Diploma Thesis

Advances in T-cell Therapy for Cancer Patients

submitted by

Magdalena Leitner

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under supervision of

Univ.-Ass. Mag. rer. nat. PhD. Julia Kargl

Univ.-Prof. Dr. med. univ. Akos Heinemann

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“Although the palliation of cancer is the daily task of the oncologist, its cure is our fervent hope”, the description of leukemia by the physician William Castle in 1950.

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, 14th September 2020 *Magdalena Leitner eh*

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List of abbreviations

ACT	Adoptive cell therapy
ALL	Acute lymphocytic leukemia
ALP	Alkaline phosphatase
AFP	Alpha Fetoprotein
ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ATP	Adenosintriphosphat
B2M	Beta-2 Microglobulin
Bcl-2	B-cell lymphoma 2
BCMA	B-cell maturation antigen
CA-125	Cancer-Antigen 125
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CCI	Charlson comorbidity index
CCR	Chimeric costimulatory receptor
CEA	Carcinoembryonic antigen
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
CR	Complete remission
CRP	C-reactive protein
CRS	Cytokine release syndrome
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated antigen-4
CDK4	Cyclin-dependent kinase 4
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERBB2IP	Erbb2 interacting protein
ETBR	Endothelin B receptor
Fab	Fragment antibody binding
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein-α
FasL	Fas-ligand
Fc	Fragment crystallizable
FDA	U.S. Food and Drug Administration
FL	Follicular lymphoma
FRA	Folate receptor-alpha
GBM	Glioblastoma
G-CSF	Granulocyte-colony stimulating factor
GD2	Ganglioside
gp100	Glycoprotein gp100
GVHD	Graft-versus-host disease

HCC	Hepatocellular Carcinoma
HER	Human epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HPV	Human papillomavirus
HSV-TK	Herpes simplex virus–thymidine kinase
huEGFRt	Truncated human EGFR polypeptide
iCARs	Inhibitory CARs
iCasp9	Inducible caspase-9
IDO	Indole 2,3-dioxygenase
IFN	Interferon
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
IL-13Rα2	Interleukin-13 receptor α2
IL-2	Interleukin-2
ITRs	Inverted terminal repeats
KIF2C	Kinesin family member 2C
L1-CAM	L1-cell adhesion molecule
MART-1	Melanoma antigen recognized by T-cells 1
MCL	Mantle cell lymphoma
MDSC	Myeloid-derived suppressor cell
MAGE-A3	Melanoma antigen A3
MHC	Major histocompatibility complex
MM	Multiple myeloma
NY-ESO-1	New York esophageal squamous cell carcinoma-1
NFAT	Nuclear factor of activated T-cell
NK	Natural killer cells
NSCLC	Non-small-cell lung cancer
OKT3	Anti-CD3 antibodies
OR	Objective response
p53	p53 transcription factor
PAMP	pathogen associated molecular pattern
PAP	Prostatic acid phosphatase
Pat.	Number of patients
PBMC	Peripheral-blood mononuclear cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
POLA2	DNA polymerase alpha subunit B
PR	Partial remission
PRAME	Preferentially expressed antigen in melanoma
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
RB	Retinoblastoma tumor suppressor gene
RBC	Red blood count
RCC	Renal cell carcinoma
Ref.	Reference
scFv	Single-chain fragment variant
SCLC	Small cell lung cancer
synNotch	Synthetic Notch receptor

TALEN	Transcription activator-like effector nuclease
TBI	Total body irradiation
TCM	Central memory-enriched T-cells
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptors
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Tn-MUC1	Tn glycoform of MUC1
Treg	Regulatory T-cell
TRUCK	T-cell redirected for universal cytokine killing
TSP-1	Thrombospondin-1
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WHO	World Health Organization
WT1	Wilms tumor

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Zusammenfassung

Die Diplomarbeit mit dem Titel „Fortschritte in der T-Zelltherapie für Krebspatienten“ beschäftigt sich mit der T-Zell Therapie in der Onkologie. Grundlagen der Krebsentstehung, des Immunsystems und der Krebstherapie wurden recherchiert. Im Fokus der Arbeit steht ein Review mit den jüngsten Entwicklungen und den derzeit laufenden Studien auf dem Gebiet der T-Zell Therapie.

Die Methodik dieser Arbeit basiert auf einer systematisch durchgeführten Literaturrecherche. Die recherchierte Literatur umfasst Bücher, Fachzeitschriften, diverse Publikationen in Journals und aktuelle Behandlungsrichtlinien. Als Datenbanken wurden PubMed und ClinicalTrials.gov verwendet. Sofern es möglich war, wurde insbesondere in den Kapiteln der verschiedenen T-Zell Therapien, aktuelle Literatur herangezogen.

Die Ergebnisse umfassen eine systematische Aufarbeitung, vor allem von den verschiedenen Formen der T-Zell Therapie in diversen Tumorentitäten. Die verschiedenen Möglichkeiten der Tumortherapie werden besprochen und ein kurzer Ausflug in die Geschichte der T-Zell Therapie wird gegeben. Ergebnissen aus Studien, die T-Zell Therapie in unterschiedlichen Krebsformen verwenden, werden besprochen. Neben klinischen Ergebnissen werden auch Nebenwirkungen und Limitationen für jede Form der T-Zell Therapie aufgezeigt, sowie der Herstellungsprozess beleuchtet. Der Einfluss von verschiedenen Faktoren wie der Tumorumgebung oder der Eigenschaften der verwendeten T-Zellen werden besprochen. Zukunftsperspektiven der T-Zell Therapie und neue Ansätze werden dargestellt. Die Ergebnisse der T-Zell Therapie werden mit Ergebnissen von Standard-Krebstherapien wie Chemotherapie oder Strahlentherapie verglichen. Kombinationsmöglichkeiten aus T-Zell Therapien und anderen Formen der Krebstherapie und deren Ergebnisse in klinischen Studien werden aufgezeigt. Bereits zugelassene Wirkstoffe werden vorgestellt und aktuell laufende Studien werden beschrieben.

Durch Fortschritte in der Immuntherapien-Forschung, haben Patienten/Patientinnen in klinischen Studien bereits Tumorregression oder Heilung durch T-Zell Therapien erfahren. Durch die Zulassung von CAR T-Zell Therapien,

ist diese bereits als Bestandteil von Krebsbehandlungen für Patienten/Patientinnen mit verschiedenen hämatologischen Malignomen implementiert. T-Zell Therapien werden auch in Zukunft Thema der Forschung sein, um irgendwann für viele Tumorpatienten/Tumorpatientinnen als Therapie zur Verfügung stehen.

Abstract

This diploma thesis with the title "Advances in T-cell Therapy for Cancer Patients" deals with the topic of T-cell therapy in oncology. Therefore, basics of cancer development, the immune system and the possibilities in cancer therapy were investigated. The focus of this thesis is a systematic review of T-cell therapy, including latest developments and current studies.

The method used for this work is a systematic literature review. The literature research includes books, various publications from journals and current treatment guidelines. Therefore, PubMed and ClinicalTrials.gov were selected as databases. Wherever possible, current literature was used, in particular in the chapters of the different forms of T-cell therapies.

The results include a systemic analysis of the different forms of T-cell therapy in various tumor entities. The possibilities of tumor therapies are discussed and a short excursion into the history of T-cell therapy is given. Results from pre-clinical and clinical studies using T-cell therapies in treatment of different forms of cancer are discussed. In addition, side effects and limitations for each form of T-cell therapy are shown. The manufacturing process of T-cell therapy products is described as well. The influence of various factors, such as the tumor microenvironment or the properties of the used T-cells, are explored. Future perspectives of T-cell therapy in form of new approaches, which might enable a widespread use, are presented. Results of T-cell therapy are compared to results of standard cancer therapies, such as chemotherapy or radiation therapy. Possible combinations of T-cell therapies with other forms of cancer treatments and their results in clinical studies are shown. Already approved T-cell therapies are presented and current ongoing clinical trials are described.

Due to advances in immunotherapy research, T-cell therapies have already successfully induced tumor regression or even cure in patients in clinical studies. With the approval of CAR T-cell therapies, they are already part of cancer treatments for patients with various hematologic malignancies. Continued research in the field of T-cell therapy will make it available for many cancer patients in the future.

1 Introduction

1.1 Cancer principles

There are different capabilities that normal cells acquire on their way to become neoplastic cells, and further, to develop to human tumors. The hallmarks of cancer describe six of these capabilities, that enables cells to grow and their metastatic dissemination. Genome instability is the underlying condition for these hallmarks. The hallmarks are sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (1). Two additional hallmarks were added to the list after one decade of research; reprogramming of energy metabolism and evading immune destruction (2). Tumors do not just consist of neoplastic tumor cells, they additionally contain a repertoire of ostensibly normal cells that are creating the microenvironment of the tumor and play a role in the acquisition of hallmark features (2).

1. Sustaining proliferative signaling

In normal cells, growth is carefully controlled and the production and release of growth promoting signals is strictly regulated. This leads to an exactly controlled cell growth and cell division cycle. These signals are dysregulated in cancer cells and thereby malignant cells acquire the ability to sustain a chronic proliferation. Growth promoting signals are often growth factors that bind to receptors on the cell surface, which contain an intracellular tyrosine kinase. The binding of ligand to the receptor leads to the start of an intracellular signaling pathway that regulates cell cycle, cell growth and influences cell survival and energy metabolism (2).
There are different options how tumor cells acquire their ability to sustain proliferative signaling. They can produce growth signals by themselves that bind to their receptor, which lead to an autocrine growth stimulation (2). An alternative way is, that cancer cells emit signals that stimulate normal cells of the tumor environment, especially fibroblasts, which subsequently supply the tumor cells with various growth factors (3,4). Another way to enhance growth signaling is to increase the levels of receptors on the tumor cells surface. Structural alterations of the receptor, which result in a ligand independent signaling pathway, can also lead to sustain proliferative signaling. Ligand independent signaling can also be

implemented by alterations of the intracellular pathways downstream the receptor (2).

2. Evading growth suppressors

Cancer cells must be able to circumvent programs that inhibit growth and proliferation of the tumor. These programs are often activated by tumor suppressor genes that limit cell proliferation and growth (2). Examples for tumor suppressor genes are RB (retinoblastoma tumor suppressor gene) and p53 (p53 transcription factor), they work as control nodes in cellular regulatory circuits. They decide whether the cell should stay in the growth and division cycle, or whether the cell should start senescence and apoptotic cell death (2,5).
The RB protein integrates intracellular and extracellular signals and decides which way the cell will pass - further cell growth or cell death. It was shown that a loss of RB function, due to RB gene inactivation in a wide range of cancers types, leads to cancer initiation and progression, because the absence of RB proteins permits persistent cell proliferation (6). P53 receives intracellular inputs in case of problems like cell stress, genome damage, suboptimal levels of glucose, oxygen, growth signals, nucleotides or other cellular abnormalities. If those abnormalities accumulate to a high degree, p53 is able to arrest the cell cycle until the problem is solved, or in case of irreparable or overwhelming damage, p53 can trigger apoptotic cell death (2).

3. Resisting cell death

The programmed apoptotic cell death is a natural barrier to the development of cancer (2). Apoptosis is triggered in response to diverse intracellular damage signals that occur in tumor genesis but also as a result of anti-tumor therapy (7). The decision of the damaged cell to undergo apoptotic death is determined by the Bcl-2 (B-cell lymphoma 2) regulatory protein family. The Bcl-2 family consists of pro-survival and pro-apoptotic members, which interfere with each other. BH3 is a domain that allows protein-protein interaction and is found on pro-apoptotic members like Bax and Bak as well as on anti-apoptotic members of the Bcl-2 family as well. The inhibitors of apoptosis are Bcl-2 and its closest relatives Bcl-x_L, Bcl-w, Mcl-1 and A1. They prevent apoptosis by their pro-survival function of binding and suppressing pro-apoptotic trigger proteins Bax and Bak. The cell damage signals

are recognized by proteins like Bax and Bak and the result is the engagement of their pro-survival relatives Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1. This interaction enables activation of Bax and Bak, because they are not inhibited by their pro-survival relatives anymore. Bax and Bak disrupt the function and integrity of the outer membrane of mitochondria. This membrane damage leads to a release of different pro-apoptotic signals, the most common is cytochrome c. Cytochrome c itself activates a cascade of caspases, all of them show proteolytic activity and are associated with programmed apoptotic cell death. The balance between anti-apoptotic and pro-survival proteins with their BH3 ligands is responsible for the regulation of tissue homeostasis. Overexpression of a pro-survival members or loss of pro-apoptotic family members can be oncogenic (7). The tumor suppressor p53 is able to sense DNA damage and induces apoptosis in case of large levels of DNA breaks or other chromosomal abnormalities. In cancer, different mutations occur that inactivate the p53 pathway and lead to a loss of p53 function (8). All in all, there are many different strategies to escape apoptosis. The most common one is the loss of the tumor suppressor function of p53, however, also an increase of anti-apoptotic regulators like Bcl-2 or downregulation of pro-apoptotic factors like Bax can lead to an inhibition of apoptosis (2).

4. Enabling replicative immortality

Normal cells of the body are only able to pass through a limited number of cell growth and division cycles. There are two mechanisms that regulate the proliferation of cells; senescence and apoptosis. Senescence is the entrance of the cell in a non-proliferative but vital state. The second mechanism is the entrance into a crisis which involves apoptotic cell death. Some cells in crisis are able to emerge and exhibit unlimited replicative potential, called immortalization, a capability that allows tumor cells to grow unlimited (2). The ends of chromosomes are formed by telomeres, which protect the chromosomes. Telomeres lose length in every cell division process because of incomplete replication of them. This is the underlying mechanism of cellular aging and represents a molecular clock. When telomeres achieve a critical length, they are not able to protect chromosomes anymore. This leads to chromosomal instability and to a loss of cell viability, which ends in senescence or apoptosis. Telomerase is a DNA polymerase that is able to elongate telomeres and thus prevent the progressive telomere erosion that would occur

otherwise. Telomerase is expressed in germ cells but is almost absent in adult stem cells and normal somatic tissue. In contrast, tumor cells express high levels of telomerase, which preserve their viability (9). Indeed, it was shown that some mammalian tumor cells do not possess telomerase but are still able to prolong their telomeres by telomerase-independent mechanisms called ALT (alternative lengthening of telomeres) (10).

5. Inducing Angiogenesis

Tumor cells need a supply with oxygen and nutrients and also a clearance from carbon dioxide and metabolic waste (2). The building of vessels is essential for the development of cancer and its growth and spread. A tumor cannot grow beyond one to two millimeters without blood vessels for nutrients and oxygen supply (11). During embryogenesis blood vessels are developed by two different ways; vasculogenesis and angiogenesis. The building of new endothelial cells and the formation of them to tubes is called vasculogenesis. Angiogenesis is the sprouting of new vessels out of existing ones. In adults, only angiogenesis occurs and is switched on only temporary. Examples for physiologic and time limited forms of angiogenesis are the female reproductive cycle or wound healing. In tumors, the angiogenic switch is activated continuously and new vessels sprout out of normal vessels to supply tumor cells and help them to expand (12). The angiogenetic switch is defined by a time-restricted event with an imbalance between pro-angiogenic and anti-angiogenic factors towards the pro-angiogenic ones (13). Some of these factors are represented by proteins, which bind to stimulatory or inhibitory receptors on endothelial cells and are thus inducers or inhibitors of angiogenesis. Eminent examples are vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) (2). VEGF is the key mediator of angiogenesis. It binds to VEGF receptors on the endothelial cell surface and promotes angiogenesis in embryogenesis and plays a role in wound healing in adults. In cancer, VEGF is upregulated by the impact of oncogene expression, of a variety of growth factors and hypoxia in the tumors microenvironment (11). TSP-1 hinders tumor growth by inhibiting angiogenesis. It binds receptors on the surface of endothelial cells and leads to production of suppressive signals that counteract pro-angiogenetic factors (14).

Angiogenesis is induced very early in the development of cancer. Even in the pre-malignant phase, microvessel density was observed to be significantly increased and correlated with the expression of VEGF (15).

6. Invasion and metastasis

Invasion and metastasis of cancer cells is a multistep process. It begins with a local invasion of tumor cells in the nearby tissue, followed by intravasation of cancer cells in nearby blood or lymphatic vessels. Thereby, tumor cells acquire access to the circulation and transit trough the body by vessels. They arrest in the capillary bed and extravasate there. In distant tissue, tumor cells grow from micrometastasis to macrometastasis (16). Metastatic tumor cells are able to develop alterations in their biology, like in their shape, their attachment-abilities to other cells or extracellular matrix (2). The loss of E-cadherin is a typical alteration in cancer cells, which are invasive or metastatic. E-cadherin is a cell-to-cell adhesion molecule and thus an anti-invasive and anti-metastatic molecule. In cancer, an inactivation of E-cadherin happens due to different mechanisms, which include mutations, epigenetic changes and increased endocytosis and proteolysis (17). In contrast, adhesion molecules that are associated with cell migration, which occur in inflammatory process or in embryogenesis, are often upregulated in cancer cells (2). An example is N-cadherin that is normally expressed in different migrating cells and which is upregulated in many invasive carcinomas (18).

7. Reprogramming energy metabolism

Uncontrolled proliferation of tumor cells is dependent on an adjustment of their energy metabolism, to ensure cell growth. Under aerobic conditions, normal cells produce energy in form of ATP (adenosintriphosphat) in two steps. First step is glycolysis, where glucose is proceeded to pyruvate in the cytoplasm of the cell. Only low levels of ATP are produced in the first step. The second step is the oxidative phosphorylation in mitochondria, where pyruvate is further processed to carbon dioxide to get large quantities of ATP. This second step requires oxygen. Therefore, under anaerobic conditions, only glycolysis is performed, because mitochondria cannot produce ATP in oxidative phosphorylation without oxygen. Thereby, lactate is produced under anaerobic conditions (2).

Otto Warburg was the first person who observed changes in the metabolism of cancer cells. His finding was, that tumor cells preferentially use glycolysis over mitochondrial oxidative phosphorylation for the production of ATP from glucose, even in the presence of oxygen. This mechanism is called aerobic glycolysis or Warburg effect (19). Tumor cells have to reprogram their metabolism to compensate the lower efficient production of ATP. Therefore, they use different strategies, as an example the upregulation of glucose import into tumor cells (20). However, aerobic glycolysis is an inefficient way of ATP production, there are also advantages for the cancer cell. Increased glycolysis gives tumor cells the opportunity to generate nucleosides or amino acids, because glycolytic intermediates can be used in those biosynthetic pathways. The nucleosides and amino acids are used to generate new cells (21). An interesting circumstance is, that some tumor cells were found to import and utilize lactate, produced by neighbor tumor cells that perform aerobic glycolysis as main energy source (22).

8. Evading immune destruction

The German scientist Paul Ehrlich was the first person who found out about the existence of immune surveillance in 1909. The theory of immune surveillance says that the immune system eradicates nascent malignant cells, before they are able to grow to clinically apparent tumors (23). This means that appearing tumors are able to circumvent this immune surveillance (2). Mouse models showed that in immunosuppressed mice, tumors arose more frequently and/or that tumors grew more rapidly than in immunocompetent mice (24,25). In human studies, it was observed that patients with tumors, which are infiltrated with large amounts of immune cells have a better prognosis than patients with cancers with a lack of lymphocyte infiltration (26). Furthermore, it was observed that immunodeficient recipients of transplantations developed cancer of the ostensibly healthy donor. This suggests, that tumor cells can remain in a dormant state in immunocompetent persons and can be reactivated when they get transplanted to an immunosuppressed individual because of a failure of immune surveillance (27). Another trial showed an increased number of virus induced cancer types in immunosuppressed persons. The reason for this cancer development might also be a defective immune surveillance (28). Although immunosuppressed patients do not have significantly higher rates of non-viral induced cancer forms. An explanation for

this issue is, that immunosuppressed patients were observed to be deficient in T-cells and B-cells, but not in all immune cells, compared to immunodeficient mouse models. Thereby, natural killer cells and other innate immune cells are still able to attack cancer cells in immunosuppressed patients (2). Highly immunogenic cancer cells are able to evade their immune destruction by disturbing components of the immune system, that otherwise would attack them. For example, tumor cells release transforming growth factor beta (TGF-β), which leads to a systemic immune suppression and an inhibition of the immune surveillance. Thereby, infiltrated immune cells, which fight against cancer cells, get paralyzed (29).

1.2 Immune system

The immune system defends and protects the body by recognizing foreign antigens, which can be found on pathogens, infected cells or malignant cells. Immune cells possess effector functions to target and destroy those cells upon the development of immunological memory. The immune system consists of many different parts like bioactive molecules, cytokines, proteins and immune cells. It is composed of two different immune response types, the innate and the adaptive immunity (30).

Innate immune system

The innate immune system is not specific and can act immediately. This kind of immune system also works when the immunostimulants have never been introduced to the body before. It consists of different cells including natural killer cells (NK), macrophages, dendritic cells (DC), granulocytes (eosinophils, basophils and neutrophils) and mast cells. The complement system, cytokines and chemokines are also part of the innate immune system. The immune response is a fast and non-specific against non-self antigens. Examples for non-self antigens are microbes, non-self proteins, non-self molecules or allergenic antigens. When immune cells of the innate immune system recognize one of these antigens, an immune response with inflammation and phagocytosis gets started. They recognizes pathogens with the help of receptors like for example the toll-like receptors (TLRs) but also with receptors that recognize specific danger associated or pathogen associated molecular patterns (DAMPs or PAMPs). Phagocytes devour cells that express non-self antigens and kill them with the release of lysosomal enzymes. Other cells of the innate immune system like eosinophils, basophils and mast cells release inflammatory mediators when they get activated and this factors

subsequently recruit more immune cells to the inflammation site. The innate immune system does not develop an immunologic memory (30,31). DC and macrophages do not only have functions in the innate immune system by phagocytosing, they are also contributed to adaptive immune response because they are antigen presenting cells (APCs), required for T-cell activation (32).

Adaptive immune system

Cells of the adaptive immune system are B- lymphocytes and T-lymphocytes or B-cells and T-cells. T-lymphocytes mediate the cellular immunity and B-lymphocytes the humoral immunity. The adaptive immune response is not rapid, it occurs over time and develops an immunological memory, in contrast to the innate immune system. The immune response takes time because naïve lymphocytes have to differentiate into effector T-cells and antibody-secreting B-cells (plasma cells), which fight against non-self. There are different subsets of T- and B-cells (30,31).

T-cells

T-cells move through the body, searching for major histocompatibility complex (MHC) peptides, which bind an antigen. These complexes of MHC and an antigen are able to activate the T-cell by binding to its T-cell receptor (TCR). Tumor specific T-cells are able to recognize tumor-associated antigens, which are presented by APCs. Interaction between a T-cell and an APC leads to activation of the T-cell. Activated T-cells are subsequently able to recognize antigens that are presented on the surfaces of tumor cells directly (33). There are two different subgroups of T-cells; $CD4^+$ T-cells and $CD8^+$ T-cells.

$CD4^+$ T-cells

$CD4^+$ T-cells are also known as T-helper cells. Naïve $CD4^+$ T-cells are activated after interaction with MHCII molecules on the surface of APCs. MHC molecules bind peptides derived from non-self antigens, altered or abnormal self antigens (34). Those activated $CD4^+$ T-cells can further differentiate into different T-cell subtypes, for example T-helper 1 cells, T-helper 2 cells or regulatory T-cells (Tregs). Each of those different $CD4^+$ T-cells subsets produces characteristic cytokines, which modulate the immune response. Thereby, the effector functions of different $CD4^+$ T-cell subgroups are mediated by the cytokines they secrete. Functions of $CD4^+$ T-cells are multiple and wide spread, including the activation of cells of the innate

immune system, B-lymphocytes and cytotoxic T-cells or the suppression of immune reactions. Tregs, for example, help to reduce the inflammation by producing specific cytokines including different interleukins (IL) like IL-10 and IL-35 or TGF-β (35). In adoptive immunotherapy it was shown that Tregs, infused to cancer patients, prevented effective immunotherapy using anti-tumor $CD8^+$ T-cells (36). Transfer of $CD4^+CD25^-$ T-helper cells with anti-tumor $CD8^+$ T-cells into $CD4^+$ T-cell-deficient hosts induced regression of melanoma. Transfer of a mixture of $CD4^+$ T-cells consisting of $CD4^+CD25^-$ T-helper and $CD4^+CD25^+$ Tregs or Tregs alone inhibited the effect of immunotherapy. T-helper cells, which are capable of interleukin-2 (IL-2) production, are needed for maintenance $CD8^+$ T-cell numbers and function. Thereby, T-helper cells can help to treat tumors. However, the absence of naturally occurring $CD4^+CD25^+$ Tregs is required for the therapy to be effective (36). The use of lymphodepletion before adoptive cell therapy eliminates these suppressive Tregs (37).

$CD8^+$ T-cells

Naïve $CD8^+$ T-cells circulate through secondary lymphoid organs. DCs, which are present antigens, are able to activate those naïve $CD8^+$ T-cells via their TCRs. After this antigen-receptor-mediated activation of the naïve $CD8^+$ T-cells, $CD8^+$ T-cells start to proliferate and to differentiate into effector cells, called cytotoxic T-cells (CTLs) (38). The activation of naïve $CD8^+$ T-cells to effector CTLs is a MHCI dependent process. Compared to MHCII molecules, that are only found on APC, MHCI molecules are presented on all nucleated cells (34). There are different mechanisms that CTLs can use to kill target cells, like the secretion of death-inducing effector molecules towards the target cell, most importantly perforin, granzymes and Fas-ligand (FasL). The production of chemokines and effector cytokines, like tumor necrosis factor (TNF), plays also a role in killing target cells (38). Most CTLs, which are generated during the primary infection, live only short and are often called effector $CD8^+$ T-cells. After antigen clearance, a large pool of effector $CD8^+$ T-cells remains. This pool typically undergoes contraction and a smaller population of so called memory $CD8^+$ T-cells stays behind. These memory cells can fast grow to a big population again in the case of a further contact with the same antigen (39).

T-cells require a costimulatory signal, additionally to the TCR stimulation, to get activated. Costimulatory signals are molecules that are expressed on activated APCs or cytokines that are produced by APCs or even by the T-cell itself. A classic example is the interaction of the co-receptor CD28, which is expressed on $CD4^+$ and $CD8^+$ T-cells, with B7-1 (CD80) and B7-2 (CD86) expressed on the surface of APCs. The results of this interaction are a higher proliferation rate, production of cytokines and better cell survival. Another interesting example is PD-1 (programmed cell death 1) that is expressed on T-cells and binds to PD-L1 and PD-L2 (programmed cell death ligand 1 and 2) on APCs. This interaction ends in inhibition of T-cell activation. Some cancer cells were observed to induce the expression of PD-L1 to suppress the immune system (40).

B-cells

The maturation and activation of naïve B-cells to antibody-secreting effector B-cells can be triggered by T-helper cell dependent and T-helper cell independent mechanisms. The results from this form of immune response are specific antibodies, also known as immunoglobulins. Immunoglobulins have a domain that can bind to antigens, called fragment antibody binding (Fab) and a fragment crystallizable (Fc) domain that is able to bind to Fc receptors. These Fc receptors are found on effector cells and binding of antibodies to them mediates effector functions, like antibody-dependent complement cytotoxicity. The functions of antibodies are neutralizing the antigen by binding it, initiating antibody-dependent complement cytotoxicity, inducing complement dependent cytotoxicity and binding to antibody receptors expressed on specific cells, to activate their effector functions (30).

NKT-cells

NKT-cells are cells similar to the NK-cells of the innate immune system. They have features of NK-cells and T-cells. NKT-cells have a TCR like conventional T-cells but they recognize lipid antigens presented by CD1d, a nonclassical MHC molecule, instead of peptide antigens presented by conventional MHC. NKT-cells play both, effector and regulatory roles in diseases and respond rapidly. In cancer, they have a mostly protective role. However, recently it was observed that they also inhibit the immunosurveillance and success of cancer immunotherapy (41).

Tandem work of innate and adaptive immune system

The innate immune system is the first line defense and starts prior the adaptive system. If the innate immune system is not sufficient, the adaptive immune system aids in antigen removal and subsequently builds an immunological memory for future exposures to the same antigen (32).

1.2.1 The immune system and cancer

Cancer is characterized by a large number of genetic changes and alterations of normal cellular regulatory processes (42). These alterations result in the expression of neo-antigens, which can be processed and presented with MHC molecules on the tumor cells surface. $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells are able to recognize these complexes and subsequently to destroy tumor cells (33,43).

The cancer immunity cycle

A cycle composed of seven steps that are iterative, is the basis of an effective response of the immune system to cancer cells. In the first step, neo-antigens are released by death cancer cells. These neo-antigens are captured by DCs or other phagocytes. The second step is, that DCs or phagocytes process those cancer antigens and present them on their surface in a complex with MHC. This presentation leads to an activation of an effector T-cell response against the tumor antigen in step three. During this step, the kind of the immune response is determined due to the balance between regulatory and effector T-cells, which are important for the tumor attack. The fourth step is the traffic from activated effector T-cells to the tumor site and the fifth step is the infiltration of these T-cells into the tumor. T-cells recognize their antigens, which are bound to MHC, with the help of their TCRs in step six. The last and seventh step is the killing of the tumor cells by T-cells. Death tumor cells release neo-antigens and the circuit starts again at step one (44-46). In cancer patients, alterations occur in this cycle, which ends in tumor growth and spread. Examples for problems that can occur are, that tumor antigens are not detected by immune cells or that tumor antigens are recognized as self rather, than foreign. Thus, a regulatory T-cell response instead of an effector T-cell response is the consequence. Further examples are, that T-cells may not properly find to the tumor sites or that they are inhibited from infiltrating cancer cells. The tumor microenvironment might also inhibit the immune response of T-cells against cancer cells (44).

Immunoediting

The immunity directed against cancer cells is called cancer immunoediting and is initiated trough the activation of innate and adaptive immune mechanisms. Immunoediting consists of three phases: elimination, equilibrium and escape (47). T-cells play a grave role in this process, especially with their ability to recognize cancer-associated antigens (31).

Elimination

In the phase of elimination, an immune response against foreign epitopes which are produced by cancer cells is started and T-cells attack and eliminate tumor cells (31). This is consistent with the theory of immune surveillance of Ehrlich, which says that the immune system scans the body for neo-antigens and eradicates nascent transformed cells before they can be clinically detected (23).
In the phase of elimination both, the innate and adaptive immune system together detect and subsequently kill early cancer cells before they become clinically apparent. Cells of the innate immune system defense with different mechanisms. Macrophages and granulocytes contribute to anti-tumor activity by releasing cytokines like IL-1, IL-12 or TNF-α. DCs take up tumor antigens and present them to T-cells which subsequently get activated (47). NK T-cells play a role in tumor defense by producing Interferon-gamma (IFN-γ). Activated $CD4^+$ T-cells and $CD8^+$ T-cells also secrete IFN-γ. IFN-γ inhibits tumor cell proliferation and angiogenesis, it is capable to induce cell death and subsequently to form cellular debris, which are transported to lymph nodes. DCs, which are presenting tumor antigens to T-cells, activate anti-tumor T-cells in the lymph nodes. Subsequently $CD4^+$ and $CD8^+$ T-cells migrate to the tumor site, to kill and eliminate cancer cells (31,44,48).

Equilibrium

Tumor cells, which manage to survive the elimination phase, enter the equilibrium phase, an immune-mediated tumor dormancy. That means that the immune system holds the cancer in a state of functional dormancy. Equilibrium is reached, when the same amount of cancer cells are killed as new grown (31,47). Under the constant pressure of the immune system, some tumor cells undergo genetic and epigenetic changes and thereby, some tumor cells become resistant to immune recognition. For example by the loss of antigens or defects in antigen-presentation. Tumor cells

can also induce immunosuppression, for example by the expression of PD-L1, which ends in immune escape and tumor growth (47).

Escape

Cancer cells that survive the elimination phase mutate over time to escape the immune system, leading to a shift from equilibrium to survival (49). Thereby, the final phase is reached, when tumors begin to grow progressively and become clinically apparent with furthermore establishing an immunosuppressive tumor microenvironment (47). There are many mechanisms known that enable tumor escape, like the reduction of immune recognition (for example due to the loss of tumor antigens, loss of MHC molecules or costimulatory signals), the increase of resistance and survival (for example due to the expression of the anti-apoptotic molecule Bcl2) and also the development of an immunosuppressive tumor microenvironment (for example due to the expression of PD-1/PD-L1) (47).

1.3 Cancer types treated with ACT

Melanoma

Melanoma is a potentially fatal malignancy that develops from melanocytes, which went through a malignant transformation. Melanocytes are pigment producing cells that are not only found within the epidermis but also in other pigment containing sites like the eyes, meninges, esophagus and mucous membranes. Thereby, there are different sites of where melanomas can arise, but the most common one is cutaneous melanoma (90 %) (50). Ultraviolet (UV) light radiation from the sun is the main risk factor for the development of melanoma because of the genotoxic effect that leads to DNA damage (51). Host risk factors are the number of melanocytic nevi acquired or congenital, genetic factors and family history (52). The incidence of cutaneous melanoma has been increasing more rapidly compared to any other cancer types annually. In 2012, it was ranked the 15th most common cancers worldwide (52). In the USA melanoma is the fifth most common cancer in men and the sixth most common cancer in women. The incidence increased by 270 % from 1973 to 2002 (51). Melanoma affects mostly young and middle-aged persons with a median age at diagnosis of 57 years, which is untypical early compared to other solid tumors (51). Melanoma has a poor prognosis, patients with

metastatic melanoma have a median survival of eight months and two-year survival rates of 10 % to 15 %. (37)

B-cell malignancies

B-cell malignancies are malignant transformations of B-cells that can originate from B-lymphocytes and plasma cells. The group of B-cell malignancies contains numerous entities, including all types of B-cell lymphomas, chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), plasma cell malignancies, multiple myeloma (MM) and many other (53).

Chronic lymphocytic leukemia

CLL is a malignancy of B-cells that is defined by mature-appearing lymphocytes which accumulate in the blood, bone marrow and lymphoid tissue. The clinical result is lymphocytosis, infiltration of the bone marrow with leukemia cells, lymphadenopathy and splenomegaly. Risk factors for developing CLL are genetic and environmental factors such as the exposure to Agent Orange or to insecticides as well as viral infections (54). CLL is the most common form of leukemia in adults in the western world. The risk for developing CLL increases with age. The median age at diagnosis is 70 to 72 years. The progression of CLL is heterogeneous ranging from patients who need a treatment soon to patients who do not need therapy for years or never (54).

Acute lymphoblastic leukemia

ALL is a malignant transformation of lymphoid progenitor cells that leads to proliferation and accumulation of those malignant cells in the bone marrow, blood and extramedullary sites, which leads to lymphadenopathy, splenomegaly or hepatomegaly. Clinical manifestations can be signs of bone marrow failure (anemia, thrombocytopenia and leukopenia) including easy bleeding, fatigue, dyspnea and infection. Also a complex of symptoms called “B symptoms”, which include fever, weight loss and night sweats can be observed (55). Risk factors are genetic disposal and exogenous or endogenous exposures. ALL occurs in children and adults, but the incidence peak is at the age between two and five years. The survival rate of ALL in children is 90 %. In infants and adults the prognosis remains poor (56). Patients older than 60 years have poor outcomes with a long-term survival of 10–15 % and less than five percent for patients over 70 years (57).

Multiple myeloma

MM is a fatal B-cell malignancy of the plasma cell and is characterized by uncontrolled and destructive proliferation of those malignant cells in the bone marrow (58). As the name indicates, a dissemination of multiple tumor cells in the bone marrow occurs. The clinical results are osteolytic lesions and fractures. Other clinical findings are elevated calcium levels, anemia and renal failure (59). Risk factors are older age, being male or being African American. An elevated risk was also observed in persons with a positive family history and a relationship between obesity could be shown (60). Environmental risk factors include farming and exposure to pesticides (61). Most patients, which are affected, are older than 65 years (60). The five-year survival rate of MM is 32 % (60).

Lung cancer

Lung cancer is one of the most common and most deadly cancer types in the world. It is the leading cause of cancer deaths worldwide among all forms of cancer and is responsible for 17.7 % of all cancer deaths per year (62). It is projected to remain the top cancer killer to 2030 (63). Lung cancer is distinguished in 85 % non-small cell lung cancer (NSCLC) and 15 % small cell lung cancer (SCLC). The group of NSCLC includes, named after the histological types, the squamous cell carcinoma, the adenocarcinoma and the large-cell carcinoma. The most common type is the lung adenocarcinoma, which is found in smokers as well as in non-smokers. Adenocarcinoma also occurs in young patients under the age of 45. The primary risk factors are age and smoking (62). The advantages of cancer therapies like surgery, chemotherapy and radiation could not significantly increase the prognosis of lung cancer over the past three decades. The five-year survival rates are about 18 % in the United States (64-66).

Pancreatic cancer

Pancreatic adenocarcinoma is a cancer type with a rising incidence and high mortality. Presently it is the 14th most common cancer type and the seventh highest cause of cancer related deaths worldwide (67). It is the fourth most common cause of cancer related mortality in the United States and it is projected to become the second most common cause of cancer related mortality by 2030 (63,68). The highest incidence is seen in the western world and the lowest in Africa and South

Central Asia (69). Risk factors for pancreatic carcinoma are smoking, obesity, alcohol, Helicobacter pylori, chronic pancreatitis and higher age or being male (67). Pancreatic cancer has a poor prognosis and treatment options are limited. More than half of the patients have an advanced disease at time of diagnosis. The median overall survival ranges from eight to eleven months with the current therapeutic approaches (70). The five-year survival rate is between two and nine percent (69). The only treatment that is potential curative is the surgical resection (pancreatico-duodenectomy, called Whipple's procedure). The adjuvant addition of chemotherapy has been shown to improve survival rates (67).

Neuroblastoma

Neuroblastoma is a childhood tumor that arises from neural crest cells (71). It is the most common extracranial solid tumor in childhood and in most cases it occurs during the first ten years of life. Neuroblastoma is responsible for eleven percent of pediatric cancer deaths in patients younger than 15 years (72). The clinical presentation of children with neuroblastoma, as well as the outcomes, are extremely variable. In newborns and infants, tumors are often diagnosed incidentally, these tumors often regress spontaneously and do not need any therapy. In toddlers and older children, the tumor is often diagnosed in a widely metastatic stage and these patients need complex therapy strategies including surgery, chemotherapy, radiotherapy, autologous stem cell transplant, differentiation therapy and monoclonal antibody-based immunotherapy (71). Outcomes of patients with localized tumors that were resected in a surgery are excellent, the event free survival rate five years after diagnosis is >90 % (73). Patients with a high risk disease, which is defined by an age older than 18 months, the extent of metastases, histologic and genetic factors, have a poor long-term survival. The five-year event free survival rate is about 50 % (71). In patients who survive, often long-term sequelae occur due to the intense tumor treatment. Examples are hearing loss, growth retardation and secondary malignancies (74).

Glioblastoma (GBM)

GBM is a primary brain tumor and it is the most common, most malignant and most aggressive of glial central nervous system tumors. The etiology of brain neoplasms is not well known, no carcinogenetic causes could be identified (75). The global incidence of GBM is less than ten per 100,000 people. The incidence is higher in

the western world compared to less developed countries. GBM can occur at any age but the peak is between 55 and 60 years. Malignant gliomas cause about two percent of cancer deaths and are the third foremost cause of cancer deaths in people at the age from 15 to 34 years (75). GBM is among the most lethal of human cancer (76). The median survival of patients with multifocal tumors is six months, in patients with solitary GBM median survival is eleven months. The two-year survival rate is about four percent for patients with multifocal tumors and 29 % for patients with a unifocal tumor (77). Conventional cancer therapies like surgery, radiation and chemotherapy do not lead to an improvement of survival rates in patients with GBM (78). At the moment, no curative treatment is known (79). GBM is invariably lethal (78).

1.4 Terms in cancer therapy

Complete response or complete remission (CR) means that all signs of cancer disappeared in response to the treatment. However, this does not always mean that the cancer has been cured (80). Partial response or remission (PR) means a decrease in the size of a tumor or a decrease in the extent of cancer in the body, in response to treatment (81). An objective response (OR) is any measurable response of the tumor to the treatment (82).

2 Aims of the study

Cancer is an important issue for all of us. Globally cancer is the second leading cause of death (83). In 2018, 18.1 million new cancer cases were diagnosed globally and 9.6 million people died of cancer (84). In Austria, every year around 40.000 people get diagnosed with cancer. After cardiovascular disease, cancer is the second most common cause of death in Austria (85). Lung cancer is the most common cancer type (with 11.6% of the total cases globally) and it is also the leading cause of cancer deaths with 18.4% of the total cancer deaths (84). The worldwide incidence of cancer is predicted to be 29.5 million in 2040 by the WHO (World Health Organization) (86). Given that these diseases are predominantly age-related, the increasing aging of the population will increase the importance of cancer in the future (85). Despite aggressive therapy regimes used today, cancer is still incurable in many patients and thus, there is a need of researching and developing new therapeutic approaches to treat cancer.

3 Material and Methods

This thesis was designed as a systemic review of the recent developments and advances in T-cell therapy for cancer patients. A comprehensive research of scientific literature was performed to select the literature for this thesis. Books, journals and guidelines were selected using PubMed, ClinicalTrials.gov and guideline publications. To ensure a review of high quality, literature that was published within the last years was used whenever possible to provide up-to-date information in this thesis. Literature citations were performed using RefWorks and the Vancouver citation style.

4 Results

4.1 Cancer therapies

Different cancer therapies exists, including the three most common ones chemotherapy, surgery and radiation therapy. These treatment strategies, which can be used either alone or in combination, can significantly impact tumor growth and even produce cures.

Surgery

The oldest form of cancer therapy is surgery and it is still a mainstay of treatment in solid tumors. While surgery is not a part of treatment of hematological malignancies, a surgical component is included in the therapy of most patients with solid tumors. Surgery, as a single treatment, cured more patients in comparison with any other form of cancer therapy. In the treatment of localized primary tumors and associated regional lymph nodes, surgery is the most effective treatment (87). However, adjuvant chemotherapy alone or in combination with radiation therapy showed an improved disease-free survival and a longer life with a better life quality for cancer patients. Especially for patients who have been treated by surgery and got rendered free of gross disease, but who still have a high risk of recurrence because of microscopic residual metastases that remain after surgery (87). The success and risk of surgery have to be evaluated for each patient individually. In NSCLC, for example, the Charlson comorbidity index (CCI) was evaluated for patients who underwent a surgery. A strong correlation of the CCI and a higher risk of surgery was shown (88). However, it was also shown that older patients with NSCLS (older than 76 years) with a good performance status and no comorbidity have a similar prognosis and perioperative mortality than younger patients. Thereby, advanced age alone is not a negative factor for surgery in elderly patients (89).

Chemotherapy

Paul Ehrlich coined the term “chemotherapy”. In investigated alkylating agents and came up with the term to describe the chemical treatment of diseases (90).The first chemotherapy was discovered during the First and Second World War, where it was observed that soldiers, who were exposed to mustard gas, experienced a decrease in leukocytes numbers. These findings led to the use of nitrogen mustard to treat lymphomas as the first chemotherapy. In the following years alkylating drugs, like

cyclophosphamide and chlorambucil were established to fight cancer (91,92). Folate antagonists, such as aminopterin and amethopterin, were developed and led to the development of methotrexate, a drug which achieved leukemia remission in children in 1948 (93). The first cancer cured with chemotherapy was reported in 1958, it was a choriocarcinoma treated with folic acid and purine antagonists (94). A significant progress was made in 1980 by combing different types of chemotherapies (cisplatin, bleomycin and vinblastine) and achieving higher cure rates in metastatic germ cancer (95). The function principle of chemotherapy is the application of drugs which kill cancer cells. There are many different mechanisms of how those chemicals work, for example, alkylating agents are able to damage the DNA and anti-metabolites replace normal parts of the DNA and RNA. Topoisomerase inhibitors inhibit enzymes that are involved in DNA replication and transcription by unwinding DNA strands. Mitotic inhibitors are able to inhibit mitosis and thus cell replication (64). Most normal cells of the body are resting in the G_1/G_0 phase of the cell cycle. Thereby, drugs that are damaging dividing cells in S or M phases are selective for cells under replication, such as tumor cells (96). Meta-analyzes showed, that combinations of chemotherapies result in better outcomes. For example, in patients with advanced NSCLC, a combination chemotherapy improved response rate and failure-free survival compared to a single-agent therapy (97).

Radiation therapy

Radiation therapy uses ionizing radiation to destroy malignant cells by a large spectrum of DNA damages. The most important effects induced by radiation are double-stranded breaks of DNA, because these breaks lead to inability of reproduction or even death of the cell. The damage can be induced by directly ionizing effects or by indirect ionization, which is the more common form. Indirect ionizing radiation is performed by free radicals that emerge from the radiolysis of cellular water. Cell growth, cell senescence and apoptosis can also be affected by radiation (98). About 60-65 % of all cancer patients need radiation therapy as treatment, either in combination with surgery or chemotherapy or alone (98). The challenge is to cure tumors without harming surrounding normal tissue. Higher doses of radiation lead to a better tumor control, but the maximum dose is limited by the risk of normal tissue damage. The success of radiation therapy is dependent on different factors, like the type of the tumor, its localization and regional extent, of

the anatomic area in which the tumor grows and the geometric accuracy with which the calculated radiation dose is administrated (98). The radiosensitivity is defined by the tumors response to the radiation therapy and is greater in cells with a high mitotic rate and undifferentiated cells, like malignant tumor cells. Examples for tumors that are highly radiosensitive are lymphoma, leukemia and dysgerminoma. In radioresistant tumors the dose required to induce tumor death is much higher than the radiation tolerance of surrounding tissue. Examples are melanomas, sarcomas and bone tumors (98).The performance status and comorbidity of the patient might also be a limitation of radiation therapy. A higher comorbidity score in patients with NSCLC is predictive for a worse outcome in the use of radiation therapy alone or in combination with chemotherapy. However, higher age alone is not associated with worse outcome (99). Technological advances enable a personalized radiation therapy with an accurate radiation dose that is customized to the anatomy and clinical parameters of the patient. These technological advances had improved the efficacy of radiation therapy. In head and neck cancer the overall survival rates have improved from about 30 %, two decades ago, to about 80 % today (100). It was shown that a combination of chemotherapy plus radiation therapy leads to better outcomes than using radiation therapy without additional chemotherapy (101,102). However, a higher rate of toxicities can be observed in patients who received both therapies, which is a limitation for the use of this combination (102).

Immunotherapies

The idea to use the immune system to attack cancer cells as a tumor treatment relies on the fact that the immune system is able to eliminate malignant cells in the process of immune surveillance (23). Although the immune system is able to recognize neo-antigens of cancer cells, some cancer cells manage to escape the immune systems attack (47). There are various approaches of immunotherapy with stimulating mechanisms and suppressive mechanisms, as listed below.

Table 1: Available immunotherapies

Strategy	Mechanism	Disadvantages	Ref.*
Cytokine IL-2	- Stimulation of the immune system - CR in some patients	- Low response rates - Immune-related adverse effects	(103-105)
Cytokine IFN-α	- Stimulation of the immune system - CR in some patients	- Low response rates - Immune-related adverse effects	(106-108)
Vaccines	- Stimulation of the immune system - Minimal toxicity	- Lack of universal antigens	(109-112)
Anti-CTLA-4 antibodies	- Unleashes pre-existing anticancer T-cell responses and possibly triggers new ones - Potent anti-tumor properties - Prolongation of overall survival	- Only a few patients obtain clinical benefit - Severe immune-related adverse events	(113,114)
Anti-PD-1 + anti-PD-L1 antibodies	- Responses in a wide range of cancer types - Limited toxicity - Sufficient clinical responses	- Only about 15-30 % of patients obtain clinical benefit (depending on tumor entity)	(115-117)
ACT	- High avidity T-cells - Lymphodepleting enhances efficacy - Genetically engineered T-cells	- Serious adverse effects - Requires time to develop the desired cell populations - Expensive	

* Reference (Ref.)

Interleukin 2

The cytokine IL-2 is a strong growth factor for T-cells and plays an important role in maintaining T-cell responses. Furthermore IL-2 is involved in regulating growth and differentiation in B-cells, NK-cells and many other cells of the immune system (118). IL-2 is mainly produced by activated $CD4^+$ T-cells but also in small amount by $CD8^+$ T-cells (119), NK T-cells (mouse model) (120), mast cells (mouse model) (121) and DCs (122). The use of IL-2 was the first successfully used cytokine immunotherapy in cancer. In 1984, an infusion of IL-2 was administrated to a female 33 year old patient suffering from metastatic melanoma, resistant to prior treatment. The result was, that within one month the tumor biopsy showed necrosis and two months after the treatment all tumor sites shrinked. A few months after the therapy no evidence of cancer could be detected anymore. The patient remained cancer-free for the past 29 years (103). IL-2 has been reported to induce complete and durable regressions in cancer patients, especially in metastatic melanoma. However, it also leads to immune-related adverse effects (104,105).

IFN-α

Interferons function as antiviral, antiproliferative and immunomodulatory cytokines (106). In cancer immunotherapy interferon-alpha (IFN-α) is used for the treatment of solid and hematologic malignancies. The use of IFN-α in patients with non-Hodgkin lymphoma leads to response rates of 54 % in low-grade disease. However, in intermediate-grade or high-grade disease the response rates were disappointing (107). The use of INF in the treatment of MM resulted in 6.6 % higher response rates and prolonged relapse free and overall survival, when INF was used for induction therapy. Used for maintenance therapy also a prolongation of relapse free and overall survival was achieved (108). However, the greatest therapeutic effect can be achieved by combining INF therapy with other immunotherapies or cancer therapies (106). As side effects flu-like symptoms such as fever and fatigue can occur, which are the major dose-limiting factors and they also limit the clinical application (107).

Cancer vaccines

Cancer vaccines mimic tumor antigens and prime T-cells to recognize tumor antigens with the goal to induce or boost a tumor-specific immune response, which will reduce tumor burden (109). Vaccination represents one of the most effective

methods to prevent diseases and on the other hand vaccines are also used to eliminate the cause of a given disease, like the elimination of cancer cells (123).
Cancer vaccines can trigger an immune response against a specific or multiple tumor antigens, depending on the vaccine-delivery system. There are different systems, like strategies using whole tumor cells, tumor lysates, purified tumor antigens, tumor cells genetically engineered to secrete immunostimulatory cytokines and tumor-associated antigens. Other strategies use DCs and tumor cell fusions or preparations of DCs loaded with tumor specific antigens (109,110). The activity of cancer vaccines is mostly dependent on cancer antigen-specific $CD8^+$ T-cells. Therapeutic vaccination aims at expanding these high avidity $CD8^+$ T-cells that are able to differentiate into CTLs, which are subsequently able to kill cancer cells and are also able to generate long-living memory $CD8^+$ T-cells. These long-living memory $CD8^+$ T-cells will act to prevent relapse (123). DCs are an important component of cancer vaccination because of their ability to capture, process and present antigens to T-cells (123). DCs can be produced *ex vivo* and loaded with different tumor antigens, activated and injected in cancer patients. Studies showed that DC-based vaccines are able to induce the expansion of circulating $CD4^+$ T-cells and $CD8^+$ T-cells, which are specific for tumor antigens (124).

The approval of Provenge® (Sipuleucel-T) for the treatment of prostate cancer in 2010 was a boost for cancer vaccines based immunotherapy. Sipuleucel-T is a cancer vaccine consisting of peripheral-blood mononuclear cells (PBMCs) which contain APCs, that have been activated with the protein PA2024 *ex vivo*. PA2024 is a recombinant protein containing the prostate antigen PAP (prostatic acid phosphatase) that is expressed on most prostate cancer cells but not on other tissues. Thus, a T-cell immune response against PAP is induced.
A randomized placebo-controlled trial showed that Sipuleucel-T improved the overall survival in patients with metastatic castration-resistant prostate cancer with a relative reduction of the risk of death of 22 %. The most common adverse effects were chills, fever, fatigue, nausea and headache (111). Results in the use of vaccines in colorectal cancer, are also promising. The vaccine activates the immune system against the colorectal cancer antigen GUCY2C. This therapy was observed to induce an antigen-specific anti-tumor $CD8^+$ T-cell response without inducing serious adverse effects (112). A trial showed, that vaccines, which are capable to

incorporate mutant epitopes on tumors, are able to induce tumor responses comparably to checkpoint blockade immunotherapy (125). It was observed, that the combination of high-dose IL-2 and a vaccine achieved better clinical responses, compared to high-dose IL-2 alone and also improved the progression-free survival (126). A limitation of vaccines is the phenomena called antigenic drift, which is performed by tumors and allows them to escape the destruction mediated by vaccinations (110).

Checkpoint inhibitors

Checkpoint inhibitor therapy is a cancer treatment using monoclonal antibodies. Immunosuppression plays a role in the genesis of cancer and it was shown, that in many patients immunosuppression is conducted by two receptors that are negative regulators of T-cells. The activation of these receptors leads to the start of mechanisms that limit immune responses against cancer cells. These receptors are CTLA-4 (cytotoxic T-lymphocyte associated antigen-4) and PD-1 (programmed death-1). Checkpoint blockades, targeting CTLA-4 or PD-1 have shown significant clinical benefits, including durable responses in patients suffering from different malignancies (125).

CTA-4 antibodies

Ipilimumab is a checkpoint inhibitor that blocks CTLA-4 to increase the anti-tumor T-cell response. It was administrated to patients with metastatic melanoma in a clinical trial and resulted in improved overall survival (113). In another trial, tumor necrosis with infiltration of immune cells was observed in patients with metastatic melanoma (114). Most common side effects are immune-related and can be severe and long lasting. Most often the affect the skin and gastrointestinal tract (113). In patients with advanced metastatic ovarian cancer a stabilization or reduction of CA-125 (Cancer-Antigen 125) levels was observed after administration of CTLA-4 antibodies. CA-125 is shed from ovarian cancer cells and thus, it is a useful marker of disease status (114). The CTLA-4 antibody Ipilimumab was the first immune checkpoint inhibitor to be tested and approved for the treatment of cancer patients, as monotherapy for metastatic melanoma and surgically respectable high-risk melanoma (127,128).

PD-1 antibodies

The FDA (U.S. Food and Drug Administration) approved three monoclonal anti PD-1 antibodies for the treatment of different cancer types, Pembrolizumab, Nivolumab and Cemiplimab (128). In patients with advanced melanoma, results of the treatment with Pembrolizumab were high rates of sustained tumor regression, with tumor response in 38 % of the patients. In patients receiving ten mg per kilogram every two weeks, even a response rate of 52 % was achieved. Adverse events of the treatment were mostly low-grade, including fatigue, rash, pruritus and diarrhea (115). Nivolumab, tested in patients with renal cell carcinoma (RCC), also showed objective responses including durable responses, which in some responders persisted after drug discontinuation. 63 % of the patients demonstrated some degree of tumor shrinkage, reaching from one percent to 100 %. Toxicities were generally reversible and manageable, most common toxicities were fatigue, rash, diarrhea and pruritus (129). In patients with advanced, previously treated squamous-cell NSCLC the efficacy and safety of Nivolumab was tested, compared to the treatment with the chemotherapy docetaxel. The results were, that the overall survival, response rate and progression-free survival were significantly better with Nivolumab than with docetaxel, regardless of PD-L1 expression level (130). Pembrolizumab is approved for the treatment of melanoma, NSLC, SCLC, head and neck squamous cell carcinoma, classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, RCC, microsatellite instability-high cancer, gastric cancer, esophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma and endometrial carcinoma (131).

PD-L1 antibodies

The ligand of the T-cell inhibitor receptor PD-1 is the PD-L1. Beside the blockade of PD-1, also PD-L1 can be blocked to enhance the anti-tumor immune function. There are three monoclonal anti-PD-L1 antibodies, Durvalumab, Atezolizumab and Avelumab, which are approved for the treatment of different types of cancer by the FDA (128). Administration of anti-PD-L1 antibodies to patients with different advanced cancers including NSCLC, melanoma, colorectal cancer, RCC, ovarian cancer, pancreatic cancer, gastric cancer and breast cancer led to a durable tumor regression (objective response rates of six to 17 %) and prolonged stabilization of disease (rates of twelve to 41 % after 24 weeks) (116).

Triple negative breast cancer is a complex breast cancer subtype with poor prognosis. Nine patients were treated with Atezolizumab. Efficacy was observed in three patients (33%), including one complete response and two partial responses (117). PD-L1 is also expressed in NSCLC tumors and thus 198 lung cancer patients, including non-squamous and squamous, were treated with anti-PD-L1 treatment. From 149 patients that were evaluable for response, the rate of objective response was 14 %. Objective response rate was higher in squamous (21 %) than non-squamous lung cancer patients (ten percent). Responses were durable with 76 % ongoing in a range from 0.1 to 35 weeks (132). Imfinzi (Durvalumab) is approved and indicated for patients with unrespectable stage III NSCLC, whose disease has not progressed following concurrent platinum-based chemotherapy and radiation therapy. Also for patients with locally advanced or metastatic urothelial carcinoma with a disease progression during or following a chemotherapy with platinum or patients who have a progression of the disease within twelve months of treatment with platinum-containing chemotherapies (133). Tecentriq (Atezolizumab) is approved for the treatment of bladder cancer, NSCLC, SCLC and triple-negative breast cancer (134). The antibody Bavencio (Avelumab) is indicated for the treatment of Merkel cell carcinoma, urothelial carcinoma and RCC (135).

Adoptive cell therapy

Adoptive cell therapy (ACT), also called adoptive T-cell therapy or T-cell therapy, is a personalized cancer therapy. Immune cells, specifically T lymphocytes with anti-tumor activity, are expanded *ex vivo* and readministrated to patients. The advantage, compared to other cancer immunotherapies, is the *in vivo* proliferation of effective anti-tumor T-cells. These T-cells can traffic to the tumor site and mediate destruction of the tumor (33).

4.2 A brief history about adoptive T-cell therapy

The field of treatment in oncology has been revolutionized by the development of therapies that harness and augment the natural capability of the immune system to fight against cancer. Very little was known about the function of T-cells until the 1960s, where T-cells were observed to be the mediators of allograft rejections in animal models (136). The first attempts to treat cancer with ACT were performed in mouse models by using lymphocytes from mice, which are heavily immunized against cancer. These lymphocytes were transferred to syngeneic mice with a

tumor. A slight effect of tumor growth inhibition was observed (137). In pre-clinical rat studies in the 1980s, it was shown that inhibitory factors of the host immune system can be responsible for the failure of ACT, by showing that lymphodepletion before cell transfer increases the effect of anti-tumor attack by transferred T-cells (138). The description of IL-2 as a growth factor for T-cells in 1976 was an important finding for the feasibility of ACT, because the use of IL-2 makes it possible to grow T-cells *ex vivo* (139). The administration of T-cells, which were grown in IL-2 containing media, mediated cure in 79 % of mice with lymphoma (140). The application of high-dose IL-2 alone to mice with pulmonary metastases and subcutaneous tumor led to tumor regression in 1985 (141). Furthermore, the application of IL-2 after the cell transfer enhances the effects of ACT to cure lymphoma in murine models (142). Based on those animal models, in 1985, patients with metastatic cancer in whom standard therapies have failed, were treated with autologous lymphocytes plus IL-2. Objective regression, defined as a regression of more than 50 % of the tumor volume, occurred in eleven of 25 patients and one complete tumor regression was achieved (143). Further approaches used lymphocytes which were taken out of the tumor mass, so called tumor-infiltrating lymphocytes (TILs). They were used to treat mice with different tumor types and the results showed that TILs are 50 to 100 times more effective than lymphocytes used before, which were taken from the blood (144). In 1986, TILs from melanomas were investigated and it was observed that they contain cells, which are able to specifically recognize tumor cells and thus showed that cancer patients raise an immune response against autologous cancer cells (145). These findings led to ACT using TILs in patients with metastatic melanoma which achieved objective cancer regression in 1988 (146). Even though promising results could be observed, the responses were often from short duration and the persistence of transferred cells was poor (136). An improvement of these problems was given in 2002 by the discovery that lymphodepletion, in form of a nonmyeloablative chemotherapy before the administration of TILs, leads to increased cancer regression and increased persistence of the transferred T-cells. In some cases, up to 80 % of the patients circulating $CD8^+$ T-cells were represented by the administered anti-tumor T-cells, even months after the administration (147). However, not all tumors give rise to TIL cultures that include T-cells, which are capable of specific anti-tumor recognition. Therefore, research was started to enable the use of ACT even though no TIL

cultures can be grown. Genetic engineering of lymphocytes, to make them express specific anti-tumor receptors, was found out to be a way of enabling more widely use of ACT (136). In 2006, genetic engineered lymphocytes were used in humans for the first time, in patients with metastatic melanoma. Normal lymphocytes were engineered to express TCRs capable to recognize MART-1 (melanoma antigen recognized by T-cells 1), an antigen found on melanoma and melanocytes. These transduced cells mediated tumor regression in some patients (148). In 2010, lymphocytes were genetically engineered to recognize CD19, a B-cell antigen, and those engineered cells were administrated to patients with advanced B-cell lymphoma. ACT led to a dramatic regression of the lymphoma and also to a selective elimination of B-cell precursors, which resulted in an absence of blood B-cells for at least 39 weeks (149).

4.3 Mechanism of T-cell therapy

T-cell therapy is a personalized anti-tumor therapy using immune cells, especially T-lymphocytes. ACT is a treatment where cell populations that have been expanded *ex vivo* are reinfused into the patient. These cells are capable to recognize tumor cells due to specific tumor antigens expressed by tumor cells. They can migrate to the tumor site and mediate cancer destruction (33).There are two different forms of ACT. One approach uses anti-tumor T-cells which are produced by the cancer patient, they are called naturally occurring autologous TILs. Special techniques allow the *ex vivo* expansion and selection of those cells and reinjection of millions of these autologous TILs to the patient. The second approach uses genetically engineered lymphocytes which are engineered to express receptors on their surface that are capable to recognize tumor antigens. There are two kinds of receptors, which can be used, the conventional TCRs or chimeric antigen receptors (CARs) (33,136).

Anti-tumor functions of T-cells

T-cells are capable to recognize antigens with their TCR. They migrate to areas where their target antigens are expressed and get activated by binding them in combination with MHC (33). Naïve $CD8^+$ T-cells get activated by interaction with MHCI bound antigens. Activated $CD8^+$ T-cells turn into CTLs and secretes death-inducing effector molecules like perforin, granzymes and FasL that lead to the death of the target cell (34,38). Due to the aspect that MHCI is found on all nucleated cells,

$CD8^+$ T-cells are theoretically able to recognize many types of tumor cells. However, it was observed that cancer cells are able to downregulate antigen processing and downregulate the expression of MHCI molecules. Subsequently, $CD8^+$ T-cells are not able to recognize and eliminate target cells anymore, this is a mechanism of immunoresistance of tumor cells (150,151). Naïve $CD4^+$ T-cells are activated after interaction with a foreign antigen bound to MHCII and presented on an APC (35). The activated $CD4^+$ T-cell produces and secretes cytokines, chemokines, anti-angiogenic factors and apoptosis-inducing molecules, which can affect the growth of tumor cells and mediate an effective anti-tumor immune response (33,35). Besides presenting antigens by MHCII molecules, APCs are also capable to process and present antigens by MHCI molecule using a special mechanism called cross-priming. This way of presenting tumor antigens might allow targeting tumor cells that have lost MHCII expression (33).

Tumor antigens

Tumor antigens are antigens found on tumor cells and can be recognized by T-cells. Tumor associated antigens are found on tumors in high extend but also on normal cells in small amounts. Tumor specific antigens are selectively expressed on tumor cells (152). Examples for tumor antigens are listed below.

Unaltered tissue-differentiation antigens

Tumor cells continue to express antigens which are characteristic of the tissue they developed from. These tissue-differentiation antigens can be targeted by T-cells used for ACT. However, this approach of ACT is only suitable for tissues that are not essential for life (33). An example for this approach is the use of CAR T-cells that target CD19, a target found on the B-cell linage. Patients with B-cell malignancies treated with CAR T-cells targeting CD19 experience tumor responses but also a depletion of normal B-cells (153). Another example is the use of ACT in patients with melanoma. Melanocyte differentiation antigens gp100 (glycoprotein 100) and MART1 are expressed by most melanomas but also by normal melanocytes of the skin, the eyes and the inner ear. ACT, especially using genetically engineered TCRs against these targets, leads to toxicity on skin, eye and ear (154). However, TILs can be administrated without causing eye or ear toxicity, despite having TCRs specific for the same non-mutated self-antigens (155).

This issue suggests that TILs that are naturally occurring in melanoma might respond to other antigens than non-mutated self-antigens (33).

Tumor-associated antigens that are products of mutated genes

The genetic instability of tumor cells generates many potential antigens that are tumor associated. These antigens may result from mutations within gene-encoding regions, from mutations in stop codons that expand the reading frames or from gene rearrangements that result in the production of fusion proteins (33). The idea that mutations might be a target of immune cells to recognize tumors exists for a long time. In 1995, it was shown that a mutated cyclin-dependent kinase 4 (CDK4) is a tumor specific antigen in melanoma and metastasis tissue (156). The identification of tumor antigens, which can be recognized and attacked by the host immune system has been pursued for decades. The identified antigens have been largely limited to native antigens, like overexpressed antigens or tumor differentiation antigens. Truly tumor-specific antigens are called tumor neo-antigens and the availability of massive parallel sequencing has enabled the identification of them. Because exclusively tumor cells express neo-antigens, they are highly desirable antigens for immune therapies (157). Experiments in mice showed that targeting mutant tumor-associated antigens can lead to a complete rejection of large tumors (158). It was shown that an increased number of mutations and subsequently neo-antigens in tumors correlates with an increased patient survival (159).

Antigens produced by epigenetic changes

Epigenetic changes can lead to expression of a category of non-mutated proteins called cancer testis antigens. Cancer testis antigens are normally found on fetal tissue and on adult testes and placenta tissue, but not on other normal tissue. However, tumor cells often express these antigens (33). The fact that they are found on tumor but not on normal cells, except testis cells that do not express MHC molecules and are subsequently not detected by T-cells (160), makes them to a good target for immunotherapy. There is evidence that epigenetic changes in cancer cells can be the driver of some tumor types. Retinoblastoma, an aggressive childhood tumor, is initiated by the loss of the RB1 (retinoblastoma protein) gene. It was shown that retinoblastoma may develop quickly due to epigenetic deregulation of cancer pathways. A proto-oncogene called SYK is needed for the

survival of tumor cells and was found out to be upregulated in retinoblastoma. Targeting SYK with a small-molecule inhibitor led to death of tumor cells (161).

Antigens on non-transformed tumor vasculature and stroma

A tumor consist of more cells than only tumor cells, it contains also cell types like stromal cells and leukocytes (33). Cells of the stroma support tumor cells to grow and are necessary for the tumors survival. Cancer cells are capable to produce and release factors that stimulate those stromal cells, subsequently the stromal cells produce ligands that stimulate cancer cells (162). There is evidence that targeting these stromal cells can lead to tumor regression or even elimination. For example, there are stromal cells in the tumor microenvironment that expresses fibroblast activation protein-α (FAP). Depletion of FAP expressing cells led to rapid necrosis of both, cancer and stromal cells, in a mouse model. Cell death was mediated by TNF-α and IFN-γ, which are cytokines that are involved in $CD8^+$ T-cell dependent killing. This finding suggests, that T-cells, which are capable to recognize these stromal cells, could also be able to promote anti-tumor effectivity without directly targeting the tumor (163). Growing tumors require neovasculature for their supply with nutrients. Neovasculature expresses higher levels of vascular endothelial growth factor receptor 2 (VEGFR2), compared to normal vasculature. This makes VEGFR-2 to a target for immune attack. A single dose of genetically engineered CAR T-cells targeting VEGFR-2 inhibited the growth of five different tumor types in mice and led to a prolonged survival (164).

Viral antigens

Cancer types that are associated with viruses can express viral antigens. These viral antigens are appropriate targets because normal tissues do not express them (33). Examples are cancer types derived from Epstein-Barr virus (EBV) (165) or human papillomavirus (HPV) (166). The first virus shown to cause cancer in humans was EBV. EBV is associated with a wide range of human tumors with different origins from epithelial cells, lymphocytes and mesenchymal cells. Representative epithelial malignancies associated with EBV are nasopharyngeal carcinoma and gastric adenocarcinoma. Example for lymphatic cancers associated with EBV are a subset of Hodgkin's lymphoma, Burkitt lymphoma, a subset of diffuse large B-cell lymphoma (DLBCL) or malignant B-cell lymphoma. EBV causes alterations in the host and viral genome by methylation. Thereby, EBV-positive neoplasms exhibit

genetic changes that are distinct from those of EBV-negative neoplasms (167). An example is EBV-positive gastric adenocarcinoma which shows PIK3CA mutations, extreme DNA hypermethylation and amplification of JAK2, CD274 and PDCD1LG2 (168). HPV infection is a frequent and usually transient infection. It is the most common sexually transmitted disease, although it is usually cured by the immune system. HPV infections cause a range of diseases like the common and anogenital warts and different types of cancer. The most prevalent HPV types worldwide are HPV 16 and HPV 18, both are the main types associated with carcinogenesis. The most frequent HPV associated cancers are cervical cancer, anal cancer, vaginal cancer, oropharyngeal cancer, vulvar cancer and penile cancer (169).

4.4 Natural occurring autologous TILs

Natural occurring autologous TILs are immune cells with anti-tumor activity, which naturally occur in tumor patients. These anti-tumor T-cells can be taken from the patient, expanded *ex vivo* and reinfused to the patient to fight against the cancer (33). It seems curious that tumors persist and even grow while they are surrounded by autologous anti-tumor T-cells. The reason may be that these T-cells experience a chronic activation and that they are surrounded and inhibited by different immunosuppressive factors *in vivo*, as different trials suggest. It was shown that T-cells from tumors are functionally impaired, with a decrease of cytokine production and an increase of inhibitory receptors like PD-1 or CTLA-4 (33). PD-1, a receptor with immunoinhibitory function, is expressed on chronically stimulated T-cells and was found to be expressed by TILs. TILs expressing PD-1 were observed to be functionally impaired, with a reduced capacity to proliferate and to produce effector cytokines which subsequently ends in an inability to lyse target tumor cells (170). The ligand of PD-1, PD-L1, is expressed by activated APCs. PD-L1 expression can also be observed on different human tumors. The bonding between receptor and ligand leads to an inhibition of the proliferation and cytokine production of the T-cell (171). However, it was shown that this functional deficiency of T-cells is reversible. T-cells, isolated from melanoma tissue, were able to restore the production of the cytokine IFN-γ in an *in vitro* culture (172).

Production of TIL therapy

General schema of TIL therapy production (136):

1. Biopsy of the tumor is taken.
2. Resected tumor is divided into tumor fragments.
3. Lymphocytes are grown from these fragments.
4. Most effective lymphocytes are selected.
5. Expansion of the selected lymphocytes.
6. Lymphodepletion is conducted.
7. Reinfusion of lymphocytes plus a T-cell growth factor.

The first step is to take a biopsy of the tumor tissue, to expand T-cells away from their immunosuppressive tumor environment. With the help of intravital imaging it could be shown, that the migration of tumor-specific T-cells gets arrested when they get in contact with their antigens. Subsequently, T-cells stay at the tumor site. This might be the reason why TIL populations can be isolated from tumor masses (33). Subsequently, the tumor tissue gets divided into multiple tumor fragments. Lymphocytes are grown from those fragments, for this reason T-cell growth factor IL-2 is added. Within two to five weeks lymphocytes overgrow and destroy the tumor fragments and a pure culture of anti-tumor lymphocytes remains. Tumor cells die or get killed by activated NK-cells or newly expanding T-cell populations (33). TIL products are heterogeneous, they differ in percentage $CD8^+$ versus $CD4^+$ T-cells and also in anti-tumor reactivity and antigen-specificity. Only a fraction (up to 30 %) of the total population is tumor reactive (173). Therefore, the most effective lymphocytes with the best ability of tumor recognition and the most effective anti-tumor function, are selected by testing for tumor reactivity using IFN-γ ELISA (Enzyme-linked immunosorbent assay), ELISpot assays or other methods like FACS (fluorescence-activated cell sorting) or magnetic bead sorting (33).

IFN-γ is a cytokine that is predominantly produced by T-cells and NK-cells in response to a variety of inflammatory or immune stimuli. In the context of cancer, TILs are the main producers of IFN-γ (174). ELISA is based on the principle of antigen-antibody reactions, exploiting the interaction between antigens and antibodies, produced by B-cells. It allows an analysis of antigens like proteins, peptides, nucleic acids, hormones, herbicides and other molecules. A second,

enzyme-labeled antibody is used, which is labeled with enzymes like for example alkaline phosphatase (ALP) or horseradish peroxidase (HRP). These enzymes are able to develop color by chemical reactions with different substrates. For instance, ALP hydrolyzes the substrate *p*-nitrophenyl phosphate to *p*-nitrophenol, which can be detected as yellow color. Thus, the antigen reacts with a specific antibody, after this a second enzyme-labeled antibody is added. By adding a substrate color develops. The developed color corresponds to the presence of the antigen (175).
Another approach uses FACS or magnetic bead sorting to select TILs with PD-1 expression. It was observed, that the expression of PD-1 is high on melanoma reactive TILs, this fact can be used to pre-select tumor reactive lymphocytes. Using this method, TILs showed more tumor reactivity compared to the PD-1 negative or non-selected TILs in three out of five tested patients (176). The chosen most effective lymphocytes are grown in a culture to up to 10^{11} lymphocytes in a few weeks and are administrated back to the patient (33,136). Those individual cultures are expanded in the presence of excess irradiated feeder lymphocytes, anti-CD3 antibodies (OKT3) and IL-2 (33). OKT3 leads to a greater proliferation of the T-cells and induced the differentiation of PBMCs into T-cells (177). The addition of anti-CD28 antibodies can augment the OKT3 induced T-cell proliferation (178). Irradiated feeder cells, from autologous or allogenic sources, release growth factors into the culture, which induce massive TILs expansion (173). APCs can be used for the repetitive stimulation of T-cells with exogenously added antigens or endogenously produced antigens (178). Prior the lymphocytes transfer, the patient receives lymphodepletion. Either chemotherapy alone or a combination of chemotherapy and total body irradiation (TBI) is used. Murine models showed that lymphodepletion enhance the anti-tumor effects of transferred T-cells (37). The next step is the transfer of the *vivo* expanded TIL populations back to the host in combination with IL-2 (33,136). Reinfused TILs, which recognize tumor cells will proliferate and expand in the patient and will increase anti-tumor immune response. They can trigger destruction and death of tumor cells, complete eradication, durable and complete remission and even the cure of established tumors (155).

Melanoma and TIL therapy

Already in 1994, two melanoma-melanocyte proteins were identified; MART-1 and gp100. These proteins are overexpressed on melanomas but also found in small amounts on normal melanocytes in the skin, eye and ear. TILs can be capable to detect one of those non-mutated melanocyte antigens (179,180). However, there are also neo-antigens that are expressed on cancer cells due to mutations. Melanomas exhibit a high mutation rate (136), more than other types of cancer, as a characteristic of damage from UV radiation (33). The fact that melanoma responses to a lot of immunotherapy approaches leads to the suggestion, that the large number of mutations might play a role and that these mutations might be the targets of TILs (136). In a study, two melanoma patients obtained complete regressions of metastases ongoing beyond five years after TIL therapy. These clinically effective TILs were screened with the aim to identify the antigens that are recognized by them. The screening of tandem minigene libraries enabled the identification of mutated KIF2C (kinesin family member 2C) antigen and mutated POLA2 (DNA polymerase alpha subunit B) antigen. KIF2C and POLA2 play important roles in cell proliferation (181). Cancer testis antigens can also be targets of autologous anti-tumor TILs like for example NY-ESO-1 (New York esophageal squamous cell carcinoma-1) (182).

Results of TIL therapy in melanoma

The use of TIL therapy in patients with melanoma is promising and successful as seen on the objective response rates listed in the table below.

Table 2: Selected clinical trials of TIL therapy in melanoma

Pat.*	OR	Comments	Ref.
13	46 %	22 % complete responses	(183)
35	51 %	3 ongoing complete responses	(184)
93	49 %, 52 % and 72 %	Chemo alone, plus 2 Gy and 12 Gy TBI	(37)
57	40 %	5 complete responses	(185)
31	48 %	7 % complete responses	(186)

* Number of patients (Pat.)

TIL therapy using naturally occurring TILs, can mediate objective cancer regressions in up to 72 % of patients with metastatic melanoma in combination with lymphodepletion regime consisting of chemotherapy plus 12 Gy TBI (37). Several clinical trials showed a durable complete response, even in patients with progressive disease refractory to standard therapies (183). Durable complete responses could be achieved in 22 % of patients who experienced a complete response, 93 % of them have been alive and disease free for more than seven years and are probably cured of metastatic melanoma. The three-year and five-year survival rates for the whole group were 36 % and 29 %. However, for the group of complete responders survival rates were 100 % and 93 % after three and five years (155). Clinical responses can also include significant shrinkage of metastatic sides (183). Even when other immunotherapies failed, TIL therapy is capable to induce durable and complete responses, in patients with refractory melanoma (185). Persistent repopulation of transferred TILs, which proliferate *in vivo* and have anti-tumor reactivity, including migration to the tumors location, can be observed (183). Tumor biopsies were evaluated before the treatment and only minimal lymphocytic infiltration could be detected. After TIL therapy the biopsies showed necrotic tumor tissue and strong lymphocytic infiltration. This finding indicates, that adoptively transferred anti-tumor cells are capable to traffic to the tumor site and infiltrating into the cancer (183). In some patients, multiple applications of treatment might be justified. In a trial 47 % of patients (eight out of 17) treated with a second course of TIL therapy, including nonmyeloablative chemotherapy, experienced an objective response. As side effect a delayed hematologic recovery occurred and may represent a hindrance to the use of multiple courses of TIL therapy in some patients (184).

Results of TIL therapy in other tumor entities

Table 3: Selected clinical trials of TIL therapy in other tumor entities

Cancer type	Pat.	OR	Comment	Ref.
Cholangiocarcinoma	1	100 %	Target: Mutated ERBB2IP*	(187)
Cervical cancer	9	33 %	Target: HPV antigens	(188)
NSCLC	131	-	Significant improvement of the overall survival	(186)

* Erbb2 interacting protein (ERBB2IP)

TILs from a patient with metastatic cholangiocarcinoma were identified using whole-exomic-sequencing and it was shown that $CD4^+$ T-cells are capable to recognize a mutation in ERBB2IP, which was expressed by the tumor cells. The patient was treated with mutation-specific anti-tumor T-cells and experienced dramatic tumor regression in liver and lung metastasis ongoing beyond one year. The therapy led to a decrease in target lesions with prolonged stabilization of disease (187). Uterine cervix cancer can be virally induced. In this case the tumor constitutively expresses the oncoproteins HPV E6 and E7. TILs that are reactive to HPV E6 and E7 were administrated to nine patients with cervical cancer. Three of six patients with HPV reactivity experienced an objective tumor response, including one partial response and two complete responses in patients with widespread metastases and chemotherapy and chemoradiation-refractory cancer. Two patients, who had no HPV reactive TILs, did not respond to therapy (188). In 1996, a study evaluated TIL therapy in 131 patients with NSCLC. The study showed a significant improvement of the overall survival in patients treated with TIL therapy compared to those treated with standard chemotherapy and radiation therapy (189). It was shown that high levels of TILs in the tumor are associated with improved recurrence-free survival (190).

Cancer types potentially treatable by TIL therapy in the future

In colorectal cancer the presence of *in vivo* TILs was examined. In 123 of 160 patients TILs were present inside cancer structures. Patients who had a lack in TILs at the invasive front of the tumor or a lack of TILs in the center of the tumor mass had a shorter disease-free survival time than those who had TILs (191). In 2018, the feasibility of utilizing TIL therapy in colorectal cancer was tested in a pre-clinical model. It was possible to expand T-cells from metastatic colorectal cancer that were capable to recognize and target tumor antigens. This data suggest the feasibility of TIL therapy for the treatment of metastatic colorectal cancer (192). The feasibility of TIL therapy in NSCLC was also investigated in 2018. TIL cultures of patients with lung cancer were successfully established and expanded to treatment levels. Two out of three NSCLC patients had at least one TIL culture with anti-tumor reactivity. These findings may be the basis for a new treatment for patients with NSCLC (60).

Table 4: Selected ongoing trials of TIL therapy

Cancer type	Description	ClinicalTrials.gov
NSCLC	TIL	NCT02133196
NSCLC	TIL and PD-1 antibody	NCT03215810
NSCLC	TIL and PD-1 antibody	NCT03903887
Colorectal cancer	TIL and PD-1 antibody	NCT03904537
Colorectal-, pancreatic-, ovarian- and breast cancer, GBM	TIL	NCT01174121
Cholangiocarcinoma	TIL	NCT03801083

Identification of mutated antigens

There are screening approaches to identify antigens, recognized by clinically effective TILs. One approach aims to identify mutations, using whole exome sequencing of tumor-normal pairs and using an MHC binding algorithm, to predict immunogenic mutations. Peptides that contain mutated amino acids are identified. All peptides are scanned and investigated, to find peptides with a high binding to MHC molecules. The peptide with the best binding is synthetized and a test is conducted to check, if this peptide is recognized by TILs. Furthermore it is tested, if these TILs are able to induced cancer regression (193). Another method is to screen mutations and to evaluate if they are recognized by TILs in one test. Using this method, there is no need to predict the peptide binding to MHC. The schema below shows how it works (181).

1. Tumor biopsy is taken from the patient.
2. Extraction of DNA of normal and malignant tissue.
3. Exome sequencing from tumor DNA and normal tissue DNA.
4. Comparison of these two DNA sequences to identify mutations in the malignant cells.
5. Production of minigenes or peptides encoding mutated amino acids.
6. Minigenes or peptides are introduced in APCs.

7. All mutations that are capable of being processed and capable to bind to any MHC class, are expressed on APC surface.
8. T-cells from peripheral blood or the tumor are extracted from the patient.
9. Co-cultures of APC and T-cells are made.
10. Whenever T-cells recognize their antigen, they express activation markers. These markers enable an identification of mutations recognized by T-cells.
11. Mutation-specific T-cells are expanded. Or in case of TCR T-cell therapy, the TCR can be isolated and inserted into an autologous lymphocyte.
12. Reinfusion of mutation-specific lymphocytes to the patient after he received a lymphodepletion regime.

With the use of this approach, it was shown that TILs from 21 melanoma patients who responded to TIL therapy, were able to recognize 45 mutations, which were presented on different MHC classes. Every mutation recognized by TILs was distinct. These findings suggest that TILs, which are able to induce responses, recognize random cancer mutations. The assumption that TILs recognize mutations can explain why some patients experience tumor response without autoimmune toxicities (136).

Toxicities of TIL therapy

In patients with melanoma, the use of anti-melanoma TIL therapy can lead to autoimmune destruction of normal melanocytes in the skin, eye and ear. Consequences can be vitiligo, uveitis or hearing loss. The treatment of hearing loss is an intratympanic steroid injection. The treatment of uveitis are local steroid eye drops (154,183,184). Posterior uveitis refractory to topical steroids accompanied by visual changes is also reported, however, intraocular steroid injections and treatment with diazoxide resolved the symptoms (37). Examples for other toxicities that can occur are pulmonary toxicity, jugular venous thrombosis, renal dysfunction attributed to thrombotic microangiopathy with elevated serum creatinine levels or somnolence that required intubation (37). The use of TIL therapy in cervical carcinoma led to serious adverse events in 22 % of the patients, including blood disorder, febrile neutropenia, infections, confusion, hypoxia and dyspnea (188).

Limitations of TIL therapy

Antigen loss

Recurrent lesions from some patients were tested after anti-melanoma TILs treatment. Some of them did not express MHCI anymore and some did not express the tumor antigen MART-1 protein anymore. These results suggest that transferred TILs eliminate antigen-expressing tumor cells (184). However, there are also nonresponding patients that express MHCI and antigens detected by TILs (184).

Obtain tumor tissue

For the production of a TIL product, an invasive procedure is necessary to obtain tumor tissue. There is a risk of postoperative morbidity, especially for tumor sites that are difficult to access. Gain tumor tissue from cancer sites like lung hilum or head of the pancreas has a potential high morbidity of surgery, which exclude the option for TIL resection (194).

Inability to isolate and grow TILs

In some patients it is not possible to produce TIL products because no TILs can be grown out of the tumor mass. In about ten to 15 % of patients with melanoma no TILs growth can be observed (195). An alternative to finding natural occurring TILs are genetically engineered T-cells (154).

Limitations of TIL production

TIL therapy is a personalized cancer therapy, as a specific infusion product needs to be produced for every patient and thus costs are relatively high (176). However, prices for TIL therapies are still considerably lower compared to treatment with checkpoint inhibitors like anti-CTLA-4 (196). The production time of a TIL product is more than one month. This may be too long for some patients with rapidly progressive disease. Another limitation point is, that highly specialized facilities and production staff are required. Therefore, investments and training are needed (176).

4.5 Genetically engineered T-cells

In the attempt to expand the range of tumors treatable by ACT, genetically engineered T-cells were developed. Gene engineering may allow it virtually to target any cancer histology, because T-cells can be genetically engineered to be capable of targeting antigens that are expressed by the tumor (33). Genes of receptors that are able to recognize tumor antigens are introduced into autologous T-cells, which are isolated from the blood of the patient. Subsequently, these anti-tumor CAR T-cells can be used for therapy (136). There are two different receptor forms that can be used, either conventional TCRs or CARs (136).

A schema of how ACT, using genetically engineered T-cells, works (136):

1. T-cells are preserved from the peripheral blood of the patient.
2. Gene transfer of genes encoding a receptor, which is capable to target tumor antigens is performed, using viral or non-viral methods. As result, T-cells express a TCR or CAR, which recognizes tumor antigens.
3. Expansion of these anti-tumor T-cells.
4. Preconditioning lymphodepletion regime.
5. Anti-tumor T-cell infusion to the patient in combination with IL-2.

Production of CAR and TCR T-cell therapies

For the transfer of genes, encoding anti-tumor receptors, two methods are known, either the use of viral vectors or the use of non-viral vectors. The aim is to introduce DNA or RNA of receptors into autologous T-cells, isolated from blood. It was shown, that the use of viral systems is more efficient compared to non-viral gene-transfer systems (31,33,197).

Viral vectors

Viruses enter host cells to use their cellular machinery to replicate and express their own genes. For the use of viruses in gene delivery, they get engineered to express the gene of interest and to deliver the gene of interest into target cells. These engineered viruses are often called viral vector (198). Viral vectors are used in most research and clinical studies because of their high transfer efficiency, a relatively short time that is needed to culture T-cells and the existence of different virus types with variant expression characteristics. The virus types used for virus vectors are

for example retroviruses (including lentivirus), adenoviruses and adeno-associated viruses (199). Retroviral vectors are most often used in gene therapy trials (198).

Retroviral vectors

A retrovirus consists of two copies of a single stranded RNA genome, with sequences that encode viral structural and catalytic proteins, like gag, pol, and env. Retroviruses interact with surface receptors of target cells to gain entry in the host cell. Inside the host cell the single stranded viral RNA genome is converted into double stranded DNA by the enzyme reverse transcriptase. Reverse transcriptase is a virally encoded enzyme. The viral DNA copy gets integrated into the genome of the host by the virally encoded enzyme integrase. At this point it is known as provirus. It is this proviral DNA, which is manipulated to produce retroviral vectors for gene transfer. With the rest of the genome, the provirus undergoes transcription and translation. The results are the expression of viral genes and the synthetization of viral proteins. These proteins are used to assemble new virus particles, which are released from the target cell to infect others cells (198,200,201).
A retroviral vector consists of proviral gene sequences that can place the gene of interest into the target cell. The retroviral vector also contains gene promoters for viral and cellular genes like the CMV promoter, to increase the expression of the gene of interest in the host cell (201). However, in most gene therapies a replication of the virus is not wanted because the virus may spread and cause adverse effects. For this reason, a helper construct is added to limit the ability of the virus to freely replicate. Helper functions can be provided in form of a cell, a virus or plasmid (198). The use of packaging cells, typically fibroblast derivatives, is an important part in vector technology. Packaging cells contain sequences of DNA plasmids. DNA plasmids are independently encoding DNA sequences, which are expressing viral gene products like gag and pol. The retroviral vector is introduced into the packaging cells and the results are virions that contain just the vector genome. These virions are released into a culture medium and infect target cells and integrate their genome. However, the retroviral vector is not able to replicate further because it does not encode viral structural proteins because they have been provided by the packaging cell (201).

Non-viral vectors

Non-viral vector based gene-transfer methods are for example the plasmid/ messenger RNA electroporation system, the transposon/transposase system and the lipid-based transfection (197). A physical process, called electroporation, occurs in cells placed between two electrodes under a very high voltage. Cells get temporally permeable due to the voltage and thus DNA plasmid, mRNA and proteins are able to enter the cell. The disadvantage of this method is that the high voltage impulse can cause damage to the cells (197).
The transposon/transposase system was discovered in 1940 and is composed of two components: a plasmid carrying the gene of interest (transposon) and another plasmid encoding the transposase. DNA transposons are used for gene delivery because they are able to integrate into the host genome through a cut-and-paste mechanism. This DNA elements encode a transposase that is flanked by inverted terminal repeats (ITRs). ITRs contain the binding sites for transposase, which are necessary for the transposition. Through this mechanism, a transposition of any gene of interest that is flanked by ITRs is possible (202).
Lipid-based transfection system is another tool used as a non-viral vector. This system is able to fuse with the cell membrane of the target cell, which is built of two lipid layers, and to release the genes of interest into this cell (197).

Production of TCR T-cells

It is possible to isolate genes, encoding a TCR, from a patient who received TIL therapy and demonstrated complete regression. With avidity testing TCRs with high affinity to tumor associated antigens can be found (148). Another method to produce TCR is to immunize humanized mice, which express human MHC molecules with human tumor antigens. T-cells which are specific for a tumor associated antigen get isolated from the mice and can get introduced in an autologous T-cell of a patient (154). The gene sequences that are encoding for the tumor specific TCR can get inserted into a viral vector. After this the virus is used to infect T-cells from the patient and as a consequence, the autologous T-cell expresses the tumor-specific receptor (33).

Avidity testing

The efficiency of engineered T-cells can be tested by coculturing the antigen expressing cancer cells and the T-cells. Biologically reactive T-cells show a specific secretion of IFN-γ (148). The cellular avidity of T-cells to their antigen can be evaluated by measuring this IFN-γ secretion (203). To measure IFN-γ, methods like ELISA, ELISPOT, intracellular staining and flow cytometry can be used (154).

Production of CAR T-cells

CAR T-cells are generated by using T-cells from the blood of the patient or blood from donors. Thereby, the first step is leukapheresis to isolate leukocytes from the blood and in the second step, T-cells are separated from the other leukocytes. Then, a culture of T-cells is produced. This step requires APCs from the patient or donors and anti-CD3/anti-CD28 monoclonal antibodies or anti-CD3 antibodies (OKT3) alone or in combination with feeder cells and growth factor. IL-2 is used most often as growth factor because it induces rapid proliferation of T-cells (199). The addition of OKT3 results in a greater expansion of the T-cells and primarily leads to the differentiation of PBMCs into $CD3^+$ T-cells (177). Anti-CD28 antibodies can increase the anti-CD3 induced T-cell proliferation (178). Feeder cells can lead to massive expansion of T-cells by releasing growth factors into the culture (173). APCs are used for the repetitive stimulation of T-cells by presenting antigens (178).

There are different ways known to engineer CARs. In the most common approach, sequences that encode the variable region of antibodies are genetically engineered to encode a single chain that is able to recognize the tumor associated antigen. This engineered sequence gets attached to the intracellular domains of the TCR, which are necessary for T-cell activation. The gene sequence for the CAR gets inserted into a viral vector, which infects human T-cells. The infected T-cells produce and express CARs and are subsequently able to recognize tumor specific antigens and fight against cancer cells (33).

Comparison of TCR and CAR T-cell therapy

Table 5: Comparison of TCR and CAR T-cell therapy

	TCR T-cells	**CAR T-cells**
Targets	Surface and intracellular antigens. Thus, TCRs can target much more antigens than CARs (204).	Only surface antigens (204)
Type of Antigens	Proteins (205)	Proteins, carbohydrates and lipids (205)
MHC	MHC expression is necessary for T-cell activation (206) .	MHC independent antigen recognition (33).
Antigen processing	Required (205)	Not required (205)
HLA* match	TCRs must be matched to patient's HLA alleles (204) .	CARs do not have to be HLA-matched (204).
Costimulatory signals	No costimulatory signals (33)	Costimulatory signals in second, third and fourth generation (199).
Sensitivity	Higher sensitivity than CARs (207)	10 to 100-fold less sensitive than TCR (207)
Avidity	Low avidity, unless engineered (208)	Avidity controllable (208)
Persistence	Lifelong persistence (208)	At least decade-long persistence (209)
Number of cells killed	Kill multiple tumor cells (210)	Kill multiple tumor cells (210)
Risk of CRS**	Lower risk because TCRs mediate release of less cytokines (207).	Higher risk because CARs mediate release of more cytokines (207).
Problems	Higher risk for "on-target, off-tumor" toxicity because of higher sensitivity (206).	CRS is more severe than in TCR T-cell therapy (207).

	“Off-tumor” toxicity is difficult to predict in pre-clinical investigations (211).	

*human leukocyte antigen (HLA)

**cytokine release syndrome (CRS)

4.5.1 TCR T-cell therapy: MHC presented peptide recognition

TCRs are expressed on the surface of T-cells. When the TCR recognizes antigens, which are presented on cells in combination with MHC molecules, T-cells get activated. After activation they are able to induce an immune response against cancer cells (148). Both, surface and intracellular proteins, can be processed and presented by MHC molecules. This is the reason why TCRs can target much more antigens than CARs, which are only able to recognize surface antigens (204). TCRs, in comparison to CARs, must be matched to the patients HLA alleles. Thus, most trials use TCRs restricted by the HLA *0201 allele, that is present in almost 50% of the US Caucasian population (204).

It is generally considered that TCRs have a lower affinity than CARs, but recent studies showed that CARs were ten to 100-fold less sensitive than TCRs. However, despite the lower sensitivity, CARs mediate the release of more cytokines and thus increases the risk for CRS (207). Higher sensitivity enables more rapid tumor destruction but increases the risk of “on-target, off-tumor” toxicity (206,212).

Structure of TCRs

TCRs consist of an alpha and beta chain and are associated with the CD3 complex on the T-cells surface. The alpha and beta chain are two different transmembrane polypeptide chains, which consist of a constant region, which fixes the chain inside the T-cell surface membrane, and a variable region, which is able to recognize and bind to the antigen presented by MHCs. The CD3 complex, composed of six CD3 chains, plays a role in T-cell activation (206). Costimulatory signals are essential for full T-cell function. An example is CD28, which is found on T-cells and is able to bind to B7 that is found on the surface of APCs. The engagement of CD18 and B7 promotes T-cell survival (206).

Results of TCR T-cell therapy in cancer

The first TCR T-cell cancer therapy was published in 2006 in patients with metastatic melanoma. By using a retrovirus that encodes the specific TCR, lymphocytes became capable of tumor recognition and led to objective response in two of 17 patients (148). However, there are different targets TCRs are capable to recognize, as listed below.

Shared unaltered tissue-differentiation antigens

Unaltered tissue-differentiation antigens are antigens, which are characteristic of the tissue from which the tumor evolved from. These antigens are shared and expressed by tumor cells and healthy cells. Often, these antigens are overexpressed in tumors and are only found in low levels on normal cells. Examples are MART-1 and gp100 (179,180). Targeting these differentiation antigens can induce “off-tumor, on-target” toxicity on healthy, normal cells.

Table 6: Results of TCR T-cell therapy in targeting MART-1 or gp100

Target	Cancer type	Pat.	OR	Comment	Ref.
MART-1	Melanoma	17	2	Lower avidity	(148)
MART-1	Melanoma	31	4	Lower avidity	(154)
MART-1	Melanoma	20	30 %	Higher avidity	(154)
gp100	Melanoma	16	19 %	Higher avidity	(154)

TCRs against MART-1 (DMF4) were used in 17 patients with metastatic melanoma. Genes encoding these TCRs were isolated from TILs, which mediated complete response in a patient, and were infused into autologous T-cell by using a viral vector. In two patients objective response occurred. No toxicities associated with TCR T-cells occurred in any patient (148). The trial was expanded to 31 patients and four of them (13 %) experienced an objective regression and none of them developed skin rash or normal melanocyte toxicity in the eye or ear (154). Because this DMF4 receptor had only moderate clinical responses, more highly reactive TCRs against MART-1 (DMF5) and gp100 were developed (154). The use of higher avidity TCR T-cells targeting MART-1 and gp100 led also to objective anti-tumor response. 20 patients with melanoma were treated with TCR recognizing MART-1 and 16 with TCR against gp100. In six of the 20 patients (30 %) treated with TCR targeting

MART-1 and three of 16 (19%) treated with TCR against gp100, an objective anti-tumor response occurred. The tumors regressed in multiple organs, including the brain, lung, liver, lymph nodes and subcutaneous sites. “Off-tumor, on-target” toxicities occurred. A correlation of clinical response with the persistence of transfused cells after one month was observed. There was no correlation found between the number of the administered cells and clinical response. And also no correlation between the duration the cells were grown *ex vivo* and the clinical response could be observed (154).

How higher avid TCRs are produced

Screening of hundreds of TIL clones from patients with melanoma revealed DMF5, a lymphocyte clone with much higher reactivity against the MART antigen than the previously identified DMF4. TCRs targeting MART-1 were isolated from TIL clones of melanoma patients. The alpha and beta TCR genes were isolated and the RNA of the TCR was electroporated into donor PBMCs and TILs. The engineered cells gained the ability to recognize MART-1-expressing tumors. High-avidity TCRs were identified by their ability to bind MART-1 independently of CD8. The response to the antigen MART-1 was determined by measuring the IFN-γ production. The TIL clones DMF4, DMF5 and JKF6 were the most highly avid T-cells, they were producing the highest levels of IFN-γ. Even in response to low concentrations of MART-1 or endogenous MART-1 expressed on tumor cells, DMF4, DMF5 and JKF6 responded with high levels of IFN-γ production. The DMF5 receptor showed the highest response (203). The avidity of DMF5 TCR engineered T-cells could be additionally improved by replacing the constant region from endogenous TCR by constant regions derived from the mouse genome. A limitation of TCR T-cell therapy is the competition with endogenously expressed alpha and beta TCR genes for appropriate alpha-beta TCR heterodimer pairing. To increase the pairing of anti-tumor TCR alpha-beta chains on T-cells, the transmembrane constant regions of each TCR chain can be replaced by constant regions from mice (203).

The high-avidity TCR against the gp100 epitope was produced by immunizing mice twice with the human gp100 peptide. One week after this procedure, splenocytes were stimulated with human gp100, IL-2 and activated transgenic mouse splenocytes. After this third stimulation, T-cells were cloned and the TCR RNA was electroporated into donor lymphocytes. The anit-gp100 TCRs with the highest anti-

tumor avidity, independently of CD4 and CD8 co-receptor, were selected. The anti-tumor activity was evaluated by coculturing the lymphocytes with cells expressing the antigen or melanomas. IFN-γ and IL-2 were measured by using ELISA, ELISPOT, intracellular staining and flow cytometry (154).

Cancer Testis Antigens

Cancer testis antigens are products of epigenetic changes. These antigens are found on fetal tissue but not on normal adult cells, except cells of the testes, which do not express MHCI. However, cancer testis antigens are found on different tumor types (33,213). Targeted therapies against cancer testis antigens can overcome "off-tumor, on-target" toxicities that are caused by immunotherapies directed against antigen, which are also expressed on normal tissues (214).

Table 7: Selected trials of TCR T-cell therapy against cancer testis antigens

Antigen	Cancer type	Pat.	OR	Comment	Ref.
MAGE-A3*	Melanoma, synovial sarcoma, esophageal cancer	9	5	Two patients died due to neurological toxicities	(212)
MAGE-A3	MM and melanoma	2	-	All patients died due to cardiogenic shock	(215)
NY-ESO-1	Synovial cell sarcoma	6	4	Two melanoma patients achieved CR over one year	(216)
	Melanoma	11	5		
NY-ESO-1 + LAGE-1**	MM	20	18	No toxicities occurred	(217)

* Melanoma antigen A3 (MAGE-A3)

** Cancer testis antigen 2 (LAGE-1)

MAGE-A3

MAGE-A3 is a cancer testis antigen that is expressed on a range of tumors but not on normal tissue. In a trial, nine cancer patients with melanoma, synovial sarcoma and esophageal cancer were treated with ACT using anti-MAGE-A3 TCR. In five patients, clinical regression occurred and in two patients, ongoing responses were achieved.

However, unfortunately unexpected neurological toxicity occurred and a few days after infusion, three patients developed changes in their mental status and two of them even fell in coma and died. In the autopsy necrotizing leukoencephalopathy with extensive white matter defects, that were associated with T-cell infiltration, were diagnosed. Subsequently, human brain samples were examined and it could be shown that human brains express MAGE-A12. This might be the cause of the TCR-mediated inflammatory process in the brain (212). In another trial the two patients were treated with an affinity-enhanced TCR targeting MAGE-A3. One patient had myeloma and the other patient had melanoma. Both patients developed a cardiogenic shock and died, after ACT. In the autopsy, sever myocardial damage and a high infiltration with T-cells was diagnosed. In further investigations, no MAGE-A3 expression could be detected in the heart tissue. However, it was shown that anti-tumor T-cells additionally recognized an unrelated peptide derived from a muscle-specific protein called titin. Titin is expressed on normal cardiac cells and thus healthy heart tissue was attacked by anti-tumor T-cells. (215).

NY-ESO-1

NY-ESO-1 is a cancer testis antigen that is expressed in 80 % of patients with synovial cell sarcoma and in about 25 % of patients with melanoma. In a trial using autologous T-cells with engineered TCRs targeting NY-ESO-1, an objective response was observed in five of eleven melanoma patients, including two complete responses, ongoing after 22 and 20 months. In patients with synovial cell sarcoma four of six patients experienced an objective response, with one lasting for 18 months. No toxicities attributed to the transferred T-cells were observed (216).

LAGE-1

The cancer testis antigen LAGE-1 is also known as cancer testis antigen 2. In 20 patients with MM, affinity-enhanced TCRs targeting NY-ESO-1 and LAGE-1 were administrated. 16 of 20 patients (80 %) experienced a clinical responses with a median progression-free survival of 19.1 months. Loss of T-cell persistence or antigen escape were associated with disease progression after the therapy. NY-ESO-1-LAGE-1 TCR T-cells were safe, there was no evidence of CRS (217).

Toxicities of TCR T-cell therapy

The line between efficacy and toxicity is thin, when targets are antigens that also occur on normal cells, not only on the tumor. Neutropenia and thrombocytopenia are side effects that are associated with the preparative regimen and also toxicities associated with IL-2 application can occur (216).

MART-1 and gp100 – Melanocyte toxicities

Beside melanoma also some normal cells express MART-1 and gp100 in low levels. These cells are melanocytes from the skin, eye and the striae vascularis of the inner ear (179,180). Erythematous skin rash and epidermal melanocytes destruction can occur as side effects, by attacking melanocytes of the skin. Eye toxicities include uveitis, which is treatable by steroids, and synechiae of the iris, which are reversible. Damage of melanocytes of the striae vascularis of the inner ear can lead to hearing loss in some patients. Hearing loss can be treated by intratympanic steroid injections, which led to improvement in all patients in a clinical trial. Another inner ear toxicity is inner ear related dizziness, which also responded to treatment in this trial (154). A fatal adverse effect occurred in a patient with metastatic melanoma that received TCR T-cells recognizing MART-1. The patient experienced a cerebral hemorrhage, epileptic seizures and a cardiac arrest. He died from multiple organ failure and irreversible neurologic damage. CRS, a T-cell-mediated inflammatory response, including extreme increased levels of C-reactive protein (CRP), procalcitonin and IL-6, occurred in this patient. Administrated T-cells could be detected in the blood, broncho-alveolar lavage, ascites, tumor sites and heart tissue. However, no cross reactivity of the infused T-cells toward cardiomyocytes was found. This findings lead to the assumption, that the elevated levels of inflammatory cytokines are a substantially cause of the lethal event (218).

MAGE-A3 – Neuro and cardiac toxicities

The use of TCR targeting MAGE-A3 resulted in changes of the mental status, coma and death because MAGE-A12 is expressed by brain cells (212). The use of affinity-enhanced TCR against MAGE-A3 in a patient with melanoma and one with myeloma, led to cardiogenic shock and death of both patients. T-cells recognized a peptide which is expressed by normal cardiac tissue (215). The pathophysiologic details are described on pages 61 and 62 in the chapter MAGE-A3.

Limitations of TCR T-cell therapy

TCRs only recognize antigens that have been processed and that are presented by MHC molecules (136). The patients HLA allele must match the TCRs (204). Targeting shared tissue-differentiation antigens might be limited by “on-target, off-tumor” toxicities. A limitation of targeting cancer testis antigens is, that only a few cancer types express them and the screening of suitable patients is expensive (204). Neo-antigens are formed by random somatic mutations that result in new peptide antigens and are specific to individual tumors. They also vary in one and the same patient when tumor cells are isolated from different sites or at different times. Targeting neo-antigens can reduce the risk of “on-target, off-tumor” toxicity but it is more expensive to produce and use them. The identification of neo-antigens sometimes requires sequencing of the whole genome of the individual tumor to identify mutated genes and to find peptides that are predicted to be presented by MHC molecules (206). The mispairing of TCR alpha and beta chains is another limitation. Mouse models showed, that lethal cytokine-driven autoimmune pathology can occur using TCR T-cell therapy. Pairing of introduced and endogenous TCR chains in TCR gene-modified T-cells led to production of self-reactive TCRs, which caused fatal autoimmunity (219). Another limitation from TCR therapy is the loss of MHCI molecules, as a strategy of tumor cells to escape the immune system (31,136,220).

Future of TCR T-cell therapy

Many ongoing clinical trials are performed in the field of TCR T-cell therapy in cancer. With a lot of new antigens, targeted by TCRs and more cancer types, treated by T-cell therapy. A few selected ongoing clinical trials, registered on ClinicalTrials.gov, are listed below.

Table 8: Examples of ongoing TCR T-cell therapy trials

Cancer type	Target	ClinicalTrials.gov
Cervical cancer, RCC, urothelial cancer, melanoma, breast cancer	MAGE-A3	NCT02111850
Melanoma, meningioma, breast cancer, NSCLC, Hepatocellular Carcinoma (HCC)	NY-ESO-1	NCT01967823
Synovial Sarcoma	NY-ESO-1	NCT01343043
NSCLC	NY-ESO-1	NCT02588612
Mesothelioma, NSCLC	WT1*	NCT02408016
HCC	AFP**	NCT03132792
Acute myeloid leukemia (AML), Myelodysplastic syndrome, uveal melanoma	PRAME***	NCT02743611
Cervical cancer, head and neck squamous cell carcinoma	HPV E6	NCT03578406
Papillomavirus infections, oropharyngeal cancer	HPV E7	NCT04044950 NCT04015336

* Wilms tumor (WT1)

** Alpha Fetoprotein (AFP)

*** Preferentially expressed antigen in melanoma (PRAME)

4.5.2 Chimeric antigen receptor T-cells in cancer therapy

CARs are artificial receptors that consist of an antigen recognition domain that is fused to TCR intracellular domain and costimulatory domains. To use CARs for T-cell therapy, they are introduced into T-cells (31). The targets of CARs are surface antigens of cancer cells because CARs are only able to recognize antigens found on the cell surface (206). In CAR T-cell therapy it is important that the targets of CARs are unique to cancer cells to avoid toxicity and autoimmune diseases (31). TCRs, in comparison, are capable to recognize intracellular antigens that have been processed and presented as complexes with MHC molecules (33). CARs recognize their antigens

independent of MHC. Thus, CARs overcome a limitation from TCR T-cell therapy, immune escape induced by MHC loss (31,136,220).

CAR T-cells have to be custom made of autologous T-cells for every patient. However, this is not always possible, especially for patients who do not have sufficient healthy T-cells (221). An alternative are CAR T-cells derived from healthy donors. Additionally, those donated cells can overcome immune defects that are associated with cancer treatment (208). CAR T-cells derived from healthy donors might be an opportunity to produce engineered T-cells simplified, cheaper and more rapid, compared to patients autologous T-cells. Perhaps, it will be even possible to provide “off-the-shelf” immunotherapy (222).

Structure and generations of CARs

CARs are proteins consisting of three parts: an extracellular, a transmembrane and an intracellular part. The extracellular part is derived from an antibody, it consists of a single-chain fragment variant (scFv) and a spacer. The scFv is formed by heavy and light chains of an immunoglobulin. Its tasks are the recognition of the antigen and the binding to the antigen. The spacer is the connection between the antigen binding domain and the transmembrane domain. The transmembrane domain connects the scFv region with the intracellular domain. The intracellular part is derived from a TCR. It consists of CD3-zeta chains, the common signal-transducing subunits of the immunoglobulin receptor and the TCR. It is often connected with costimulatory molecules. The aim of costimulatory domains is, to influence the proliferation and the persistence of T-cells (199).
There are four generations of CAR T-cells. The first generation does not have any costimulatory domains, it just consists of a scFv of an antibody, linked with zeta chains of the immunoglobulin receptor and the TCR (223). These first-generation CARs, expressed on genetically engineered T-cells, showed antigen-specific binding and lysis of target cells *in vitro*. However, *in vivo* the anti-tumor efficiency was limited in mouse models (224). Thus, a second generation of CAR T-cells was developed, which possesses a costimulatory domain, fused to the intracellular part. The third generation possesses two costimulatory domains (199).
The fourth generation CAR T-cells are also called T-cells redirected for universal cytokine killing (TRUCK). These CARs were generated by adding IL-12 to the second generation constructs. TRUCKs enhance the activation of T-cells and attract

and activate cells of the innate immune system, to eliminate antigen-negative cancer cells. Thus, this CAR generation is able to overcome a limitation of the other generations, because antigen-negative cancer cells, which are not recognized by CARs, are able to give rise to tumor relapse (225).
An example for a costimulatory domain is CD28. The co-binding of CD28 on activated T-cells to its ligands CD80/CD86 on tumor cells or APCs, plays roles in the production of IL-2, promoting survival and preventing senescence.
The use of CD28 as a costimulatory domain in CAR T-cells in mouse model led to enhanced efficacy against tumors and prolonged persistence of CAR T-cells (226).
Another example for a costimulatory domain is 4-1BB. In a mouse model, significantly increased anti-tumor activity, *in vivo* survival and persistence of CARs, compared to inclusion of the CD3-zeta chain alone occurred (227). Also, in human cancer those CARs showed efficiency (228).

Results of CAR T-cells in cancer therapy

Different malignancies are treatable with CAR T-cell therapy, targeting different tumor antigens. Results of CAR T-cell therapy in different cancer types and associated antigens are discussed in this chapter.

Results of CAR T-cell therapy in B-cell malignancies

Autologous T-cells, engineered to express CARs against different B-cell linage antigens, can be very efficient in patients with different B-cell malignancies.
In B-cells, different targets are established to be detected by CAR T-cells.

CD19

CD19 is a surface molecule on B-cells. It is expressed on normal B-cells as well as on malignant B-cells. However, it is not found outside the B-cell lineage (229).
CD19 occurs in an early stage of B-cell differentiation in the bone marrow and remains until the differentiation into the plasma cell (230). CAR T-cells against CD19 can induce high remission rates in patients with B-cell malignancies and are able to achieve lasting complete remissions. Even in chemotherapy refractory cases or in patients, in which even stem-cell transplantation had failed, CAR T-cell therapy can lead to remission (231).

Table 9: Results of CAR T-cell therapy targeting CD19 in B-cell malignancies

Type of B-cell malignancy	Pat.	Results	Ref.
B-cell lymphoma or CLL	8	1 CR, 5 PR	(232)
B-ALL in children and adults	30	27 (90 %) remission in the first month, 19 remained in remission	(231)
B-ALL in children	21	14 CR	(233)
B-ALL	5	5 remission (100 %), 1 relapse after 90 days	(234)
Different B-cell malignancies	14	8 CR, 4 PR, 1 stable lymphoma	(235)
Different B-cell malignancies	10	1 CR, 1 PR	(236)
Different B-cell malignancies	20	6 CR, 2 PR	(237)
B-ALL	2	2 CR, 1 ongoing for 12 months	(238)
B-ALL	75	61 (81 %) CR	(239)
CLL	14	4 CR (no patient relapsed), 4 PR	(153)

CAR T-cells against CD19 are capable to proliferate *in vivo* and they are detectable in blood, bone marrow and cerebrospinal fluid of patients, who show a response to the anti-tumor therapy (240). Thus, CAR T-cells are also capable to reach the central nervous system (CNS). In patients with CNS Leukemia, blast cells can be found in the cerebrospinal fluid. After CAR T-cell therapy, no blast cells were detectable in the cerebrospinal fluid anymore (231,233).
The expansion of CAR T-cells *in vivo* correlates with the clinical response. In patients with a response, high levels of CAR T-cells can be detected. Conversely, in patients with no response, only low levels of CAR T-cells are observed (153,231,237). The importance of peak blood levels versus persistence of CAR T-cells is unknown (235). In patients with sustained remission, CAR T-cells could be detected even two years after application by PCR, and in another trial even beyond four years after application (153,231).

CD20

CD20 is another antigen found on B-cells and a potential target for immunotherapies. CD20 is expressed on pre-B-cells and mature B-cells and on the surface of malignant B-cells. However, it is not expressed on normal cells of the B-cell linage (241).

Table 10: Results of CAR T-cell therapy targeting CD20 in B-cell malignancies

Type of B-cell malignancy	Pat.	Results	Ref.
DLBCL	7	1 CR, 3 PR, 1 stable disease	(242)
MCL (mantle cell lymphoma) **FL (follicular lymphoma)**	4	1 PR, 2 stable diseases	(243)
FL	7	2 CR, 1 PR, 4 stable diseases	(244)

Trials showed, that CAR T-cells targeting CD20 can induce complete response in B-cell malignancies like DLBCL and FL (242,244). An *in vivo* increase of $CD8^+$ T-cells, as well as a decrease of $CD20^+$ B-cells can be observed after an anti-CD20 CAR T-cell infusion (242). In a study in 2019, the cytotoxic efficacy of anti-CD20 CAR T-cells therapy in Burkitt's lymphoma was evaluated. Anti-CD20 CAR T-cells were observed to be reactive against Burkitt's lymphoma cell lines with CD20 expression *in vitro*. After the confirmation of anti-CD20 CAR T-cells reactivity *in vitro*, they were tested *in vivo* using mice. Mice were systemically engrafted with Raji cells (Burkitt's lymphoma cells) and a group of mice was treated with CAR T-cells against CD20. CAR T-cell treated mice showed a longer survival time, compared to the mice who did not receive CAR T-cells (245). Trials have shown that patients with B-cell malignancies relapse after anit-CD19 CAR T-cell therapy due to the emergence of CD19-negative leukemic cells. Thus, anti-CD19/anti-CD20 CARs were developed and used in a mouse model. These CARs were observed to be able to control wild-type and CD19 mutant B-cell lymphomas *in vivo.* This approach might be a strategy to overcome the limitation of antigen escape in ACT for B-cell malignancies (246).

CD22

The antigen CD22 is exclusively expressed on the B-cell linage, including malignant B-cells. Thereby, it is another target suitable for CAR T-cell therapy in cancer patients (247).

Table 11: Results of CAR T-cell therapy targeting CD22 in B-cell malignancies

Type of B-cell malignancy	Pat.	Results	Ref.
B-ALL (naive or resistant to anti-CD19 CAR T-cell therapy)	21	12 (57 %) CR	(247)
B-ALL (resistant to anti-CD19 CAR T-cell therapy)	34	24 (70 %) CR	(248)
B-ALL (anti-CD19 plus anti-CD22 CAR T-cells)	24 (1x) 16 (2x)	1x: 20 (83.3 %) CR 2x: 100 % responders (15 CR and 1 PR)	(249)

Higher doses of T-cells lead to higher complete response rates. In a study, one group of patients was treated with a higher dose of T-cells and complete response was achieved in 73 % of the patients. Complete response occurred in nine of ten patients who received prior anti-CD19 CAR T-cell therapy. The median complete response rate of all groups together was 57 %. The median duration of remission was six months. An association between relapse and reduced density of the CD22 antigen could be observed (247). It was shown that patients with B-ALL, relapsed or refractory to previous anti-CD19 CAR T-cell therapy, can experience complete response when they are treated with CAR T-cells targeting CD22 (247,248). The combination of CAR T-cells targeting CD19 plus CD22 can induce high response rates in patients with relapsed B-ALL. After one T-cell infusion, 20 of 24 (83.3 %) patients experienced a complete response. In the 16 of 24 patients who received two applications, the objective response rate was 100 %. 15 patients experienced complete responses and one patient a partial response (249).

BCMA

B-cell maturation antigen (BCMA) is an antigen that is only expressed on normal and malignant plasma cells. In most cases of multiple myeloma, BCMA expression can be observed. (250).

Table 12: Results of CAR T-cell therapy targeting BMCA in MM

Type of B-cell malignancy	Pat.	Results	Ref.
MM	12	2 very good PR, 1 CR	(251)
MM	33	85 % OR, 45 % CR	(252)
MM	25	5 PR, 5 very good PR, 2 CR	(253)

The first in human clinical trial of CAR T-cells targeting BCMA was a dose-escalation trial in 2016 and included twelve patients. Six patients, which were treated with the two lowest doses of 0.3 × 10^6 CAR T-cells/ kg body weight, experienced limited antimyeloma activity and only mild toxicity. One patient in the third dose level group of 3 × 10^6 CAR T-cells/ kg body weight, experienced very good partial response. Two chemotherapy-resistant patients were treated with the fourth dose level of 9 × 10^6 CAR T-cells/ kg body weight. Bone marrow plasma cells decreased form 90 % to undetectable in one patient and he experienced a complete response that lasted for 17 weeks. The second patients bone marrow plasma cells decreased form 80 % to undetectable and he experienced a very good partial remission. In both patients toxicity, including CRS, occurred (251). Another phase I study treated 25 patients in three cohorts. Cohort one was 1 × 10^8 to 5 × 10^8 anti-BCMA CAR T-cells alone. Cohort two was cyclophosphamide 1.5 g/m^2 plus 1 × 10^7 to 5 × 10^7 anti-BCMA CAR T-cells and cohort three was cyclophosphamide 1.5 g/m^2 plus 1 × 10^8 to 5 × 10^8 anti-BCMA CAR T-cells. Responses were observed in four of nine (44 %) patients in cohort one, in one of five (20 %) patients in cohort two, and in seven of eleven (64 %) patients in cohort three. Those twelve responses included five partial, five very good partial and two complete responses. On residual MM cells a decrease in BCMA expression could be observed (253). It was shown, that higher peak levels of CAR T-cells correlate with a higher antimyeloma responses (251).

Results of CAR T-cell therapy in solid tumors

Beyond CAR T-cells targeting B-cell malignancies also CAR T-cells that recognize a variety of solid tumor antigens have been developed. Tumor antigens of solid cancers are for example human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor variant III (EGFRvIII), carbonic anhydrase IX (CAIX), carcinoembryonic antigen (CEA), ganglioside (GD2), interleukin-13 receptor α2 (IL-13Rα2), mucin-1 (MUC1) and Mesothelin.

Human epidermal growth factor receptor 2

The human epidermal growth factor receptor (HER) family has four main members; HER1, HER2, HER3 and HER4 (254). HER1 was the first discovered HER and was found in 1978 (255). Today we know that the HER family plays a role in pathogenesis of several human tumors (254). HER2, also known as erb-b2 receptor tyrosine kinase 2 (ERBB2), is a receptor with tyrosine kinase activity. The dimerization of the receptor, after binding its ligand, results in autophosphorylation of tyrosine. Subsequently, an intracellular signaling pathway is initiated. These signaling pathways play a role in regulating cell growth and cell proliferation, cell survival and cell differentiation (254). In normal cells, the activity of HER2 is strictly controlled and only a few HER2 molecules are expressed on the cell surface. Thereby, growth signals are relatively weak and controllable. In some malignant tumors, an overexpression of HER2 can be observed, subsequently, multiple heterodimers are formed in the presence of ligands and resulting in enhanced cell growth and differentiation. This enhanced growth explains why HER2 overexpression is a marker of poor prognosis in cancer patients (256,257). Not all tumors do overexpress HER2, which is the requirement for a HER2-based therapy. An overexpression is common in breast cancer but also occurs in a variety of other cancer types like colorectal cancer (258), cancer of the gastrointestinal tract (259), ovarian cancer (260), sarcoma (261) or GBM (262). An efficient clinical treatment approach is the use of an anti-HER2 monoclonal antibody called trastuzumab (Herceptin®). Most common, trastuzumab is used in combination with chemotherapy in HER2 positive breast cancer (263).

The first patient treated with CAR T-cell therapy targeting HER2 had colon cancer with metastases in the lungs and liver, refractory to multiple standard treatments. After CAR T-cell therapy, the patient died due to fatal toxicity including pulmonary failure followed by hypotension and organic failure. After further investigations, it could be shown, that low levels of HER2 are expressed on lung epithelial cells. With the use of high affinity and high dose anti-HER2 CAR T-cell therapy, pulmonary failure and massive CRS were induced. Lung toxicity occurred within 15 minutes after the application (258). CARs in lower doses and lower affinity were used to treat patients with metastatic or recurrent sarcoma. Four of 17 patients achieved a stable disease, with three of them who had their tumor removed. The treatment was safe, no toxicity occurred (261). There are many ongoing trials registered on ClinicalTrials.gov in the field of CAR T-cell therapy targeting HER2 for the treatment of different tumor entities.

Table 13: Ongoing trials of CAR T-cell therapy targeting HER2

Malignancies	**Target**	**ClinicalTrials.gov**
Lung cancer	HER2, Mesothelin, Lewis-Y, PSCA, MUC1, PD-L1	NCT03198052
Breast-, ovarian-, lung-, gastric-, colorectal- and pancreatic cancer, glioma	HER2	NCT02713984
Different HER2 positive solid tumors	HER2	NCT01935843

Human epidermal growth factor receptor 3

EGFRvIII is the most common variant of mutant EGFR observed in human tumors (264). EGFRvIII is the result of a tumor specific gene mutation, in form of a deletion. The product of this mutation is EGFRvIII, a ligand-independent constitutively active protein (265). In about 30 % of GBM cases, EGFRvIII is overexpressed and it was observed that the expression of EGFRvIII is a negative prognostic indicator (266). In 2017, the first in human study of intravenous application of CAR T-cells targeting EGFRvIII was conducted in ten patients with GBM. Only one of ten patients

experienced a stabilization of the disease, ongoing for over 18 months. The therapy was proven to be safe without evidence of “off-tumor” toxicity or CRS (79).

Table 14: Ongoing trials of CAR T-cell therapy targeting EGFRvIII

Malignancies	**Target**	**ClinicalTrials.gov**
Colorectal cancer	EGFR	NCT03152435
Advanced solid tumors	EGFR	NCT03182816, NCT01869166

Carbonic anhydrase IX

The murine monoclonal antibody G 250 was observed to detect an antigen that is expressed on malignant cells of RCC, but not on normal renal epithelium. Thereby, G 250 can be used as a marker for RCC. Later, it was shown that the antigen of the G 250 antibody is the transmembrane protein CAIX, which is frequently overexpressed on RCC (267,268). CAR T-cells, targeting the tumor antigen CAIX, were used to treat patients with metastatic RCC. As a side effect, the treatment led to disturbance in liver enzymes. Liver biopsies where performed and showed that there is also an expression of CAIX on bile duct epithelium, which led to infiltration and destruction of this epithelium by CAR T-cells (269). Another group of patients with CAIX positive RCC was pretreated with monoclonal antibodies against CAIX to prevent those toxicities. These patients experienced no liver toxicities. However, no clinical responses were achieved (269). An ongoing trial (ClinicalTrials.gov NCT01826877) uses CAIX transduced DCs for the treatment of patients with metastatic kidney cancer.

Carcinoembryonic antigen

CEA is a tumor associated antigen that is overexpressed in a range of tumors, such as gastrointestinal malignancies including colon cancer, stomach cancer, rectum cancer, pancreas cancer and esophagus cancer (270). Immunotherapies using vaccines against the tumor antigen CEA resulted in objective response in some patients (271). CAR T-cells targeting CEA were tested in CEA positive gastrointestinal malignancies in 14 patients. However, no objective clinical response could be observed in any patient. As a side effect, acute respiratory toxicity

occurred, which led to premature close of the trial. It is likely that the cause of this toxicity was an expression of CEA on lung epithelium (270).

Table 15: Ongoing trials of CAR T-cell therapy targeting CEA

Malignancies	Target	ClinicalTrials.gov
Liver metastasis	CEA	NCT02850536, NCT02416466
Lung-, colorectal-, gastric-, breast- and pancreatic cancer	CEA	NCT02349724

GD2 ganglioside

In 1984, GD2 was identified as a tumor antigen that is overexpressed neuroblastoma, a childhood cancer (272). GD2 is physiologically found in developing brains and in lower levels in the brain of healthy adults, especially in the cerebellum, basal regions and in peripheral nerves (273,274).
It was shown that neuroblastoma patients possess significantly elevated GD2 levels, compared to healthy children and compared to children with other cancer types (272). Further, it was observed that the serum level of GD2 correlates with progression of the disease (272). Today, we know that GD2 is not only overexpressed in neuroblastoma but also in melanoma and types of pediatric sarcoma (272,275). In a pre-clinical neuroblastoma model, high avidity CAR T-cells against CD2 led to lethal CNS toxicities. CAR T-cells migrated, infiltrated and proliferated in the CNS and led to neuronal destruction. Especially in the regions, were low amounts of GD2 are physiologically expressed, fatal encephalitis occurred (276). Ongoing trials are testing CAR T-cell therapies in lung and cervical cancer.

Table 16: Ongoing trials of CAR T-cell therapy targeting GD2

Malignancies	Target	ClinicalTrials.gov
Lung cancer	GD2, MAGE-A1, MAGE-A4, MUC1, Mesothelin, novel cancer antigens	NCT03356808
Cervical cancer	GD2, PSMA, MUC1, Mesothelin	NCT03356795

Interleukin-13 receptor alpha 2

The receptor IL13Rα2 is a tumor-associated antigen that is expressed in GBM and only a little, if any, in normal human brain (277). A patient with recurrent multifocal GBM was treated with CAR T-cell therapy targeting IL13Rα2. CAR T-cells were administrated multiple times locally into the resected tumor cavity and the ventricular system. No higher grade toxicities occurred and regressions of all intracranial and spinal tumors were observed. However, the patient had a recurrence after seven months (76).

Table 17: Ongoing trials of CAR T-cell therapy targeting IL13Rα2

Malignancies	Target	ClinicalTrials.gov
Malignant glioma	IL13Rα2	NCT02208362
Malignant glioma	IL13Rα2, EGFRvIII, HER2, GD2, CD133	NCT03423992

Mucin-1

MUC1 is a membrane mucin, which is expressed by normal glandular epithelial cells. When these cells transform to malignant cells, the expression of MUC1 is increased (278). In cancer cells, changes in glycosylation of MUC1 occur and result in the Tn glycoform of MUC1 (Tn-MUC1). These changes have been observed to increase tumorigenesis and metastasis. Tn-MUC1 is expressed as a neo-antigen on a variety of cancer types (279,280). The use of anti-Tn-MUC1 CAR T-cells in mouse models of T-cell leukemia and pancreatic cancer led to a specific tumor attack, including successfully controlled tumor growth (280).

Table 18: Ongoing trials of CAR T-cell therapy targeting MUC1

Malignancies	Target	ClinicalTrials.gov
MUC1 positive solid tumors	MUC1	NCT03330834
Lung cancer	MUC1, HER2, Mesothelin, Lewis-Y, PSCA, PD-L1	NCT03198052
Lung cancer	MUC1, MAGE-A1, MAGE-A4, GD2, Mesothelin, novel cancer antigens	NCT03356808
Cervical cancer	MUC1, GD2, PSMA, Mesothelin and others	NCT03356795
HCC, NSCLC, pancreatic cancer, triple-negative invasive breast cancer	MUC1	NCT02587689

Mesothelin

Mesothelin is expressed on mesothelial cells, such as cells of the pleura, pericardium, peritoneum, fallopian tubes, trachea, tonsils and cornea (281). Mesothelin is a potential target for immunotherapy because of its low expression on normal mesothelial cells and high expression on a wide range of solid tumors (282). As observed in mouse models, mesothelin seems not to have any essential biological functions. Mesothelin knockout mice showed normal development, reproduction and blood cell count (283). However, the aberrant expression of mesothelin was observed to play a role in tumor development and to influence the aggressiveness of tumors by promoting proliferation, local invasion and metastasis and by giving resistance to apoptosis (284,285). Examples for cancer types that overexpress mesothelin are mesothelioma, pancreatic cancer, ovarian cancer, cholangiocarcinoma, breast cancer or gastrointestinal cancer types (281,282).

The use of short living CAR T-cells targeting mesothelin, to reduce toxicities, led to stable disease and to a partial clinical response in one patient with malignant pleural mesothelioma and one patient with pancreatic cancer. No evidence of “off-tumor, on-target” toxicity was observed (286).

Table 19: Ongoing trials of CAR T-cell therapy targeting mesothelin

Malignancies	Target	ClinicalTrials.gov
Lung cancer	Mesothelin, MAGE-A1, MAGE-A4, MUC1, GD2, novel cancer antigens	NCT03356808
Cervical cancer	Mesothelin, GD2, PSMA, MUC1	NCT03356795
Mesothelin positive tumors	Mesothelin	NCT02930993
Pancreatic cancer	Mesothelin	NCT02465983

Other targets tested in ongoing trials of CAR T-cell therapy

Currently there are several ongoing clinical trials registered on ClinicalTrials.gov, which are assessing the efficacy and safety of CAR T-cell therapy in solid tumors, targeting different antigens.

Table 20: Some selected ongoing clinical trials of CAR T-cell therapy in solid cancer

Malignancies	Target	ClinicalTrials.gov
NSCLC	PD-L1	NCT03330834
HCC	GPC3	NCT02723942, NCT02715362, NCT03198546, NCT03130712, NCT02395250, NCT02905188
HCC	ET1402L1	NCT03349255
Colorectal-, ovarian-, breast- urothelial-, and pancreatic cancer	NKR-2	NCT03370198, NCT03018405
Colon-, esophageal-, hepatic- pancreatic-,	EpCAM	NCT03013712, NCT02915445

prostate-, gastric-, and nasopharynx cancer		
Pancreatic cancer	PSCA	NCT02744287
Liver- and stomach cancer	EPCAM	NCT02729493, NCT02725125
Sarcoma, osteoid sarcoma, Ewing sarcoma	Sarcoma-specific targets	NCT03356782
Prostate cancer	PSMA, TGFβRDN	NCT03089203
Bladder- and urothelial cancer	PSMA, FRa	NCT03185468
Melanoma, renal cancer	VEGFR2	NCT01218867
Nasopharyngeal cancer	LMP1	NCT02980315
Liver-, pancreatic-, brain-, breast-, ovarian- and colorectal cancer, AML, ALL	CD133	NCT02541370
Melanoma, breast cancer	cMET	NCT03060356, NCT01837602
NSCLC, triple negative breast cancer	ROR1	NCT02706392
Pancreatic-, renal-, breast- and ovarian cancer, melanoma	hCD70	NCT02830724

Toxicities of CAR T-cell therapy

Cytokine release syndrome

CRS is a systemic inflammatory response. The causes of this inflammatory process are elevated levels of cytokines, which are released by immune cells. Examples for those cytokines are IFN-γ, IL-6 or TNF (231). Sever CRS occurred after different forms of CAR T-cell therapies, such as CARs targeting CD19, CD22 or BCMA (232,234,235,239,240,247).

Pathophysiology: The available data suggest that CAR T-cells are expressing these high levels of cytokines when they get in contact with their targets. For example, when anti-CD19 CAR T-cells get in contact with normal and malignant

B-cells. It could be shown that CAR T-cells are the producers of these cytokines because it was observed that peripheral blood T-cells produced TNF and IFN-γ in a CD19-specific manner after the anti-CD19 T-cell infusions (232).

Symptoms of the CRS: There are different manifestations of CRS. From a mild and self-limitation form with slight symptoms such as fever and myalgia, to severe and life threatening manifestations with symptoms like vascular leak, hypotension, tachycardia, respiratory insufficiency, renal failure, cytopenias and coagulopathy (231,232,234,235,237,239,240).

Associations: The manifestation of the acute toxicities correlates with the serum levels of IFN-γ and TNF (232). There is also a correlation between the severity of CRS and the blood levels of CRP and IL-6. Thereby, CRP might be a biomarker to predict sever CRS (233). An association between the severity of CRS and the disease burden before T-cell administration could be observed in patients with ALL (231,234). The dose of administrated CAR T-cells also correlates with the severity of CRS (233).

Treatment: Patients with CRS often require hospitalization. In mild forms of CRS, patients receive supportive medication. Sever forms of CRS require intensive care, including respiratory support and vasopressor support. An effective way of treating sever CRS is the use of anti-IL-6 receptor antibodies (tocilizumab). Tocilizumab leads to a rapid decrease of fever and to a stabilization of the blood pressure. Glucocorticoids, as a high dose lymphotoxic steroid therapy, can also be used to treat CRS. Corticosteroids decrease the inflammatory response and reduce levels of cytokines through a reduction of transduced T-cells (231,233,234,239). Methotrexate may also help to limit complications of CRS (31).

Prognosis: In nearly every case, CRS symptoms are fully reversible (233). The serum levels are only elevated temporally with a peak during the first weeks after T-cell application and completely resolve over time (232,240).

An approach to minimize toxicity could be the reduction of inflammatory cytokines levels, because it is proven that levels of cytokines correlate with the severity of CRS (232). Decreased levels of cytokines were observed in CAR T-cell therapies using a costimulatory signaling domain called 4-1BB, instead of CD28. Thereby, this may be one approach to reduce CRS in the future (228).

Neurotoxicity and encephalopathy

Neurological toxicities occurred in patients who received CAR T-cells targeting CD19 and BCMA (231,233,235,239).

Pathophysiology: It is not clear if a connection between CRS and neurotoxicity exists. It is not known if neurotoxicity is an extreme manifestation of CRS, or if a separate mechanism is the cause of neurological toxicities (208). However, in patients with severe neurotoxicity after anti-CD19 CAR T-cell therapy, more sever CRS occurred. There is evidence of endothelial activation, accompanied by disseminated intravascular coagulation, capillary leak and increased blood-brain-barrier permeability. Due to the higher permeability, higher concentrations of cytokines can traffic into the cerebrospinal fluid and cause endothelial activation and endothelial injury there. These findings indicate an association between neurotoxicity, vascular dysfunction and high concentrations of serum cytokines. However, in nine percent of the patients with neurological adverse effects no CRS occurred, but in these cases neurotoxicity was mild (287).

Symptoms: The neurological symptoms range from delirium and confusion to hallucinations, aphasia and seizures. Facial paresis occurred in one patient and there are reports of patients with an abnormal MRI. However, these abnormalities resolved within two weeks (231,233,235,239). Platelet microthrombi, hemorrhage and edema can occur in severe cases (287).

Associations: There was a correlation observed between the penetration of CAR T-cells in the cerebrospinal fluid and neurotoxicity. Patients with neurotoxicity have evidence for CNS trafficking of CAR T-cells (233). Neurotoxicity is also associated with higher tumor burden and the dose of CAR T-cells administered (287). Patients with more sever neurotoxicity also suffered from more sever CRS (287).

Treatment: As in patients with CRS also in patients with neurotoxicity, tocilizumab, an IL-6 monoclonal antibody is used as treatment. Tocilizumab can be administered in combination with corticosteroids (287).

Prognosis: The neurotoxicity that occurred in patients after anti-CD19 CAR T-cell therapy was self-limiting and reversible in almost every patient (231,233,235,239). In the autopsy of patients who died after anti-CD19 CAR T-cell therapy, multifocal microhemorrhages, platelet microthrombi and parenchymal necrosis in the pons, medulla and spinal cord were diagnosed (287).

Pre-clinical: In a pre-clinical neuroblastoma model, high avidity CAR T-cells against CD2 led to lethal central nervous toxicity. Especially in regions of the brain where low amounts of GD2 are expressed, the fatal encephalitis occurred, as discussed in more detail on page 75 (276).

B-cell aplasia

The absence of B-cells is called B-cell aplasia. In the context of CAR T-cell therapy, it is caused by targeting CD19 positive cells. However, in some studies the level of B-cell aplasia was not determined because of a preexisting B-cell depletion due to the chemotherapy regime (235).

Pathophysiology: Almost every patient with a response to anti-CD19 CAR T-cell therapy develops B-cell aplasia. In patients who did not respond to CAR T-cell therapy, no evidence of B-cell aplasia could be observed (153,231,239). Since all types of B-cells are depleted by anti-CD19 CAR T-cells, hypogammaglobinemia occurs, this means that the total serum immunoglobulin or antibody levels decline (231,288). However, it was shown that some immunoglobulins, that are specific to pathogens or vaccines, remained in the serum. Antibodies are short-lived proteins with a half-life time from one week to one month in serum. This fact indicates, that plasma cells, which produce these antibodies, must be present long-term. This can be maintained by long living plasma cells or by the development of plasma cells out of long living memory B-cells.
In bone marrow biopsies of patients who received CAR T-cells against CD19, plasma cells, capable to produce antibodies, could be found months after the administration, despite the absence of CD19 positive B-cells. This suggests, that there is a long lasting immunity through long living plasma cells, which keep on working independent from memory B-cells. Subsequently, it could be shown that two fractions of plasma cells exist: CD19 positive and CD19 negative plasma cells, whereas the CD19 positive fraction varies from approximately 55 % to 90 % of all plasma cells. These findings led to the assumption that anti-CD19 CAR T-cells spare the fraction of CD19 negative plasma cells, leaving previously established humoral immunity intact (288).

Treatment and prognosis: For the management of B-cell aplasia intravenous immunoglobulin are administrated (230-232). B-cell aplasia was observed to can persist for years (288).

Tumor lysis syndrome

Tumor lysis syndrome is a potentially life threatening condition, that especially develops in patients with a large tumor burden. In most cases, it occurs due to cytotoxic therapies, but it can also arise spontaneously because of massive tumor cell lysis (289). Clinical characteristics of tumor lysis syndrome are acute renal failure, hyperuricemia, hyperkalemia, hyperphosphatemia, hypocalcemia and increased lactate dehydrogenase levels (240,289).

Organ toxicities

Lung toxicities: High affinity CAR T-cells against HER2 were administrated to a patient with colon cancer, metastatic to the lungs and liver. T-cell therapy led to pulmonary failure, because low levels of ERBB2 are expressed on lung epithelial cells, as review in detail on pages 72 and 73 (258).
Anti-CEA CAR T-cells in a higher dose induced acute respiratory toxicity. It is likely that CEA is also expressed on lung epithelium (270).
Liver toxicity: CAR T-cells against CAIX used to treat patients with kidney tumors, led to disturbance in liver enzymes. The reason for this toxicity is, that CAIX is also expressed on epithelium of the bile duct (269). This clinical trial is further described on page 74.

Limitations of CAR T-cell therapy

Different factors are known to be limitations for a successful CAR T-cell therapy in different cancer types. Including that only surface antigens can be recognized by CARs, antigen loss that can occur after CAR T-cell therapy, lack of persistence in CAR T-cells or the development of antibodies against CARs.

Only surface antigens

CARs, compared to TCRs, are only able to recognize antigens, which are expressed on the cell surface. However, most antigens are expressed intracellular and only 28 % on the surface. Thereby, this is a limitation for CARs because this big group of intracellular antigens cannot be detected by them (206).

Antigen loss

The loss of the targeted antigen is another limitation of CAR T-cell therapy, which can be observed. The antigen loss leads to resistance of the tumor to the therapy

and ends in a relapse of tumor growth. For example, in patients with B-cell malignancies treated with CAR T-cells targeting CD19, a loss of CD19 expression in tumor cells occurred in some patients. Subsequently, therapy resistance and relapse of the malignant disease was the consequence (231,233,238,239). In CAR T-cells targeting CD22, an association between relapse and reduced density of the CD22 antigen on tumor cells was described (247).

In patients with GBM, treated with anti-EGFRvIII CAR T-cells, the expression levels of EGFRvIII declined in five of seven patients. These patients undergo tumor surgery after T-cell therapy and thereby the tumor mass could be analyzed post-infusion. The highest levels of CAR T-cells in the tumor were detected at early points, within 14 days after infusion. These levels were consistent with levels in the peripheral blood. Three months after the infusion, no CAR T-cells were detectable in the tumor, despite continued low levels in the blood. These findings led to the assumption that antigen expression decreased in the tumors of some patients. Additionally, an increase of immunosuppressive factors like PD-L1 and increased numbers of Tregs were observed in the tumor microenvironments (79). After CAR T-cell therapy targeting IL13Rα2 in patients with GBM, only IL13Rα2 negative tumor cells remained and expanded (76).

In patients with MM treated with CAR T-cells therapy against BCMA, a progression of BCMA negative MM cells could be observed in some patients. In a trial with twelve patients, one patient developed a partial loss of BCMA (251). In another trial a decreased BCMA expression was observed on residual MM cells (253). A potential solution for this issue might be combinatorial surface targeting, as described in the chapter “new approaches in adoptive cell therapy” on pages 89 and 90.

Lack of persistence of transferred T-cells

The lack of persistence of CAR T-cells after infusion is another limitation of this approach of tumor treatment (208). However, there are solution strategies to overcome this issue, like the use of memory cells. In a trial conducted in patients with B-cell non-Hodgkin lymphoma, autologous central memory-enriched T-cells (TCM) were engineered to express anti-CD19 CARs. Disease assessments were implemented at baseline and at 60 days, six months, twelve months, 18 months and 24 months after the conditioning regimen. Four of eight patients (50 %) treated with $CD8^+$ TCM were progression free after one year. And six of eight patients (75 %)

treated with $CD4^+$ and $CD8^+$ TCM were progression free after one year. No CRS or delayed hematopoietic engraftment were observed in any patient, thereby this trial demonstrate the safety and feasibility of anti-CD19 CAR TCM therapy (290).

Another way to affect the persistence and effectivity of CAR T-cells is the selection of the costimulatory signals. In a mouse model with B-cell ALL, CAR T-cell therapy against CD19 was conducted. It was shown that T-cells expressing CARs, which were containing the domain 4-1BB, led to the greatest anti-tumor efficacy and to prolonged (more than six months) survival *in vivo*. These CARs were significantly more effective than CARs containing TCR-zeta alone or CD28 costimulatory signals. This study suggests that the use of the 4-1BB domain in CARs can improve the persistence of administrated cells and thus increase their anti-tumor activity (291).

Development of antibodies

The development of antibodies against CARs, containing binding domains from murine monoclonal antibodies (murine scFvs), results in shorter persistence of transferred T-cells. These antibodies can lead to a premature elimination of CAR T-cells and can thus increase the risk of tumor relapse (208).

A solution for this issue is the use of humanized scFv. In a trial scFv of fully human origin specific to CD19 were developed by screening for human anti-CD19 scFvs in human Ab-chain-libraries. Several CARs were constructed form several scFvs and were transduced into human T-cells. These CAR T-cells were able to eliminated human lymphoma xenografts in immunodeficient mice. The human CARs were superior to murine derived CARs. For a further reduction of the immunogenicity of these CARs, fusion sites between different CAR components were modified, because these fusion sites can be potentially immunogenic. The binding to MHCI molecules is a requirement for peptides to be recognized by cytotoxic T-cells, peptides of these fusion sites were analyzed for their potential to bind to human MHCI using the NetMHC prediction algorithm. The fusion site between CD28 and 4-1BB costimulatory domain was identified and to reduce the potential of this site to provide immunogenic epitopes, CD28 was modified and tested *in vitro*. No significant differences between the original and the new fusion site in cytotoxicity, CRS and proliferation could be observed, which demonstrate that these optimized CARs can be generated and used. Thereby, this trial suggests that fully human

CARs may reduce the rejection by the immune system, compared to murine-based CARs (291).

CAR T-cell production

CAR T-cells have to be custom made out of autologous T-cells for every patient. This is not always possible, especially for patients who do not have sufficient healthy T-cells. For example, in heavily pretreated cancer patients or infants (221). CAR T-cells derived from healthy donors can be an alternative (208), as discussed in detail in the chapter “Universal CAR T-cells” on pages 86 to 88.

4.6 New approaches in adoptive cell therapy

There are several approaches to expand and improve the use of adoptive transferred T-cells in cancer therapy.

Universal CAR T-cells

The access to CAR T-cell therapies is still limited because widespread application needs specialized manufactories, expertise and logistical support to gather, manipulate and consign these genetically engineered cells (221). Autologous T-cells are required for the production of CAR T-cells. However, in patients who do not have sufficient healthy T-cells, like heavily pretreated cancer patients or infants, it is not always feasible to produce effective CAR T-cells (221). In some cases it is possible to use donor T-cells from HLA-matched allogeneic hematopoietic stem cell donors. The use of allogeneic T-cells, genetically engineered to express a CAR targeting CD19, led to remission in eight of 20 treated patients. The patients were pretreated with allogeneic hematopoietic stem cell transplantation and the allogenic T-cells were also taken from the donors (292). Other issues that occur in cancer patients are immune defects associated with cancer treatment, like chemotherapy. These immune defects can be overcome by using healthy donor CAR T-cells (208). To overcome the limitation that suitable T-cells for therapy are only T-cells, which are either autologous or MHC-matched allogeneic, the idea of universal CAR T-cells arose. These universal CAR T-cells would also simplify the manufacturing process, make the production more rapid and less expensive. Thus, these CAR T-cells may be used even as “off-the-shelf” medications. Therefore, this new approach of engineering CAR T-cells uses T-cells derived from non-HLA-matched donors (208).

A pre-clinical study showed that allogeneic T-cell precursors can be transferred to irradiated individuals independently of MHC inequality. These allogeneic T-cell precursors gave rise to host-MHC-restricted and host-tolerant T-cells, and resulted in improved survival and enhanced anti-tumor responses in irradiated recipients. In the pre-clinical model, bone marrow stroma cells were used for the *in vitro* generation of T-cell precursors. To determine the effectivity of these cells, BALB/c mice were treated with hematopoietic stem cells plus the *in vivo* generated T-cell precursors derived from C57BL/6 mice. The control group were mice that received hematopoietic stem cells alone. Transferred T-cells showed tolerance to host and donor. The anti-tumor activity was significantly increased in the study group compared to recipients of hematopoietic stem cells only, as determined by survival and *in vivo* bioluminescence imaging (293). Additional anti-tumor activity could be conducted by using CARs in precursor T-cells. Therefore, T-cell precursors were further engineered to express a CAR targeting CD19. The results showed that engineered T-cell precursors give rise to antigen-specific host-tolerant T-cells with cytotoxic activity, migration to the site of antigen expression and persistence for at least two months. These findings suggest that *ex vivo* generated MHC-disparate lymphoid precursor cells from any donor can be transferred to any individual irrespective of MHC disparities universally as “off-the-shelf” immunotherapy (293).
The use of donor-derived T-cells, engineered to express CARs, can lead to problems because the TCRs on the infused allogeneic T-cells may recognize MHC antigens of the recipient and thus induce a graft-versus-host disease (GVHD). Therefore, the idea to generate a universal allogeneic T-cell from one donor that might be used to treat multiple patients arose. For this reason, genetically engineered T-cells were produced by introducing an anti-CD19 CAR to the T-cell. Subsequently, the alpha or beta TCR chains were removed with designer zinc finger nucleases. The aim was to prevent GVHD without compromising the effector functions of the CAR T-cells. Results showed specificity of the T-cells for CD19 without responding to TCR stimulation, no TCR activity could be observed (294). A pilot trial using TALEN (transcription activator-like effector nuclease) demonstrated the feasibility of “off-the-shelf” universal CAR T-cell therapy specific to CD19. Universal CAR T-cells derived from T-cells of non-HLA-matched, healthy donors were used. Gene editing of T-cells using TALEN was performed to edit the alpha chain of the TCR to reduce the risk of GVHD. A second TALEN was used to

disturb the CD52 expression, which enables infused cells to escape the depletion effects of alemtuzumab (an anti-CD52 antibody used for lymphodepletion) and enhances the survival of transferred cells. The approach led to molecular remission in the two treated patients with B-ALL after infusion of universal TALEN gene-edited CAR T-cells from non-HLA-matched donors. GVHD occurred in one patient, which received mismatched MHC T-cells. The GVHD was associated with the expansion of non-edited T-cells that still expressed endogenous TCRs. These results suggest that a more complete editing will be required in the future. Engraftment was limited in both patients (221).

It was observed that MHCI deficient cells are recognized and eliminated by NK-cells, despite immunosuppression using alemtuzumab. Thus, a limitation of the engraftment of administrated cells is the consequence. Disruption of the B2M gene (Beta-2 Microglobulin) eliminates the surface expression of all MHCI molecules. $B2M^{-/-}$ human pluripotent stem cells were used in studies to eliminate the expression of MHCI, to prevent the stimulation and activation of allogeneic $CD8^{+}$ T-cells (295). Further, it was observed in mice models, that MHCI negative cells are lysed by NK-cells because of "missing-self" response (296). This limitation of "missing-self" response can be prevented by the expression of minimally polymorphic HLA-E molecules. The use of HLA-E expression without surface expression of HLA-A, HLA-B and HLA-C on universal CAR T-cells prevented administrated T-cells from NK-cell attack and reduced the stimulation of allogeneic T-cells, which would have induced GVHD otherwise (297). Due to the rapid progress of universal CARs it is likely, that universal CAR T-cells will be widely used in cancer patients in the future. The question is, if these T-cells will be able to serve as a stand-alone therapy or if they will act as a bridge for a definitive therapy, such as autologous CAR T-cell therapy or stem cell transplantation (208).

Genome editing

Many technologies, which allow genome editing by inducing double strand breaks in the DNA and inserting or deleting genes afterwards, are known. Examples for these gen editing tools are TALEN, CRISP-cas9 nucleases and zinc finger nucleases. All of them have been successfully applied to engineer T-cells (208). With the use of genome editing it is possible to eliminate immunosuppressive signals such as CTLA-4 and PD-1, which results in enhanced T-cell function.

This might be possible without inducing toxicities, which occur by global blockade of these immune checkpoint molecules (298). Target antigens that are not only expressed by the tumor but also by T-cells, are a limitation of CARs. The elimination of these target genes with help of gene editing may be an approach to overcome this restriction. In a study, the feasibility of targeting the CS1 antigen in patients with MM was evaluated. CS1 is a cell-surface glycoprotein which is highly expressed on tumor cells of MM. However, CS1 is also expressed on normal $CD8^+$ T-cells, which can result in attack of those cells by CAR T-cells and thereby, impacting the number and the phenotype of the final CAR T-cell population. As a solution for this problem, TALEN gene-editing technology was used to delete CS1 in CAR T-cells. The results showed that non-gene-edited T-cells, which were engineered to express anti-CS1 CARs, showed limited cytolytic activity against MM cells and resulted in a progressive loss of $CD8^+$ T-cells. In comparison, the CS1-gene-edited CAR T-cells were observed to have significantly increased cytolytic activity and the percentage of $CD8^+$ T-cells remained unaffected. Additional experiments in mice with MM showed that CS1-gene-edited CAR T-cells are capable to induce an *in vivo* anti-tumor activity. Subsequently, this strategy was tested in “off-the-shelf” CAR T-cells, which have an inactivated TCR-alpha gene to reduce GVHD. The results showed that multiple genome editing is practicable. This approach of developing double knockout T-cells (TCR-alpha and CS1) allows the production of allogeneic, non-alloreactive CS1 specific T-cells with enhanced anti-tumor activity in a large scale. These allogenic T-cells could be easily available to treat many MM patients (299). The introduction of a CAR into the locus of a TCR using CRISPR/Cas9 genome editing was performed in another genome editing trial (300). By targeting the CAR coding sequence to the TCR locus, the CAR gets placed under the control of endogenous regulatory elements. Results showed that this approach prevents chronic CAR signaling, leads to internalization and re-expression of the CAR after single or repeated exposure to the antigen and delays the exhaustion of effector T-cells. Especially these CAR T-cells showed increased therapeutic potency in a mouse model of ALL, compared to conventional CAR T-cells. This study demonstrated the impact of a tight regulation of CAR expression, on an effective tumor eradication. Another advantage of this approach is, that the risk of TCR-induced alloreactivity is limited (300).

Targeting more antigens

Some antigens recognized by anti-tumor CAR T-cells are also expressed on healthy tissues in small amounts. Therefore, approaches which only detect one antigen are limited because of their inability to discriminate between tumor and normal tissue. Another limitation is the issue of antigen loss, which is a problem when T-cells are only targeting one antigen. To increase the specificity of ACT to tumors, approaches were developed which target two or more antigens and thus increase the “on-target” activity and decrease the “off- tumor” toxicity (208).
A strategy to produce T-cells that are specific for tumor cells, even in the case when no tumor-specific antigens are known, was developed. For this reason, T-cells which express two separate CARs were designed. One CAR, that recognizes an antigen and another chimeric costimulatory receptor (CCR), that recognizes a second antigen, were used. The antigens used in this trial were the prostate tumor antigens PSMA (prostate-specific membrane antigen) and PSCA (prostate stem cell antigen). These antigens are found on metastatic prostate cancer, however, they are not absolutely prostate-specific. They are also found in the kidney, liver, colon, and brain astrocytes. Therefore, the combination of targeting PSCA and PSMA was expected to increase prostate cancer specific targeting and to reduce reactivity against healthy tissues expressing one antigen alone. The results showed that co-transduced T-cells only destroy tumors that express both antigens. Tumors that are only expressing one antigen alone were not affected by the engineered T-cells. This strategy of “tumor-sensing” may help to achieve a widespread applicability of CARs and may help to avoid some of the side effects induced by “off-tumor” targeting (301). Another approach to enhance the “on-target” activity of T-cells is the use of a synthetic Notch receptor (synNotch), which also leads to T-cells that requires two antigens for activation. The synNotch receptor is specific for one antigen and induces the expression of a CAR that recognizes a second antigen, when it gets activated. When ligand A, which is recognized by the synNotch receptor, binds to the extracellular part of this synNotch receptor, an intracellular transcription factor gets released from the intracellular part of the receptor. However, unlike CARs, binding of the target antigen does not trigger T-cell activation. Rather, this transcription factor traffics to the nucleus of the cell and regulates the transcription of genes encoding for a CAR, which is specific to a second antigen B. The CAR protein is translated and expressed on the cell surface. When ligand B

binds the expressed CAR, the T-cell gets activated. Therefore, the T-cell gets only activated in the presence of a tumor cell expressing both antigens, A and B. These combinatorial T-cells showed discrimination in mouse models, they distinguished between single antigen tumors, which were spared and combinatorial antigen tumors, which were efficiently eradicated (302).

Inducible apoptosis – suicide switch

In some patients, treated with ACT, severe adverse events occurred (258,287). To reduce the risk of toxicities, approaches to induce death of the transfused T-cells, called suicide switch, were developed. This switch enables a quick elimination of the infused cells in case of severe toxicities. (208).
There is one approach using the suicide gene inducible caspase-9 (iCasp9). The iCasp9 gene consists of the sequence of FKBP12, connected to the gene encoding pro-apoptotic caspase 9. FKBP12 is a binding protein that binds to a small-molecule dimerizing agent, called AP1903, with high affinity.
In the presence of the drug AP1903, two iCasp9 promolecule dimerizes and thus the intrinsic apoptotic pathway gets activated and ends in cell death.
This approach was tested in patients who received donor T-cells to enhance immune reconstitution after allogenic stem cell transplants and patients who developed GVHD afterwards. Patients were treated with infusions of iCasp9-expressing donor T-cells. Only one dose of a dimerizing drug led to an elimination of more than 90 % of the modified T-cells within 30 minutes and it led to a stop of the GVHD without a relapse. Therefore, this findings suggests that the use of the iCasp9 system may enhance the safety of ACT and expand their clinical applications because there is the ability to stop GVHD (303). Engineered iCasp9 cells were detectable in patients for over two years after the transfer, what indicates that this construct is not immunogenic (304). Another suicide gene approach is the use of herpes simplex virus-thymidine kinase (HSV-TK). The insertion of these genes into T-cells makes them susceptible to the antiviral medication ganciclovir. The approach was used successfully in patients who developed GVHD after allogeneic transplantation in 1997. Eight patients with relapsed lymphoma after bone marrow transplantation were treated with donor lymphocytes that were transduced with the HSV-TK suicide gene. In three patients GVHD occurred but it could be controlled by ganciclovir-induced elimination of the transduced cells (305). A limitation of this approach is, that HSV-TK is a highly immunogenic virus-derived protein and

therefore cells which are expressing it can be immunologically rejected, what can result in limited cellular persistence (306). Another disadvantage is, that the mechanism to activate the suicide gen requires interference with DNA synthesis, resulting in prolonged delay in clinical effects because cell killing may take a few days (307). Truncated human EGFR polypeptide (huEGFRt) is another suicide gene. HuEGFRt is a shortened epidermal growth factor receptor gene and does not have extracellular ligand binding domains and intracellular receptor tyrosine kinase activity anymore. However, is has an intact binding site for the anti-EGFR monoclonal antibody cetuximab. T-cells transduced with huEGFRt additionally to a CAR result in T-cells that are a target for cetuximab-mediated cellular cytotoxicity. Thus, an *in vivo* elimination of these cells is possible (308). In pre-clinical studies the use of cetuximab led to antibody-dependent cytotoxicity of huEGFRt T-cells. Clinical trials incorporating huEGFRt T-cells are currently enrolling patients (309).

"On-switch" CARs

In "on-switch" CARs the antigen-binding and intracellular-signaling domains of the receptor are separated into two components. Both parts of this receptor contain heterodimerization domains that interact after binding of a heterodimerizing small-molecule. Thereby, those two parts only get together in the presence of the small-molecule dimerizer. This allows physicians to control timing and dosage of T-cell activity and thereby limit toxicity, by using pharmacologic small-molecule dimerizer (310). In mouse models *in vitro* and *in vivo* killing of target cells could be observed using "on-switch" CAR T-cells (311).

Temporary receptor activation

To enhance the safety of ACT, strategies to control the duration, location and timing of engineered receptor activity were developed. One approach is the electroporation of the receptors RNA into T-cells, because RNA does not integrate into the cells genome and is a short-lived molecule *in vivo*. Results are T-cells with self-limited receptor expression. If toxicities occur due to the ACT, the receptor expression on transferred cells will stop spontaneously within several days (309). This approach was tested in a pre-clinical model, where RNA encoding a CAR against mesothelin was transduced into T-cells. Multiple applications of these CAR T-cells led to anti-tumor effects, significantly prolonged survival and reduced tumor burden (312). In the first in human study of anti-mesothelin mRNA CAR T-cells in

patients with solid mesothelin-expressing malignancies, clinical evidence of tumor responses and induction of an anti-tumor immune response could be observed (313).

Inhibitory CARs

CARs, which are containing antigen-specific inhibitory signals are called inhibitory CARs (iCARs). These antigen-specific iCARS were developed with the use of CTLA-4 and PD-1 as inhibitory signals. To generate iCARS, the signaling domains of coinhibitory receptors like CTLA-4 or PD-1, are attached to a scFv region that recognizes structures on normal tissues. These iCARs, which are targeting antigens of healthy tissue, and CARs, which are targeting the actual tumor target, get introduced into the same T-cell. The results are T-cells with “on-tumor” and “off-target” activity. When iCARs recognize their antigen, an acute inhibitory signaling is induced to limit T-cell response, despite simultaneously binding of the CAR to its tumor antigen. Therefore, this approach makes use of the natural physiology of T-cell and regulates T-cell responses in an antigen-selective manner. Thus, iCARs make it possible to discriminate between target and “off-target” cells. *In vitro*, it was observed that these CTLA-4 or PD-1 based iCARs are able to suppress cytokine release, cytotoxicity and cellular proliferation after their exposure to targets, that express antigens which are recognized by both receptors, the stimulatory CAR and the inhibitory iCAR (314).

4.7 ACT in solid tumors

ACT is able to achieve good clinical responses and results, especially in hematologic malignancies and melanoma (37,231). However, the use of CARs in epithelial solid cancers, which account for ~90 % of all cancer fatalities, is limited because suitable targets, that are exclusive found on cancer cells, are lacking (136). However, T-cell therapy was also tested in solid tumor types in several clinical trials. Four examples of ACT in solid tumors are discussed below; lung cancer, pancreatic cancer, the childhood cancer neuroblastoma and glioblastoma.

Lung cancer

Lung cancer cells have a high mutation burden, which makes them vulnerable to T-cell attack. Following melanoma it has the highest mutation rate and the result of these mutations is the expression of neo-antigens, which can be recognized and attacked by T-cells (315).

TIL therapy in lung cancer

In 1996 a study evaluated TIL therapy in 131 patients with NSCLC. The results showed a significant improvement of the overall survival in patients treated with TILs, compared to those treated with standard chemoradiotherapy (189). In a trial in 2018 the feasibility of ACT using TIL in NSCLC was investigated. TIL cultures of patients with lung cancers were successfully established and expanded to treatment levels. TILs from lung cancer were observed to expand similar to TILs form melanoma. For the evaluation of the anti-tumor reactivity of these TILs, autologous tumor cell cultures were established and co-cultured with the TILs, subsequently the IFN-γ secretion was measured. Two out of three NSCLC patients had at least one TIL culture with anti-tumor reactivity. These findings may be the basis for a new treatment of patients with NSCLC (62). Additionally it was observed that high levels of TILs in the tumor masse are associated with improved recurrence-free survival in stage 1A NSCLC and with a reduction of the likelihood of systemic recurrence (190).

Genetically engineered T-cells in lung cancer

Cancer testis antigens are potential targets for engineered T-cells in ACT. Cancer testis antigens are expressed by a variety of malignant tumors and also by lung cancers. Examples for cancer testis antigens expressed by NSCLC are MAGE, NY-ESO-1, SP17 or CABYR (213). However, it was observed that immune responses to these cancer testis antigens are rare in patients with lung cancer, due to low levels of expression of them, below the threshold for recognition by the immune system (316). A strategy to overcome this limitation is the upregulation of the cancer testis antigens expression by chromatin-remodeling agents. This strategy might improve the immunogenicity of lung cancer cells and thus enhance their eradication by adoptively transferred T-cells (317).

FRA (folate receptor-alpha) is an antigen that lung cancer cells express, these findings suggests that FRA might be a target for CAR T-cell therapy against the majority of lung cancers, especially adenocarcinomas (318,319). Other antigens that might be potential targets for CAR T-cell therapy are EGFR, HER2, mesothelin, GPC3, mucin 1, immunomodulatory antigens like PD-L1 and many others (205).

Clinical results of ACT in lung cancer

CARs were used to treat patients with EGFR-positive NSCL. The anti-EGFR CAR T-cells led to stable disease in five of 11 patients and two patients obtained a partial response. In tumor biopsies an eradication of EGFR-positive cancer cells could be detected. The therapy was well tolerated and no severe toxicity occurred (320). In another trial CAR T-cells against PSCA and MUC1 were tested in mouse models of human NSCLC. The trial showed specificity and efficacy of CAR T-cells targeting PSCA and MUC1 *in vitro*. Additionally it could be shown that PSCA-targeting CAR T-cells can efficiently suppress the growth of NSCLC in mice. Subsequently, it was observed that combinatorial targeting of both antigens can further enhance the anti-tumor effect (321). There are several ongoing trials testing T-cell therapy in lung cancer registered. A few examples are listed in the table below.

Table 21: Ongoing trials testing T-cell therapy in lung cancer

Malignancies	**ACT**	**Target**	**ClinicalTrials.gov**
NSCLC	CAR	MUC1	NCT03525782, NCT02587689,
NSCLC	CAR	ER2, Mesothelin, PSCA, MUC1, Lewis-Y, or CD80/86	NCT03198052
Advanced lung cancer	CAR	PD-L1	NCT03330834
Lung cancer	CAR	CEA	NCT02349724
Squamous cell lung cancer	CAR	GPC3	NCT03198546
Adenocarcinoma	CAR	Mesothelin	NCT03054298

NSCLC	TCR	NY-ESO-1	NCT03029273

Clinical results of other immunotherapies in lung cancer

Immunotherapy using checkpoint inhibitors showed clinical effects in patients with lung cancer. Especially the combination of chemotherapy plus ipilimumab, a CTLA-4 antibody, resulted in improved progression-free survival (322). However, another trial showed that the overall survival is not prolonged, compared to chemotherapy alone in patients with advanced squamous NSCLC (323). The use of the checkpoint inhibitor nivolumab, an antibody against PD-1, as a monotherapy shows clinically meaningful activity and can lead to durable responses and improved survival rates in patients with NSCLC (324,325). Currently many clinical trials are evaluating combinations of PD-1/PD-L1 and CTLA-4 checkpoint inhibitors (326).

Pancreatic cancer

Pre-clinical results of CAR T-cell therapy in pancreatic cancer

In pancreatic cancer many tumor associated antigens are known. Many trials tested T-cells, engineered to express CARs against these antigens, in pre-clinical models. Mesothelin is an antigen that is overexpressed in pancreatic cancer. A study observed the naturally occurring T-cell response against mesothelin and showed that mesothelin-specific T-cell responses are significantly increased in patients with cancer and thus that mesothelin is a target for tumor-specific T-cells (327). In a mouse model using anti-mesothelin CAR T-cells, growth suppression in pancreatic cancer cells was observed (328). Another potential target for CAR T-cell therapy in pancreatic cancer is CEA that is expressed in up to 70 % of pancreatic tumors. In a mouse model of CAR T-cells targeting CEA in mice with CEA positive pancreatic cancer, a reduction of the tumor size under the limit of detection was observed in all mice. In 67 % of the mice a long-term tumor eradication was achieved (329). PSCA is a tumor-associated antigen that is expressed in more than 70 % of pancreatic cancers. In a pre-clinical model anti-PSCA CAR T-cells were investigated and it could be shown that anti-PSCA CAR T-cells are able to kill specifically PSCA-expressing pancreatic tumor cells (330). CAR T-cells targeting HER2 or CD24 were also tested in a mouse model of pancreatic cancer. CAR T-cells were directly injected intratumoral. This intratumoral therapy led to a complete elimination

of the tumor in most animals. Intravenous application reduced the tumor size and improved the survival of the mice, over 50 % of them seemed to be disease-free for more than two months. CAR T-cells stopped growth and metastasis of the tumor (331). MUC1 is also an antigen, often expressed on tumors including pancreatic carcinoma. In a mouse model anti-Tn-MUC1 CAR T-cells induced target-specific toxicity and controlled tumor growth successfully (332). CD47 is a glycoprotein that is often overexpressed on pancreatic cancer. Anti-CD47 CAR T-cells effectively killed pancreatic cancer cells in pre-clinical models and blocked tumor growth (333).

Clinical results of CAR T-cell therapy in pancreatic cancer

Different clinical studies were conducted using CAR T-cells targeting the antigen mesothelin in patients with pancreatic cancer. A patient with metastatic pancreatic carcinoma was treated with anti-mesothelin CAR T-cell therapy with only transient CAR expression for better safety. The CAR T-cells were mesothelin-specific and evidence of anti-tumor activity could be observed (286). In another trial, patients with chemotherapy-refractory pancreatic carcinoma were treated with a mesothelin-specific CAR T-cell therapy. The therapy showed preliminary evidence of anti-tumor efficacy and was well tolerated. No CRS, pleurisy, pericarditis or peritonitis occurred (334). Mesothelin-specific CAR T-cells were administrated to six patients with pancreatic cancer in another clinical study. The therapy led to a disease stabilization in two patients, with progression-free survival times of 3.8 and 5.4 months. No CRS, neurologic symptoms or dose-limiting toxicities occurred. A FDG-PET was used to monitor the metabolic active volume of the tumor. The metabolic active volume was stable in three patients and even decreased in one patient by 69.2 %, this patients experienced a complete reduction in FDG uptake in all liver lesions (335). Many ongoing trials of CAR T-cell therapy in pancreatic cancer are registered on ClinicalTrials.gov, a few examples are listed in the table below.

Table 22: Ongoing trials using CAR T-cell therapy in pancreatic cancer

Target	ClinicalTrials.gov
Mesothelin	NCT03323944, NCT02580747, NCT02930993, NCT03497819
Mesothelin, PSCA, CEA, HER2, MUC1, EGFRvIII and others	NCT03267173
CEA	NCT02850536, NCT02349724
PSCA	NCT02744287
hCD70: CAR that engages CD70 using its natural ligand CD27	NCT02830724
MUC1	NCT02839954
CLD18	NCT03302403, NCT03159819
CD133	NCT02541370

Immunotherapies using antibodies, which are targeting PD-1, PD-L1 and CTLA-4, have not shown any clinical activity in pancreatic cancer (70).

Neuroblastoma

In neuroblastoma two tumor associated antigens are in interest of immunotherapy; GD2 and CD171.

CD2

GD2 is a tumor associated antigen in neuroblastoma and is expressed highly and nearly universally on neuroblastoma (71). CAR T-cells targeting GD2 were infused to 19 children with high-risk neuroblastoma, eight of them in remission and 11 with active disease. The therapy could induce complete remission in three of the 11 patients with active disease (295). CTLs engineered to express a CAR directed to GD2 were administrated to children in another trial. The therapy was safe and was associated with necrosis or tumor regression in half of the patients (336). However, the approach is not successfully in all patients, 11 patients were treated with CAR T-cells targeting GD2 and anti-tumor responses were modest with six patients who had progressive disease and five who had a stable disease (337).

CD171

CD171 is the other target that is overexpressed in neuroblastoma. It is also known as L1-CAM (L1-cell adhesion molecule) and is an adhesion molecule (71). A first-generation CAR T-cell therapy targeting L1-CAM showed pre-clinical activity in xenograft models of neuroblastoma (338). CE7R is specific for an epitope of L1-CAM and thus anti-CE7R CAR T-cells were used in a clinical study. The aim of the trial was to evaluate the feasibility of isolating and the safety of infusing those CAR T-cells. However, only one patient (this patient had limited disease burden) of six patients experienced a significant clinical response and all other patients with a higher disease burden had a progressive disease. All patients died of their disease (339). To improve the activity and persistence of CARs targeting L1-CAM, second generation CAR T-cells were developed. They were tested to be safe in pre-clinical mouse models (340). A phase I trial at Seattle Children's Hospital is currently testing these second generation CAR T-cells and also a third generation of T-cells in children with refractory high-risk neuroblastoma (NCT02311621). Other ongoing trials are listed below.

Table 23: Ongoing trial of CAR T-cell therapy in neuroblastoma

Target	**ClinicalTrials.gov**
CD171	NCT02311621
GD2	NCT03373097, NCT03294954, NCT02761915, NCT02765243

Glioblastoma

In GBM a few tumor antigens are known and of interest in immunotherapy including IL13Rα2, EGFRvIII and HER2.

IL13Rα2

IL13Rα2 is a tumor-associated antigen that is expressed in patients with GBM and only in small amounts, if any, in normal human brain (277). In 2015, the first human safety and feasibility trial of $CD8^+$ T-cells expressing CARs against IL13Rα2 for the treatment of recurrent GBM was conducted. Three patients were treated with up to twelve local infusions of CAR T-cells. The therapy was well tolerated with

manageable and temporary inflammation of the brain. Evidence of transient anti-tumor responses was observed in two patients. The tumor tissue was analyzed before and after the treatment in one patient. Reduced IL13Rα2 expression within the tumor could be observed after the treatment (341).
A case report of a patient with recurrent multifocal GBM who was treated with CAR T-cell therapy targeting IL13Rα2 was published in 2016. The patient received multiple applications of CAR T-cells over 220 days through routes through the brain, locally into the resected tumor cavity and the ventricular system.
After the treatment a regression of all intracranial and spinal tumors sites was observed. The radiologic response was dramatic with a shrinkage of the lesion from 70 to 100 %. No higher grade toxicities occurred. These results are encouraging but nevertheless 7.5 months after the initiation of the therapy the patient had a recurrence. It is assumed that the tumor recurred due to immunoediting of the tumor, only IL13Rα2 negative cells remained and expanded (76).

EGFRvIII

In about 30 % of GBM cases EGFRvIII is overexpressed and it was observed that the expression of EGFRvIII is a negative prognostic indicator (266).
EGFRvIII is the result of a tumor-specific gene mutation which results in the production of this unique protein. The mutation, in form of a deletion, results in a ligand-independent constitutively active protein (265). It could be shown, that the expression of EGFRvIII enhances tumorigenicity, promotes cellular motility and induces resistance to radiation therapy and chemotherapy (342).
In 2012, the recognition of glioma stem cells by CAR T-cells targeting the antigen EGFRvIII was conducted in a pre-clinical trial. Sequencing of the EGFRvIII-specific deletion was performed and based on this information monoclonal antibodies targeting EGFRvIII were developed and introduced into CARs.
The CAR T-cells were evaluated and it could be observed that they are able to produce the effector cytokine IFN-γ and to lyse the antigen-expressing target cells (342). Based on this information, EGFRvIII CAR T-cells were produced for clinical use (79). CAR T-cells directed to EGFRvIII were administrated intravenous in ten patients with EGFRvIII positive recurrent GBM. This approach was proven to be safe with no CRS or neurotoxicity. However, the results were disappointing, the overall survival of the patients did not seem to be affected.

Only one patient had a stable disease for over 18 months. Nevertheless, all patients had transient EGFRvIII cells in peripheral blood. The tumor tissue and environment were analyzed from seven patients and it was shown that the expression of EGFRvIII by tumor cells decreases in five of seven patients. At the same time, an upregulation of immunosuppressive factor was observed in the tumor microenvironment (79). These mechanisms of immuno-escape will need to be overcome to achieve effective immunotherapy for this disease.

HER2

In up to 41 % of all GBM an overexpression of HER2 can be observed (343). HER2 or ERBB2 is a receptor with tyrosine kinase activity and is overexpressed in some malignant tumors including GBM. The overexpression of HER2 leads to multiple heterodimers that are formed in the presence of ligands and this results in enhanced cell growth and differentiation (256,257).
In a phase I clinical study, autologous T-cells were engineered to express a second generation HER2 CAR and administrated to 17 patients, including six children and eleven adults with progressive HER2-positive GBM. The therapy was tolerated well without severe adverse events or CRS. Administered cells were found in peripheral blood for up to twelve weeks post infusion. 16 patients were evaluable and eight of them had objective responses and eight had progressive disease. In the group of responders one patient experienced a partial response with a 62 % reduction of the tumors volume that lasted for nine months. Seven patients experienced a stable disease for eight weeks to 29 months. Therefore, this trial showed that CAR T-cells targeting HER2 in GBM are safe and a durable clinical benefit can be induced in 38 % of the patients (344,345).
In another approach NK-cells were engineered to express a HER2 specific CAR. *In vivo* activity of those NK-cells could be observed in mice with orthotopic GBM, including extension of symptom-free survival. In immunocompetent mice the local therapy with NK-cells led to cures of four from five mice with subcutaneous tumors and five of eight mice with intracranial tumors, they became tumor free. Additionally, induction of endogenous anti-tumor immunity and long-term protection against tumor re-appearance at distant sites could be observed (343).

Table 24: Selected ongoing trials of CAR T-cell therapy in GBM

Target	Application	ClinicalTrials.gov
IL13Rα2	systemic	NCT02208362, NCT04003649
EGFRvIII	intracerebral	NCT03283631
EGFRvIII	systemic	NCT02664363, NCT03726515
HER2	intracerebral	NCT03389230, NCT02442297
EphA2	systemic	NCT02575261–

There are also ongoing trials testing TILs in progressive GBM (NCT01174121, NCT03347097). T-cells, engineered to express TCRs targeting mutated neo-antigens, are also tested in an ongoing trial on GBM patients (NCT03412877).

4.8 Differentiation status of infused T-cells

Different subsets of $CD8^+$ or $CD4^+$ T-cells exist and can be potentially used as T-cells for ACT. Therefore, it is important to find the ideal T-cell population for the treatment of cancer patients.

$CD8^+$ T-cells

$CD8^+$ T-cells are able to destruct tumor tissue in mice and humans and can be categorized into different subsets according to their differentiation states. The phenotype of T-cells progressively changes after antigenic stimulation. In the process of differentiation they gain but also lose some abilities. It was shown that $CD8^+$ T-cells are following a progressive pathway of differentiation, from naïve T-cells to stem cell memory T-cells, to central memory T-cells, to effector memory T-cells. Stem cell-like memory T-cell cells have a genetic program that allows them to proliferate and to differentiate further into central memory and effector memory T-cells (33,136). Pre-clinical mouse models evaluated the efficiency of the ACT using different differentiation stages of $CD8^+$ T-cells. Studies showed a better anti-tumor response in T-cells of an early differentiation status, such as central memory $CD8^+$ T-cells (346). It was shown that naïve and early effector T-cells led to eradication of large tumors. Paradoxically, despite enhanced *in vitro* anti-tumor efficiency of more differentiated effector T-cells, they were less effective *in vivo* (347). A population of stem-cell-like T-cells showed increased proliferation capacity and superior anti-tumor responses in a mouse model (348).

CD27 is a marker that is associated with cells in an early stadium of differentiation. A study showed a strong correlation between the expression of CD27 and clinical response (155). Immunological memory depends on stem-cell-like lymphocytes with self-renewing and long-living features that are able to differentiate into effector cells in case of re-exposure to antigens. Therefore, it is relevant to identify these human populations to design more effective T-cell therapies (348).

$CD4^+$ T-cells

Many existing publications in ACT research have focused on $CD8^+$ T-cells. However, $CD4^+$ T-cells control $CD8^+$ T-cells effector functions, memory and maintenance. Therefore, the anti-tumor effect of $CD8^+$ T-cells depends on $CD4^+$ T-cells. They provide them with growth factors for example IL-2 and are capable to mediate the destruction of tumor cells. $CD4^+$ T-cells have also an effect on the innate immune system, they can activate APCs and other innate immune cells. Furthermore, it was shown that $CD4^+$ T-cells have a more direct role in tumor elimination than only enhancing the function of $CD8^+$ T-cells or activating the innate immune system (33). Several trials illustrated the potential of tumor-specific $CD4^+$ T-cells for ACT because of their direct anti-tumor effects. For example, a trial used $CD4^+$ T-cells against a mutated antigen in metastatic epithelial cancer and a regression was induced. In a patient with metastatic cholangiocarcinoma TILs were examined using whole-exome-sequencing. $CD4^+$ T-helper cells were identified to recognize a mutation in ERBB2IP, which is expressed by cancer cells (187). Another trial used autologous $CD4^+$ T-cell clones that are specific to NY-ESO-1. The cells were administrated to a patient with refractory metastatic melanoma and the transferred $CD4^+$ T-cells mediated a durable clinical remission. No evidence of disease or recurrence was found in the follow ups 22 months after the treatment. Because only 50 to 75 % of the tumor cells expressed NY-ESO-1 but the whole tumor regressed, it was investigated and proven that the treatment also led to endogenous responses against melanoma antigens other than NY-ESO-1 such as MART-1 and MAGE-3 antigen. T-cells specific to MART-1 and MAGE-3 were undetectable before the infusion of NY-ESO-1-specific $CD4^+$ T-cells (182). It is important to distinguish between the two forms of $CD4^+$ T-cells, T-helper cells and Tregs. Transfer of T-helper cells in combination with anti-tumor $CD8^+$ T-cells induced cancer regression. In contrast transfer of a mixture of $CD4^+$ T-helper and

Tregs or the transfer of Tregs alone prevented an effective ACT. The supportive effect of T-helper cells on $CD8^+$ T-cells depends on their ability to produce IL-2. Therapy failed when using T-helper cells derived from IL-$2^{-/-}$ mice. These findings lead to the assumption that T-helper cells can help in treating established tumors through a mechanism that is dependent on IL-2, but to achieve an effect, absence of naturally occurring Tregs is required (36). Another trial suggests, that the effects that $CD4^+$ T-cells are able to achieve, depend on their polarization, which is determined by their expression of transcription factors (136).

4.9 The role of the tumor microenvironment

To achieve an effective immunotherapy not only cancer-specific T-cells that attack tumor cells are required, it is also necessary that these T-cells physically contact the tumor cells. In some cancer patients a coexistence of cancer cells and anti-tumor T-cells indicates that the cancer cells many exhibit an immune privilege, which protects the cancer tissue from an attack by the immune system. Solid tumors are a mixture of not just tumor cells, they are surrounded by a local microenvironment consisting of different cell types that support tumor growth and progression. These different cell types are for example inflammatory cells, lymphocytes, cells of blood vessels and fibroblastic cells as well as collagen and proteoglycans, which build the extracellular matrix (349).
In several pre-clinical mouse models of cancer, it was shown that the major stromal cell types of the tumor microenvironment (TME), such as cancer-associated fibroblasts and different myeloid-derived suppressor cells (MDSCs), are responsible for the limitation of the accumulation of T-cells in the surrounding of cancer cells (349). MDSCs are cells that accumulate during the formation of tumors and are a part of the immunosuppressive TME, which blocks the attack of anti-tumor T-cells. They facilitate immune escape of tumor cells and thus enable tumor progression and tumor growth. It was shown that MDSCs are limiting the efficacy of immunotherapy by regulating the traffic of activated $CD8^+$ T-cells to the tumor site. These results suggest that targeting of MDSCs might enhance outcomes in ACT (350). In a pancreatic cancer mouse model, it was observed that mice had cancer cell specific $CD8^+$ T-cells but did not respond to two checkpoint inhibitors, which promote the function of T-cells. It was shown that the depletion of carcinoma-

associated fibroblasts, which express FAP, led to an immune control of the cancer and also to anti-tumor effects of the checkpoint inhibitors (351).

Mechanisms of TME-induced immune suppression

There are different mechanisms known of how the TME induces immune suppression, as discussed in detail below.

Disturbed recruitment of anti-tumor T-cells

In lymph nodes that drain the tumor, cancer-specific T-cells get activated and afterwards they traffic to the tumor site by the circulation system. Different studies observed, that the TME plays a role in the accumulation and traffic of T-cells in tumors at the first step; the interaction of T-cells with local blood vessels (349). For example, the recruitment of T-cells to the tumor site can be blocked by nitration of the chemokine CCL2. The reactive nitrogen species is produced by MDSCs within the TME. The CCL2 chemokine nitration hinders T-cell infiltration and results in the trapping of tumor-specific T-cells in the stromal extracellular matrix, which surrounds the cancer cells (352). In animal models an inhibition of CCL2 nitration enhanced the accumulation of TILs and resulted in improved efficacy of ACT (349).

Tumor vasculature excludes T-cells

Even in the case, that there are appropriate chemotactic signals for the extravasation and T-cells are recruited to the tumor, the vasculature can actively exclude T-cells. The expression of the apoptosis inducer FasL by endothelial cells in the vasculature of human and mouse solid tumors was observed, but was not found in normal vasculature. The FasL expression in endothelial cells was associated with low $CD8^+$ T-cell infiltration and a predominance of Tregs. Endothelial cells expressing FasL, mediate killing of effector $CD8^+$ T-cells but not Tregs, because they express higher levels of c-FLIP, an anti-apoptotic regulator. FasL is expressed in the tumor vasculature of many different tumor types including human breast cancer, colon cancer, renal cancer, bladder cancer, prostate cancer and ovarian adenocarcinoma (353). Another ligand found to be expressed by tumor blood vessels is B7-H3, an immune regulatory ligand that weakens immune responses by co-inhibition. A correlation of B7-H3 overexpression and poor clinical outcome and a decreased number of tumor-infiltrating T-cells could be observed in

various carcinomas like ovarian cancer (354). The expression of the endothelin B receptor (ETBR) is another strategy of the tumor endothelium to prevent T-cell infiltration. An association between the overexpression of ETBR and the absence of TILs and short patient survival time was shown (355). Pharmacological inhibition of ETBR was tested in mouse models and resulted in increased T-cell adhesion to endothelial cells in an intercellular adhesion molecule-1 (ICAM-1) dependent manner and subsequently resulted in increased T-cell infiltration in tumors. Tumor responses to an otherwise ineffective anti-cancer vaccine could be observed after ETBR inhibition (355). FasL inhibition is another method to improve the efficacy of ACT. An increased influx of anti-tumor T-cells, enhanced T-cell infiltration and impaired growth of the tumor after ACT was observed in mice models (353). Anti-angiogenic therapies using anti-angiogenic inhibitors targeting VEGF and its receptor VEGFR2, are also a potential way to enhance the efficacy of immunotherapies (356).

Intratumoral inhibition of T-cell proliferation

For an effective attack of anti-cancer immune cells, locally expansion of T-cells is required. It was shown that the TME may be the major site of expansion of anti-tumor T-cells (349). The intratumoral T-cell proliferation can be directly reduced by the TME via the Indole 2,3-dioxygenase (IDO) pathway. IDO can be expressed by DCs, MDSCs and cancer cells, it catabolizes tryptophan and generates kynurenine. Both the withdrawal of tryptophan and the production of its metabolic product lead to an inhibition of the clonal expansion of T-cells (357). Additionally, the conversation of naïve T-cells to Tregs and an increase of IL-6 expression, which augments the functions of MDSCs, is promoted by IDO (358). IDO is also able to block the reprogramming of Tregs in helper-like cells, which is induced by downregulation of the transcription factor Eos (Ikzf4). IDO is able to suppress this loss of Eos and thus the reprogramming, which is another reason why IDO promotes immune suppression within the TME (359). In mouse models of lung and breast cancer a genetic IDO deficiency showed to be associated with reduced tumor burden and metastasis and an enhanced survival (358). A combination therapy of a checkpoint inhibitor (anti-CTLA-4) with the inhibition of IDO was administrated in a mouse model. In IDO knockout mice, which were treated with anti-CTLA-4 antibody, a prominent delay in growth and progression of melanoma and increased overall survival was observed, compared

to wild-type mice. Further, it was shown that CTLA-4 blockade with IDO inhibitors lead to enhanced infiltration of anti-tumor effector T-cells and increased the effector-to-regulatory T-cell ratios in the tumors (360). Another immune suppressive component of the microenvironment are cells, which produce FAP. In a mouse models, FAP expressing cells were depleted and rapid necrosis of cancer cells as well as stromal cells in immunogenic tumors occurred, induced by preexisting cancer-specific T-cells (361). However, the depletion of FAP positive stromal cells is not a therapeutic option because these cells are necessary for several other healthy tissue types. Experimental ablation of these cells in models led to loss of muscle mass and to a reduction of B-lymphopoiesis and erythropoiesis resulting in cachexia and anemia (349).

B-cells that suppress T-cells

Regulatory B-cells (Bregs), a subset of B-cells, can suppress diverse cell types including T-cells, by the secretion of anti-inflammatory mediators like IL-10. They can also induce the conversion of T-cells into Tregs and thereby weaken anti-tumor immune responses (362). It was observed in pre-clinical models that B-cells are able to regulate the phenotype of macrophages, resulting in suppression of $CD8^+$ T-cells. It was shown that B-cell deficient mice, bearing squamous cell carcinoma, had a reduced tumor growth (363). Mice with squamous cell carcinoma were treated with B-cell-depleting CD20 monoclonal antibodies additionally to chemotherapy and an improved chemotherapy responsiveness was the result. This enhanced efficiency of chemotherapy depended on an altered expression of chemokines by macrophages, which subsequently promoted and induced the infiltration of $CD8^+$ T-cells into the tumor (363). However, B-cells can also play roles in anti-tumor efficiency by producing antibodies directed against tumor antigens, acting as antigen presenting cells that are driving T-cell expansion and by releasing cytokines and chemokines that promote anti-tumor immunity (362).

T-cells trapped in the tumor stroma

The TME regulates the distribution of T-cells within the different tumor areas. An increased number of cancer-specific T-cells in the tumor is not enough for a successful anti-tumor response, especially when these T-cells are restricted to the stroma. T-cells need to get access to the vicinity of cancer cells to induce an

anti-tumor response. As a study showed, stromal extracellular matrix influences the anti-tumor response of T-cells, by controlling the positioning and migration of T-cells into the tumor site. Using real-time imaging in viable slices of human lung tumors it was observed, that the density and the orientation of the extracellular matrix of the stroma, play a key role in the migration of T-cells to cancer cells. The result showed an active T-cell motility in regions with loose fibronectin and collagen. In contrast, in regions with dense matrix, T-cells migrated poorly. Fibers in areas around the tumor cells restricted T-cells to contact cancer cells. By editing collagenase to reduce matrix rigidity, an increased ability of T-cells to move out of the stromal area and get into contact with cancer cells occurred (364).

4.10 Lymphodepletion

The preparative lymphodepletion induces a temporary suppression of the immune system of the patient. This is a very important step in ACT according to persistence of transferred cells and clinical response. Before lymphodepletion was used, engraftment and persistence of transferred T-lymphocytes was a rarity (365). In trials conducted after the implementation of lymphodepletion, transferred cells rapidly grew *in vivo* and persisted as a main part of $CD8^+$ T-cells in the circulation of the patients (183).

Different forms of lymphodepletion

Lymphodepletion can be performed by chemotherapy only or in combination with TBI. The most common chemotherapy regime consists of 60 mg/kg Cyclophosphamide for two days followed by 25 mg/m^2 Fludarabine for five days. After this, T-cells get administrated to the patient, accompanied by IL-2 as a T-cell growth factor *in vivo* (136). TBI is the second method to achieve a lymphodepletion. TBI is always combined with chemotherapy. Studies showed that response rates of the tumor correlates with the TBI dose (37,155).

Effects of lymphodepletion

Depletion of immune elements before the application of ACT can improve the efficacy of transferred anti-tumor T-cells dramatically by mechanisms discussed in this chapter (37). Homeostatic cytokines such as IL-7 and IL-15 are required for augmenting the function and anti-tumor activity of T-cells. Removal of endogenous, competitive cells, that are responsive to those cytokines, results in an enhanced

anti-tumor effect and improves the efficiency of ACT. Lymphodepletion removes these cells, which act like sinks for cytokines and subsequently more cytokines are left to augment the activity of anti-tumor $CD8^+$ T-cells. These findings lead to the assumption that restricted availability of homeostatic cytokines can be a cause of peripheral tolerance and limited effectiveness of ACT (366). Thereby, after lymphodepletion increased levels of IL-7 and IL-15 can be observed. The stronger the lymphodepletion, the higher the blood levels of those parameters (37). Microbial translocation leads also to an increased ACT efficiency. Microorganisms that colonize the gastrointestinal tract are necessary for health and disruption of the homeostatic balance is a part of the pathogenesis of different diseases. Cancer treatments using lymphodepletion and ACT are capable to disturb the host/microflora relationship. Bacteria possess molecular patterns like LPS that can be bound by pattern recognition receptors like TLRs. Engagement of TLRs on APCs with those bacteria patterns, leads to maturation and trafficking of those activated APCs to lymph nodes, where they activate antigen-specific T-cells. TBI causes injuries of the mucosal barrier and as a result translocations of microorganisms and systemic liberation of LPS occur. The TLR engages to LPS and subsequently an increased DC activation occurs, which leads to an enhanced self/tumor-specific $CD8^+$ T-cell activation. This whole process causes greater tumor regression but also more autoimmune effects (367). Tregs decrease the anti-tumor immune response and can be found in higher levels in the tumor microenvironment and circulation. In patients with metastatic melanoma, an accumulation of Tregs in the tumor compared to the blood levels was shown (368). Increased levels of Tregs in the blood and tumor in patients with oral squamous cell carcinoma were also observed (369). Peripheral blood cancer patients also show slight increased levels of Tregs, as well breast- and pancreas cancer patients (370). High levels of Tregs in ovarian cancer were shown to be associated with a poor prognosis and inferior survival (371). There was an association found between the clinical response and the Tregs levels. These findings lead to the assumption that Tregs have a negative effect on cancer therapy (372). In a trial three different groups of lymphodepletion-regimes were administrated, nonmyeloablative chemotherapy with or without TBI. Patients who received TBI had lower levels of Tregs than those without TBI and the level of Tregs correlated with the dose of TBI (372).

CD25, also known as interleukin-2 high-affinity receptor alpha chain (IL-2Rα), is an antigen expressed on Tregs. However, CD25 is not specific to Tregs, it is also expressed by activated T-cells and B-cells, monocytes and some DCs (373). Immunosuppressive lymphodepletion can be combined with antibodies against CD52 to eliminate Tregs. To avoid the depletion of transferred T-cells by the antibodies, transferred T-cells can be genetically engineered to have a failure of CD52 expression (221).

Comparison of the different forms of lymphodepletion

In patients with metastatic melanoma three different lymphodepletion regimes were tested. Chemotherapy alone, chemotherapy in combination with 2 Gy TBI and chemotherapy in combination with 12 Gy TBI. The results were an objective response in 49 % of the patients in the chemotherapy only group, 52 % and 72 % of the patients, which were treated with additionally 2 Gy or 12 Gy TBI. The differences of the overall response rates between the three different groups were not significant. However, there was a relation between an increase of complete response and a higher dose of irradiation (37,155).

Toxicities associated with lymphodepletion

Hematologic toxicities are side effects that often occur after lymphodepletion. However, they are transient in almost every case. Most patients develop cytopenias following lymphodepleting regimes (233). Low red blood counts (RBC), low platelet counts, low absolute neutrophil counts, low absolute lymphocyte counts and the extended depression of $CD4^+$ lymphocyte counts can be observed in nearly every patient (37). Opportunistic infections are also side effects that occur in some patients after lymphodepletion. Examples are infections with herpes zoster, pneumocystis carinii, EBV and respiratory syncytial virus (184). There is also a case of death reported due to a sepsis in a neutropenic patient, who had an unrecognized diverticular abscess (37). Multiple administrations of lymphodepletion and TILs in patients with melanoma lead to a delayed hematologic recovery. This may represent a limitation of the use of repeated courses of lymphodepletion in some patients (184). It was shown that chemotherapy induced depression of neutrophil counts can be treated with granulocyte-colony stimulating factor (G-CSF). As G-CSF administration significantly improved neutrophil recovery by non-impacting the clinical response of ACT (184).

A rare non-hematologic toxicity, associated with the lymphodepleting chemotherapy fludarabine, is progressive multifocal neuropathy, which can lead to cortical blindness (184).

4.11 Factors associated with the success of T-cell therapy

Different factors are known to affect the success of ACT in cancer patients. Lymphodepletion is a very important step in all forms of T-cell therapy for achieving better response rates. The most effective lymphodepletion regime is a combination of chemotherapy plus 12 Gy TBI. This regime leads to the best response rates in comparison to chemotherapy alone and to chemotherapy plus 2 Gy irradiation (155). The length of telomeres correlates with clinical responses. This fact leads to the assumption that the proliferative potential of the infused T-cells is a relevant factor of their ability to initiate a response and their ability to destruct tumor cells (37). Metastatic melanoma patients who experienced an objective response received lower doses of IL-2 than patients with no clinical response. The reason might be that IL-2 causes more side effects in patients with a response because it increases the activity of the administrated T-cells and thus limits the IL-2 dosing (155). Another trial in patients with metastatic melanoma showed similar results, nonresponding patients tolerated higher IL-2 doses than responding patients (184). The number of the CD8+ CD27+ memory T-cells was significantly larger in responders and the persistence of the transferred cells *in vivo* was also significant higher in melanoma patients with an objective response, compared to non-responders (155).

Factors that do not affect the success of the ACT

In patients with metastatic melanoma, no difference in the number of infused cells could be found, compared in clinical responders and nonresponders (155,184). However, the dose of administrated CAR T-cells correlates with the severity of toxicities (233). Additionally, it was shown that the likelihood of achieving a complete response was the same in all groups independent of the prior treatment. The factors sex, age, HLA type, metastatic stage or lactate dehydrogenase levels did not make a significant difference in objective responses (155).

4.12 Advantages and disadvantages of T-Cell therapy

Advantages of ACT

T-cell therapy makes it possible to induce tumor regressions in patients, who did not response to previous, standard cancer treatments. For example, TIL therapy can induce a durable complete response even in patients with progressive disease refractory to standard therapies (183). Another example are CAR T-cells targeting CD19, which are able to induce complete remissions in chemotherapy-refractory B-cell malignancies (235). CAR T-cell therapy can lead to higher rates of remission and longer survival compared to standard cancer treatments, for example in ALL (374). There are several advantages of genetically engineered T-cells including the opportunity for the therapist to choose the properties of the cell population, for example cells at an early differentiation status (348), as well as the properties of the used receptor (375). Beside the new antigen reactivity, which is inserted to the recipient T-cells by genetically engineering, also genes that improve the efficacy of these T-cells can be inserted. Examples for genes, which improve the anti-tumor effects of T-cells, are genes encoding molecules involved in costimulation, prevention of apoptosis, induction of inflammation or homeostatic proliferation and also the encoding of chemokine receptors that promote homing of T-cells (33).

Disadvantages of ACT

A disadvantage of ACT is that the access to this specialized cancer therapy is still limited because widespread application needs specialized manufactories, expertise and logistical support, which is associated with high costs (221).
A great challenge in developing T-cell therapies is the little amount of pre-clinical models to evaluate the safety and efficacy of these therapies before human studies (208). Unexpected toxicities in from of “off-tumor, on target” toxicities are another problem and disadvantage of ACT. These toxicities result from recognition of antigens found not only on tumor cells, but also on normal healthy tissue. For example, CAR T-cells against ERBB2 were administrated to a patient with patient with colon cancer and led to pulmonary failure, because low levels of ERBB2 are expressed on lung epithelial cells (258).

4.13 Outcome comparison of ACT to other cancer therapies

Melanoma

In melanoma, standard chemotherapy induces objective responses in less than 20 % and rarely leads to complete responses, additionally benefits last only months (376). TIL therapy is arguably more successful, with objective response rates from up to 72 % (37) and durable complete response rates of 20 % in patients with metastatic melanoma resistant to other therapies (155).

DLBCL

DLBCL is the most common subtype of non-Hodgkin lymphoma (377). In a retrospective study the outcomes of 636 patients with refractory or relapsed DLBCL were investigated. Refractory DLBCL was defined as progressive disease (received at least four cycles of first-line therapy) or stable disease (received two cycles of later-line therapy) as best response to chemotherapy or relapse within twelve months after autologous transplantation. Included patients must have received an anti-CD20 monoclonal antibody and anthracycline chemotherapy. The response rate to the next line of therapy was 26 % including 7 % complete responses. The median overall survival was 6.2 months and 20 % of the patients were alive after two years (378). In comparison, the use of tisagenlecleucel, an anti-CD19 CAR T-cell therapy, in adult patients with relapsed or refractory DLBCL led to better results and high rates of durable responses were produced. In a trial initiated to evaluate the safety and efficacy of tisagenlecleucel, a total of 93 patients with relapsed or refractory DLBCL, who were ineligible for or had disease progression after autologous hematopoietic stem cell transplantation, received an infusion of tisagenlecleucel. The overall response rate was 52 %, including 40 % of patients with complete responses and 12 % with partial responses. The rate of relapse-free survival was estimated to be 65 % (79 % among patients with a complete response) twelve months after the initial response (379).

Table 25: Outcome comparison of Tisagenlecleucel and conventional therapy in DLBCL

Therapy type	Response rate	Complete response	Ref.
Conventional therapy	26 %	7 %	(378)
Tisagenlecleucel	52 %	40 %	(379)

ALL

CAR T-cells targeting CD19 were compared to chemotherapy in patients with relapsed or refractory ALL. The complete response rate was higher in the CAR T-cell therapy group with 90 %, compared to the chemotherapy group with 37.9 %. The overall survival rate was also higher in the CAR T-cell therapy group (60.9 %) compared to the group, who received chemotherapy (10.1 %). This data demonstrates, that ALL patients treated with CAR T-cell therapy targeting CD19 acquired higher rates of remission and longer survival (374).

Table 26: Outcome comparison of anti-CD19 CAR T-cell therapy and chemotherapy in ALL

Therapy type	Response rate	Survival rate	Ref.
Chemotherapy	37.9 %	10.1 %	(374)
anti-CD19 CAR T-cell therapy	90 %	60.9 %	(374)

In B-ALL in pediatric patients and young adults under the age of 25 years, tsagenlecleucel appears to be significantly more effective than the current standard of care treatment (380). Compared to a control datasets on blinatumomab chemotherapy (381) and clofarabine chemotherapy (382), Tisagenlecleucel has demonstrated a benefit in event-free and overall survival (380).

NSCLC

Adoptive immunotherapy in form of TIL therapy plus IL-2 was compared to standard chemotherapy in patients with resected NSCLC, as a postoperative treatment. From the tissue samples from the surgically removed cancer, TILs were expanded *in vitro*. 113 patients were randomized to receive ACT plus IL-2 or standard chemoradiotherapy. The results showed a significantly better three-year survival in patients who underwent ACT. However, ACT induced no benefit in patients with Stage II NSCLC but was observed to be potentially useful to patients with

Stage IIIa NSCLC and significantly advantageous to patients with Stage IIIb NSCLC. For patients with Stage III NSCLC the rate of local relapse was significantly reduced in patients who received ACT (189).

4.14 Combination of T-cell therapy with other cancer therapies

Conventional cancer therapy in combination with ACT

Conventional cancer therapies like chemotherapy and radiation therapy induce apoptotic cell death of cancer cells. Apoptotic cell death leads to the release of more tumor antigens, which might result in a more effective treatment with immunotherapy like ACT (383). The dying tumor cell releases antigens that can be acquired by APCs which subsequently process and present these antigens to tumor-specific T-cell populations. This process can lead to the priming and activation of adoptively transferred anti-tumor T-cells and also endogenous anti-tumor T-cells (33,384). However, studies showed that the results of TIL therapy in heavily pretreated patients with metastatic melanoma do not depend on the prior form of cancer therapy (155).

Targeted therapy in combination with ACT

A targeted therapy can be used to induce apoptosis of tumor cells, as described above. Additionally, treatments with cytokines and chemokines, which stimulate the immune system, may also improve the efficiency of the anti-tumor T-cell response. Targeted therapies toward immunosuppressive factors or immunosuppressive cells, found in the tumors microenvironment, might also increase the response of anti-tumor T-cells (33,384). An example for a targeted therapy is vemurafenib which is an inhibitor of the oncogenic BRAF pathway. The use of BRAF inhibitors in melanoma leads to an increased antigen expression like gp100 and MART-1, on tumor cells. Peripheral blood lymphocytes of melanoma patients were engineered to express TCR against MART-1 and gp100 and an improved tumor recognition by MART-1 specific and gp100 specific TCR T-cells was observed (383).

Checkpoint inhibitors in combination with ACT

The combination of ACT in form of CAR T-cells combined with an immunomodulatory reagent for blocking immunosuppression in form of a PD-1 blockade was tested in mouse models of breast cancer and sarcoma,

two different HER2 positive tumors. Results demonstrated that ACT in combination with PD-1 blockade leads to significantly enhanced anti-tumor effects compared to each intervention alone. Therefore, in conclusion this study showed that blocking PD-1 immunosuppression can potently enhance CAR T-cell therapy (385). The first clinical trial, which is testing the combination of CTLA-4 blockade with CAR T-cells, is still ongoing. The checkpoint inhibitor is combined with CAR T-cells targeting CD19 and applied to patients with B-cell lymphoma, CLL and ALL (NCT00586391).

Vaccination in combination with ACT

In mouse models it could be observed that a short activation of T-cells, by stimulating their TCR, can augment their anti-tumor function because these T-cells undergo a programmed activation and expansion in response to this stimulus (386). *In vivo*, this T-cell stimulation can be achieved with the help of antigen-specific vaccination (387)

4.15 Quality of life of cancer patients

The remaining quality of life is a major concern of many patients after the diagnosis of cancer. Different factors affect quality of life and in a study, that included 768 cancer patients, quality of life was assessed by interviews (388). A total of 82.3 % of the patients had low quality of life scores, according to this study. In the category general well-being very low levels of quality of life were observed in 738 patients (96.1 %). Also, the physical well-being was at very low levels in 555 patients (72.3 %) and was mainly affected by pain in 560 patients (72.9 %), sleep problems in 551 patients (71.7 %) and fatigue in 705 patients (91.8 %). The psychological well-being was also observed to be at very low levels of quality of life in 411 cancer patients (53.5 %) and was affected by depression in 418 (54.4 %) of the participants. 755 patients (98.3 %) reported to be not comfortable in attending the social functions, 585 (76.2 %) patients had a fear of recurrence and 658 patients (85.7 %) were not satisfied with their body image. 755 (98.3 %) patients felt that their income status was reduced due to their disease. Therefore, this financial constraint was reported as a major issue among patients as well as family caregivers. This finding demonstrates that many symptoms and factors affect the quality of life of cancer patients and that a multi management of all health professionals is necessary to improve the quality of life of each patient (388).

T-cell therapy limits quality of life in cancer patients under T-cell therapy by causing side effects and toxicities. In melanoma patients who receive TCR T-cell therapy or TIL therapy, quality of life might be effected by the destruction of normal melanocytes in the skin, eye and ear resulting in vitiligo or uveitis, hearing loss (154,183,184). A common adverse effect of CAR T-cell therapy is CRS with symptoms like fever and myalgia which effects the quality of life of patients, but also severe and life threatening effects can occur like hypotension, respiratory insufficiency or coagulopathy which effects also the survival of patients (232,237,239,240). Limitations of quality of life also occur due to the lymphodepletion regime that is necessary for a successful T-cell therapy. Especially opportunistic infections may decrease the quality of life (184).

4.16 Approved T-cell therapy medications

The first FDA approved CAR T-cell therapy was Tisagenlecleucel (Kymriah), a CAR T-cell therapy targeting CD19, indicated for children and young adults with B-cell ALL that is refractory or have relapsed at least twice (389). The European Medicines Agency (EMA) recommended granting marketing authorizations for the first two CAR T-cell therapies in the European Union in 2018. Tisagenlecleucel and Axicabtagene Ciloleucel (Yescarta), two anti-CD19 CAR T-cell therapies for the treatment of different B-cell malignancies. Tisagenlecleucel is indicated for the treatment of relapsed or refractory DLBCL and relapsed or refractory ALL. Axicabtagene Ciloleucel is intended for treatment of relapsed or refractory primary mediastinal B-cell lymphoma and relapsed or refractory DLBCL (390).

Tisagenlecleucel (Kymriah)

Tisagenlecleucel is an anti-CD19 CAR T-cell therapy, with the costimulatory domain 4-1BB. It led to high response rates and high rates of complete remission in children and young adults with relapsed or refractory B-cell ALL (239). However, it was also associated with toxic effects which were mainly reversible. Every patient had at least one side effect during the study, most common side effect were CRS (77 %), pyrexia (40 %), decreased appetite (39 %), febrile neutropenia (36 %) and headache (36 %) (239).

Axicabtagene Ciloleucel (Yescarta)

Axicabtagene Ciloleucel is also an autologous anti-CD19 CAR T-cell therapy that is generated by introducing the CAR construct into primary T-cells with a retroviral vector (391). The costimulatory domain of CAR T-cells is CD28, compared to 4-1BB at Tisagenlecleucel (392). In the ZUMA-1 study, a phase I study, Axicabtagene Ciloleucel showed efficacy in patients with refractory large B-cell lymphoma after the failure of conventional therapy. An objective response rate of 82 % was achieved including 54 % complete response. The median follow-up was 15.4 months with 42 % of the patients continued to have a response, including 40 % continuing to have a complete response. The overall rate of survival after 18 months was 52 %. The most common side events of grade three or higher were neutropenia (78 %), anemia (43 %) and thrombocytopenia (38 %) (391). Two-year follow-up data from the ZUMA-1 study including 101 patients, showed 84 patients (83 %) who had an objective response and 59 (58 %) who had a complete response. These observations suggest that Axicabtagene Ciloleucel can induce durable responses and a median overall survival of greater than two years (393).

5 Discussion

The field of immunotherapy in cancer is one of the great success stories in medicine of the past decade. ACT has emerged as a promising advance in cancer treatment. Data from many pre-clinical and clinical studies have increased the understanding of underlying mechanisms of successful immunotherapies. Additional gene engineering made it possible to expand the potential target population that could benefit from T-cell therapies. This knowledge has helped scientists to identify and engineer most effective T-cell populations and to achieve promising results in many cancer types (33).

Major limitations of ACT are high costs, the need of specialized cell-production facilities and highly trained medical and laboratory personnel (33). The requirement of ACT to develop highly personalized therapies for each patient does not fit into the paradigm of most pharmaceutical companies because they are interested in “off-the-shelf” therapies that can be widely distributed (136). A “new drug” must be developed for each patient, including weeks of cell culture and patient preparation (394). The custom manufacturing processes used to design highly personalized engineered T-cell therapies result in high costs. For example, the costs for TIL therapy for the treatment of melanoma are around €60,000 per patient (395). However, TILs compared to ipilimumab, a monoclonal antibody against CTLA-4, are less costly and more effective (395). The first FDA and EMA approved T-cell therapies Tisagenlecleucel and Axicabtagene Ciloleucel cost $373,000 each (396). However, the cost of the manufacturing process of CAR T-cells is expected to decrease (208).

Regulations of the EMA and the FDA are set up for drugs that are produced and tested in advance of their medical need and for medication that can be manufactured in a single production run to treat thousands of patients. The quality control systems of the pharmaceutical industries are intended for a large amount of drugs of the same quality (397). The cell culture process of T-cells requires highly skilled scientists, which perform extensive manipulation on these cell cultures. Thereby, another question is, how T-cell therapies will be able to achieve the mass production that is needed to treat the large numbers of cancer patients. A more automated production of T-cell therapies will be the answer.

By making use of equipment and facilities, developed for blood banks and stem cell laboratories, it will be possible to make T-cell therapies widely available to many patients (397). Another approach to ensure mass-produced tumor-specific T-cells might be the use of allogenic cells that could be grown in blood banks, central facilities or even by commercial enterprise (299). Several pharmaceutical and biotechnological companies started research in this field with the aim to develop commercial-scale manufacturing of T-cell therapies for a worldwide application to cancer patients (397). Bruce L. Levine and Carl H. June are optimistic that the pharmaceutical industry, with its resources and expertise, will be able to create an infrastructure that is needed for a widespread use of T-cell therapies in cancer patients (397).

It seems clear that drug-based tumor treatments alone will generally be not able to kill all cancer cells, with the notable exception of some hematological malignancies and germ cell tumors. After drug therapy, residual tumor cells will ultimately grow back, with potentially lethal consequences. However, the immune system is capable of achieving sterilizing immunity and is able to induce long-term and durable responses that are probably curative. Therefore, the use of adoptive T-cell therapies to eradicate cancer, as a nexus of basic immunology and clinically meaningful therapy, can be able to achieve cure of cancer (33).

Developing T-cell therapies, that are available for a large number of patients, will be challenging but it is justified because they have the power to cure cancer (397).

“Although the palliation of cancer is the daily task of the oncologist, its cure is our fervent hope”, the description of leukemia by the physician William Castle in 1950.

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