized anti-human CCR7 CAR [(rVH)-linker-pHVL(T14P)-IgG1Δ]
M29+_CCR7 scFv_[(rVH)-linker-pHVL(S22T)-IgG1Δ]	GeneArt plasmid containing partially humanized anti-human CCR7 CAR [(rVH)-linker-pHVL(S22T)-IgG1Δ]
M30+_CCR7 scFv_[(rVH)-linker-pHVL(D40N)-IgG1Δ]	GeneArt plasmid containing partially humanized anti-human CCR7 CAR [(rVH)-linker-pHVL(D40N)-IgG1Δ]
M31+_scFv_[(rVH)-linker-pHVL(K45R)-IgG1Δ]	GeneArt plasmid containing partially humanized anti-human CCR7 CAR [(rVH)-linker-(K45R)-IgG1Δ]
M28-MP71 CCR7 [rVH-pHVL(T14P)28z_P2A_mTq]	Retroviral vector MP71 expressing anti human CCR7 [rVH-linker-pHVL(T14P)-IgG1Δ-CD28-CD3ζ-P2A-mTq]

M29- MP71 CCR7 [rVH- pHVL(S22T) _ 28z_P2A_mTq]	Retroviral vector MP71 expressing anti human CCR7 [rVH-linker-pHVL(S22T) -IgG1Δ-CD28-CD3ζ-P2A-mTq]
M30- MP71 CCR7 [rVH- pHVL(D40N)_28z_P2A_mTq]	Retroviral vector MP71 expressing anti human CCR7 [rVH-linker - pHVL(D40N)-rVL-IgG1Δ-CD28-CD3ζ-P2A-mTq]
M31- MP71 CCR7 [rVH- pHVL(K45R)_28z_P2A_mTq]	Retroviral vector MP71 expressing anti human CCR7 [rVH-linker-pHVL(K45R)-IgG1Δ-CD28-CD3ζ-P2A-mTq]
pALF-MLV 10A1	Expression vector encoding murine leukemia virus 10A1 (MLV) env gene
pcDNA3.1gag/pol	Expression vector encoding MLV gag and pol genes
pLP1	Expression vector encoding HIV-1 gag and pol genes
pLP2	Expression vector encoding HIV-1 Rev protein
pCMV-VSV-g	Expression vector encoding G glycoprotein of the vesicular stomatitis virus (VSV-g) (env)
pFU-Luc-eGFP	Lentiviral vector expressing luciferase and eGFP

3.1.3 Antibodies

Table 4: List of antibodies for flow cytometry

Specificity	Fluorophore	Clone	Isotype	Provider	Dilution
IgG	PE		Polyclonal	Southern Biotech	1:600
IgG	APC		Polyclonal	Southern Biotech	1:400
CCR7 (CD197)	PE	3D12	Rat IgG2a	BD Biosciences	1:5
CD107a (LAMP-1)	PE	H4A3	Mouse IgG1	Biologend	1:100
CD56 (NCAM)	AF647	5.1H11	Mouse IgG1	Biologend	1:300
CD19	PE	HB19	Mouse IgG1	Biologend	1:100
CD19	PerCP-Cyanine 5.5	UCHL1	Mouse IgG1	Biologend	1:100
CD45	BV421	2D1	Mouse IgG1	Biologend	1:100
CD45	AF488	Hi30	Mouse IgG1	Biologend	1:100
CD3	APC-Cy7	HIT3a	Mouse Ig2a	Biologend	1:200
CD7	FITC	CD7-6B7	Mouse Ig2a	Biologend	1:100
CD7	PE	CD7-6B7	Mouse Ig2a	Biologend	1:100
CD2	PE-Cy7	RPA-2.10	Mouse IgG1	Biologend	1:100
CD5	APC	L17F12	Mouse Ig2a	Biologend	1:100
CD5	BV421	L17F12	Mouse Ig2a	Biologend	1:100

3.1.5 Primary cells and culture media

Table 5: Primary cells and culture media

Primary cells	Description	Provider	Medium
HUVEC	Human Umbilical Vein Endothelial Cells	PromoCell	Endothelial cell growth medium and supplements (PromoCell)
HUAEC	Human Umbilical Artery Endothelial Cells	PromoCell	Endothelial cell growth medium and supplements (PromoCell)
HA	Human Astrocytes	ScienCell	HA medium and supplements (ScienCell)
HPNC	Human Perineurial Cells	ScienCell	Fibroblast medium and supplements (ScienCell)
HCoEpiC	Human Colonic Epithelial Cells	ScienCell	HCoEpiC medium and supplements (ScienCell)
HCerEpic	Human Cervical Epithelium Cells	ScienCell	HCerEpic medium and supplements (ScienCell)
HUCc	Human urothelial cells	ScienCell	HUC medium and supplements (ScienCell)

3.1.6 Cell lines and culture media

Table 6: Cell lines and culture media

Cell line	Description	Provider	Medium
293VecGalV	Packaging cell line	Quantum Biotechnologies Inc. (Canada)	DMEM high glucose complete
HEK-293T	Human embryonic kidney	Quantum Biotechnologies Inc. (Canada)	DMEM high glucose complete

HEK-293	Human embryonic kidney	ATCC (USA)	DMEM high glucose complete
DEL	Anaplastic Large Cell Lymphoma (ALCL), t (2;5)-positive	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
DL-40	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-negative	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
FE-DP	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-negative	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
Mac-1	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-negative	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
JB6	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-positive	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
SU-DHL-1	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-positive	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
Karpas-299	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-positive	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
Mac-2a	Anaplastic Large Cell Lymphoma (ALCL), t(2;5)-negative	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
HuT-78	Cutaneous T Cell Lymphoma, Sézary Syndrome (SS)-derived	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
Se-Ax	Cutaneous T Cell Lymphoma, Sézary Syndrome (SS)-derived	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
MyLa	Cutaneous T Cell Lymphoma, Mycosis Fungoides (MF)-derived	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
HH	Cutaneous T Cell Lymphoma, Mycosis Fungoides (MF)-derived	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete

REH	B Acute Lymphoblastic Leukemia (B-ALL)	Dr. Stephan Mathas (MDC, Berlin)	RPMI-1640 complete
Jurkat	T Cell Acute Lymphoblastic Leukemia (T-ALL)	DSMZ (Braunschweig)	RPMI-1640 complete
HepG2	Hepatocellular Carcinoma Cell	DSMZ (Braunschweig)	DMEM high glucose complete
SW-620	Colon Adenocarcinoma Cell	CLS Inc. (Eppelheim)	DMEM high glucose complete
NK-92 (CRL-2408)	Natural Killer Cell Lymphoblastic Leukemia/Lymphoma	ATCC (USA)	α -MEM complete
YTS	Natural Killer Cell Lymphoblastic Leukemia/Lymphoma	A. Temme, University Clinics Carl-Gustav-Carus, Dresden	RPMI-1640 complete (YTS)

RPMI-1640 complete: medium supplemented with 10% FCS, 1x penicillin/streptomycin

DMEM high glucose complete: medium supplemented with 10% FCS, 1x penicillin/streptomycin, 1x non-essential amino acids solution, and 1 mM sodium pyruvate

α -MEM complete: medium supplemented with 12.5% horse serum, 12.5% FBS, 0.2mM inositol, 0.1mM 2-mercaptoethanol, 0.02 mM folate, 100 U/ml recombinant IL-2 (Peprotech), 2 mM L-glutamine, 100 U/ml penicillin, 0,1 mg/ml streptomycin (Gibco), 1% sodium pyruvate

RPMI-1640 complete (YTS): medium supplemented with 10% v/v heat-inactivated FBS (Gibco), 2 mM L-glutamine (Biochrome), 10 mM Hepes (Gibco), 100 U/ml penicillin, 0,1 mg/ml streptomycin (Gibco).

3.1.7 Kits

Stratec:	Invisorb Fragment Cleanup (1020300300)
	Invisorb Spin Plasmid Mini Two (1010140300)
Qiagen:	QIAGEN Plasmid Midi Kit (12143)
Miltenyi Biotech:	Human NK Cell Isolation (130-092-657)

Human NK Cell Activation/Expansion Kit (130-094-483)
Human Pan B Cell Isolation Kit (130-101-638)
Human Naïve Pan T cell Isolation Kit (130-097-095)
BD Biosciences: OptEIA™ human IFN-γ ELISA Set (555142)
Quantibrite PE Phycoerythrin Fluorescence Quantitation Kit (340495)

3.1.8 Instruments

PeqLab: Nanodrop Spectrophotometer 1000 ND 1000
Vilber Lourmat: Quantum Gel Documentation Imaging
Eppendorf: Mastercycler pro
BioTek Instruments: PowerWave X Select microplate UV/VIS reader
BD Biosciences: FACSCanto II Cell Analyzer
FACSAria Fusion Cell Sorter
FACSymphony A1 Cell Analyzer
Miltenyi Biotech: MACSQuant Analyzer 10
PerkinElmer: IVIS Spectrum In Vivo Imaging System

3.1.9 Software

Dotmatics: SnapGene 6.2.2
Adobe: Illustrator 24.0.1
Microsoft: Excell 16.76, Word 16.76.1
BD: FACS DIVA
FlowJo 10.4
GraphPad: Prism 8
ImageJ: ImageJ 2.0.0
PerkinElmer: Living Image 3.2/4.5

3.1.10 Mice

NOD.Cg-Prkdc^{scid} Il2rg^{tm1 Wjl}/SzJ (NSG) mice were housed and bred at the animal facility of the Max-Delbrück-Center for Molecular Medicine, Berlin, Germany. All animal studies received approval from the Berlin State review board at the Landesamt für Gesundheit und Soziales, Berlin under the registration number Landesamt für Gesundheit und Soziales TVV G 0279/19 and G 0331/19. The institutional guidelines of the MDC were observed for all animal testing.

3.2 Methods

3.2.1 Molecular biology

3.2.1.1 General workflow for cloning MP71 CAR constructs

Polymerase chain reaction (PCR)

The generation of plasmids containing novel MP71-CAR constructs involved using overlapping primers in two PCR reactions (PCR A and PCR B), yielding two overlapping DNA products A1-A2 and A3-A4 incorporating the designated DNA sequence. Invisorb® Spin DNA Extraction Kit (Strattec) was used to purify the DNA fragments. Primer sequences are listed in the Material section.

Reaction mix	10 µl	5X Q5 Reaction Buffer (NEB)
	1 µl	10 mM dNTPs (ThermoFisher Scientific)
	2.5 µl	10 µM FWD Primer (Eurofins MWG Operon)
	2.5 µl	10 µM REV Primer (Eurofins MWG Operon)
	5 ng	Template DNA
	0.5 µl	Q5 Hot Start High-Fidelity DNA Polymerase (NEB)
	Up tp 50 µl	Nuclease-Free H2O

Thermocycling conditions	Initial denaturation	98°C	30 sec
	25-35 cycles	98°C (denaturation)	10 sec
		50°C - 72°C (annealing)*	20 sec
		72°C (extension)	30 sec/kb**
	Final extension	72°C	2 min

*The annealing temperature was customized on each primer pair and determined using NEB Tm calculator for calculation.

**The extension time depended on the size of the DNA fragment.

A third PCR (PCR 3, annealing PCR) merged these fragments via annealing, resulting in an insert that integrated the desired modification which was subsequently amplified using primers.

Reaction mix	10 µl	5X Q5 Reaction Buffer (NEB)
	1 µl	10 mM dNTPs (ThermoFisher Scientific)
	50 ng*	A1-A2 DNA fragment
	50 ng*	A3-A4 DNA fragment
	0.5 µl	Q5 Hot Start High-Fidelity DNA Polymerase (NEB)
	Up to 50 µl	Nuclease-Free H ₂ O

*The DNA fragments A1-A2 and A3-A4 were added to the reaction at a 1:1 molar concentration. NEBioCalculator was used to determine the necessary DNA mass of the shortest fragment. For the longest fragment, 50 ng of DNA was utilized.

Thermocycling conditions	Initial denaturation	98°C	30 sec
	10 cycles	98°C (denaturation)	10 sec
		50°C - 72°C (annealing)* Cool down from 95°C to 45°C, 5°C every 5 sec	20 sec
		72°C (extension)	30 sec/kb**
	Final extension	72°C	2 min

*The annealing temperature was customized on each primer pair and determined using NEB Tm calculator for calculation.

**The extension time according the size of the DNA fragment.

Following the annealing reaction, 10 μ M FWD Primer, 10 μ M REV Primer and 0.5 μ l of Q5 Hot Start High-Fidelity DNA Polymerase (NEB) were applied to the mix and PCR was carried out as outlined above. The resulting DNA product underwent purification by Invisorb® Spin DNA Extraction Kit (Stratec). To verify the correct length of the PCR product, agarose gel electrophoresis (1% agarose, 0.5 μ g/mL of ethidium bromide, EtBr) was prepared and the 1 Kb Plus DNA Ladder (NEB) was used for length determination. Ultraviolet (UV) light excitation was employed to visualize DNA bands intercalating EtBr.

Restriction digestion, vector dephosphorylation and DNA fragment ligation

The enzymatic restriction digestion was conducted to either generate DNA fragments for ligation (preparative digest) or to confirm the correct orientation and insertion post-ligation (analytical digest). FastDigest enzymes were incubated for 30 minutes at 37°C. FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific) was added to the and incubated for an additional 30 minutes at 37°C to the reaction mix. This step aimed to prevent re-ligation of the vector by phosphorylating the 5' phosphate residues following linearization. DNA fragments were separated based on their size in base pairs via agarose gel electrophoresis as described above and fragments of interest were excised from the gel and purified with the use of Invisorb® Spin DNA Extraction Kit (Stratec). For ligation of vector and insert, the Quick Ligation Kit (NEB) was used. The reaction mix was incubated for 30 minutes at RT.

Preparative digest mix	5 μ l	10X Fast Dig Buffer (ThermoFisher Scientific)
	1 μ l	FastDigest enzyme 1
	1 μ l	FastDigest enzyme 2
	3 ng	DNA
	Up to 50 μ l	Nuclease-Free H ₂ O
	1 μ l	FastAP

Analytical digest mix	5 μ l	10X Fast Dig Buffer (ThermoFisher Scientific)
	1 μ l	FastDigest enzyme 1
	1 μ l	FastDigest enzyme 2
	200 ng	DNA
	Up tp 20 μ l	Nuclease-Free H ₂ O

Ligation mix	10 μ l	2X Quick ligation reaction buffer (NEB)
	50 ng	Vector
	1:3*	Insert
	1 μ l	Quick ligase (NEB)
	Up tp 20 μ l	Nuclease-Free H ₂ O

*The linearized insert and vector were added to the reaction at a 1:3 (insert:vector) molar concentration. NEBioCalculator was used to determine the necessary DNA mass of the insert. For the vector, 50 ng of DNA was utilized.

Transformation of competent cells and isolation of plasmid DNA

The chemically competent Mach1T1® (Thermo Fisher Scientific) *E. coli* strain was subjected to plasmid DNA transformation. 4 μ l of ligation mix was added to 50 μ l of bacteria cells and incubated on ice for 30 minutes, followed by a 30-second heat shock at 42°C, and a rapid cooling on ice. Afterward, 450 μ l of SOC medium were added and the mixture was incubated for 1 h at 37°C for 60 minutes under constant shaking (200 rpm). The resulting transformation mix was applied to LB-agar plates supplemented with either 100 μ g/ml ampicillin (Roth) or kanamycin (Roth). The Invisorb® Spin Plasmid Mini Two was used for plasmid DNA extraction from Mach1T1® (small scale DNA preparation). For large scale preparations, the DNA Maxi Kit (Qiagen) was employed. Verification of the plasmid sequence was performed by restriction enzyme digestion and subsequent Sanger sequence (Eurofins Genomics).

SOC medium: 2.5 mM KCl, 0.5% yeast extract, 20 mM glucose, 10 mM MgSO₄, 2 % tryptone, 10 mM NaCl (Roth), ddH₂O (up to 1 l);

Luria-Bertani broth (LB) culture medium: 1% NaCl, 0.5% yeast extract, 1% tryptone (Roth), ddH₂O (up to 1 l);

Luria-Bertani broth (LB) agar: 1.5% agar (Roth) in 1 l of LB culture medium.

3.2.1.2 Generation of the rat anti-CCR7 CAR construct

The scFv was constructed using the sequences of the variable heavy (VH) and light (VL) chains from the rat anti-human CCR7 monoclonal antibody (clone 3D12). The VH sequence was fused to an immunoglobulin K (IgK) leader sequence and connected to the VL sequence through a Whitlow linker (18 aa). A second-generation CAR backbone was selected, incorporating an IgG1 (266-503 aa) derived spacer bearing mutated Fc- γ binding domains, the transmembrane and cytoplasmic regions of CD28, and CD3 ζ activation module. The codon-optimized sequence of the scFv was synthesized by GeneArt (Thermo Fisher Scientific), and amplified with a pair of primers harboring the enzymatic restriction sites NotI. The sequence of the CAR backbone was amplified from the MP71-CXCR5 CAR construct [194] (Bunse et al., 2021) with a pair of primers harboring the enzymatic restriction sites BsiW. The two fragments were fused via overlapping PCR. The MP71-CXCR5 CAR was enzymatically digested at the NotI and BsiW restriction sites to obtain the MP71 retroviral vector scaffold. The CCR7 scFv-CAR insert harboring NotI/BsiW compatible sticky ends was subsequently cloned into the MP71 retroviral vector scaffold which bears a fluorescent reporter, the mTurquoise2 (mTq) in tandem.

3.2.1.3 Generation of the humanized anti-CCR7 CAR constructs

The humanization of the rat anti-human CCR7 scFv (clone 3D12) was accomplished by complementary determining regions (CDR) grafting onto human framework region (FRs). The immunogenetic database IMGT/V-QUEST was used to identify the human immunoglobulin (Ig) sequences with the greatest similarity in amino acid (aa) sequence with 3D12 VH (rVH) and VL (rVL) chains. For VH, the human V region IGHV3-73*01, the J region IGHJ4*01, and the D region IGHD5-12*01 were selected as acceptors FR. For VL, the V region IGKV2-40*01 and the J region IGKJ1*01 were chosen. A fully humanized version (fH, M8; 28 mutations) and a partially humanized version (pH, M9; 6 mutations) were designed. All rat FR aa residues were modified in M8; residues with dissimilar physiochemical properties were retained in M9; FR rat hypermutations were conserved. Chimeric variants of either pH or fH chains combined with parental chains (M17-20) were generated by overlapping PCR. M28-M31 scFv variants bearing single-point mutations were designed. The full sequence of M8-9-28-

29-30-31 encoding the scFv and CAR cassette was codon optimized and synthesized by GeneArt. Subsequent enzymatic digestion at NotI and BspEI restriction sites allowed subcloning of the complete CAR cassette into the MP71 retroviral vector scaffold bearing a fluorescent reporter (mTq) in tandem.

3.2.2 Cell culture

3.2.2.1 Cryopreservation and maintenance of cell lines

Cells were centrifuged for 5 minutes at 350g. Supernatants were removed, and the cell pellets were resuspended in a freezing medium containing FCS (Gibco) with 10% DMSO (SigmaAldrich). 1 ml aliquots were distributed into cryovials and stored and frozen at -80°C in a cryotube holder (CellCamper) for 24-48 h and transferred into liquid nitrogen at -150°C. Frozen samples were rapidly thawed in a water bath at 37°C for 2 minutes, transferred into 15 ml tubes containing cold medium, and centrifuged at 350 g for 5 minutes. Supernatants were discarded and cells were resuspended in fresh medium. Adherent cell lines were cultivated in DMEM supplemented with 1X Penicillin/Streptomycin (Pen/Strep), 10% FCS, sodium pyruvate and non-essential amino acid solution (MEM) and sodium pyruvate (NaPy) and passaged upon confluency upon application of trypsin/EDTA for 5 minutes. Suspension cells were maintained in RPMI complete containing RPMI 1640 with 1X Pen/Strep and 10% FCS and passaged every second or third day. The cell lines were grown at 37°C in a Binder CB210 incubator (Thermo Fisher Scientific) with a 5% CO₂.

3.2.2.2 Cell count determination

Mouse spleen and bone marrow (BM) cells were diluted 1:100 in CASYton and counted by the CASY Cell Counter TTC. The number of human primary lymphocytes and cell lines was assessed using a Neubauer hemocytometer in PBS with 10% of trypan blue or in PBS with 7AAD (1:200) with the MACSQuant Analyzer 10 (Miltenyi Biotech).

3.2.2.3 Culture of primary cells

Primary cells were handled in accordance with the manufacturer's instructions.

3.2.2.4 Transient transfection

Transient transfection was performed by using the calcium phosphate precipitation method. One day prior to the transfection, HEK293T cells (7.5×10^5 /well) were seeded in 3 mL of HEK medium into a 6-well tissue culture plate to achieve an optimal confluency of 60–70% at the time of transfection. The precipitation mixture was prepared in a 15-ml polystyrene tube.

Precipitation mixture/well:	6 μ g	MLV gag/pol
	6 μ g	10A1 env
	6 μ g	Retroviral CAR
	15 μ l	CaCl ₂ 250 mM
	Up to 150 μ l	ddH ₂ O

Subsequently, 150 μ l of transfection buffer [(1% HEPES (Sigma), 270 mM NaCl (Roth), 1.5 mM Na₂HPO₄ (Sigma), 10 mM KCl (Roth) in 1 l ddH₂O pH 6.76)] were slowly applied dropwise to the precipitation mixture while vortexing. After 20 minutes of incubation time at RT, the formed DNA precipitates were distributed in small drops over the cells and incubated for 6-8 hours. The medium was renewed and incubated for a further 48 hours.

3.2.2.5 Virus production and generation of stable retrovirus-producing packaging cell lines

HEK293T packaging cell lines were transiently transfected with retroviral plasmids as described in 3.2.2.4 for the production of small-scale retroviral supernatant. Alternatively, 18 μ g of retroviral vector per well (6 well-plate) was transfected into 293VecGalV packing cells which stably express retroviral the genes GalV (gag, pol and env). For lentiviral supernatant production, HEK293T cells were transiently transfected with 4.5 of each packaging DNA plasmid (pLP1, pLP2 and pCMV-VSV-G) and 4.5 μ g of pFU-Luc-eGFP per well (6 well-plate). After 48 hours, the viral supernatant was harvested and subsequently filtered through a 0.45 μ m filter and utilized for transduction. Stable retrovirus-producing packaging cell lines were generated for large-scale retroviral supernatant. 293VecGalV (6.5×10^5 /well) cells were seeded in 3 mL of HEK medium into a 6-well tissue culture plate one day prior to transduction. After discarding the medium, 3 ml/well of viral supernatant mixed with

polybrene (1ug/mL) was added. Plates were centrifuged at 800 g for 90 minutes at 32°C. A second round of transduction was repeated the next day following the same procedure. CAR-transduced 293VecGalV cells were sorted by flow cytometry twice for the top 5% CAR-expressing cells, expanded, and frozen in stocks. For the production of retroviral supernatant, CAR-293VecGalV cells (1×10^7) were plated in a T175 culture flask. After 24 hours, the medium was replaced. The viral supernatant was harvested after an additional 24 hours, filtered (0.45µm), and either used immediately for transduction or frozen at -80°C.

3.2.2.6 Isolation of primary human NK, T and B cells from PBMC of healthy donors

Human PBMCs were obtained either from the peripheral blood of healthy donors through density gradient centrifugation or utilizing leukapheresis material from healthy blood donors. Voluntary blood donor recruitment adhered to the principles of the Declaration of Helsinki and complied with local ethical guidelines. Enrichment of CD56+ CD3- NK cells was achieved by magnetic separation, employing the "NK Cell Isolation Kit, human" (Miltenyi Biotec). NK cells were stimulated with "NK Cell Activation/Expansion Kit beads" (Miltenyi Biotec), and further cultured in NK MACS medium (Miltenyi Biotec) supplemented with 5% human AB serum (Sigma-Aldrich) and 500 U/ml IL-2, 20 ng/ml IL-15 (Peprotech). NK cells were cultured for 14-21 days. Untouched naive T cells and B cells were isolated from PBMCs of healthy donors by magnetic separation using the "Naive Pan T Cell Isolation Kit" (Miltenyi Biotec) and "Pan B Cell Isolation Kit" (Miltenyi Biotec). Cells were immediately used for functional assays.

3.2.2.7 Transduction of primary human NK cells and cell lines

Primary NK cells were transduced with γ -retroviral vectors five days after isolation. 24-well non-tissue treated culture plates were incubated with 500 ul of RetroNectin[®] solution (recombinant human fibronectin fragment, Takara) at a concentration of 12.5 µg/ml per well and incubated at 4°C overnight. The RetroNectin solution was removed and the plates blocked with 500 ul per well 2% bovine serum albumin (BSA) in PBS for 30 minutes at 37°C and washed twice with 2 ml of ice-cold PBS. 500 ul of viral supernatant was added and the plates were centrifuged at 3000 g at 4°C for 90 minutes. After discarding the supernatant, wells were loaded with 1×10^6 cells, 750 ul

of fresh complete medium supplemented with IL-2 (500U/ml) and IL-15 (20 ng/ml), and 250 μ l of fresh viral supernatant. For spinoculation, plates underwent centrifugation at 800 g and 32°C for 90 minutes. NK-92 (1×10^6 cells/well), YTS (1×10^6 cells/well), and Jurkat (2×10^5 cells/well) cells were transduced with γ -retroviral vectors and HuT-78 (2×10^5 cells/well) with as described above. After 24 hours later, 1 ml of medium was exchanged with 1 ml of fresh viral supernatant, and the plate was re-centrifuged at 800 g and 32°C for 90 minutes. Two consecutive spinoculations were applied to HuT-78 (2×10^5 cells/well) with 3 ml of lentiviral supernatant for 90 minutes at 800 g and 32 °C. 72 hours after transduction, the expression of the transgene encoded by the virus was assessed by flow cytometry. CAR+ NK-92 and YTS cells were enriched by cell sorting. Hut-78^{GFP_LUC} cells were sorted twice for the top 5% GFP-expressing cells.

3.2.3 Immunological techniques

3.2.3.1 Flow cytometry

Cells were adjusted to 5×10^5 - 1×10^6 in a 96-well plate. Prior to antibody staining, cells were blocked for 10 minutes at RT with either 25 μ l of 5 % goat or human serum in FACS buffer (PBS, 2% FCS, 10 mM EDTA, pH 8.0). Antibody mixes were prepared in FACS buffer. 25 μ l of the solution was added to the cells and incubated for 20 minutes at RT. Specific staining of the antibodies was confirmed through isotype control staining or by fluorescence minus one (FMO) staining. Two washing steps with FACS buffer were applied after staining. Cells were then resuspended in 200 μ l FACS buffer for measurement. Stained samples were exposed to 7-AAD (1:200; Biolegend) 5 to 10 minutes for living and dead cells discrimination. Fluorescence signals were acquired with a FACSCantoll or Symphony A3 (BD Bioscience) or MACSQuant Analyzer 10 (Miltenyi Biotech) and further analyzed with the FlowJo software (TreeStar). The absolute number of membrane-bound molecules of CCR7 per cell was estimated by PE Phycoerythrin Fluorescence Quantibrite beads according to the manufacturer's instructions (BD Biosciences). Cell sorting was carried out using either FACSAriaIII or FACSAria Fusion instruments (BD Biosciences). Before sorting, cells were filtered with a 70 μ m cell strainer (Miltenyi Biotec). The sorted cells were collected

in FCS supplemented with Gibco antibiotic antimycotic (ThermoFisher) to prevent bacterial contamination. The sorting purity was confirmed by flow cytometric analysis.

3.2.3.2 Cellular cytotoxicity assay

The cytolytic activity of CAR NK-92 cells was analyzed by flow cytometry. CAR-engineered NK-92 were seeded in 96-well U-bottom plates and mixed with a fixed number of 5×10^4 GFP+ target cells. Effector-to-target ratios were titrated in the range of 5:1, 1:1, 0.5:1, 0.25:1, and 0.125:1. Co-cultures in RPMI medium were kept for 16 hours and stopped by the inclusion of ice-cold FACS buffer. Cells were washed and resuspended in 5% of human serum in FACS buffer for 10 minutes. To distinguish effector and target cells, anti-human CD56-AF647 (5.1H11, BioLegend) antibody in combination with 7-AAD for dead cell exclusion were used. Samples were acquired on a FACSCanto II Cell Analyzer (BD Biosciences) and further analyzed with FlowJo v. 10.0.8 software. The relative percentage of specific target cell killing is determined using the formula: $\% = (1 - (\text{tumor cells in coculture} / R)) \times 100$. 'R' depends on the E:T ratio: 33,3 (2:1); 50 (1:1); 66,7 (0.5:1); 80 (0.25:1); 89,9: (0.125:1); 94,1(0.0625:1). Dasatinib was applied to the culture at the beginning of the coculture at a concentration of 100 nM in specific experiments. CAR (PB)-NK cells were cocultured with target cells at 0.5:1 E:T ratio as described above. For autologous CAR (PB)-NK cells cocultures, autologous PB-NK cells were retrovirally transduced, enriched for CAR+ cells by cell sorting and cocubated with freshly isolated autologous T and B cells at at 1:1 E:T ratio as described. Samples were acquired on a MACSQuant Analyzer 10 (Miltenyi Biotech). Absolute quantification of residual target cells was expressed as cells/well. Alternatively, relative killing rate was displayed as percentages of absolute residual counts of target cells normalized to SP6 counts.

3.2.3.3 Degranulation assay

CAR-transduced NK-92 or primary NK cells were cocultured with various T-NHL cell lines in a 96-well U-bottom plate in a 1:1 ratio (5×10^4 cells per well each) for 2 hours at 37°C. Anti-human CD107a-PE antibody (H4A3, Biolegend) was included throughout the duration of the coculture. Thereafter, cells were washed in ice cold FACS buffer. 5 % human serum in FACS buffer was applied for 10 minutes at RT for cell blocking. Cells were then stained with anti-human CD56-AF647 (5.1H11,

BioLegend) to distinguish effector cells and the target cells. Degranulation rate was calculated as gMFI of CD107a-positive CD56⁺mTq⁺ NK-92 or primary NK cells. For maximal stimulation, cells were incubated with phorbol myristate acetate (PMA; 5 ng/ml)/ionomycin (Iono; 1 μ M), while minimal degranulation was assessed in effectors incubated without target cells.

3.2.3.4 Enzyme-linked immunosorbent assay (ELISA)

5x10⁴ CAR NK-92 cells were coincubated with an equal number of target cells for 16 hours at 37°C in a U-bottom 96-well plate. Adherent target cells were seeded in flat-bottom 96-well plates one day prior to coculturing. After cell removal, IFN- γ concentration in the supernatant was assessed using the BD OptEIA™ ELISA kit on Nunc MaxiSorp 96-well plates. Serial dilutions of the standard were prepared in duplicates. Measures were acquired on a PowerWave X Select microplate UV/VIS reader (BioTek Instruments, USA). For maximal release, effectors were treated with phorbol myristate acetate (PMA; 5 ng/ml)/ionomycin (1 μ M) in the absence of target cells. NK cells seeded without target cells served as a control for minimum release. In selected experiments, Dasatinib (100 nM) was added at the beginning of the culture.

3.2.4 Animal experiments

3.2.4.1 Xenograft mouse model

For xenograft experiments, eight- to ten-week-old NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1 Wjl}/SzJ, Jackson laboratories) were used. The mice were intravenously (i.v.) injected in the tail vein with HuT-78 cells expressing firefly luciferase and eGFP (1x10⁶/mouse/100 μ l). One dose of 1.5 x10⁷ total NK cells/mouse (3.75 x10⁶ CAR-positive cells) was intravenously inoculated after nine days. Mice were intraperitoneally (i.p.) supplemented with IL-2 (1 μ g/mouse) and IL-15 (1 μ g/mouse) every 2-3 days. Tumor growth was analyzed weekly with an IVIS Spectrum imaging system (Perkin Elmer). Bone marrow, spleen, and blood were collected and tumor (%CD45+GFP+) and NK cell (%CD56+CD45+) load in each compartment by FACS analysis.

3.2.4.2 *In vivo* imaging

Mice were anesthetized via exposure to isoflurane (2% respiration anesthesia in oxygen) in the XGI-8 Gas Anesthesia System (Caliper Life Science), prior to intraperitoneal (i.p.) application of luciferin (Biosynth) (150 µg/mouse in PBS). After 10 minutes, the anesthetized mice were transferred to the imaging platform of the Xenogen IVIS 2000 (Caliper LifeScience). Imaging was conducted at different detector exposure times ranging from 1 to 300s and images were captured using small binning. Subsequent analysis was performed using the LivingImage 4.5 analysis software (Caliper Life Science).

3.2.4.3 Isolation of bone marrow, spleen and blood

At the endpoint of animal experiments, mice were euthanized by administering an overdose of Isoflurane (Abbott Laboratories). Bone marrow (BM), spleen, and blood were harvested for flow cytometric analysis. For BM analysis, the femur was dissected, muscles and tendons were carefully removed and the bone was opened by cutting at the epiphysis. BM was flushed with PBS. Spleens were homogenized in FACS buffer using a 5 ml syringe plunger. Isolated bone marrow and spleen cells were strained through a 100 µl cell filter in FACS buffer and centrifuged at 350 g for 5 minutes. Subsequently, red blood cell lysis was performed for 5 minutes using a hypotonic lysis buffer 10x lysis buffer: 1 g KHCO₃, 8.29 g NH₄Cl, 37.2 mg Na₂-EDTA, 1 l H₂O with pH 7.3). The remaining cells were stored in FACS buffer. PBMC from blood were obtained through heart puncture. To prevent blood clogging, blood was mixed with 50 µl 0.5 M EDTA. Erythrocytes were removed by hypotonic lysis. The cells that remained cells were resuspended in FACS buffer and prepared for FACS analysis.

3.2.5 Statistics

Prism software Version 6.0 (GraphPad) was used for data analysis and to assess the statistical significance of differences between groups. Results are displayed as arithmetic means ± standard error of the mean (SEM). The Mann-Whitney U test was applied to calculate p-values. Statistical significance was attributed to values of p<0.05.

4 Results

4.1 Exploiting the T cell homing receptor CCR7 as a T-NHL selective target

4.1.1 CCR7 is expressed on T-NHL cell lines

The expression pattern of CCR7 in T cell malignancies has been described in a number of studies. In the case of T cell leukemia, CCR7 has been found highly expressed on the surface of several T Cell Acute Lymphoblastic Leukemia (T-ALL) cell lines and primary samples [184, 185] and on a high proportion of T Cell Prolymphocytic Leukemia (T-PLL) cases [186]. In Cutaneous T Cell Lymphoma (CTCLs), evidence of CCR7 protein expression in samples is scarce and in Anaplastic Large Cell Lymphoma (ALCL), CCR7 expression remains controversial and poorly studied [190, 191]. To validate and broaden the expression data, numerous T-NHL cell lines were tested for CCR7 surface expression by flow cytometry. The cell lines were stained with the rat anti-human CCR7 antibody (clone 3D12) or its corresponding isotype control. Exemplary FACS histograms displayed robust and uniform CCR7 expression on cell lines originating from patients with CTCL such as Sézary Syndrome (SS) (HuT-78, Se-Ax) and Mycosis Fungoides (MF) (MyLa, HH) (**Figure 4a**, top panel). Cell lines derived from ALCL ALK- entities (Mac-1, Mac-2a) exhibited high CCR7 levels, whereas the (ALCL)-ALK+ cells were negative, JB6, or showed a modest expression, DEL (**Figure 4a**, bottom panel). Within the CTCL group, the highest expression was detected on My-La, while Se-Ax, and HuT-78, showed intermediate levels of CCR7. The lowest CCR7 surface expression was detected on HH cells (**Figure 4b**). Among the ALCL cell lines, highly variable CCR7 densities were detected on ALK- cells, ranging from high, Mac-1, to intermediate, Mac-2a, to low, FE-DP, DL-4, levels. In most cases of ALK+ subtypes, CCR7 was absent with the exception of DEL.

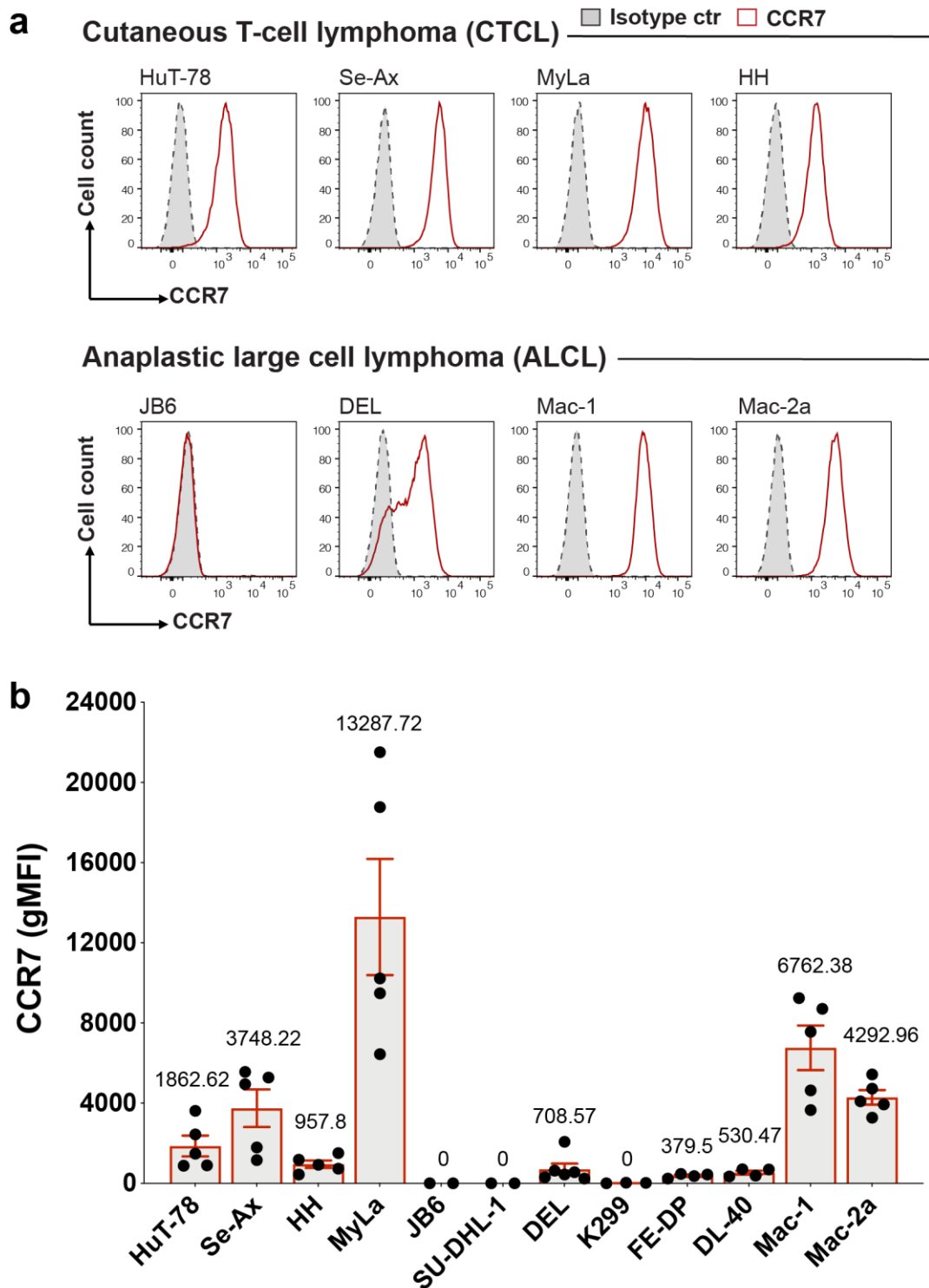


Figure 4: Flow cytometric analysis of surface expression of CCR7 on T-NHL cell. CTCL (HuT-78, Se-Ax, HH, MyLa) and ALCL (JB6, DEL, Mac-1, Mac-2a)-derived T-NHL cell lines were stained with the rat anti-human CCR7-PE antibody (clone 3D12) (red lined) or the corresponding isotype control (filled grey lines). (a) Representative FACS histograms in the upper row show CTCL cell lines. ALCL cell lines are depicted in the lower panel (b). CCR7 surface density is expressed as gMFI (mean \pm SEM). Data were pooled from 4-6 independent measures. n=4: MyLa, FE-DP, DL-40; n=5: HuT-78, Se-Ax, HH, JB6, SU-DHL-1 FE-DP, Mac-1, Mac-2a; n=6 DEL.

4.1.2 T cell neoplasms exhibit a wide range of CCR7 surface expression levels

Differences in CCR7 expression levels on distinct T-NHL entities were detected. Therefore, QuantiBRITE PE calibration method was employed to calculate the number of CCR7 molecules on the surface. In the QuantiBRITE assay, cell lines were immunostained with an anti-CCR7 PE-coupled antibody and analyzed by flow cytometry. Predetermined quantities of four PE-labeled bead populations (high, medium-high, medium-low and low) were included in each sample for calibration. The precise number of surface molecules per cell was calculated by linear regression on the bead fluorescence intensities. Within the CTCL-derived cell line group, the highest CCR7 expression was measured for MyLa (46040 ± 5604 molecules/cell). HH (2631 ± 1028 molecules/cell), HuT-78 (7014 ± 1724 molecules/cell), Se-Ax (12419 ± 997 molecules/cell) exhibited medium to high antigen densities. Among ALCL entities, Mac-2a (10819 ± 937 molecules/cell) and Mac-1 (19303.5 ± 2962.5 molecules/cell) showed medium CCR7 surface expression, whereas FE-DP (1061 ± 88.5 molecules/cell), DEL (2377 ± 2247.5 molecules/cell), DL-40 (1255 ± 474.5 molecules/cell) revealed low to medium values. In this analysis, the B-ALL cell line REH was used as negative control (**Figure 5**).

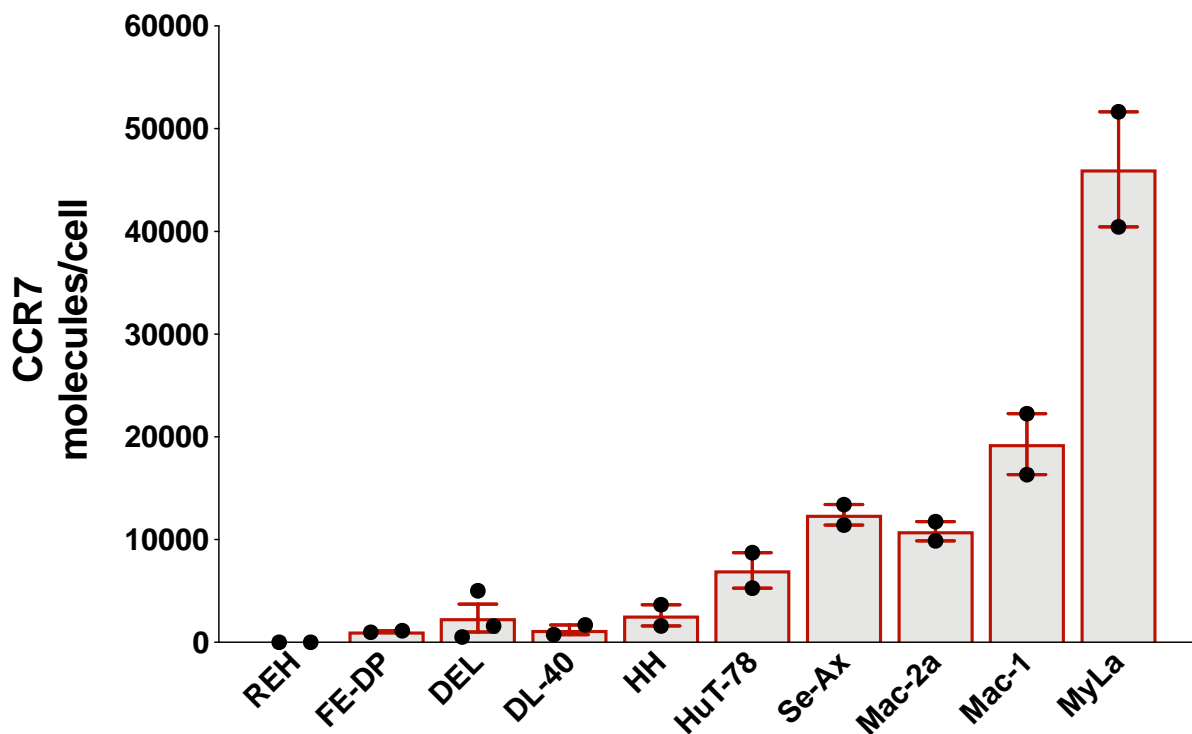


Figure 5: Quantification of CCR7 surface density on selected T-NHL cell lines. The amount of CCR7 molecules per cell was determined by flow cytometry-based QuantiBRITE approach. Various T-NHL cell lines (FE-DP, DEL, DL-40, HH, HuT-78, Se-Ax, Mac-2a, Mac-1, MyLa) were stained using the rat anti-human CCR7-PE antibody (clone 3D12). A B-NHL cell line (REH) served as a negative control. CCR7 protein quantification is expressed as CCR7 molecules/cell. Values are displayed as mean \pm SEM (n=2-3). n=2: REH, FE-DP, DL-40, HH, HuT-78, Se-Ax, Mac-2a, Mac-1, MyLa, DEL; n=3:

Taken together, CCR7 was consistently and exclusively found on a wide range of mature T-NHL cell lines, originating from CTCL including Sézary syndrome and Mycosis Fungoides, and ALCL. On T/B cell malignancies, such as mature B-NHL and precursor T cell leukemia cell lines, CCR7 was not detected.

4.1.3 A curated microarray database revealed CCR7 expression in various T-NHL malignancies

CCR7 was detected on a wide panel of T-NHL lymphoma cell lines, including CTCL and ALCL subtypes by flow cytometry. To confirm CCR7 expression in T-NHL samples, a published microarray database of primary human cancers was interrogated using Genevestigator software (Nebion). The HS_AFFY_U133PLUS_2-1 microarray

Collectively, flow cytometry data of T-NHL cell lines and RNA expression data supported the notion that targeting CCR7 can be a promising therapeutic approach in T-NHL.

4.1.4 CCR7 is highly expressed on primary T-NHL samples

Next, CCR7 surface expression on primary malignant cells derived from T-NHL patients was validated. As shown in 4.1.3, RNA-seq expression data confirmed CCR7 expression in various malignancies of T cell origin. Relatively few studies report surface expression of CCR7 in relapsed T Cell Acute Lymphoblastic Leukemia (T-ALL), T Cell Prolymphocytic Leukemia (T-PLL), Adult T cell Leukemia/Lymphoma (ATLL), Mycosis Fungoides (MF) and Sézary Syndrome-derived CTCL, ALCL and peripheral T cell lymphoma, not otherwise specified (PTCL-NOS) cases [193]. Six patient biopsies of P-TLL (#451, #681, #54, #913, #1334, #53) origin and two biopsies derived from Sézary Syndrome (#1112) and T Cell Large Granular Lymphocytic Leukemia (T-LGL) (#388) patients were kindly provided by Prof. Dr. Jörg Westermann (Charité-Universitätsmedizin Berlin). Generally, the diagnosis of putative T-NHL cases relies on differential antigen expression from normal cells by flow cytometric immunophenotyping [195]. In the presented analysis, a panel of antibody combinations was employed to discriminate T-NHL cells from normal T cells. After excluding singlets and dead cells, cells were gated for SSC-A low and further gated on CD45+ as CD19-CD3+CD7+CD2+CD5+. CCR7 expression was assessed in this subpopulation (**Figure 7a**). PBMC from healthy donors were used as positive control for CCR7 staining. Histograms indicated substantial CCR7 density on the surface of most samples. In detail, four out of six P-TLL samples exhibited homogeneous and robust CCR7 expression with a gMFI of 637.7 up to 5153.8. Two P-TLL patient samples displayed biphasic (#913: 576.8 gMFI) or minor (#53: 142 gMFI) CCR7 expression. CCR7 was also highly expressed on Sézary cells (#112: 780.3 gMFI), whereas on T-LGL cells the level was low (#388: 36.82 gMFI) (**Figure 7b**).

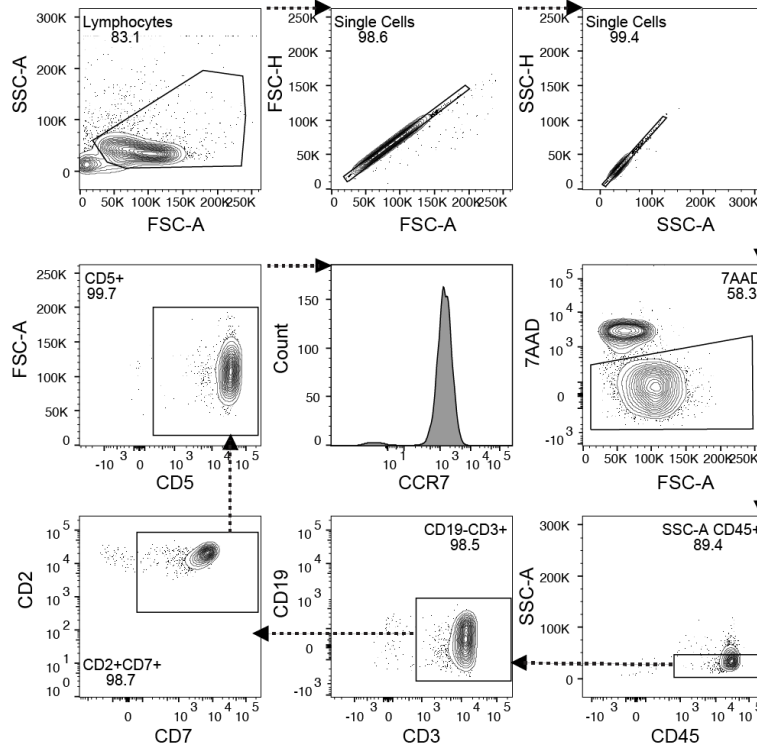
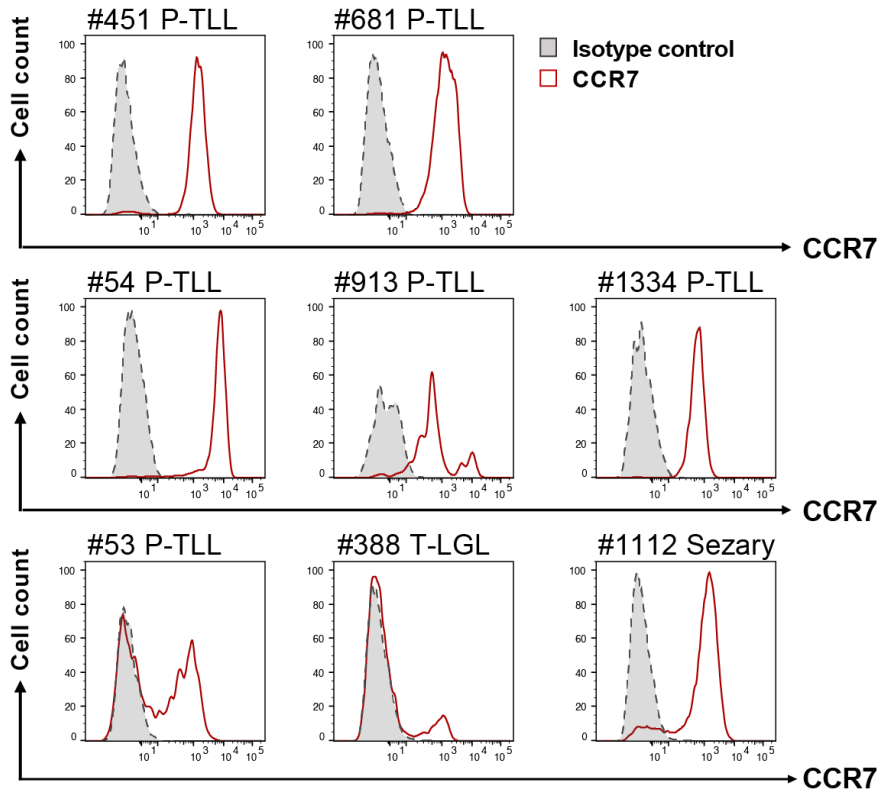
a**Gating strategy****b**

Figure 7: CCR7 surface expression on patient-derived primary T-NHL samples. PBMC derived from eight T-NHL patients, including P-TLL, Sézary Syndrome, and T-LGL, were stained with the rat anti-human CCR7-PE antibody (clone 3D12) (red lined) or the corresponding isotype control (filled grey lines). The gating scheme employed in this analysis is illustrated in (a) Singlets and dead (7AAD⁺) cells were excluded. Cells were gated for SSC-A low and CD45⁺ and further on CD19-CD3⁺. Double-positive CD7⁺CD2⁺ cells were additionally discriminated within this gate. CCR7 expression (gFMI) was calculated on CD3⁺CD7⁺CD2⁺CD5⁺ cell population. FACS histograms in (b) show CCR7 surface expression.

Altogether, CCR7 was validated on almost all analyzed patient biopsies and it could be shown that CCR7 was found on primary samples.

4.2 Construction of CCR7 CAR

4.2.1 *In silico* design of the CCR7 CAR construct

In this study, a novel CAR construct directed against human CCR7 was developed to target CCR7 on T-NHL. The single chain variable fragment (scFv) sequence was derived from the rat hybridoma cell line (clone 3D12) secreting monoclonal antibodies that was developed at the Max Delbrück Center (MDC). Variable heavy chain and (VH) variable light chain (VL) of the 3D12 clone were sequenced by ProMab Biotechnologies. To construct the scFv, the VH sequence was fused to an immunoglobulin K (IgK) leader sequence and connected to the VL sequence through a Whitlow linker [196]. The resulting scFv sequence was codon optimized for *Homo sapiens* using the GeneArt® algorithm and subsequently synthesized by GeneArt® Gene Synthesis. For efficient and stable gene transfer, the gamma-retroviral MP71 vector was chosen and retroviruses were derived thereof [197]. The MP71 harbors an optimized 5' untranslated leader region and showed exceptional transgene expression in human primary T cells, thereby offering a suitable tool for gene therapy applications [198, 199]. The codon-optimized scFv construct was incorporated into a second generation CAR backbone encoding an IgG1Δ spacer (Hinge-CH2-CH3, 237 aa), a CD28 transmembrane region and co-stimulatory domain, followed by the CD3ζ activation module. A fluorescent reporter mTurquoise2 (mTq) was additionally included in tandem using a P2A self-cleaving element (**Figure 8**).

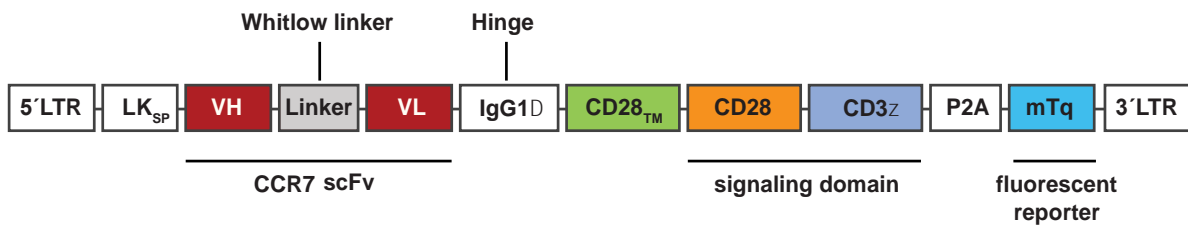


Figure 8: Schematic representation of the CCR7 CAR construct. Long terminal repeat (LTR), leader kappa (LK) signal peptide (SP), variable chain (VH) heavy (H) and light (L) VH, IgG1 Δ spacer (Hinge-CH2-CH3), transmembrane (TM) domain, co-stimulatory domain, activation domain, 2A self-cleaving peptide (P2A), mTurquoise2 fluorescent reporter (mTq).

4.2.2 Cloning of the CCR7 CAR construct

The DNA sequence encoding the scFv was amplified from the plasmid obtained from GeneArt® with a pair of primers harboring the enzymatic restriction sites NotI. The MP71-CXCR5 CAR construct (Bluhm, Dissertation 2018) was used to amplify the second generation CAR backbone with a pair of primers harboring the enzymatic restriction sites BsiW. (**Figure 9a**). Fragments of interest had a size of 846 bp (A1-A2) for the scFv and 1618 bp for the CAR backbone (A3-A4). The two fragments were fused together via overlapping PCR, resulting in a 2422 bp gene insert (A1-A4). The MP71-CXCR5 CAR was enzymatically digested at the NotI and BsiW restriction sites to obtain the MP71 retroviral vector scaffold of 5130 bp. The A1-A4 gene insert harboring NotI/BsiW compatible sticky ends was subsequently cloned into the MP71 retroviral vector scaffold in order to obtain the final CCR7 CAR construct (**Figure 9b**). This construct was additionally modified to include the mTq fluorescent reporter downstream to the CAR cassette via overlapping PCR (not shown). A control CAR containing the SP6 scFv, which recognized the hapten 2,4,6-trinitrophenyl, was cloned in the same backbone and configuration.

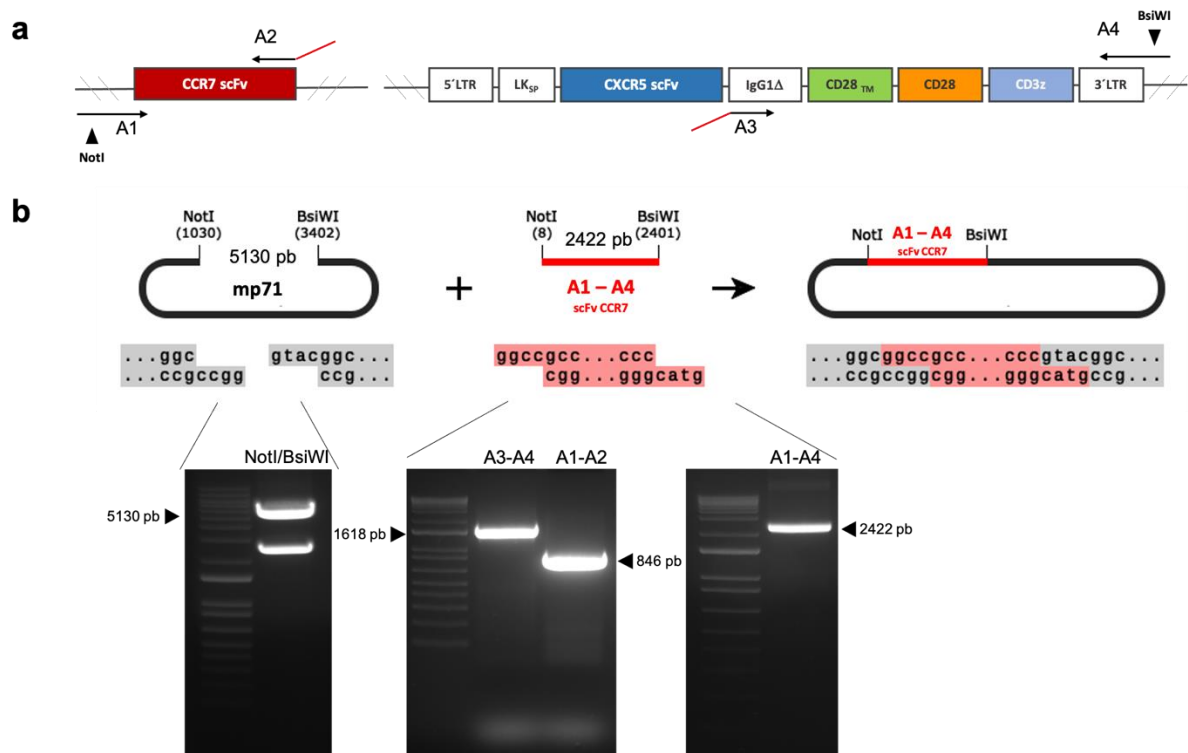


Figure 9: Cloning of CCR7 CAR construct. (a) The illustration represents the cloning strategy of the synthesized anti-CCR7 scFv from GeneArt® gene synthesis into the mp71-CAR vector. (b) Individual fragments were amplified via overlapping PCR and subsequently digested with NotI/BsiWI restriction enzymes and ligated into the retroviral vector, harbouring compatible ends (middle panel). Gel electrophoresis of digested MP71-CAR vector, amplified scFv (A1-A2), CAR backbone (A3-A4), and fused insert (A1-A4). Fragments of interest are indicated by arrows.

4.2.3 The CCR7 CAR was efficiently expressed on the surface of Jurkat cells

The plasmid was amplified by large-scale DNA preparation for transfection. HEK293T cells were transiently transfected with the CAR construct using the calcium phosphate method. The aforementioned SP6 CAR retroviral vector was included as a positive control for transfection and transduction as well as for downstream functional analysis. The retroviral supernatants were harvested and used for the subsequent transduction of the T-ALL cell line Jurkat. Jurkat cells do not express CCR7. Therefore, this cell line was chosen to test whether the CCR7 CAR could be expressed on its surface. Expression of the CAR constructs was assessed by flow cytometry 72 h post-transduction. CAR-transduced cells were stained with an IgG antibody that binds to the hinge moiety of the CAR construct. Flow cytometry analysis revealed successful

surface expression of the CCR7 CAR, indicating proper folding and surface delivery of the chimeric receptor with efficient transduction rates for both SP6 (50.1%) and CCR7 CARs (31.3%) (**Figure 10**).

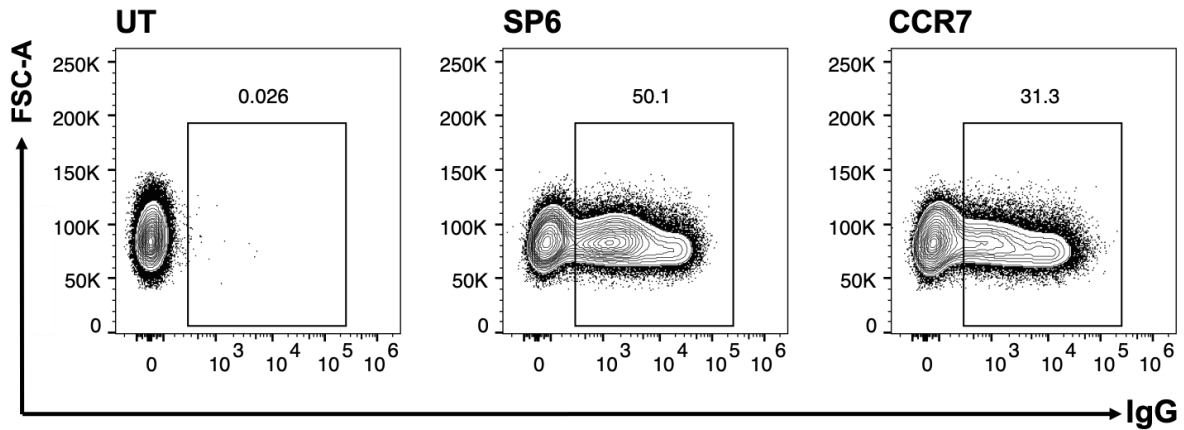


Figure 10: The CCR7 CAR construct was efficiently expressed on the surface of Jurkat cells. Jurkat cells were transduced with CCR7 CAR using transient γ -retroviral supernatants. Untransduced (UT, negative) and SP6 CAR served as controls. UT (left) and SP6 (middle) or CCR7 CAR (right)-transduced Jurkat cells were stained 3 days post-transduction with an anti-IgG-PE antibody that detects the extracellular hinge domain of the CAR.

Taken together, the CCR7 CAR was successfully expressed in Jurkat cells line at profound rates.

4.2.4 Construction of humanized versions of the rat CCR7 scFv

The scFvs of CARs are typically derived from murine monoclonal antibodies. However, the use of foreign proteins as CAR binders carries the risk of inducing an anti-CAR antibody response, which can limit the therapeutic efficacy. To avoid this, non-human scFvs can be humanized by grafting the complementarity determining regions (CDRs) onto human framework regions (FRs). The first step in humanizing the rat scFv from the CCR7 CAR was to identify those human Ig sequences that had the highest degree of similarity to the rat sequences. This was achieved by performing a V-QUEST search of the immunogenetics database IMGT for human Ig sequences. As a result, the human V region IGHV3-73*01, the J region IGHJ4*01 and the D region IGHD5-12*01 were selected as acceptors of the rat heavy chain variable sequences (rVH). Similarly, the V region IGKV2-40*01 and the J region IGKJ1*01 were selected as acceptors for

the rat variable light chain (rVL). The flowchart summarizes the construct variants that were generated for the humanization of the rat scFv (**Figure 11**). Initially, two versions of the rat CCR7 scFv were constructed: i) a fully humanized version (fH, M8) with 28 mutations and a partially humanized version with 6 mutations (pH, M9). In the fH scFv variant, all rat FR aa residues were mutated, whereas in the pH scFv variant, aa residues with dissimilar and very dissimilar physiochemical properties were left unchanged. In addition, rat hypermutations in the framework regions were left unchanged in both constructs. In either fH or pH configurations, the mutations interfered with proper folding and surface delivery of the CARs. To evaluate the surface expression of each chain individually, either partially or fully humanized chains were subsequently combined with the parental rVH and rVL chains, resulting in four different chimeric variants (M17-20). Only the constructs M18 and M20 comprising humanized VHs and rat VLs resulted in successful CAR surface expression. Functional testing of both CARs showed that only the partially humanized VH with two mutations, but not the fully humanized VH with 14 mutations, mediated antigen-specific reactivity. This suggests that some of the 12 mutations that differ between M18 and M20 are critical for antigen recognition. To further identify the critical mutations, additional constructs harbouring a fraction of these mutations would be required. With respect to the VL, neither the fully (14 mutations) nor the partially (4 mutations) humanized VLs of constructs M17 and M19 were surface expressed on HEK293T cells. Therefore, each mutation of the partially humanized VL was tested individually and the mutation D40N of the M30 construct was identified as critical for surface expression. The next steps would be to test the M28, M29 and M31 constructs for functionality and then to generate a new partially humanized VL containing all three mutations. This new pHVL could then be combined with the already tested pHVH to generate a partially humanized scFv with a total of 5 mutations compared to the parental rat scFv. Considering that the total difference between rat and human FR is 28 aa residues, there would still be a great potential to humanize further residues and additional constructs would need to be tested. Therefore, it was decided at this point to prioritize the functional characterization of CCR7 CAR for the original rat scFv and potentially return to the humanization at a later stage.

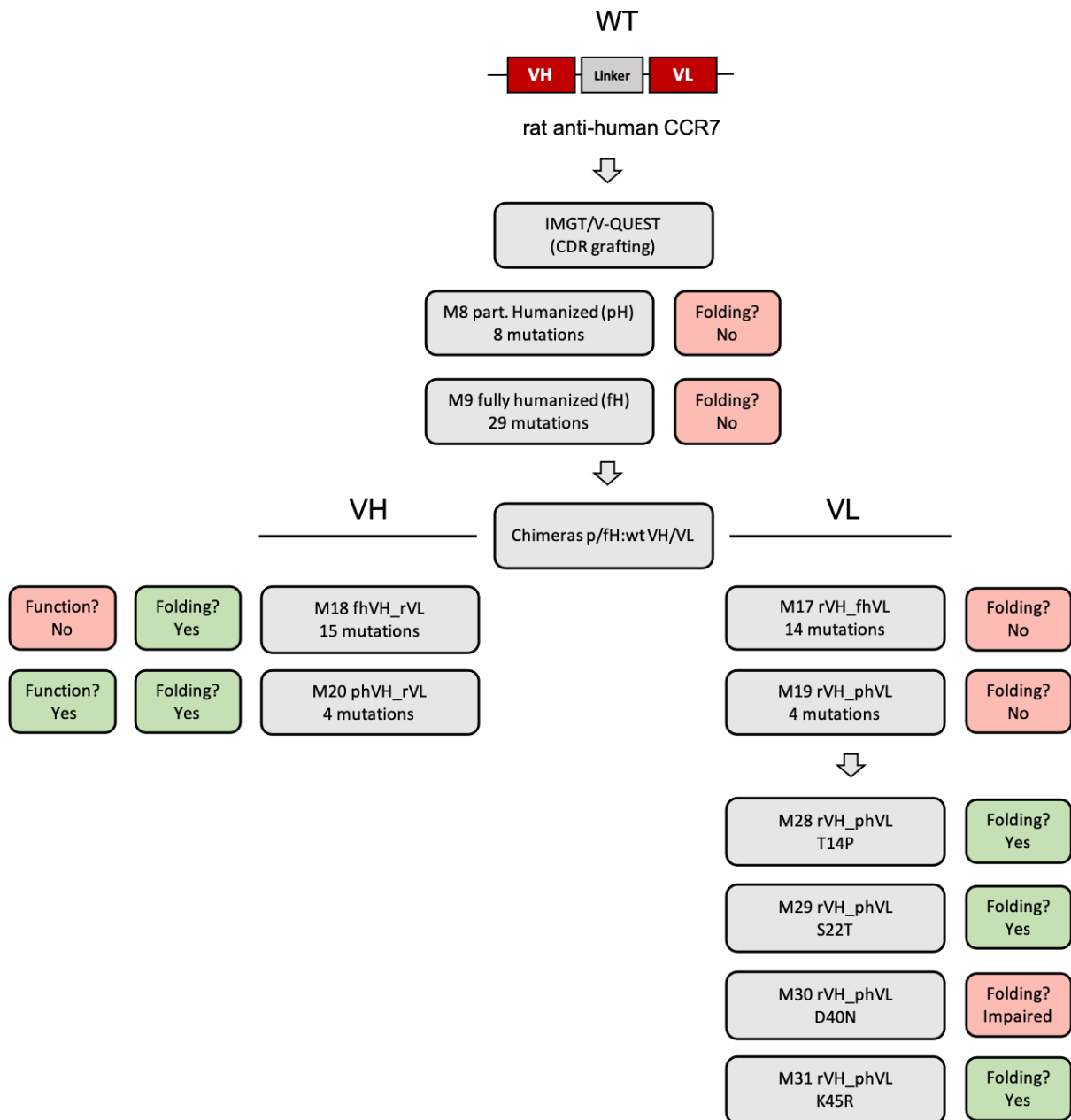


Figure 11: Workflow of humanization of the rat CCR7 scFv. Humanization of variable heavy chain and (VH) variable light chain (VL) of the rat CCR7 scFv clone by grafting the complementarity determining regions (CDRs) onto human framework regions (FRs). A V-QUEST was submitted to the immunogenetics database IMGT to identify human Ig sequences with the highest degree of similarity to the rat sequences. A fully humanized version (fH, M8, 28 mutations) and a partially humanized version (pH, M9, 6 mutations) were constructed. Subsequently, either partially or fully humanized chains were combined with the parental rVH and rVL chains in four chimeric variants (M17-20). Four additional pH constructs (M28-M31) with point mutations in the VL were generated. Surface expression was evaluated by flow cytometry. Functionality of the CARs was assessed in cocultures of CAR engineered NK-92 cells against CCR7 positive HuT-78 cell line.

4.2.5 Generation of stable retrovirus-producing packaging cell lines

A high titer retroviral supernatant is key for achieving suitable transduction rates in NK cells for CAR-NK cell therapy. Retroviral titers obtained with transient transfection of HEK293T cells were not sufficient to reach transduction efficiencies >15% in NK cell lines. To overcome this challenge, a stable retrovirus-producing packaging cell line was generated. 293VecGalV cells stably express retroviral genes, namely GalV env and gag/pol which are indispensable for the production of functional retroviral particles [200]. Amphotropic retroviral vectors carrying either the SP6 control or the CCR7 CARs were produced in HEK293T cells. These viral supernatants were subsequently used to transduce 293VecGalV cells. CAR-transduced 293VecGalV were subjected to cell sorting for the highest 5% CAR-expressing cells. Untransduced (UT) 293VecGalV served as negative control (**Figure 12**). After expansion, this procedure was repeated. The newly generated cell lines enabled production of large high-titer viral supernatants.

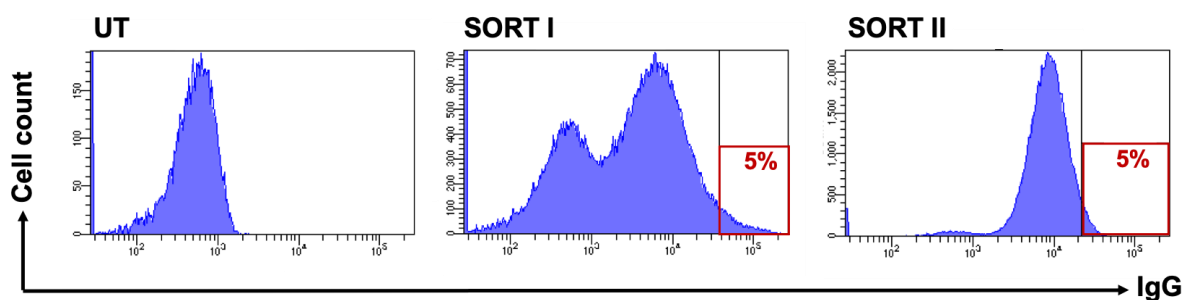


Figure 12: CAR surface expression on stable retrovirus-producing packaging 293VecGalV cells. Amphotropic retroviral supernatants containing the CAR gene were produced by transient transfection of HEK293T packaging cells. 293VecGalV cells were subsequently transduced with these viral supernatants for generation of stable retrovirus-producing packaging CAR-293VecGalV cell lines. Cells were immunostained with an anti-IgG antibody that binds the extracellular hinge domain of the CAR. Histograms depict the sorting strategy applied for enriching the top 5% CAR-expressing cells. Two consecutive rounds of sorting were performed (SORT I, SORT II) by flow cytometry. Untransduced (UT) 293VecGalV cells were used as negative control.

4.2.6 Stable transduction of NK-92 and YTS effector cells with CCR7 CAR

Targeting T cell malignancies with CAR-T cell based approaches remains challenging due to similarities between normal, malignant, and therapeutic T cells. CCR7 is physiologically expressed on subpopulations of benign T cells. Employing T cells as

immune effectors for CAR cell therapy might lead to the self-targeting of therapeutic T cells during manufacturing and compromise the therapeutic efficacy of the product. As a solution, NK cells could be employed as cellular vehicles. NK cells can be obtained from multiple allogeneic sources for CAR engineering including PBMC, umbilical cord blood (UCB), induced pluripotent stem cells (iPSC) and cell lines. Seven human NK cell lines have been established to date which are derived from patients suffering from NK or NKT malignancies. Currently, NK-92 is the only FDA-approved entity for *in vivo* adoptive cell therapy. This cell line is IL-2 dependent and phenotypically mirrors activated blood-derived NK cells. Amphotropic retroviral vectors produced in either SP6 CAR-293VecGalV or CCR7 CAR-293VecGalV cells were used to transduce NK-92 cells. After one centrifugal spinoculation, cells were expanded and immunostained for the CAR 72 hours post-transduction. High transduction rates were achieved as seen below. 40.8% (SP6 CAR) and 45.9% (CCR7 CAR) of the cells were double positive for IgG hinge moiety and mTq fluorescent reporter (**Figure 13a**). A second NK cell line, YTS, was employed. This cell line exhibits a distinct phenotypical and functional profile when compared to the CD56^{bright} NK-92 cells, namely a rather CD56^{dim} like-phenotype [201]. Comparable levels of either SP6 (44.4%) or CCR7 (43.3%) CAR were achieved in YTS cells (**Figure 13b**). CAR+ cells were enriched by cell sorting, expanded, and used to functionally validate the anti-tumor efficacy of CCR7 CAR against CCR7 positive T-NHL cell lines and primary samples *in vitro*.

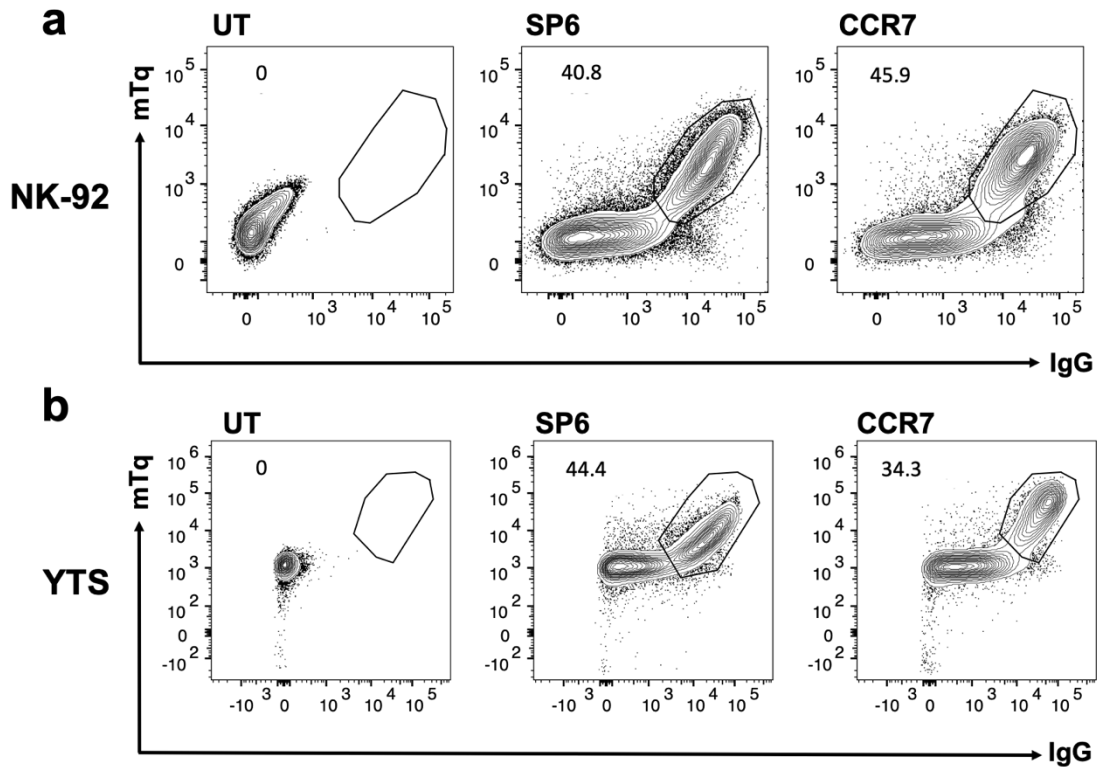


Figure 13: Efficient retroviral vector-mediated gene transfer into NK-92 and YTS cell lines. NK-92 (a) and YTS (b) were transduced using retroviral supernatant from SP6-293VecGalV or CCR7-293VecGalV stable retrovirus-producing packaging cell lines. UT (negative) and SP6 CAR control served as controls. UT (left) and SP6 (middle) or CCR7 CAR (right) transduced cells were stained 3 days post-transduction with an anti-IgG-PE antibody which binds the IgG1 Δ -derived spacer of CARs. Percentage of CAR+ cells are indicated as IgG+mTq+ double positive cell population. Exemplarily dot plots are shown.

In summary, CCR7 CAR-armed NK-92 and YTS cell lines were successfully generated.

4.3 CCR7 CAR enhances NK-92 effector functions against CCR7 positive cell lines *in vitro*

4.3.1 CCR7 CAR NK-92 cells mediated cellular cytotoxicity towards CCR7 expressing tumor entities

NK cells exert their cytotoxic functions without prior immunization or MHC restriction by releasing lytic granules containing perforin and granzyme. Upon formation of the immunological synapse (IS) with the target cells, direct secretion of lytic granules

occurs at the IS interface within minutes, triggering pro-apoptotic pathways in target cells. NK cells can also eliminate tumor cells by engaging death receptors like TRAIL and Fas-L in a granule-independent fashion. The cytotoxicity of CCR7 CAR NK-92 cells was investigated using selected CCR7 positive T-NHL cell lines HuT-78, HH, and My-La. CCR7 negative JB6 cells were included as a negative control. Previously, the selected target cells were retrovirally engineered with a GFP fluorescent protein and enriched for GFP+ cells by cell sorting. Target cells were cocultured with SP6 or CCR7 CAR-engineered NK-92 cells for 16 hours at various effector-to-target ratios (E:T). The highest E:T ratio was 2:1, which was gradually adjusted to E:T ratio of 0.0625:1. Thereafter, the proportion of residual target cells was analyzed by flow cytometry. **Figure 14** illustrates exemplarily FACS plots of SP6 or CCR7 CAR NK-92 cells (CD56+GFP-) coincubated with the correspondent target cells (CD56-GFP+) at 0.5:1 ratio. Target cell frequencies displayed within individual gates show robust CCR7 CAR-mediated cellular cytotoxicity towards CCR7 positive cells. Relative quantifications of viable residual target cells demonstrated that CCR7 CAR NK-92 cells exerted higher cytolytic activity against HuT-78, HH and MyLa cells compared to the SP6 CAR NK-92 control cells at E:T ratio of 2:1, 1:1, 0.5:1 and 0.25:1. CCR7 CAR NK-92 cells also showed higher cytotoxicity against JB6 cells with “undetectable” CCR7 level at 2:1, 1:1 and 0.5:1 E:T ratios. Interestingly, these data also revealed substantial target cell killing or ‘background killing’ in the SP6 CAR cocultured samples in an antigen-independent manner that was strictly dependent on each cell line. These results can be explained by the different grade of allogeneic NK cell stimuli towards the tested lymphoma cells. For this reason, it was not possible to correlate tumor lysis rates with surface density of CCR7.

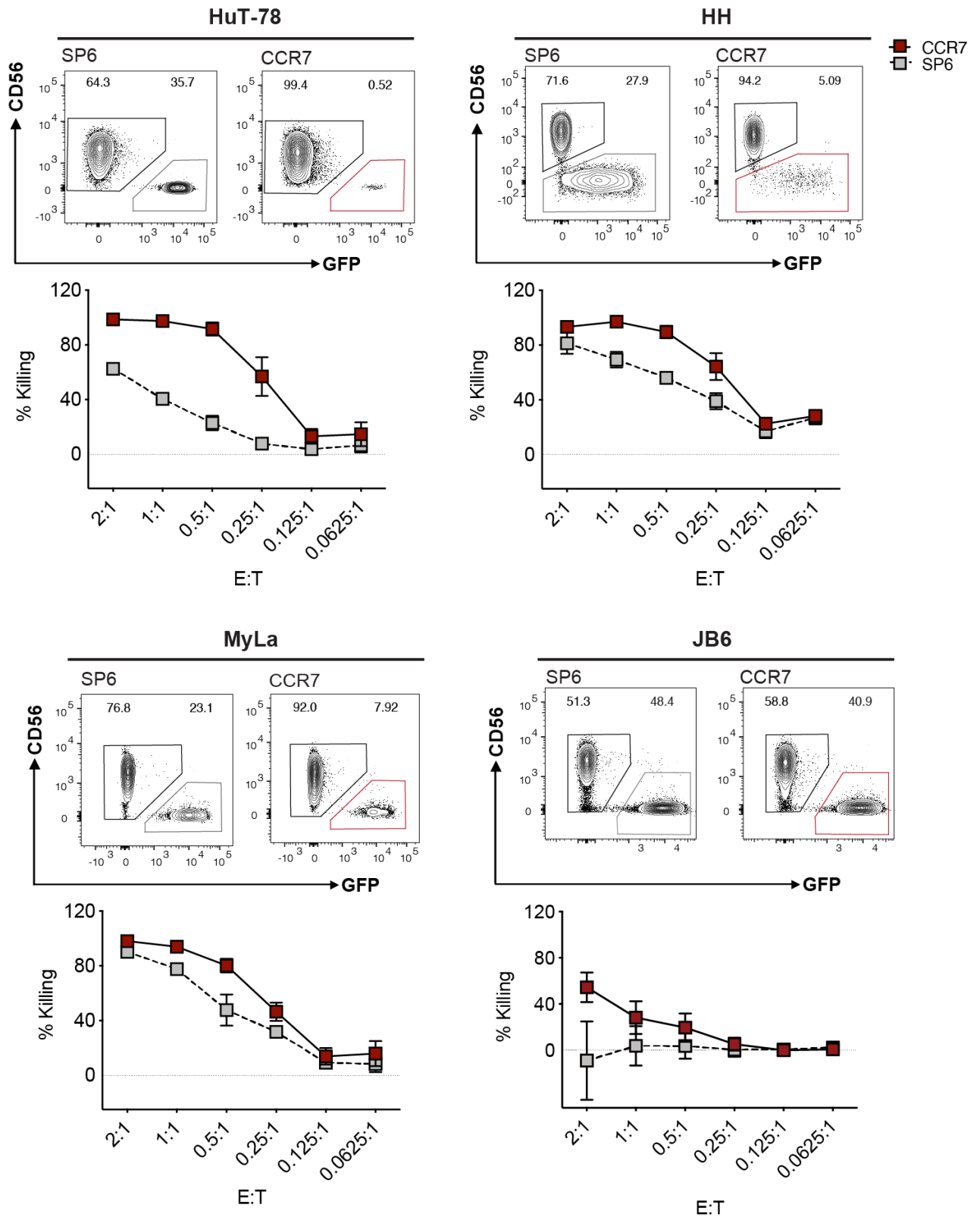


Figure 14: The CCR7 CAR specifically retargets NK-92 cells against T-NHL cell lines *in vitro*. The cytotoxic activity of CAR NK-92 cells was analyzed by conducting a flow cytometry-based cytotoxicity assay. Either SP6 or CCR7 CAR-transduced NK-92 cells were coincubated with four T-NHL target cell lines for 16 hours at the indicated E:T ratios. JB6 with

“undetectable” level of CCR7 served as a control. Representative FACS plots depicts cocultures between target cells (CD56-GFP+) and SP6 or CCR7 CAR-transduced NK-92 cells (CD56+GFP-) at 0.5:1 E:T ratio. Relative percentage of specific target cell killing is determined using the formula: $\% = (1 - (\text{tumor cells in coculture} / R)) \times 100$. 'R' depends on the E:T ratio: 33.3 (2:1); 50 (1:1); 66.7 (0.5:1); 80 (0.25:1); 89.9: (0.125:1); 94.1(0.0625:1). Singlets and dead cells were excluded. Values displayed are mean \pm SEM (2-4 independent experiments): HuT-78 (n=3), HH (n=2), MyLa (n=3), JB6 (n=5).

Together, these results indicated that CCR7 CAR conferred the NK-92 cells with antigen-specific cytolytic efficacy.

4.3.2 Cytolytic granules are secreted by the CCR7 CAR NK-92 cells in an antigen-dependent fashion

Target cell recognition activates the polarized exocytosis of perforin and granzyme-containing lytic granules. The pore-forming perforin enables granzymes to enter the cytoplasm of the target cell, initiating the cleavage of multiple downstream effectors, including caspases. The end result is cellular death. The secretory lysosome-associated protein CD107a or LAMP-1 lines the membrane of these cytotoxic granules and its surface expression is considered a marker of degranulating NK cells following activation. A CD107a degranulation assay was conducted to corroborate whether the CCR7 CAR equipped NK-92 cells recognized the cognate antigen. CCR7+ (HuT-78, HH, SeAx, MyLa) and “undetectable”-CCR7 (JB6) target cells were cocultured with either SP6 or CCR7 CAR-engineered NK-92 at 1:1 E:T ratio. CD107a expression was measured on effector cells (CD56+mTq+) by flow cytometry. Representative FACS plots in **Figure 15a** show higher proportions of CD107a+ NK-92 cells expressing CCR7 CAR following 2 hours cocultures with HuT-78 and MyLa cells compared to SP6 CAR control. CD107a surface density was increased on CCR7 CAR NK-92 fraction solely upon incubation with CCR7 positive target cells (**Figure 15b**). This indicates that CCR7 recognition induces the rapid mobilization of lytic granules following CCR7 CAR engagement in NK-92 cells. Of note, the level of antigen-independent degranulation in the SP6 cocultured samples paralleled the aspecific background killing of the cell lines detected in previous cytotoxicity assays.

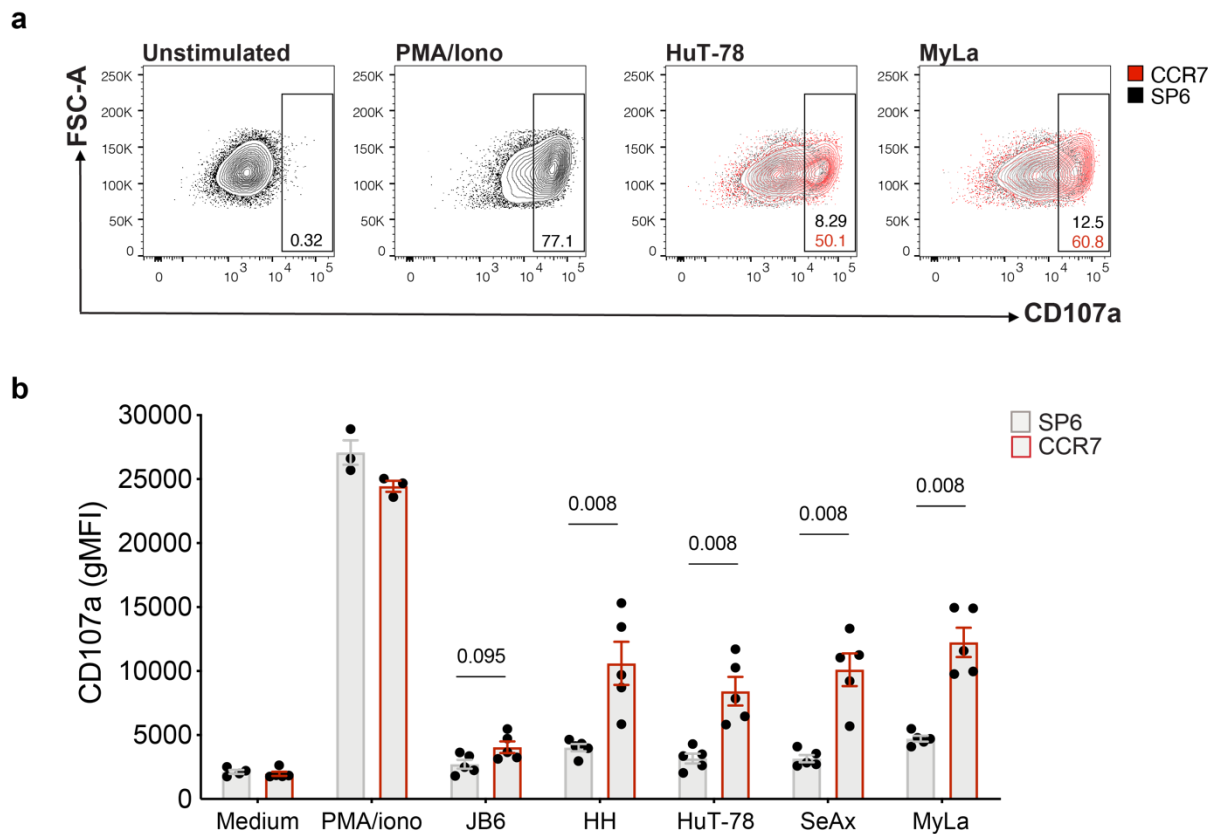


Figure 15: CCR7 CAR-engineered NK-92 cells lyse CCR7 positive target cell lines *in vitro*. (a) Representative FACS plot of a degranulation assay detecting CD107a expression on CAR NK-92 cells after 2 hours in coculture with five T-NHL target cell lines at 1:1 E:T ratio. Singlets and dead cells were excluded. CD107a⁺ NK-92 cells were pre-gated on CD56⁺mTq⁺ double-positive cells. Frequencies of CD107a⁺ CAR-SP6 (grey plot) cells are overlaid onto CD107a⁺ CAR-CCR7 cells (red plot). Unstimulated (minimum) and PMA/ionomycin stimulated (maximum) NK-92 cells were used as controls. (b) Quantification bar plots of CD107a expression (gMFI) on SP6 (grey) or CCR7 (red) CAR+ NK-92 cells. Values displayed are mean \pm SEM (5 independent experiments). Statistics was calculated by Mann-Whitney U test (n=5).

Taken together, CD107a upregulation in CCR7 CAR NK-92 cells correlated with NK cell-mediated specific lysis of positive target cells.

4.3.3 Effector cytokines are released upon coincubation of CCR7 CAR NK-92 cells with CCR7 positive cell lines

Upon target tumor cell engagement, activated NK cells secrete various effector molecules, such as cytokines (IFN- γ , IL-10 and TNF- α) as well as chemokines and growth factors, thereby mediating adaptive immunity [101]. IFN- γ plays a role in CD4 Th1 cell priming [202] and in the induction of CD8⁺ T cell memory responses [203].

To determine whether CCR7 CAR triggers the release of effector cytokine IFN- γ in NK-92 cells upon CCR7 recognition, co-culturing was performed, and secretion was assayed in the cell-free supernatants. As observed in **Figure 16**, higher levels of IFN- γ were elicited exclusively towards the CCR7 expressing T-NHL compared to the SP6 control CAR NK-92 cells in an antigen-dependent fashion. Minor levels of IFN- γ were detected in JB6 cocultured samples. This analysis showed that the production of IFN- γ is in line with CCR7 CAR-NK cell-mediated specific lysis and degranulation. Substantial background antigen-independent IFN- γ secretion of the SP6 CAR correlated with the 'background killing' previously reported only in the case of MyLa cells.

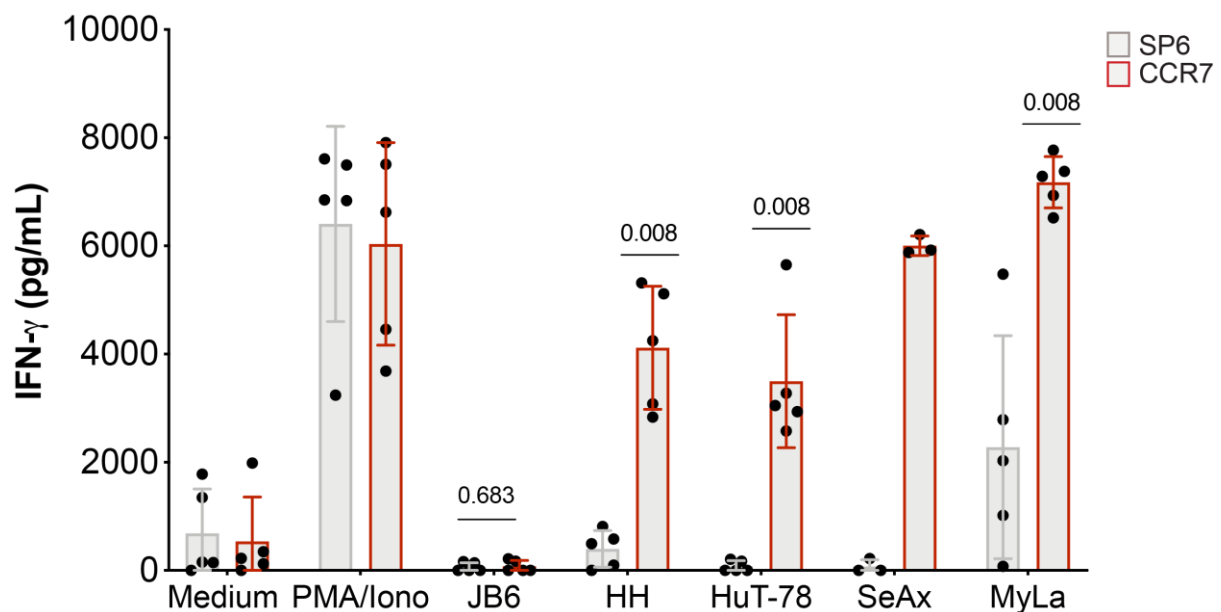


Figure 16: CCR7 CAR NK-92 cells react against antigen positive T-NHL cell lines by secreting IFN- γ in vitro. IFN- γ release of SP6 (grey) or CCR7 (red) CAR NK-92 cells upon 16 hours coincubation with five T-NHL cell lines at 1:1 E:T ratio was quantified by ELISA. Data are expressed as pg/mL and bars represent mean \pm SEM of 3-5 independent experiments: JB6 (n=5), HH (n=5), JB6 (n=) HH (n=5), HuT-78 (n=5), SeAx (n=3), MyLa (n=5).

Mann-Whitney U test was used to calculate p values. Unstimulated (minimum) and PMA/ionomycin stimulated (maximum) NK-92 cells served as controls.

Collectively, these data indicated that the CCR7 CAR was fully functional within NK-92 immuno-effectors and conferred antigen-selective redirected and enhanced activity.

4.4 CCR7 CAR (PB)-NK cells exhibit specific cytolytic efficacy against T-NHL cell lines *in vitro*

The NK-92 cell line mirrors the characteristics of human NK cells and provides an unlimited source of allogeneic effectors for CAR-NK cell therapy. Nonetheless, a major drawback arises from the requirement of lethal irradiation before infusion, aimed at preventing cell growth and tumor transfer to patients. This drastically limits cell persistence after infusion. Human primary NK cells can be obtained from various allogeneic sources: peripheral blood (PB), umbilical cord blood units (CB), hematopoietic stem cells (HSCs) or induced pluripotent stem cells (iPSCs).

4.4.1 CCR7 CAR can be successfully expressed in PB-NK cells

Primary human NK cells were derived from peripheral blood mononuclear cells (PBMCs) of healthy donors. Alternatively, buffy coats from healthy donors were utilized. Following gradient centrifugation, CD56⁺ NK cells were enriched through magnetic separation and subsequently stimulated using anti-NKp46 and CD2 beads. The presence of residual T (CD56⁻CD3⁺) and NKT (CD56⁺CD3⁻) cells was assessed via flow cytometry immediately after isolation and compared to the starting population. The cells were cultivated in the presence of 500 U/mL recombinant human (rh) IL-2 and 20 ng/mL rhIL-15. Five days post-isolation, amphotropic retroviral vectors produced in either SP6 CAR-293VecGalV or CCR7 CAR-293VecGalV cells were used to transduce the cells through a single centrifugal spinoculation (**Figure 17a**). After five days post-transduction, flow cytometry was applied to assess CAR expression. **Figure 17b** illustrates exemplarily FACS plots of SP6 or CCR7 CAR-NK cells (mTq⁺). The transduction rate was evaluated for each NK cells batch, resulting in transduction efficiencies of 20.77 ± 3.24 for the SP6 construct and 24.01 ± 4.14 for the CCR7 construct (**Figure 17c**). Functional assays were conducted after a period of 14 days after isolation.

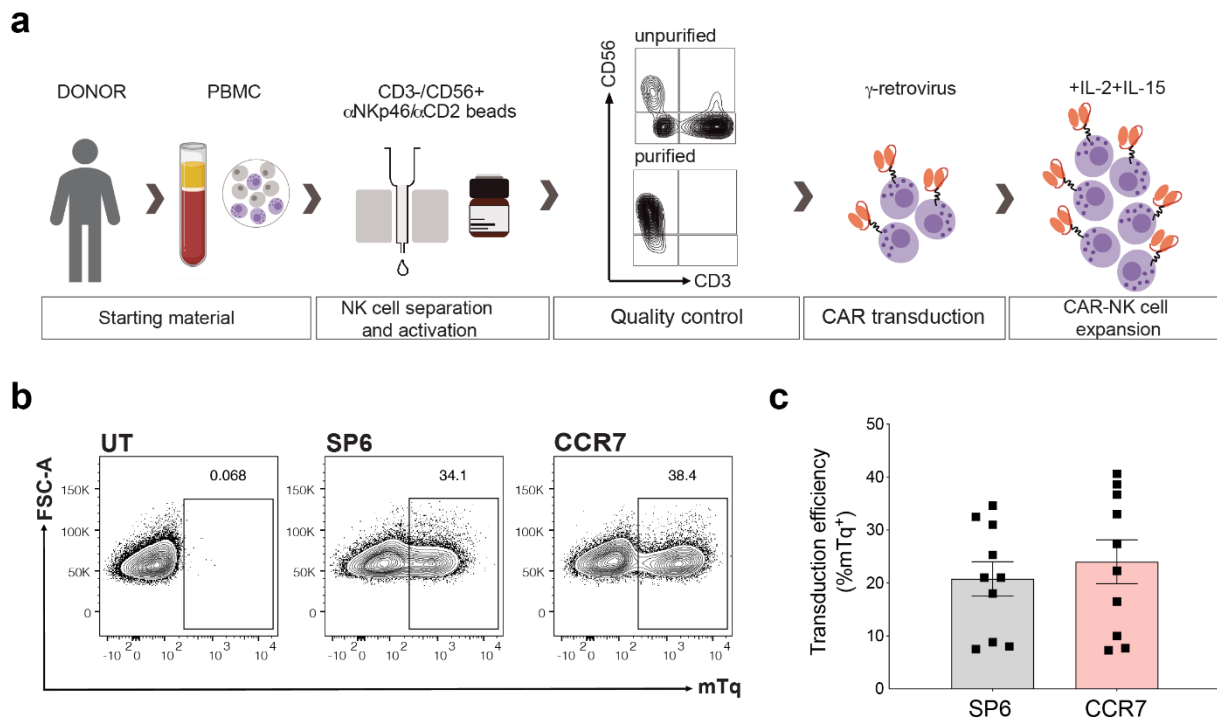


Figure 17: The CCR7 CAR construct was successfully transduced into human NK cells. (a) Workflow for the generation of CAR-(PB)-NK cells. Primary human NK cells were obtained from PBMC or leukapheresis of healthy donors through negative enrichment (CD3-CD56+) employing an NK cell isolation kit. NK cells were stimulated with NK cell activation/expansion kit beads (α NKp46 and α CD2) and grown in the presence of 500 U/ml rh IL-2 and 20 ng/ml rh IL-15. Purity was assessed via FACS (“purified”). The starting material was used as a control (“unpurified”). Residual T (CD56-CD3+) and NKT (CD56+CD3-) cells. After 5 days, cells were transduced using retroviral supernatant from SP6-293VecGalV or CCR7-293VecGalV stable retrovirus-producing packaging cell lines through spinoculation. (b) UT (negative) and SP6 CAR control served as controls. UT (left) and SP6 (middle) or CCR7 CAR (right) transduced cells were analyzed by flow cytometry on day 5 after transduction. Percentage of CAR+ cells are indicated as mTq+ cells. Representative dot plots are shown. (c) The transduction rates of the SP6 and CCR7 CAR constructs transduced into human NK cells 5 days after transduction are shown. Bars represent mean \pm SEM of 11 independent experiments.

4.4.2 CCR7 CAR endows PB-NK cells with specific effector functions towards T-NHL cells lines *in vitro*

The cytolytic efficacy of CCR7 CAR (PB)-NK cells towards CCR7 positive T-NHL cell lines was analyzed in coculture. Either SP6 or CCR7 CAR-NK cells were cocultured with Se-Ax and MyLa for 16 hours at 0.5:1 E:T ratio. A CCR7 negative B-ALL cell line, REH, was included as a negative control. Absolute cell counts of residual tumor cells (CD56-mTq-) at the end of the coculture were analyzed by flow cytometry and compared to viable tumor cells in coculture with the SP6 CAR-NK control cells for each

donor. The results presented in **Figure 18a** revealed variability among the donors. CCR7 CAR-NK cells exerted 1.68-fold and 1.71-fold greater cytolytic activity against Se-Ax and MyLa cell lines, respectively, when compared to the SP6 CAR-NK control cells for most of the donors, with the exception of one. The REH cells were killed to a similar extent (1.09-fold) as for the control CAR. Subsequently, a CD107a degranulation assay was performed to confirm that CCR7 CAR equips PB-NK cells with enhanced antigen-dependent cytolytic efficacy. CCR7⁺ (HuT-78, Se-Ax, MyLa), “undetectable” (JB6) and CCR7⁻ (REH) target cells were cocultured with either SP6 or CCR7 CAR-NK cells at 1:1 E:T ratio for 2 hours. CD107a expression was measured on effector cells (CD56+mTq⁺) by flow cytometry. Despite donor variability, higher levels of CD107a on the surface of CCR7 CAR-NK cells were detected upon incubation with positive target cells compared to SP6 control group. There was no difference in CD107a expression between SP6 and CCR7 CAR-NK cells in response to JB6 CCR7 “undetectable” and REH negative cells (**Figure 18b**).

These findings indicated that CCR7 CAR endowed PB-NK cells with antigen-specific effector functions. The observed inter-donor variations in cytotoxic responses can be attributed to a varying sensitivity towards different target cell lines. Importantly, different allogeneic settings contributed to variable levels of NK cell activation.

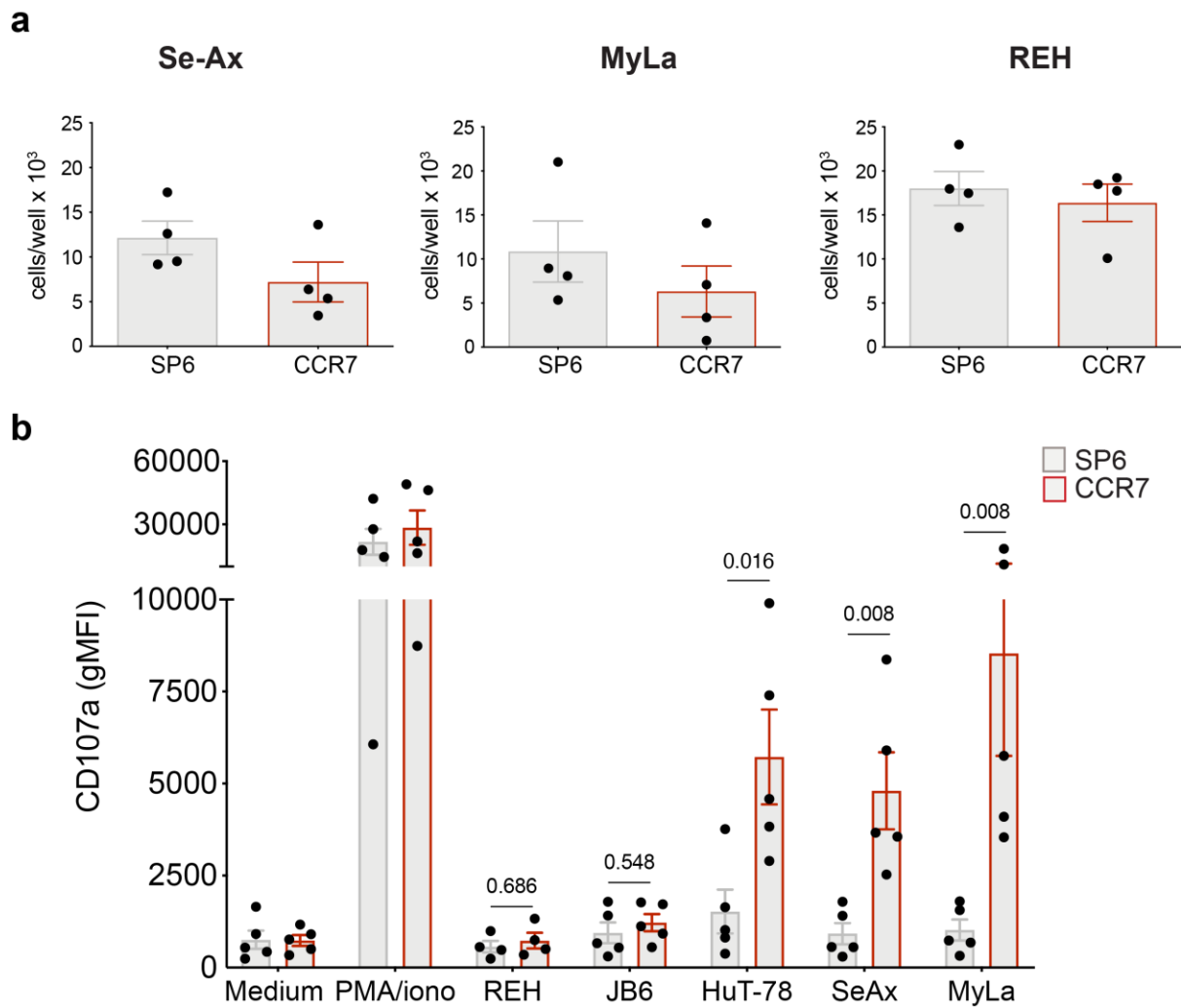


Figure 18: CCR7 CAR (PB)-NK cells mediated cellular cytotoxicity against CCR7 expressing tumor entities. (a) CCR7 CAR (PB)-NK cytolytic efficacy was investigated in a flow cytometry-based cytotoxicity assay upon 16 hours of coculture (0.5:1 E:T ratio) with CCR7+ (Se-Ax, MyLa) and CCR7- (REH, negative control) target cell lines (4 independent donors, transduction efficiencies: 20%-40%). SP6 CAR-NK cells served as a control. Singlets and dead cells were excluded. Absolute quantification of residual target cells (CD56-mTq-) was expressed as cells/well. **(b)** CD107a staining (1:1 E:T ratio) was assessed by flow cytometry after 2 hours of incubation of targets (CCR7+: HuT-78, Se-Ax, MyLa; CCR7 undetectable: JB6; CCR7-: REH, JB6) and effector cell. CD107a+ NK cells were pre-gated on CD56+mTq+ double-positive cells. Quantification bar plots of CD107a expression (gMFI) on SP6 (grey) or CCR7 (red) CAR+ NK cells. Unstimulated (minimum) and PMA/ionomycin stimulated (maximum) NK cells were used as controls. Values are displayed as mean \pm SEM (5 independent donors, transduction efficiencies: 20%-40%). Statistics was calculated by Mann-Whitney U test.

4.5 Patient-derived T-NHL samples are potently eradicated by CCR7 CAR-NK cells *in vitro*

4.5.1 CCR7 CAR-NK cells kill primary patient-derived T-NHL cells

CCR7 CAR redirected NK-92 cells and primary human NK cells were shown to recognize their antigen on T-NHL cell lines, endowing them with selective and enhanced effector functions. These CCR7-expressing cell lines homogenously expressed the antigen and were ideal for proof-of-concept *in vitro* testing. Primary patient-derived T-NHL cells of P-TLL and Sézary Syndrome backgrounds were also shown to exhibit CCR7 on their surface. The cytolytic efficacy of CCR7 CAR NK-92 cells towards the patient-derived T-NHL biopsies was assessed in cocultures. Either SP6 or CCR7 CAR NK-92 cells were incubated with the lymphoma samples for 16 hours at 3 different E:T ratio (2:1, 1:1, 0.5:1). Since CCR7 is expressed by subpopulations within peripheral blood lymphocytes, PBMCs from 3 healthy donors were included as a positive control for specific reactivity. Frequencies of T-PLL, Sézary Syndrome, and T-LGL cells in cocultures were analyzed by flow cytometry according to the gating scheme presented in **Figure 19a**. Cells were gated from SSC-A low CD45+ for CD19-CD3+CD7+CD2+ tumor fraction within the CD56-mTq- target cells. Absolute cell counts of residual tumor cells at the end of the coculture revealed high variability among patient samples. Therefore, residual tumor fraction was compared to viable tumor cells in coculture with SP6 CAR-transduced NK-92 control cells. Results shown in **Figure 19b** revealed tumor eradication in T-PLL samples and Sézary Syndrome samples at all E:T ratios. Complete tumor removal was achieved in the case of P-TLL #451. The CCR7 CAR NK-92 killing was weak against the T-LGL sample that exhibited minor CCR7 expression.

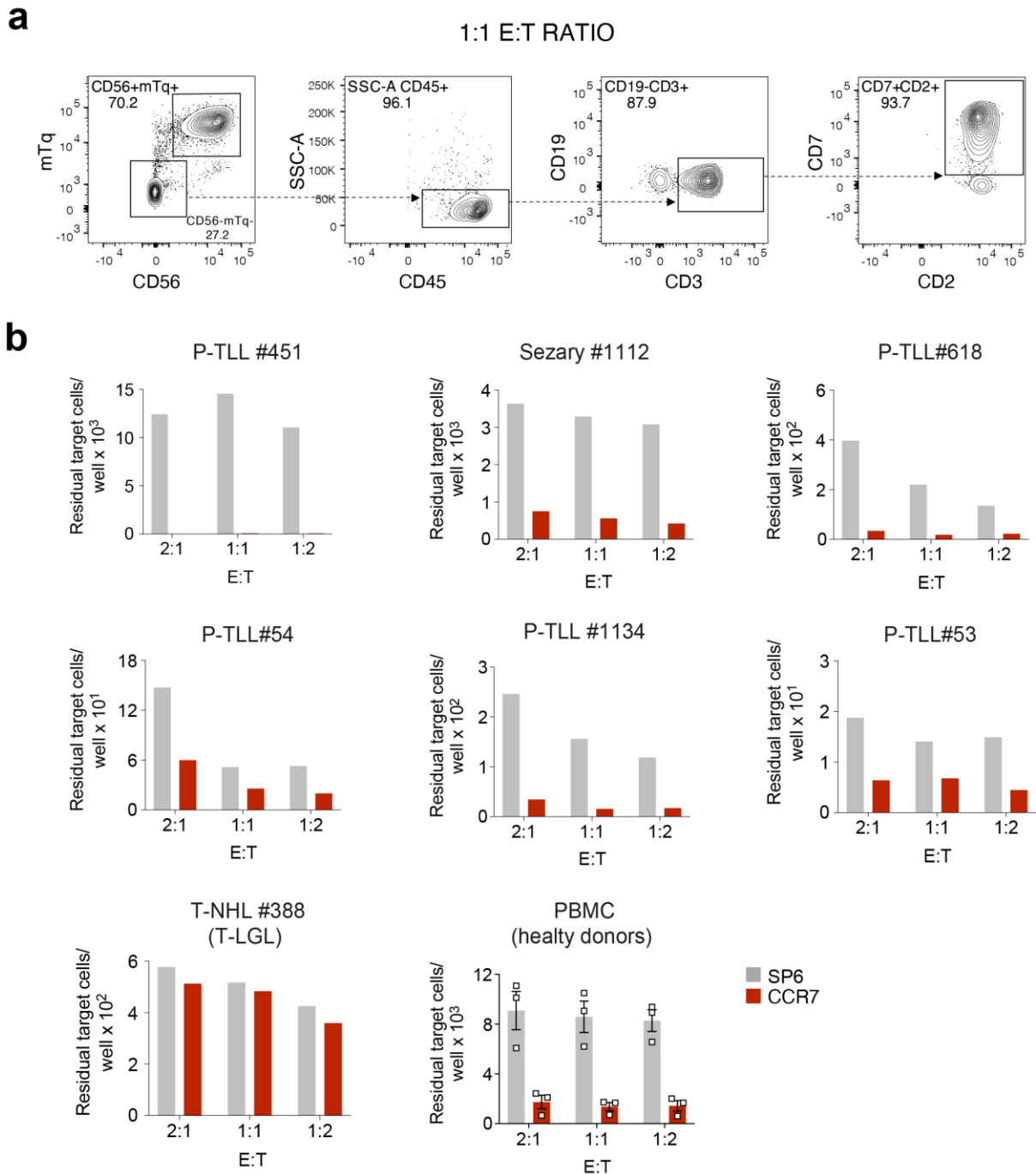


Figure 19: CCR7 CAR-engineered NK-92 cells lyse primary T-NHL samples expressing CCR7. CCR7 CAR NK-92 cell-mediated cytolytic efficacy against patient-derived T-NHL cells was evaluated by a flow cytometry-based cytotoxicity assay. PBMC derived from seven T-NHL patients were coincubated with either SP6 (grey) or CCR7 (red) engineered CAR NK-92 cells for 16 hours at the indicated E:T ratios. PBMC from 3 healthy donors was used as a positive control. The gating strategy used to define the residual target cell population is shown in (a). After exclusion of singlets and dead cells, target cells (CD56-mTq-) were gated on SSC-A low CD45+ and further on CD19-CD3+. Within this gate, double positive CD7+CD2+ cells were additionally discriminated. Absolute quantification of residual target cells

(CD3+CD7+CD2+) was expressed as cells/well (**b**). All PBMC were derived from archived frozen stocks.

4.6 Expression pattern for the target antigen CCR7

In CAR cell therapy, unwanted on-target/off-tumor toxicities can arise if the candidate targeted antigen is expressed on non-malignant tissues. Whether this leads to life-threatening organ damage or clinically manageable loss of tissues must be addressed by preclinical investigation of the target expression across human tissues.

4.6.1 CCR7 is selectively expressed in the hematopoietic compartment

To investigate whether CCR7 CAR-NK cells might cross-react with cells of the hematopoietic compartment, CCR7 expression was assessed on peripheral blood-derived human lymphoid and myeloid cells by flow cytometry. Freshly isolated PBMC were prepared and cells were stained with the rat anti-human CCR7 monoclonal antibody (mAb) (clone 3D12) (that is the monoclonal antibody from which the binding moiety of the anti-CCR7 CAR, the scFv, is directly derived). Because this mAb and the CAR share the same epitope binding specificity, those immune cells stained positive for CCR7 with this mAb would be targeted by the CCR7 CAR-NK cells as well. In this analysis, CCR7 expression was assessed on B cell, T cell and NK cell subpopulations. These three subpopulations were differentiated based on the relative expression of specific lineage markers: i) CD19 for B cells; ii) CD3 for T cells; iii) CD56 for NK cells iv) CD3 and CD56 for NKT cells of the lymphocytic compartment. CD8+ (CD8+CCR7+ T) or CD4+ T (CD4+CCR7+ T) cells were further discriminated within the CD3+ cell population and Treg cells (CD4+CD25+CCR7+) gated utilizing the relative expression of CD25. In addition to lymphoid cells, CCR7 expression was also investigated in the myeloid compartment, encompassing monocytes, dendritic cells, and granulocytes. Within the PBMCs, DCs, and monocytes were gated by their characteristic SSC-A low and HLA-DR expression. Plasmacytoid DC (pDC) (CD303+/CD11c-), myeloid DC (mDC) (CD11c+/CD1c+/CD14-) and monocytes (CD11c+/CD1c-/CD14+) were discriminated within this gate. The monocytic compartment was further differentiated in classical monocytes (CD45+/SSC-A low/CD14+HLA-DR+/CD16-CD24+), intermediate monocytes (CD45+/SSC-A

low/CD14+HLA-DR+/CD16^{low}CD24⁺) and non-classical monocytes (CD45+/SSC-A^{low}/CD14+HLA-DR+/CD16⁺CD24^{low}). Within the granulocytic compartment, neutrophils (CD45+/SSC-A^{high}/CD16⁺CD24^{low}) and eosinophils (CD45+/SSC-A^{high}/CD16⁻CD24⁺) were discriminated based on their characteristic SSC-A^{high}, CD16 and CD24 expression. Within the CD45+SSC-A^{low} cell population, basophils (CD14-HLA-DR-/CD123⁺) were further gated by using the relative expression of CD14, HLA-DR, and CD123. **Figure 20** shows lineage-specific subpopulations within the lymphoid and myeloid compartments and their respective CCR7 expression. Within the CD8 lineage, CCR7 was predominantly found on naïve T cells (T_n) and only minor expression was detected on stem cell-like memory T (T_{scm}) and central memory T (T_{cm}). Effector T (T_e) and effector memory T (T_{em}) cells were stained negative. Within the CD4 lineage, T_n, T_{scm}, Treg (CD4+CD25⁺), T_{cm} compartments exhibited gradually diminished expression of CCR7. CD56^{bright} (CD56⁺⁺⁺) NK cells and B cells exhibited lower CCR7 expression levels in comparison with T cells. CD56^{dim} (CD56⁺) NK cells and NKT cells showed no expression. Within the myeloid compartment, CCR7 was solely detected on the surface of pDC, in contrast to monocytes, mDC, and granulocytes which were negative.

Collectively, these data confirmed that CCR7 expression is restricted to B, T_{scm}, T naïve, T_{cm}, Treg, CD56^{bright} NK and pDC cells as previously reported.

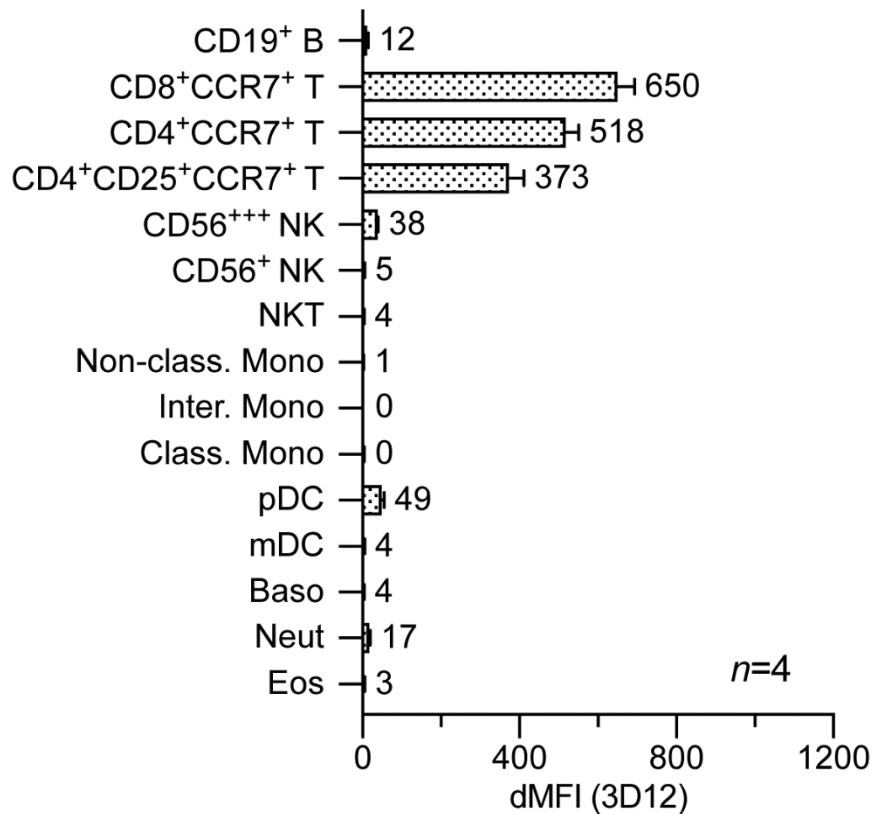


Figure 20: Analysis of CCR7 expression on peripheral blood lymphocytes and myeloid cells. PBMC were isolated from the peripheral blood of healthy donors and cells were stained using the rat anti-human CCR7-PE antibody (clone 3D12) or the corresponding isotype control antibody. Lineage-specific antibody panels were applied to distinguish lymphoid and myeloid cells. Within the lymphoid compartment, CCR7 was assessed on B (CD19⁺ B), CD8⁺ (CD8⁺CCR7⁺ T) or CD4⁺ T (CD4⁺CCR7⁺ T), T-regulatory cells (CD4⁺CD25⁺CCR7⁺), CD56⁺⁺⁺ NK, CD56⁺ NK and NKT cells. Within the monocytic compartment, non-classical (Non-class), intermediate (Inter.) and classical (Class.) monocytes were analyzed for CCR7 expression. Plasmacytoid DC (pDC) and myeloid DC (mDC) were further distinguished. Within the granulocytic compartment, basophils (Baso), neutrophils (Neut) and eosinophils (Eos) were gated. Data are displayed as delta MFI (dMFI): gMFI (CCR7 mAb) – gMFI (isotype control); bars represent mean ± SEM of 4 donors (performed by Dr. Mario Bunse).

4.6.2 Autologous CCR7 CAR (PB)-derived NK cells react towards benign B and naïve T cells *in vitro*

Besides malignant cells, CCR7 was also detected on the surface of benign mature B cells and T cell subsets (naïve and central memory). Recognition of B cells by CCR7 CAR-NK cells would cause the elimination of B cells in patients. This medical condition, referred to as B cell aplasia, is frequently observed in patients treated with CD19 CAR-T cells and can be managed through immunoglobulin supplementation

[204]. Removal of T cells with the concomitant onset of T cell aplasia after CCR7 CAR-NK cell therapy can also be predicted. Therefore, to assess to which extent the CCR7 CAR-NK cells reacted towards these immune compartments, autologous coculture systems were performed. The term “autologous” refers to the fact that blood-derived NK cells used as immune effectors and primary naïve T cells and B cells used as targets were obtained from the same donor. This approach was chosen because CAR-specific reactivity might be masked by the effect of “background killing” or antigen-independent killing due to allogeneic stimulations of the NK cells. **Figure 21a** depicts the experimental design. Primary NK cells were purified from the peripheral blood and retrovirally transduced with either SP6 or CCR7 CAR. After expansion, CAR+ NK cells were enriched by cell sorting. On the day of the assay, primary B cells and naïve T cells (CD45RO- CCR7+) were freshly isolated from the peripheral blood of autologous donors by using a negative selection kit. Autologous B and naïve T cells were coincubated with either autologous SP6 or CCR7 CAR (PB)-NK cells for 16 h at 1:1 effector-to-target ratio. The CCR7 expressing HuT-78 T-NHL cells were included as a positive control for killing. Residual absolute cell counts of B, naïve T, and HuT-78 cells in cocultures were analyzed by flow cytometry. Relative killing rates of CCR7 CAR (PB)-NK cells were calculated by normalizing target cell counts in the samples incubated with CCR7 CAR (PB)-NK cells to target cell counts in the samples incubated with SP6 transduced CAR (PB)-NK cells. Results shown in **Figure 21b** revealed that CCR7 CAR (PB)-NK cells reacted towards the target cells to different extents. The highest cytotoxicity was measured against HuT-78 T-NHL cells (82%) followed by naïve T (67%) cells and B cells (41%). This showed that B cells were less depleted than naïve T cells. In detail, B cells were less killed than T cells by a factor of 3.85 (donor 1, circle) and 1.45 (donor 3, rhombus). In the case of donor 2 (square), B cells and T cells were killed at comparable rates. The previous flow cytometric analysis of PBMC (4.6.1) demonstrated that B cells expressed low antigen levels.

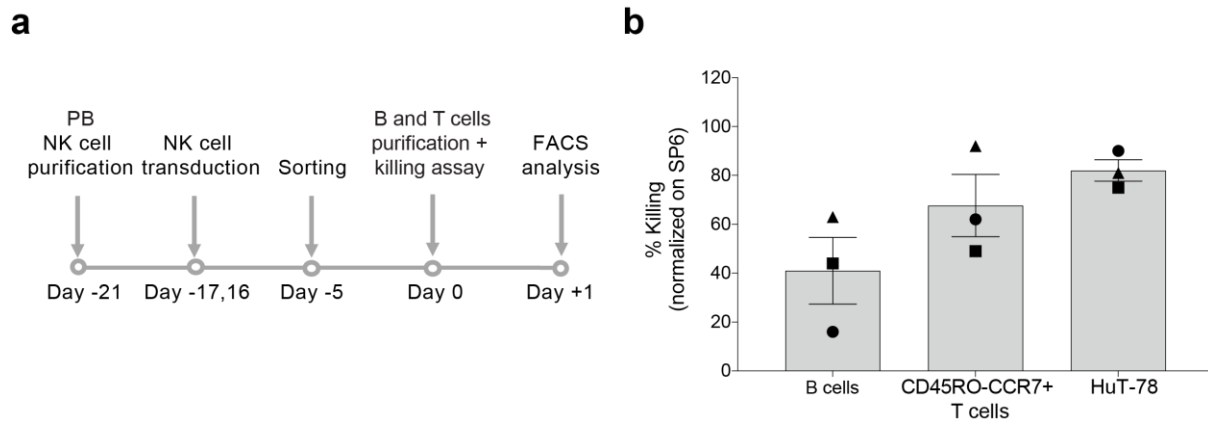


Figure 21: Autologous CCR7 CAR (PB)-NK cells reactivity towards benign naïve T cells and B cells. (a) Scheme of the *in vitro* evaluation of CCR7 CAR (PB)-NK cells cytotoxicity against B and naïve T (CD45RO- CCR7+) cells isolated from matched donors. (b) SP6 or CCR7 (PB)-NK cells reactivity was analyzed via flow cytometry after 16 hours at 1:1 E:T ratio. Relative killing rate is displayed as percentages of absolute residual counts of target cells (B cells: CD56-CD19+, T cells: CD56-CD3+, HuT-78: CD56-CD5+) normalized to SP6 counts. HuT-78 T-NHL cells served as positive control. Bars displayed are mean \pm SEM (n=3). Donor 1 (circle), donor 2 (rectangle), donor 3 (rhombus).

Altogether, these results supported the hypothesis that targeting T-NHL with the CCR7 CAR-NK cell product would induce the removal of B and T cell subsets, but with a milder impact on the B cells.

4.6.4 CCR7 is absent on non-hematopoietic primary cells and cell lines

To exclude potential sites of on-target/off-tumor toxicities outside the hematopoietic compartment, the expression pattern of CCR7 was investigated on various primary cells and cell lines originating from non-hematopoietic tissues. A flow cytometric analysis was conducted on 10 different human cell types. The vascular endothelium is the first tissue site that CCR7 CAR-NK cells would encounter upon infusion. Therefore, primary umbilical vein endothelial cells (HUVEC) and umbilical artery endothelial cells (HUAEC) were tested for CCR7 expression to exclude that targeting CCR7 could effectuate vascular damage. In the analysis of the HS_AFFY_U133PLUS_2-1 microarray dataset presented in chapter 4.3.1., high CCR7 mRNA levels were revealed on a number of metastatic cancers originating from various epithelial compartments, including, urinary, reproductive and gastro-intestinal (GI) tracts. Thus, CCR7 expression was evaluated at the protein level on primary urothelial cells (HU), embryonic kidney cell line (HEK-293), primary cervical epithelial

cells (HCerEpiC), primary colonic epithelial cells (HCoEpiC), hepatocellular carcinoma (HepG2) cell line and colorectal carcinoma cell line (SW620). Cells were labeled with the rat anti-human CCR7 antibody (clone 3D12) or the corresponding isotype control antibody. As shown in **Figure 22**, CCR7 could not be detected on the surface of any of these samples by flow cytometry. Infused CCR7 CAR-NK cells travel to tumor sites via the blood circulation. Because of the possibility that NK cells cross the blood-brain barrier (BBB), where CCR7 CAR-NK cells make direct contact with cells of the central nervous system (CNS), CCR7 negativity had to be ensured in this compartment to exclude the possible onset of neurotoxic events. Gomez-Nicola et al. found that CCR7 was expressed on astrocytes in the normal mouse brain by immunohistochemistry (IHC) and western blot (WB). To investigate CCR7 expression in the human CNS, primary human perineurial cells (HPNC) and human astrocytes (HA) were screened. Flow cytometry data revealed that CCR7 was absent on these cell lines compared to REH^{CCR7+} B-NHL lymphoma cells that served as positive control.

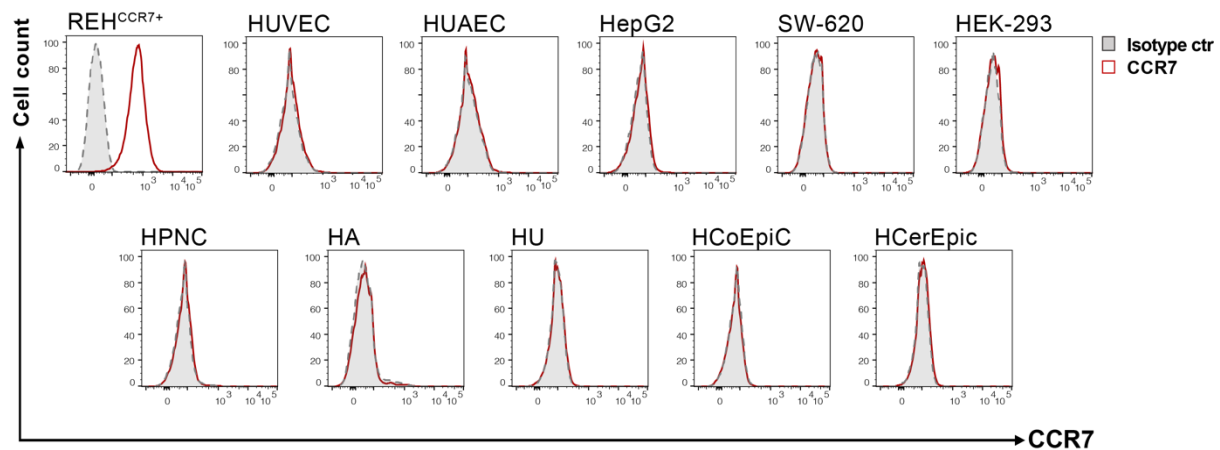


Figure 22: CCR7 is not expressed on unrelated primary cells and cell lines. The indicated primary cells and cell lines were labeled with the rat anti-human CCR7-PE antibody (red lined) or the corresponding isotype control (filled grey lines) and analyzed by flow cytometry. Exemplarily FACS histograms of two independent experiments were chosen. Singlets and dead cells were excluded. CCR7 expression was plotted for human umbilical vein endothelial cell (HUVEC), human umbilical artery endothelial cell (HUAEC), human hepatocellular carcinoma (HepG2), colorectal carcinoma (SW620) and embryonic kidney (HEK-293) cell lines, human perineurial cells (HPNC), human astrocytes (HA), human urothelial cells (HU), human colonic epithelial cells (HCoEpiC), human cervical epithelial cells (HCerEpiC). REH^{CCR7+} B-NHL lymphoma cells were used as positive control.

4.6.5 Coincubation of CCR7 CAR-NK cells with CCR7 negative primary cells and cell lines does not trigger the release of effector cytokines

As demonstrated by flow cytometry, CCR7 was not detected on the surface of primary cells and cell lines derived from non-hematopoietic tissues. This excluded potential on-target/off-tumor toxicities that might arise upon CCR7 CAR-NK cell therapy. A second type of off-target toxicities (off-tumor off-target toxicities) might also occur due to the cross-reactivity of CCR7 CAR-NK cells to epitopes of unrelated proteins expressed on benign CCR7 negative tissues. Therefore, cocultures were performed to address potential off-tumor/off-target effects. The analyzed non-hematopoietic primary cells (HUVEC, HUAEC, HUC, HPNC, HCoEpiC, HCEpiC HPNC and HA) and cell lines (HEK-293, HepG2 and SW620) were incubated either with SP6 (grey) or CCR7 (red) engineered CAR NK-92 cells for 16 h at 1:1 E:T ratio. The target cells were plated 24 hours prior to the cocultures to enable them to adhere. After 16 hours of incubation, IFN- γ released in the supernatants was measured by ELISA. **Figure 23** illustrates that the CCR7 CAR NK-92 cells did not secrete IFN- γ in response to CCR7 negative primary cells and cell lines. On the one hand, this corroborates the absence of CCR7 on these target cells, as revealed by flow cytometry. On the other hand, this excludes potential cross-reactions of CCR7 CAR NK-92 cells towards unrelated antigens on the target cells. Three CCR7 expressing T-NHL cell lines (HuT-78, HH, MyLa) and one CCR7 B-NHL negative cell line (REH) were used to validate CCR7 CAR NK-92 cell functionality.

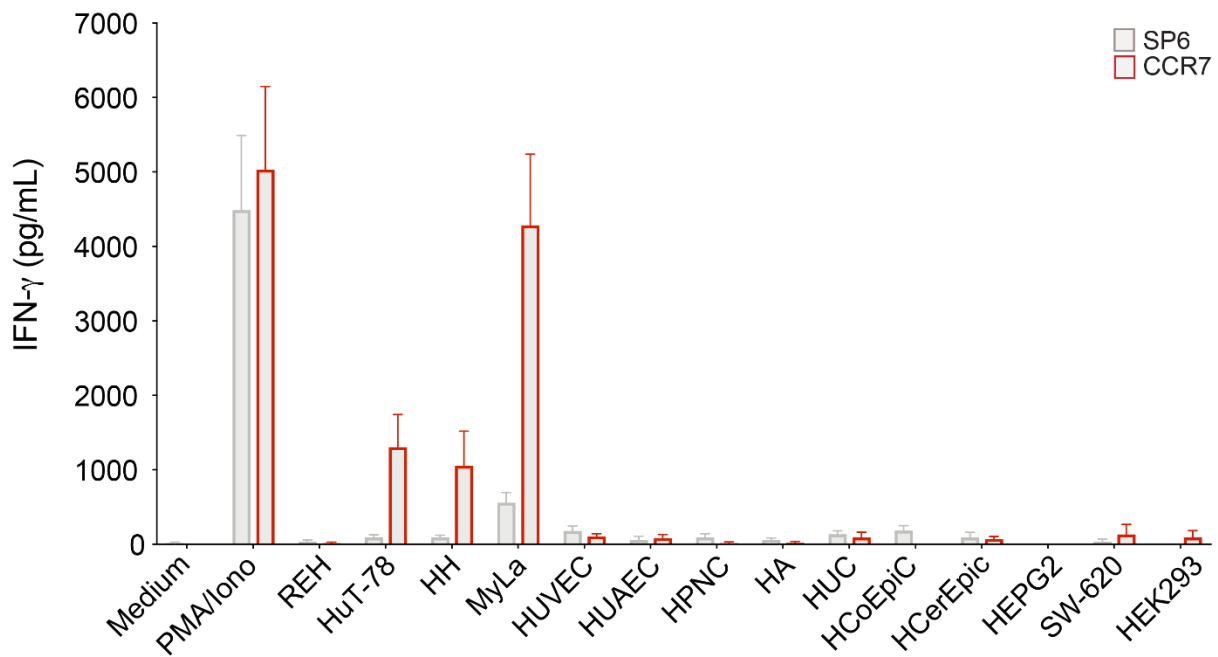


Figure 23: CCR7 CAR-NK cells do not exhibit *in vitro* reactivity against CCR7 negative primary cells and cell lines. Quantification of IFN- γ secreted in supernatants of SP6 (grey) or CCR7 (red) CAR NK-92 cells upon 16 hours of cocultures with primary human umbilical vein endothelial cell (HUVEC, n=6), human umbilical artery endothelial cell (HUAEC, n=4), human perineurial cells (HPNC, n=7), human astrocytes (HA, n=6), human urothelial cells (HUC, n=7), human colonic epithelial cells (HCoEpiC, n=7), human cervical epithelial cells (HCEpiC, n=7), human hepatocellular carcinoma (HepG2, n=4), colorectal carcinoma (SW620, n=4) and embryonic kidney (HEK-293, n=4) cell lines at 1:1 E:T ratio was performed by ELISA. HuT-78, HH, MyLa T-NHL cell lines were used as positive control. REH B-NHL cells were the negative control. Minimum (unstimulated NK-92); maximum (PMA/ionomycin stimulated NK-92). Values expressed pg/mL and bars are mean \pm SEM.

In summary, the comprehensive analysis of CCR7 expression across the human body demonstrated that CCR7 expression is restricted to the hematopoietic compartment. Moreover, it was shown that CCR7 CAR-NK cells exerted neither on-tumor off target effects nor off-tumor off-target effects against non-hematopoietic tissues *in vitro*, indicating that CCR7 presents a safe antigenic target for CAR-NK cell immunotherapy of T-NHL.

4.8 Establishment of a T-NHL xenograft model

NOD.Cg-Prkdc^{scid} Il2rgt^{m1 Wjl}/SzJ (NSG) mice are devoid of B and T lymphocytes, as well as innate immune functions. These mice allow the engraftment of cells of human origin, including immortalized cell lines and primary cells derived from patients, thereby

enabling preclinical testing of antigen-specific anti-tumor effects of human CAR-NK cells. *In vivo* imaging platforms, such as the IVIS Spectrum systems, enable non-invasive monitoring of luciferase-engineered tumor cells within living animals over time. Upon luciferin application, luciferized cells emit a bioluminescent signal which is detected by a highly sensitive optical system.

4.8.1 HuT-78 lymphoma cells can be efficiently engrafted in NSG mice

This study demonstrated that CCR7 CAR-NK cells effectively recognized and eliminated T-NHL cell lines and patient-derived samples in an antigen-dependent fashion *in vitro*. This prompted further investigation into validating these findings in a T-NHL xenograft *in vivo* model. The HuT-78 Sézary syndrome (SS)-derived CTCL cell line was selected as a tumor target cell line. To enable monitoring of localization and tumor growth kinetics, the cell line was lentivirally transduced with a firefly luciferase-eGFP construct. The two genes are connected via an IRES sequence, allowing eGFP expression to correlate with luciferin catalysis. Following transduction, 10% of the highest expressing eGFP HuT-78 cells were enriched twice by FACS sorting. **Figure 24a** depicts the experimental design. 1×10^6 HuT-78 luciferized cells (HuT-78^{GFP_LUC}) per mouse were intravenously (i.v.) administered to twelve-week-old NSG mice. Tumor progression was monitored by IVIS bioluminescence imaging (BLI) at seven-day intervals for 21 days upon application of luciferin. Animals developed progressive lymphoma between day 7 and 14. Bioluminescent signals were detected mostly in the cranium and femur (**Figure 24b**). At the endpoint of the experiment, bone marrow (BM) of the femur, spleen, and blood were harvested. FACS analysis confirmed that the tumor cells were located in the bone marrow of the femur and disseminated through the blood circulation with comparable CCR7 expression levels. Tumor cells detected in the spleen were minor (**Figure 24c**).

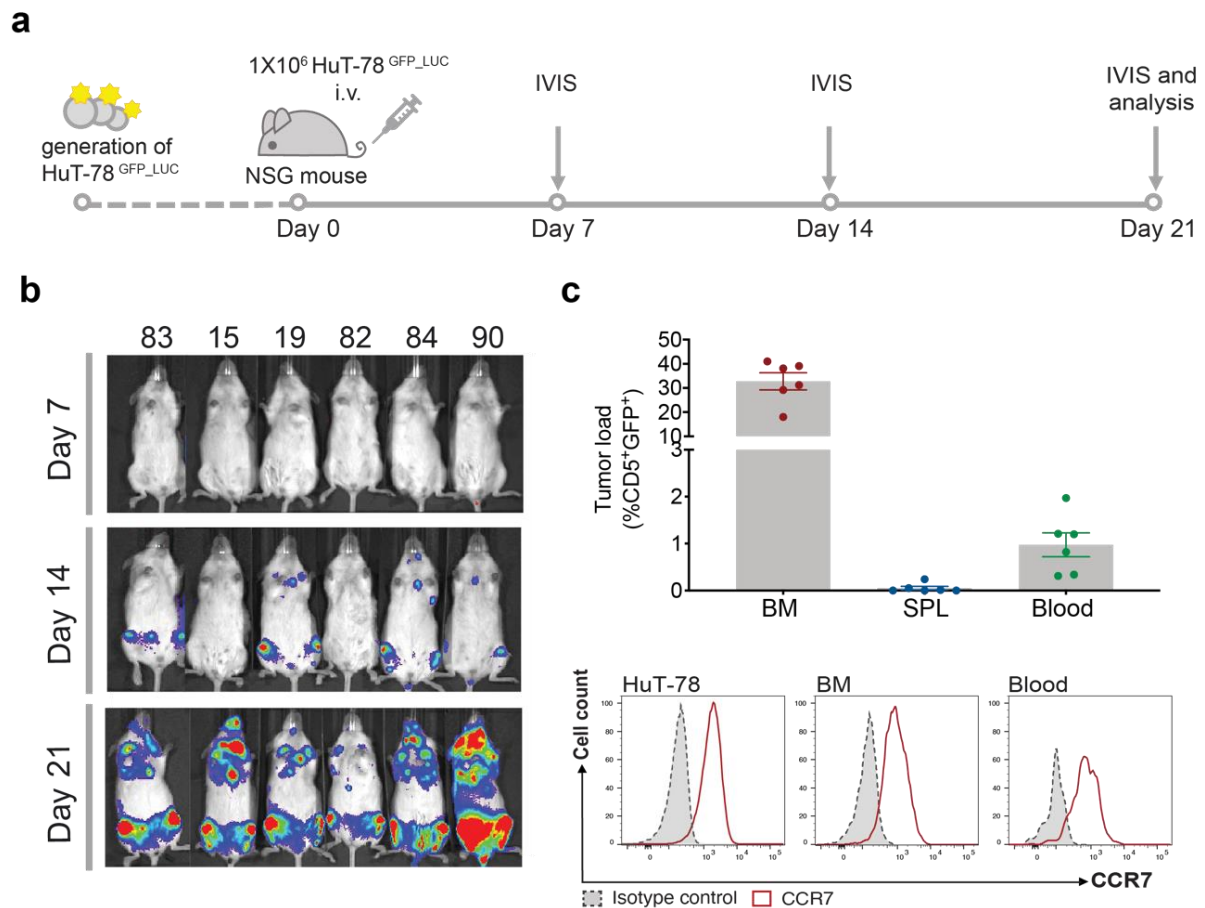


Figure 24: Development of a mouse xenograft model for T-NHL. (a) 1×10^6 HuT-78 firefly-luciferase-labelled (GFP-LUC) cells were intravenously (i.v.) transplanted into twelve-week-old NOD/SCID/Gamma (NSG) mice ($n=6$). (b) Tumor growth was visualized via IVIS bioluminescence SCAN weekly (day 7, 14 and 21) upon intraperitoneal (i.p.) application luciferin (i.p. $150 \mu\text{g}/\text{mouse}$). 120s exposure time is shown. Pixel intensities: 1×10^5 (min)- 1×10^6 (max). (c) Tumor load (CD5+GFP+) was assessed in the indicated organs and expression of CCR7 was measured on HuT-78 tumor cells in the CD5+GFP+ population by flow cytometry in individual mice. Bone marrow (BM), spleen (SPL). Histograms show geometric mean values (gMFI) of fluorescence intensity. Grey filled lines represent isotype controls and red lines are CCR7.

In summary, an *in vivo* model of T-NHL was established for further evaluation of the anti-lymphoma activity of CCR7 CAR-NK cells.

4.8.2 A single dose of CCR7 CAR (PB)-NK cells revealed a moderate anti-tumor efficacy in HuT-78 engrafted NSG mice

CCR7 CAR (PB)-NK cells were manufactured using PBMCs from healthy donors as allogeneic NK cell source. CAR-NK cells were cultured as previously described for 21 days. **Figure 25a** depicts the experimental design. Twelve-week-old NSG mice were xenotransplanted with 1×10^6 HuT-78 luciferized (HuT-78^{GFP_LUC}) cells. SP6 and CCR7 CAR-(PB)-NK cells were adjusted to the same transduction rate, and one dose of CAR-NK cells was intravenously inoculated (3.75×10^6 CAR-positive cells, 1.5×10^7 total NK cells/mouse) nine days later. Serial bioluminescence imaging (BLI) measurements indicated a trend for a delay in tumor growth in mice treated with CCR7 CAR-NK cells as compared to the control group (**Figure 25b**, upper panel). However, applying a single dose of CCR7 CAR (PB)-NK cells did not control the expansion of malignant cells over 14 days (**Figure 25b**, bottom panel). When animals were sacrificed, flow cytometry analysis of flushed bone marrow confirmed the presence of substantial numbers of residual HuT-78^{GFP_LUC} cells (**Figure 25c**, left panel). Remarkably, in both treated groups, PB-NK cells proliferated and persisted *in vivo* for 14 days, repopulated the BM and spleen (not shown), and disseminated into the blood circulation (not shown) (**Figure 25c**, right panel). Furthermore, upon application of the CAR-NK cells, the animals did not show weight loss (not shown), revealing that this xenotransplantation protocol was well tolerated.

These results suggested that repetitive administration of the CCR7 CAR (PB)-NK cells might be required for effective and prolonged tumor eradication.

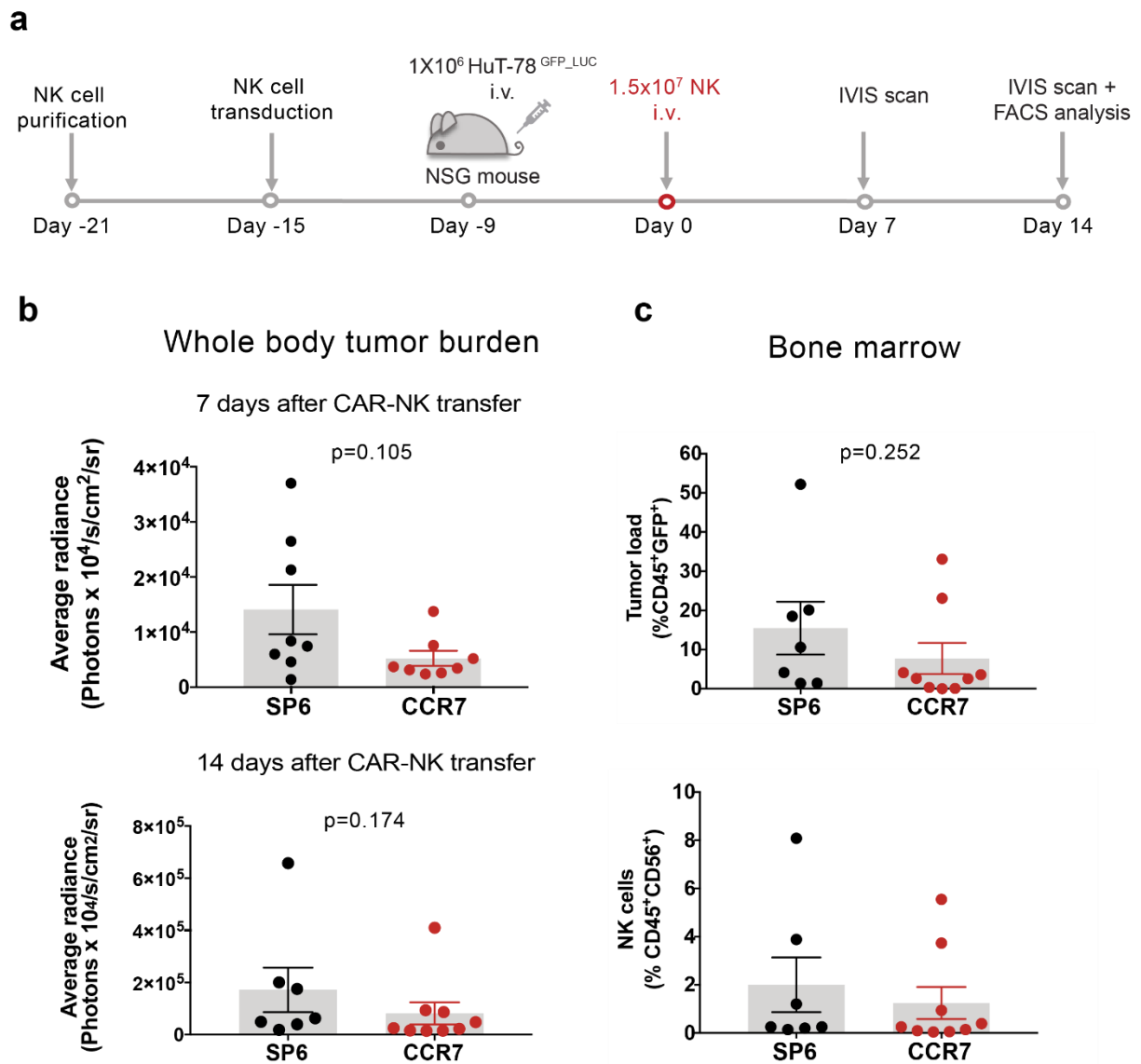


Figure 25: Antitumor activity of CCR7 CAR-(PB) NK cells against T-NHL xenograft in vivo. (a) Scheme of the *in vivo* evaluation of a single treatment with SP6 or CCR7-CAR (PB)-NK cells (1.5×10^7 NK i.v.) in HuT-78^{GFP_LUC} xenografted NSG mice (SP6: n=7; CCR7: n=9). (b) Tumor-bearing mice were IVIS scanned on days 7 (upper panel) and 13 or 14 (bottom panel) after CAR (PB)-NK transfer. Mean values of the bioluminescence imaging (BLI) intensities obtained with an exposure time of 30 sec from the entire body tumor burden are plotted for each group. Pixel intensities: 1×10^5 (min) - 1×10^6 (max). (c) Tumor (%CD45⁺GFP⁺) and NK cell (%CD56⁺CD45⁺) load was assessed in the BM by flow cytometry in individual mice. Histograms show means \pm SEM and *p* values were calculated by unpaired Mann-Whitney test.

4.9 Pharmacological control of CCR7 CAR-NK cells using Dasatinib

In addition to malignant cells, CCR7 expression was detected in subpopulations of benign cells within the hematopoietic compartment, as presented in Chapter 4.6. Consequently, when treating patients with CCR7 CAR-NK cells, there is a concern about potential side effects arising from the CAR on-target/off-tumor activity. Safety switches can be utilized to provide control over CAR-NK cells and their effector functions in real time through the course of adoptive immunotherapy.

4.9.1 100 nM Dasatinib ablates cytolytic activity and cytokine secretion

Mestermann et al. demonstrated that the tyrosine kinase inhibitor (TKI) Dasatinib (**Figure 26a**), which is an FDA-approved medication used to treat Philadelphia chromosome-positive CML and ALL, interferes with the lymphocyte-specific protein tyrosine kinase (LCK), thereby abrogated phosphorylation of CD3z and z-chain of T cell receptor-associated protein kinase 70 kDa (ZAP70) [96]. Consequently, this abolishes signaling in CAR constructs that include either CD28-CD3z or 4-1BB-CD3z activation modules. To assess the impact of Dasatinib on CAR-NK cell functions *in vitro*, CCR7 CAR NK-92 cells were coincubated with HuT-78 target cells for 16 hours at various effector-to-target ratios (E:T) upon application of 100 nM Dasatinib or vehicle only (DMSO). The proportion of residual target cells (CD56-GFP+) was assessed by flow cytometry and release of IFN- γ was evaluated in the cell-free supernatants. Relative quantifications of viable residual target cells demonstrated that treatment with 100 nM Dasatinib induced complete blockade of target cell lysis across all E:T ratios as compared to control samples (**Figure 26b**). Similarly, Dasatinib interfered with the ability of CCR7 CAR NK-92 cells to secrete IFN- γ upon antigen stimulation. (**Figure 26c**). Provided that these observations are validated in pre-clinical animal models and clinical trials, Dasatinib could serve as a readily available pharmacological safety switch that might result in the complete functional inhibition of CAR-NK cells and thus, might mitigate the risk of adverse on-target/off-tumor effects caused by persistent CAR-NK cells.

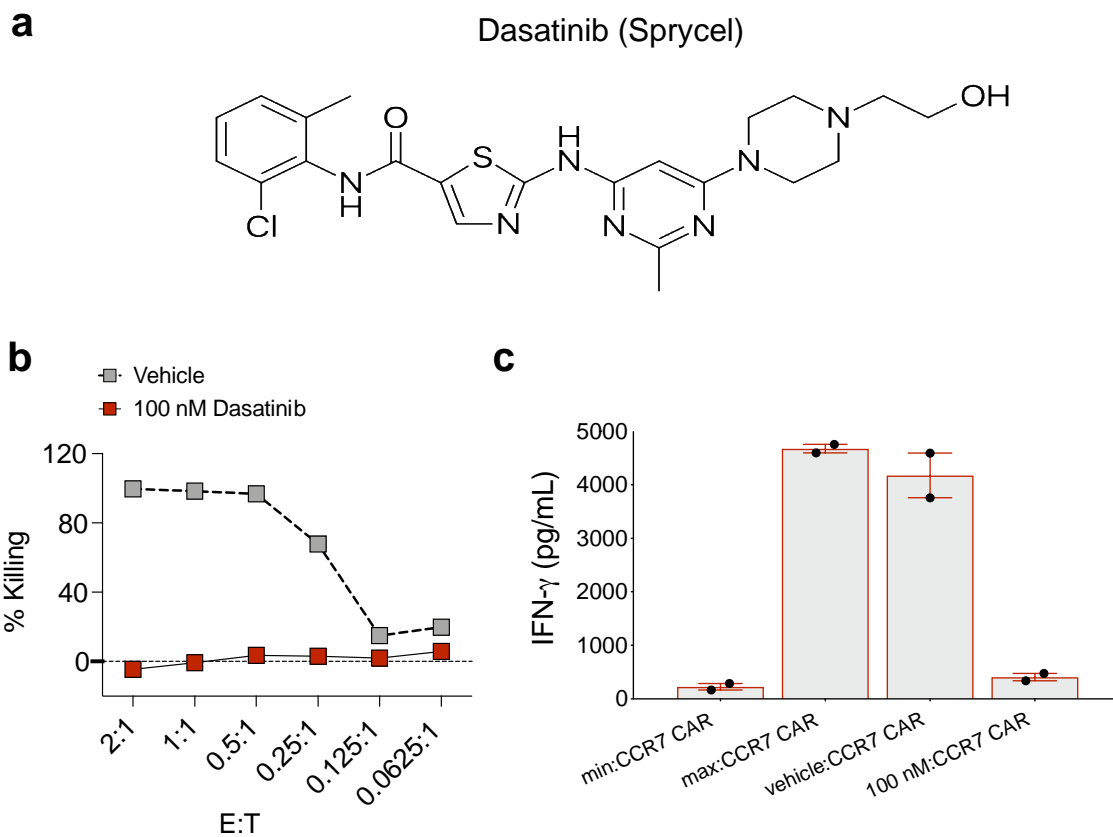


Figure 26: 100 nM Dasatinib-mediated inhibition of cytolytic activity and cytokine secretion. (a) Chemical structure of the tyrosine kinase inhibitor (TKI) Dasatinib (Sprycel). (b) CCR7 CAR NK-92 cells were cocultured with HuT-78 target cells at a 1:1 E: T ratio for 16 hours in presence or absence (vehicle) of 100 nM Dasatinib. CCR7 CAR specific cytotoxicity was evaluated by flow cytometry. Relative percentage of specific target cell killing is determined using the formula: $\% = (1 - (\text{tumor cells in coculture} / R)) \times 100$. 'R' depends on the E:T ratio: 33.3 (2:1); 50 (1:1); 66.7 (0.5:1); 80 (0.25:1); 89.9: (0,125:1); 94.1(0.0625:1). Singlets and dead cells were excluded. (c) IFN- γ release was determined by ELISA. Unstimulated (minimum) and PMA/ionomycin stimulated (maximum). Data are expressed as pg/mL and bars represent mean \pm SEM of 2 independent experiments.

5 Discussion

5.1 The chemokine receptor CCR7 as a target

T cell malignancies encompass a large and heterogeneous group of diseases that vary in clinical behavior and overall prognosis. In general, limited effective treatments are available for these patients, especially in the frontline. This often leads to frequent relapses and high morbidity, primarily due to the lack of effective targeted treatments, with a dismal overall prognosis. For example, patients with Peripheral T Cell Lymphoma (PTCL) face survival rates of 5 months when refractory and 11 months when relapsed. Even less aggressive subtypes, like Cutaneous T Cell Lymphoma (CTCL), become difficult to treat when they advance with tumor involvement or systemic disease. One promising approach to improve the current therapeutic options is CAR-based cellular immunotherapy. This technology has proven effective in treating refractory or relapsed (R/R) B cell malignancies by redirecting T cells using chimeric antigen receptors (CARs) that target CD19. Challenges have arisen in expanding the application of CAR T cell therapy to T cell malignancies due to the shared expression of numerous potential target antigens between normal and cancerous T cells. As a consequence, the therapeutic CAR-modified T cells undergo self-destruction with concomitant elimination of normal peripheral T cells. To mitigate these challenges, the selection of appropriate target antigens combined with the use of alternative immune-effectors becomes critically important. One such potential target is the tumor-associated chemokine receptor CCR7, which holds promise for CAR cell therapy in T cell non-Hodgkin lymphoma (T-NHL).

5.1.1 Physiological and pathophysiological expression of CCR7

The chemokine receptor CCR7 drives naïve lymphocyte homing to secondary lymphoid organs such as lymph nodes (LN) or spleen. In various lymphomas and leukemias, CCR7 is expressed due to their derivation from the lymphoid lineage and their specific stage of maturation, mirroring the expression patterns found in their healthy counterparts. A growing body of evidence suggests that CCR7-mediated cell trafficking is fundamentally involved in the pathophysiology of blood cancers. In the majority of blood cancers, CCR7 expression is associated with LN or spleen

involvement, as seen in Chronic Lymphocytic Leukemia (CLL), Burkitt's Lymphoma (BL), and Classical Hodgkin's Lymphoma (cHL). In other conditions such as Mantle Cell Lymphoma (MCL), B Cell Acute Lymphoblastic Leukemia (B-ALL), T Cell Prolymphocytic Leukemia (T-PLL), and T Cell Acute Lymphoblastic Leukemia (T-ALL), CCR7 expression is linked to central nervous system (CNS) infiltration. In Cutaneous T Cell Lymphoma (CTCLs), CCR7 expression has been considered an indicator of advanced Mycosis Fungoides (MF) and a central player in the spread of skin lesions to lymphoid compartments. Skin biopsies from individuals with advanced disease were shown to express high CCR7, correlating with subcutaneous extension of lymphoma cells. CCR7 is also highly expressed in Sézary Syndrome (SS) clinical samples and cell lines [22, 205–207]. Its role is described in regulating the skin egress of T lymphocyte and migration into the draining LNs. Interestingly, Cristofolletti et al. reported a significant correlation between the high level of CCR7 in circulating SS cells and the degree of epidermal infiltration [189]. Furthermore, CCR7 is found on other types of T cell neoplasms, including ALK-negative Anaplastic Large Cell Lymphoma (ALCL) [190, 208] and T cell lymphoma not otherwise specified (PTCL-NOS) [191]. In the presented thesis, a comprehensive analysis of CCR7 expression in both T cell lines, and primary T-NHL patient samples not only corroborated but also broadened the expression pattern previously documented. CCR7 expression was investigated at the protein level on cell lines originating from various T-NHL subtypes. Within the Cutaneous T Cell Lymphoma (CTCL)-derived entities, CCR7 was revealed to be expressed on the surface of the two Sézary syndrome (SS) cell lines analyzed as well as on the two originating from Mycosis Fungoides (MF). Among the four analyzed Anaplastic Large Cell Lymphoma (ALCL) ALK- cell lines, CCR7 was detected on all of them but at variable densities. In contrast, three out of four (ALCL)-ALK+ cell lines were negative, consistent with previous reports. CCR7 expression was confirmed for a substantial number of primary human malignancies of T cell origin by querying genevestigator.com, a large repository of microarray data. High CCR7 expression was found on T-PLL, Adult T cell Leukemia/Lymphoma, Primary Cutaneous Anaplastic Large T Cell Lymphoma, Angioimmunoblastic T Cell Lymphoma, Mature T Cell Lymphoma-NOS and Mycosis Fungoides. Subsequently, CCR7 surface expression was validated on primary malignant cells derived from T-NHL patients. Among the P-

TLL samples, four out of six P-TLL samples displayed homogeneous and robust CCR7 expression, while two P-TLL patient samples displayed biphasic or minor CCR7 expression. CCR7 was also highly expressed in one biopsy of Sézary Syndrome, while one T-LGL sample exhibited substantially low CCR7 levels, in line with previous studies.

5.1.2 Adoptive immunotherapy for T cell malignancies

CAR-T cells have demonstrated significant efficacy in the treatment of B cell neoplasms. Notably, the use of anti-CD19 CAR T cells in treating B cell cancers stands as a major advancement in cancer therapy, achieving sustained and deep remissions in R/R patients. Considering the relatively similar characteristics of B and T cell malignancies, it is conceivable that CAR T cells could offer similar benefits in treating T cell malignancies. Nonetheless, targeting T cell antigens with CAR-T cell therapy while sparing endogenous T cells presents major obstacles, such as self-targeting of therapeutic CAR T cells, immunosuppression of healthy T cell function *in vivo*, and product contamination with cancerous T cells. To tackle these hurdles, one strategy consists of targeting antigens expressed on subpopulations of T cells, or which are downregulated upon T cell activation. Examples of targets include CD5, CD7, CD2, CD4, CD3, CD37, CCR4, CD30 and the 2 alleles of the T cell receptor beta chains (TRBC1/TRBC2). The first published CAR developed for T cell malignancies targeted CD5, a protein expressed by thymocytes, peripheral T cells, and a subset of B lymphocytes. CD5 CAR-equipped T cells swiftly developed resistance to self-targeting shortly after transduction, minimizing T cell fratricide during manufacturing and ensuring the generation of a functional CAR T cell product generation. This was attributed to both effector CAR T cells and bystander normal T cells downregulating CD5 from the cell surface [209]. The ease of CD5 downregulation raises concerns about potential antigen escape. Additionally, while CD5 is expressed in most T-ALL cases, its expression is limited to a minority of mature T cell lymphomas.

Among the proposed targets, CD7 stands out as the most extensively studied, largely owing to its abundant expression in hematological malignancies with T cell origin. Numerous autologous and allogeneic CD7-targeting CAR-T therapeutic products are being developed and tested in preclinical and clinical studies. In the NCT04572308

trial, patients received treatment with "naturally selected" CD7 CAR (NS7CAR) T cells, where NS7CAR-T cells are those that survive fratricide during production [83]. However, durable responses were limited, presumably due to compromised cell fitness resulting from self-targeting of residual CD7-positive T cells. Subsequently, Cooper and colleagues engineered allogeneic CD7-targeting CAR-T cells which they termed "UCART7". Concomitant genetic deletion of CD7 and T cell receptor alpha chain (TRAC) was applied, enabling the generation of fratricide-resistant CAR-T cell product for the treatment of CD7-positive cancer with no risk for GvHD [210]. Safety and efficacy of UCART7 against T-ALL were shown in preclinical testing. A phase 1/2 clinical trial is presently undergoing rigorous evaluation of the product (WU-CART-007, NCT04984356) [211]. Despite encouraging results obtained for T-ALL, concerns regarding therapeutic resistance and relapse arise for certain mature T cell tumors. This is particularly due to endogenous absence of the target or its downregulation that can occur as a result of the therapy selective pressure [212–214].

CD2 has been recently proposed as an alternative target to CD7, providing effective targeting for T cell leukemia and lymphomas with low or negative CD7 expression. CD2 is a transmembrane glycoprotein expressed on NK cells and T cells, including early T cell progenitors, ranking among the surface markers with the least aberrant expression pattern in T cell malignancies [140]–[142]. The group developed UCART2, an allogeneic CAR-T cell product directed against CD2, wherein the genetic removal of CD2 and TRAC was implemented to prevent fratricide and GvHD. However, it was reported that CD2 deletion led to a reduction of CAR-T efficacy in a mouse model [144]. Another therapeutic strategy involves the selection of antigenic targets which are expressed on subpopulations of T cells. For example, CD30, a member of the TNF-receptor superfamily, is physiologically expressed on subsets of activated T and B lymphocytes and found in Hodgkin Lymphoma, some PTCLs and T-ALLs [216–218]. A second example is TRBC1 and TRBC2. Maciocia et al. exploited the clonal expression of either TRBC1 or TRBC2 on malignant T cells. Given that the expression of TRBC1 and TRBC2 is mutually exclusive in TCR-positive T cell lymphomas and healthy T cells, a CAR targeting TRBC1 could selectively deplete TRBC1-positive cancerous cells while preserving TRBC2-positive normal T cells [219]. Although both approaches hold promise in preserving a sufficient number of healthy T cells, only a

subset of patients might experience therapeutic benefits due to limited target expression.

In addition to the aforementioned antigens, targeting CCR7 offers an alternative therapeutic option for patients with T cell malignancies. One notable advantage lies in the biology of the target antigen. CCR7 plays a crucial role as a regulator of benign and malignant T cell homing to survival niches expressing CCL21/CCL19, directly impacting the lymphomagenesis process. These niches include the T cell zones of secondary lymphoid organs as well as the cerebrovascular endothelium and the choroid plexus of the CNS [209, 220]. This makes it less likely for the CCR7 antigen to be lost under the selective pressure induced by CAR targeting. As already pointed out, recent evidence indicates additional pathogenic functions of the receptor in blood cancer beyond cell lymphotropism, involving neurotropism, epidermotropism, interstitial migration, and regulation of cell survival.

5.1.3 Safety of anti-CCR7 therapies of T-NHL

For clinical application, newly identified CAR cell target antigens have to exhibit a safe and well-tolerated expression pattern in patients. The ideal target antigen should be tumor-specific with expression primarily limited to malignant cells. However, this is rarely the case. Tumor-associated antigens, such as CD19, are more common, but their application leads to side effects due to their expression on non-cancerous tissues. The first CAR products approved for B cell malignancies by the U.S. Food and Drug Administration were designed to target the CD19 antigen, which is present on both malignant and benign mature B cells. This led to profound aplasia of normal B cells as a result of such on-target/off-tumor toxicity. Nevertheless, extensive supportive care measures, including immunoglobulin supplementation, prove effective in mitigating the consequences for patients in a variety of ways. Overall, the impact of CD19 CAR therapy on healthy compartments showcased the fact that targeted therapy of lymphoid malignancies with CAR T cells may certainly affect normal lymphocyte populations.

Targeting chemokine receptors in cancer immunotherapy is an emerging field. Currently, monoclonal antibodies like Mogamulizumab, which targets CCR4, and the CXCR4 antagonist AMD3100 are already in clinical use for hematological

malignancies. The concept of immunotherapeutic targeting of CCR7 was initially introduced in 2006. The authors demonstrated that a mouse anti-human CCR7 monoclonal antibody effectively induced complement-dependent cytotoxicity (CDC) against CLL cells while sparing normal T lymphocytes from the same patients. Additionally, it blocked the migration of CLL cells in response to its ligand CCL19 *in vitro* [221]. In a follow-up study, the *in vivo* efficacy of the anti-CCR7 therapy was demonstrated in two separate xenograft models of MCL. This treatment significantly extended the survival of the animals, which was attributed to reduced infiltration of MCL cells into various tissues [222]. More recently, a humanized anti-CCR7 blocking antibody, CAP-100, (Catapult Therapeutics) was developed and was shown to mediate potent anti-tumor activity in various *in vitro* and *in vivo* preclinical models [186]. This antibody is currently undergoing clinical trials in patients with relapsed and/or refractory Chronic Lymphocytic Leukemia (CLL) (NCT04704323). A second product targeting CCR7, an antibody-drug conjugate (JBH492, Novartis) will soon be tested in a phase-I trial (NCT042140704).

The main concerns surrounding the potential use of CCR7 as a therapeutic target arise from its central role in initiating adaptive immune responses and in generating regulatory T cells (Treg) that control the development of self-reactive cells, thus preventing autoimmunity. Numerous mouse models have demonstrated that a deficiency in CCR7 signaling does not pose a life-threatening condition, as it was linked to a modest impact on immunity. T cell and B cell responses were retarded but preserved, particularly against infections. In the long term, these mice tend to develop generalized multi-organ autoimmunity, primarily affecting mucosal tissues. However, they had a normal lifespan under laboratory conditions [223].

In this thesis, it was demonstrated that CCR7 is selectively expressed in cell subsets in the hematopoietic compartment. By flow cytometry, it was corroborated that CCR7 was restrictively expressed on naïve (Tn), central memory (Tcm) and Treg cells, B cells, and a subpopulation of NK cells in the peripheral blood of healthy donors, as previously reported. Surprisingly, low density detection of CCR7 on B cells and on a subset of NK cells was revealed by using the 3D12 mAb, which is the basis for the CCR7 CAR. Additionally, the functional analysis presented in the thesis showed *in vitro* reactivity of CCR7 CAR-NK against Tn and B cells. In line with the expression

data, the depletion of B cells was less pronounced as compared to T cells. These results suggest that patients undergoing anti-CCR7 therapy may experience low-to-mild immunosuppression in the serum response. Targeting CCR7 may potentially impede new immunization processes reliant on Tn cells, but it should not impact memory effector responses to infections. It is important to consider that patients affected by mature T-NHL are typically elderly, and there is evidence indicating a shift in T cell subset distribution from Tn cells to more differentiated subsets due to continuous antigen stimulation and thymic involution [224]. Therefore, the extent of T cell depletion of anti-CCR7 therapy may vary depending on individual patient factors. Notably, CCR7-negative effector and effector memory T cells, crucial for acute effective anti-tumor responses, will remain unharmed. Another cell population that could be impacted by anti-CCR7 treatments is Treg cells. As previously mentioned, the absence of CCR7 signaling in Treg cells resulted in generalized multiorgan autoimmunity in CCR7 deficient mice. Whether anti-CCR7 therapies will produce similar effects in patients remains uncertain until the first evidence in clinical studies becomes available. Based on clinical data obtained with Mogamulizumab, an antibody that targets CCR4+ T cell subsets, including Treg [225], it seems reasonable to assume that anti-CCR7 treatment may be safe and tolerable. Consistent with this, in preclinical syngeneic mouse models of cancer, autoimmunity, GvHD, and inflammation, there were no treatment-related side effects reported. Toxicology studies with CAP-100 in non-human primates (NHP) showed no significant toxicities or autoimmune reactions, reinforcing the good tolerability of this innovative therapy [226]. Targeting CCR7 could also have some effects on B cell depletion. Although we expect moderate depletion of mature B cells, B cell BM precursors and plasma cells [227] will remain unaffected due to their lack of CCR7 on the surface. This implies that CCR7 therapy should not hinder B cell lymphopoiesis or immunoglobulin secretory function.

In CAR cell therapy, undesired toxicities may arise if the targeted antigen is expressed on unrelated tissues. To address the effect of on-target/off-tumor toxicities beyond the hematopoietic compartment, CCR7 expression was assessed on the protein level on various primary cells and cell lines originating from critical non-hematopoietic tissues. CCR7 negativity could be ensured by flow cytometry for various epithelial

compartments, including vascular, urinary, reproductive, and gastro-intestinal tracts. In the literature, it was described that CCR7 was expressed on astrocytes in the mouse brain [228]. If CCR7 was expressed in human nervous tissues, it could trigger neurotoxic events in patients undergoing anti-CCR7 therapy. However, the results outlined in this thesis could not confirm the presence of CCR7 on primary astrocytes and pericytes of the human central nervous system. In summary, the extensive investigation of CCR7 expression throughout the human body unveiled its restricted expression within the hematopoietic compartment.

Previous reports have shown that CAR-T cell can exhibit greater sensitivity in detecting ultra low levels of target antigens on tumor cells hardly detectable by flow cytometry [229]. Consequently, relying solely on flow cytometry with the CCR7 antibody is insufficient. For example, the JB6 ALCL-ALK+ cell line was stained negative by flow cytometry. However, cytotoxicity assays unveiled specific cytolytic activity of CCR7 CAR NK-92 cells against JB6 at high E:T ratio. This suggests that the CCR7 density on JB6 cells is below the detection limit of flow cytometry. Hence, numerous assays were applied to exclude potential off-target effects of CCR7 CAR-NK cells towards antigen-negative non-hematopoietic target cells. CCR7 CAR-NK cells coincubated did not secrete IFN- γ in the absence of CCR7 *in vitro*. These results rule out ultra low CCR7 expression on these tissues and indicate CCR7-targeted NK cells do not react towards unrelated structures on the target cells *in vitro* (off-tumor off-target effects).

5.2 CAR-NK cells for T-NHL immunotherapy

Reprogramming T cells to selectively eliminate cancerous T cells while preserving healthy T cell compartment poses a multifaceted problem. Although a wide variety of targetable antigens have been proposed for treating T cell malignancies, tackling them with CAR-redirectioned T cells often leads to fratricide and T cell aplasia. Researchers are exploring a number of sophisticated gene-editing strategies to decrease antigen expression in therapeutic T cells, thereby circumventing fratricide. For example, CRISPR/Cas9 genome editing has proven successful in multiple preclinical and clinical trials focused on CAR T cells. However, editing multiple gene loci through DNA

double-strand break induction poses potential risks if breakage occurs at off-target sites, potentially resulting in unintended genome alterations. Therefore, addressing safety concerns of edited CAR-T cell products is essential to mitigate potential adverse events for patients undergoing CRISPR-based therapies. While targeting T cell antigens with gene-edited CAR T cells may resolve fratricide, concerns remain about T cell aplasia and long-term immunodeficiency linked to CAR T cell persistence. Shifting to NK cells as cellular vehicle for CARs has the potential to decrease the risk of prolonged depletion of the T cell compartment, as NK cells function as short-lived effectors. Additional editing to prevent fratricide is unnecessary for NK cells, provided that the manufacturing protocol does not induce upregulation of the target antigen on the CAR-NK cell product. Furthermore, utilizing NK cells as immune effectors addresses the challenge of separating healthy T cells from malignant ones in autologous CAR T cell therapy for treating T cell malignancies. This ensures the CAR-NK cell product is free from contamination with cancerous T cells.

5.2.1 CAR-NK cells offer advantages over CAR T cells in the treatment of T-NHL

Natural killer (NK) cells have emerged as a promising cellular source for CAR cellular therapies, providing unique biological properties that make them potentially safer and more effective CAR drivers compared to T cells. NK cells are endowed with a potent ability to lyse cancerous cells with no requirement for previous contact or immunization.

Managing cytokine release syndrome (CRS) and neurotoxicities that may arise upon CAR-T cell therapy presents an additional challenge in clinical practice. NK cells are considered a safer cellular platform for CAR-based immunotherapies, due to the release of a different class of cytokines upon cell activation. Activated NK cells typically secrete IFN- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), circumventing the potential for CRS and severe neurologic adverse events associated with pro-inflammatory cytokines like TNF α , IL-1 β , IL-2, and IL-6 observed in CAR-T cell therapy [230]. IL-6 is the central mediator in the development of CRS [231]. Tocilizumab, an interleukin-6 receptor (IL-6R) antagonist, is commonly employed for the rapid clinical management of CRS associated with CAR T cell therapy [232].

Adoptively transferred NK cells have a limited *in vivo* persistence of about 2 weeks [233]. This implies that if on-target/off-tumor toxicity arises, it can naturally resolve with the disappearance of CAR-NK cells. This is especially beneficial for T cell malignancies, as the short lifespan of the cellular product may prevent the potential onset of prolonged T cell aplasia.

A notable proportion of individuals exhibit loss of antigenic target expression on malignant cells following CAR-T cell treatment, developing tumor resistance towards the therapy. This phenomenon represents a prominent mechanism behind the ineffectiveness of CAR-T therapies [234] and occurs, most likely, due to extended immunoselection.

Target recognition solely occurs in a CAR-mediated fashion in CAR-T cells. Contrarily, NK cells possess intrinsic CAR-independent killing capacities. Cancerous cells can be directly killed upon release of cytotoxic granules containing perforin and granzymes. Alternatively, NK cells can induce apoptosis through death receptors, such as FASL and TRAIL, or ADCC through CD16. After activation, numerous pro-inflammatory cytokines and chemokines are secreted by the NK cells, thereby orchestrating downstream innate and adaptive immune responses. These innate mechanisms persist even in scenarios where tumors escape CAR therapy due to therapy resistance. Hence, redirecting the cytotoxicity of NK cells through a CAR has the potential to eliminate tumors exhibiting a heterogeneous phenotype, thereby tackling the risk of relapse through mechanisms both dependent and independent of the CAR.

The source of immune cells for CAR-NK cell therapy represents another key advantage compared to CAR-T cell manufacturing which requires the use of the patient's cells. Currently, CAR-T cell therapy is not a frontline treatment. Instead, it is utilized for patients with R/R disease who had not responded to multiple prior lines of therapy. For example, the two FDA approved CAR T cell therapies for BCMA-positive tumors are administered to MM patients who have progressed after fourth-line therapy, including a proteasome inhibitor, immunomodulators and an anti-CD38 antibody therapy [235]. Therefore, T cells obtained from patients may exhibit compromised functionality and restricted numbers, particularly in cases where patients have undergone extensive treatments prior to CAR-T therapy [167]. The complex and lengthy manufacturing process of CAR-T cell therapy, from apheresis collection to

administration, adds an additional challenge for patients in urgent need of treatment due to rapidly advancing disease [237].

Genetic removal of the T cell receptor (TCR) is required when utilizing T cells from healthy donors for CAR-T cell manufacturing, thus reducing the likelihood of allogeneic rejection [238]. In contrast to T cells which detect and respond against non-self through the MHC pathway, NK cells are independent of this mechanism, leading to a reduced risk of graft-versus-host-disease (GvHD). Consequently, the collection of NK cells from a patient or a specific HLA-matched donor is not required for CAR-NK cell manufacturing. Therefore, NK cells are readily available as allogeneic effectors, paving the way for a potentially off-the-shelf product for cell therapy.

There are various allogeneic sources available for obtaining NK cells [239]. Large quantities of NK-92 cells can be expanded from a GMP- cryostored master cell bank. However, their requirement for irradiation limits *in vivo* persistence to approximately 48 hours, impeding the establishment of a sustained clinical impact. Third-party donor peripheral blood (PB)-NK cells exhibit a mature phenotypic signature, yet the variability among donors poses challenges for dose standardization, given the absence of a single, uniform, and renewable source for PB-NK cells [150].

Umbilical cord blood is a rich source of NK cells, stored off-the-shelf in frozen umbilical cord blood (UCB) banks. However, using unmanipulated UCB-NK cells for CAR immunotherapy faces challenges due to scarce NK cell yield in a single UCB unit and the lower cytotoxicity of resting UCB NK cells compared to PB-NK cells [240]. Nonetheless, these limitations can be addressed through *ex vivo* expansion using cytokines and engineered feeder cells [241].

Both UCB and PB-derived NK cells encounter limitations in providing a genetically edited, homogeneous NK cell population suitable for use in multi-dosing strategies, as obtaining cells from a single, renewable source is impractical.

On the contrary, human induced pluripotent stem cells (iPSC) can be indefinitely expanded in a homogenous manner and differentiated into iNK cells. The iNK cell platform offers the possibility of multi-gene editing prior to NK cell differentiation from iPSCs, allowing the selection of optimally edited clones. This enables the creation of

large-scale, off-the-shelf therapeutic doses of homogeneous master cell banks of NK cell products, readily stored and available on demand [242].

5.2.2 CAR-NK cell therapies

Multiple preclinical and clinical trials have investigated the efficacy of CAR-NK cells in treating hematological malignancies, with an initial focus on B cell-derived cancers. In the first-in-human phase 1/2 trial conducted in 2020 by Liu et al., HLA-mismatched UCB derived NK cells expressing a second generation anti CD19 CAR, IL-15, and an inducible caspase-9 suicide gene were administered to patients with R/R B cell malignancies. The study demonstrated a 73% objective response rate. Within the cohort, 7 patients achieved complete remission with a median follow-up of 13.8 months. Notably, there were no significant adverse events such as CRS, neurological toxicities, or GvHD, except for transient myelotoxicity. In addition, the UCB-NK cells persisted in patients for up to 1 year, demonstrating their enduring potential [243].

Fate Therapeutics is pioneering the field of iPSC-NK cells with multiple off-the-shelf CAR-NK products. One notable example is FT596, engineered with a second generation anti CD19 CAR, a high-affinity CD16, and IL-15/IL-15R α [244]. Results of a phase I dose escalation clinical study (NCT04245722) showed the safety of the product without dose-limiting toxicities. In this study involving 20 patients with R/R B cell lymphoma (BCL), 10 received FT596 alone (regimen A) and 10 received FT596 in combination with rituximab (Regimen B) with an OR of 63% (5/8) in Regimen A and 44% (4/9) in Regimen B [245]. Cichocki and colleagues developed a quadruple gene-edited iPSC-NK cell product, iDuo-MM-CAR-NK cells, targeting BCMA in MM. Cells were engineered with an NK cell optimized CAR for BCMA, human non-cleavable (hn) CD16 and IL-15/IL-15R α . In addition, CD38 was knocked out to prevent fratricide upon application of Daratumumab (CD38 mAb). The product demonstrated sustained tumor control comparable to BCMA CAR-T cells with no toxicities, such as GvHD. In combination with Daratumumab, iDuo-MM-CAR-NK cells exhibited enhanced ADCC and anti-MM efficacy [246].

CAR retargeted NK cell therapies towards CD3, CD4, CD5, and CD7 were developed for T cell malignancies. Third generation CD3 CAR NK-92 cells were generated by Chen et al., demonstrating effective lysis of CD3-positive primary human PTCL

samples and T cell leukemia cell lines and tumor control in xenogeneic mouse models of T-ALL [97]. In a separate study, the functionality of two second generation 4-1BB and 2B4 based anti CD5 CAR were compared by Xu et al. Their results indicated that the 2B4 could augment the cytotoxicity of CD5 CAR-NK cells, in direct comparison to the T cell-associated activating receptor 4-1BB [247]. Finally, You et al. equipped an IL-2 independent NK-92 cell line with a third generation CD7 CAR showing specific cytotoxicity against primary T-ALL samples and inhibition of tumor growth in a patient-derived xenograft (PDX) mouse model [99]. Presently, only one clinical trial (NCT02742727) for T cell malignancies is enlisted to assess the safety and efficacy of CD7 CAR NK-92 cell therapy in patients with CD7-positive R/R lymphoma and leukemia. However, the study results are not available yet.

5.2.3 CCR7 CAR-NK cell therapy

In this thesis, CCR7 was proposed as a novel target for CAR-NK cellular therapy of T cell neoplasm. To our knowledge, no anti-CCR7 CARs have been previously described. The scFv amino acid sequence was obtained from a rat anti-human CCR7 antibody. VH and VL were linked by a Whitlow sequence and incorporated into a retrovirally-encoded second generation CAR backbone with CD28 and CD3z as signaling modules. The CD3z subunit is fundamentally involved in T- and NK cell signaling and activation [248, 249]. Though CD28 is widely acknowledged for its role in T cell costimulation [250], its involvement in NK cell activation has not been fully elucidated. Nonetheless, the expression of CD28 in human fetal NK cells and various NK cell lines, along with their ability to eliminate CD80/CD86-expressing tumors, suggests a potential role for CD28 in NK cell activation [251, 252].

In this study, the CCR7 CAR conferred NK-92 cells with high avidity, enabling them to i) recognize, ii) be activated against, and iii) kill T-NHL cell lines with high, intermediate, or low CCR7 surface expression in various *in vitro* coculture assays. These findings were corroborated in PB-derived NK cells. Furthermore, the CCR7 CAR-NK cells efficiently killed CCR7-positive patient-derived T-NHL cells. Additional pre-clinical *in vivo* testing of the CCR7 CAR activity against xenotransplanted T-NHL mice will substantiate the preliminary results presented here. This study unveiled the unique *in vitro* properties of the product. The anti-CCR7 CAR binder used in this thesis confers

NK cell reactivity towards CCR7 positive benign and transformed T cells but to a much lesser extent when the antigen was expressed in benign and transformed B cells, thus reducing on-target/off-tumor side effects on B cells.

The potential initial patient population for CCR7 CAR-NK cell therapy would likely include individuals with progressive disease who have failed multiple lines of standard-of-care therapies. This cohort is expected to have undergone extensive lymphotoxic therapy and may present low peripheral lymphocyte counts. Therefore, allogeneic NK cells obtained from third-party donors might be a more suitable option for manufacturing the CCR7 CAR-NK cell product. This approach eliminates the necessity for additional genetic editing of the NK cells, such as removing the TCR to mitigate the risk of graft-versus-host disease (GvHD) or eliminating the antigen itself to prevent fratricide during manufacturing. Additionally, choosing donor-derived NK cells circumvents the risks linked to autologous CAR-T cell therapies, specifically the potential contamination of the final product with malignant T cells from the patients themselves. CCR7 CAR-NK cell therapy has the potential to be administered to patients suffering from various T cell malignancies characterized by pathogenic cells expressing CCR7, including both immature subtypes like T-ALL and more mature diseases such as P-TLL, MF, SS, ALCL, and PTCL-NOS. Administration of the CCR7 CAR-NK cell product might be an effective approach to hinder the homing of leukemic T cells to protective niches. Particularly, the treatment may prove effective in cases of Alemtuzumab-relapsed/refractory disease or for individuals with low levels of CD52 expression [253] or absence of CD52 [254, 255]. For instance, the percentage of T-PLL patients responding to Alemtuzumab is minimal in cases of CNS disease [256] and/or bulky lymph node masses [257, 258]. In this regard, CCR7 CAR-NK cell therapy has the potential to effectively eliminate tumor cells from various sites, including LN and CNS. In other clinical scenarios, the application of CCR7 CAR-NK cell therapy could tackle minimal residual disease in SLOs and allow previously ineligible patients to proceed to stem cell transplantation. Additionally, CCR7 CAR-NK cell therapy could be beneficial for patients with co-morbidities that limit further chemotherapies or for elderly patients unable to tolerate traditional chemotherapy. Opposite to current alternative cell products for T cell neoplasms, such as allogeneic CD7 or CD2 CAR-T cell therapies, neither prolonged T cell aplasia nor CRS and

neurotoxicity are of concern for CCR7 CAR-NK cell therapy. In addition, in contrast to CD7, CCR7 is absent on common myeloid progenitors. Therefore, we expect no reactivity of our CAR-NK cell product towards physiological myeloid cell lineages and their precursors.

Therapy resistance is less likely to happen upon CCR7 CAR-NK cell application as selective pressure will only be transient due to the short persistence of the product in a patient, thereby circumventing the risk of potential relapses. Contrasted with antibody therapy, CCR7 CAR-NK cell therapy might exhibit potential efficacy even when the antigen density on target tumor cells is minimal, a scenario where antibodies may prove ineffective. Moreover, CCR7 CAR-NK cells might be applicable as monotherapy, unlike antibody therapy.

5.3 Next generation CARs

CAR-engineered T cells have demonstrated significant success in addressing hematological malignancies, as evidenced by the approval of multiple CAR-T cell products by the EMA and FDA. However, numerous challenges have limited the efficacy of CAR-T cell therapies for the treatment of solid tumors, which constitute the majority of cases. Key hurdles include i) the potential for serious on-target, off-tumour toxicity due to CAR T cells reactivity against non-malignant tissues bearing the antigen; ii) antigen escape and heterogeneity; iii) the highly immunosuppressive nature of the TME. The synergistic application of multidimensional gene and cell engineering strategies combined with a deeper understanding of the complex TME will facilitate the rational design of next generation CAR-based cellular products with improved safety and efficacy.

5.3.1 Improving safety through the integration of control switches

Differently from conventional cancer treatments (small molecules and antibody-therapies), adoptively transferred CAR-T cells function as a living drug, being able to expand, differentiate, and persist for years within the human body. Constitutive expression of the CAR may prove undesirable in case of anticipated toxicities towards healthy tissues expressing the antigenic target. ON/OFF switches offer precise

temporal control over the transgene expression and cytotoxic function of the CAR-T cell product. CAR-destabilizing domains (DD) exemplify this model. A DD is incorporated within the CAR encoding transgene which places it in an inactive state (OFF state) with swift protein degradation. Activation is achieved upon administration of small molecules that stabilize the CAR protein [259]. On the contrary, in small molecule–assisted shutoff (SMASh) CAR systems, the transgene is fused to a protease binding sequence along with the protease and protease destabilizing element (degron) at the C-terminus that triggers CAR degradation. In the ON state, the degron is cleaved off, thereby preserving the stability of the CAR protein. Adding protease inhibitors results in degron stabilization and consequent CAR protein degradation (OFF state) [260]. Similarly, spatially controlling CAR expression involves, for example, splitting the scFV and signaling elements of the CAR into two separate nonfunctional units (Split CARs), incorporating a heterodimerization domain. The CAR protein assembles when exposed to a dimerizing agent [261]. Alternatively, switchable CARs (sCARs) feature a neoepitope-specific scFv (e.g., PNE). The switchable component is a soluble antigen-specific fragment tagged with a peptide neoepitope [262]. Adjusting the timing and amount of the protein switch fine-tunes T cell response intensity, while exchanging the protein switch itself alters the antigen specificity of the effector cells. To our knowledge, no reports about real world applications in patients of the aforementioned technologies are available yet.

5.3.2 Implementing logic-gated CARs for combinatorial antigen targeting

Most tumor-associated antigens are often co-expressed on non-cancerous cells, posing a significant risk of morbidities due to on-target, off-tumor effects of the CAR. A way to ameliorate CAR specificity is by AND-gate logic CARs which requires binding of multiple targets for activation. An example is the synthetic Notch (synNotch) system, functioning as AND-gate when binding of the first antigen by the synNotch receptor drives downstream activation of a transcription factor, thereby triggering the expression of a CAR specific for second antigen [263]. A second approach to circumvent off-target toxicity is the use of AND-NOT logic CARs, e.g., iCARs, targeting antigens expressed on healthy tissues. iCARs comprise Inhibitory signaling domains derived from, e.g., PD-1 or CTLA-4, are incorporated in iCARs such that suppression

of T cell responses occurs upon recognition of healthy cells expressing the target. *In vitro* and *in vivo* studies demonstrated that AND-NOT logic gated CAR T cells coexpressing a CD19 CAR and an inhibitory CAR (iCAR) equipped with the intracellular module of PD1 or CTLA-4 exhibit high anti-tumor efficacy against cancerous B cells while sparing benign B cells [264, 265]. AND and AND-NOT logic gating strategies are effective in preventing potential toxicities. However, they do not address evasion mechanisms, such as tumor antigen loss and downregulation. CARs with OR-gate design were developed for concomitant targeting of two antigens, enabling activation of CAR-T cells upon engagement with either target. Bispecific CARs equipped with two scFVs connected in tandem (tandem CARs) are examples of this category. T cell expressing CD19/CD22 and CD19/CD20 OR-gate CARs were shown potent in controlling cancerous cells escaping CD19 CAR-T cell therapy [266, 267]. Numerous clinical studies have reported encouraging preliminary outcomes [268–270]. Alternative OR-gate approaches such as dual CARs (engineered with two separate CAR proteins in the same cell) or CAR-pooling (application of two separate CAR-T cellular products) have also been preclinically explored. However, in head-to-head comparison studies, tandem CARs configurations have consistently shown superior anti-tumor activity [271–273].

5.4 Limitations of the study

In this study, a novel CCR7 CAR was developed for CAR-NK retargeting in the treatment of T-NHL. The sequence of the CAR targeting moiety, the scFv, was derived from a rat hybridoma. As the use of foreign proteins as CAR binders may induce an anti-CAR antibody response, scFv humanization was attempted utilizing a rational method, the "CDR grafting", based on the scFv sequence information. A small set of variants was generated to be tested for binding. Analysis of the scFv structure by computer-assisted molecular modeling (e.g., root-mean-square deviation) could have been integrated to provide guidance in the identification humanized sequences that preserve the original CDR conformation. A second limitation of the study concerns the assessment of CCR7 expression on primary T-NHL samples, where only circulating tumor cells were analyzed. Including cells obtained from primary tumor, e.g., skin biopsies bone marrow aspirates or lymph node biopsies would further support the

validation of CCR7 as a promising target in the treatment of T-NHL. Finally, the expansion protocol employed in this work solely relied on interleukins for NK cell expansion. However, the obtained cellular yields were insufficient for repetitive applications of the CCR7 CAR (PB)-NK cells to the NSG xenografted mouse model of T-NHL. Opting for a feeder cell-based expansion protocol may help manufacture suitable doses for finalizing the *in vivo* testing of the efficacy of CCR7 CAR (PB)-NK cells.

5.5 Future directions

Integrating the innate capability of NK cells to eliminate cancerous cells with the targeted cytotoxicity provided by the CAR has proven technically feasible. Preliminary and early clinical data on the use of CAR-NK cells have shown efficacy and safety in the treatment of a variety of cancers and antigens, thereby addressing some of the constraints associated with CAR T cell therapies. However, the broad therapeutic application CAR-NK cells remains challenging due to the inability to harvest sufficient yields for adoptive transfer. NK cells constitute a minor fraction in PBMC or UCB, exhibit a poor *ex vivo* expansion and have relatively short life spans *in vivo*. In contrast to the standardized culture of T cells, NK cell cultures exhibit high variability depending on the employed expansion protocol. Efforts are underway to develop expansion methods compatible with good manufacturing practices, aiming to produce sufficient yields for clinical use from a single source and selectively expand NK cell subsets for optimal efficacy. Despite the utilization of diverse cytokine cocktails, including IL-2, IL-15, IL-18 and IL-21 for activating and expanding allogeneic NK cells, these approaches fail to generate an adequate quantity of NK cellular products suitable for clinical applications. Implementing feeder-cell-based systems has resulted in the greatest fold expansion of CAR-NK cells to date. Various cell types are utilized, such as EBV-transformed lymphoblastoid cell lines or autologous irradiated PBMCs, which achieved promising outcomes. However, K562-based approaches have shown superiority in both the extent and speed of expansion. Liu et al. engineered K562 cells into a universal antigen-presenting cell (uAPC), lacking MHC class I and equipped with CD48, 4-1BBL, and membrane-bound IL-21. This yielded over a 900-fold expansion of CAR UCB-derived NK cells within two weeks of coculture with high purity

and no signs of exhaustion or senescence [241]. The downstream processing of the obtained cellular product for the remote storing of the allogeneic doses presents an additional bottleneck for CAR-NK cell therapy. Cryopreservation has proven problematic for NK cells, resulting in decrease in cell viability, cytotoxicity and motility following thawing [274]. The development of effective cryopreservation protocols that maintain both NK cell number and functionality is of central importance to propel this field forward. The limited *in vivo* persistence of NK cells, particularly in the absence of cytokine support, poses an additional barrier to their effective utilization. To overcome this challenge, cytokine genes, e.g., IL-2 and IL-15, or cytokine/receptor fusion complexes, e.g. IL-15/IL-15Ra, can be incorporated in the CAR construct to provide autocrine or paracrine support for the CAR-NK cells. An alternative strategy for enhancing NK cell persistence consists of differentiating them into cytokine-induced memory-like NK (MLNK) cells through the combination of IL-12, IL-18 and IL-21. Cooper et al. reported that murine NK cells show higher IFN- γ secretion following a short stimulation with IL-12, IL-18 and IL-21 in comparison to non-preactivated NK cells upon cytokine restimulation [275]. Similar responses were observed in human NK cells [276]. In both *in vitro* and *in vivo* settings, CD19 CAR MLNK cells displayed enhanced persistence and greater anti-lymphoma efficacy than conventional CAR-NK cells [277].

5.6 Conclusions

In the presented work, a novel CAR targeting the human chemokine receptor CCR7 has been developed and characterized for CAR-NK cell therapy in the treatment of T-NHL. Comprehensive preclinical *in vitro* analysis strikingly showed that the CCR7 CAR confers human NK cells with high cytotoxic activity against CCR7-positive T-NHL cell lines and primary cells. Extensive expression studies on protein level were conducted, confirming the limited expression of CCR7 within the hematopoietic compartment. Toxicity analysis showed that CCR7 CAR-NK cells preferentially target T cells, minimizing the effect on B cells, with no off-target effects on CCR7-negative benign tissues. CCR7 CAR-NK cell therapy could constitute a promising treatment option for patients suffering from various T cell malignancies characterized by pathogenic cells expressing CCR7, with progressive disease unresponsive to multiple lines of

standard-of-care therapies. The treatment could also be beneficial for patients i) with multidrug resistance; ii) ineligible for allogeneic stem cell transplantation; iii) with comorbidities that prevent further chemotherapies. Taken together, CCR70 CAR-NK cell is a promising immunotherapy for T-NHL whether applied as monotherapy or integrated into a multimodal treatment regimen.

6 References

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7 Statutory Declaration

“I, Marialucia Massaro, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “Targeting CCR7 with CAR engineered NK cells for the treatment of T cell non-Hodgkin's lymphoma (T-NHL)” – “CCR7 als Zielstruktur gentechnisch entwickelte CAR-NK Zellen zur Behandlung von T Zellen-Non-Hodgkin-Lymphome (T-NHL)”, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date

Signature

8 Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

9 Publications

“CXCR4 has a dual role in improving the efficacy of BCMA-redirected CAR-NK cells in multiple myeloma” Michael W. Moles*, Henry Erdlei*, Lutz Menzel*,

Marialucia Massaro, Agnese Fiori, Mario Bunse, Moritz Schrimpf, Kerstin Gerlach, Venugopal Gudipati, John Reiser, Ketan Mathavan, Jode Goodrich, Johannes B. Huppa, Jan Krönke, Bahram Valamehr, Uta E. Höpken, Armin Rehm (Submitted to *Frontiers in Immunology*, 2024). *equal contribution

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11 Certificate of the accredited statistician



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Bescheinigung

Hiermit bescheinige ich, dass Frau Massaro innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgender Beratungstermin wurde wahrgenommen:

- Termin: 18.01.2024

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Achsen wenn möglich nicht abschneiden, da es das Ergebnis visuell verfälscht
- P-Werte nicht konfirmatorisch interpretieren, nur die Werte berichten. Alle Werte unter 0.01 sollten auch so angegeben werden ($p < 0.001$)
- Bei kleinen Fallzahlen (z.B. $n < 30$) sollten nicht-parametrische Tests bevorzugt verwendet werden, aufgrund einer möglichen Verletzung der Normalverteilungsannahme

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 19.01.2024



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