Targeting tumors using T-cell receptor gene transfer: a balance between efficacy and safety

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GENERAL INTRODUCTION AND AIM OF THE THESIS

GENERAL INTRODUCTION

IMMUNOTHERAPY IN CANCER

The immune system is an effective and specific system that protects the human body from invasion by bacteria, viruses, and pathogens that appear harmful and non-self. The immune system may also protect the human body from cancer. Upon recognition of non-self antigens expressed on tumor cells, a network of immune cell types can induce active tumor immunosurveillance.[1] The immune system is a complex network of innate and adaptive immune cell types that must interact in order to induce an effective immune response. The main effector cells in tumor control are T cells and natural killer (NK) cells, both cell types are able to recognize tumor cells and directly eradicate them. Antigen presenting cell (APC) types, such as B cells and dendritic cells, are required to optimally activate or stimulate T cells. Patients showing signs of an active anticancer immune response often demonstrate a more favorable prognosis.[2] Especially the presence of infiltrated T cells in tumors has demonstrated a prognostic value.[3,4] Unfortunately, one of the hallmarks of cancer is the evasion of immunological destruction.[5,6] As tumor cells grow uncontrolled or spontaneously develop into less-immunogenic variants, the immune system may not be capable of detecting all tumor cells and thus reduce tumor growth. These less-immunogenic variants exhibit downregulation of tumor-specific antigens or inhibition of antigen presentation in general. Furthermore, the immune response is often suppressed in tumors, demonstrated by production of immunosuppressive factors, recruitment of immunosuppressive cells and an immunosuppressive tumor microenvironment.

Immunotherapy uses components of the immune system to target and eradicate tumor cells, aiming to control cancer in patients. In general, these therapies try to overcome immune evasion of tumors, by modulating the patient's immune response or by editing immune cells. Several types of immunotherapy exist, including anti-cancer vaccination, use of immune checkpoint inhibitors, infusion of tumor infiltrating lymphocytes (TILs), allogeneic stem cell transplantation (alloSCT), and adoptive transfer of effector cells genetically engineered with chimeric antigen receptors (CARs) or T-cell receptors (TCRs). Most of these immunotherapies directly stimulate, expand or adjust T cells. Which therapy is suitable for a patient, depends on the cancer type and the presence of tumor-specific T-cell infiltration in the tumor. Tumors with low T-cell infiltration are called 'cold' tumors and tumors with high T-cell infiltration are called 'hot' tumors.[7] The tumor mutation burden (TMB), or mutational load, is considered an important factor for the infiltration of T cells.[8] Tumors with a high TMB present a high number of mutation-derived T-cell epitopes that can be recognized by T cells as non-self and harmful. These T-cell epitopes can consequently result in a high number of infiltrated tumor-specific T cells. On average, tumors with the highest TMB are melanoma and lung cancer. Both tumors are often a direct result of DNA damage induced by exposure to UVB irradiation and tobacco, respectively.[9] Therapies using immune checkpoint inhibitors or infusion of TILs

require the presence of endogenous tumor-reactive T cells and are thus suitable in patients with 'hot' tumors. In patients with 'cold' tumors, redirecting T cells to target non-mutated antigens using anti-cancer vaccines and CAR or TCR gene transfer therapies, is considered more suitable. These targeted therapies are often developed for a specific cancer type.

BIOLOGY OF T CELLS

T cells go through several development stages, they originate from bone marrow progenitors, home to the thymus and mature after multiple checkpoints and selection steps.[10] Throughout adult life, development of new T cells slows down and initially T cells circulate between the blood and lymphatic system as functional inactive cells, referred to as naïve T cells. Upon recognition of an antigen, T cells proliferate and differentiate into functional effector T cells. T cells can be distinguished by their TCR determining the antigen specificity and other functional markers determining the T-cell type. The major T-cell types are helper-T cells (CD4+) and cytotoxic-T cells (CD8+). Once activated, CD8+ T cells produce pro-inflammatory cytokines to stimulate other immune cells and release cytotoxic proteins aiming to kill antigen positive cells.[10] CD4+ T cells provide helper function to CD8+ T cells by releasing cytokines and activating APCs. Multiple interactions are required for the activation of T cells.[11] Co-receptors CD4 or CD8 on the T cells stabilize and optimize the interaction of the TCR and antigen-presenting molecules on the target cell. Costimulatory molecules CD80 and CD86 on APCs, binding to CD28 on T cells, provide essential costimulatory signals influencing T-cell differentiation and fate. Various other costimulatory molecules and pathways are involved as well.[12] Absence of costimulatory signals leads to death, anergy or differentiation into T-regulatory cells. Furthermore, cytokines influence T-cell differentiation as well and are especially important for the development of strong effector functions and T-cell survival.[13]

The antigen specificity of a T cell is determined by the TCR, a membrane bound heterodimeric protein that forms a complex with CD3. Most TCRs consist of an α and β chain, both consisting of a variable region forming the antigen-binding domain and a constant region providing structural integrity. Within the variable region, gene rearrangement of three complementary determining regions (CDRs) result in diversity of the TCR repertoire and thus antigen-binding diversity.[14] The CDR3 of the α chain consists of a variable (V) and joining (J) segment, whereas the CDR3 of the β chain consists of a V, J and diversity (D) segment. The number of different TCRs potentially generated is estimated to exceed 10^{15}, although only 10^{7} are actually expressed in one individual at a given time point.[14] This diversity is essential for an effective immune reponse.[15] The TCR rearrangement takes place during the early development of T cells, in the thymus.

MHC, HLA AND THYMIC SELECTION

Major histocompatibility complex (MHC) molecules are cell surface proteins involved in antigen presentation by presenting peptides of protein fragments that can interact with the

TCRs on T cells. MHC class I molecules are ubiquitously expressed on most human cells, whereas MHC class II molecules are predominantly expressed on dendritic cells, B cells and macrophages. MHC class I molecules typically present peptides of 8-11 amino acids (aa) and CD8+ T cells can interact with these peptide-MHC (pMHC) class I complexes.[16] The peptide presentation on MHC class I molecules depends on intracellular processing of endogenous proteins into peptides.[17] Proteasomes within the cytosol degrade proteins into peptide fragments. The transporter associated with antigen processing (TAP) delivers these peptides into the endoplasmic reticulum (ER) where it can bind to MHC class I in the peptide loading complex (PLC). After stable binding, the pMHC complex is released from the PLC and transported to the cell surface. MHC class II molecules typically present 12-25 aa peptides and CD4+ T cells can interact with these pMHC class II complexes.[16] The peptide presentation on MHC class II molecules starts with the uptake of extracellular proteins by endocytic vesicles and the degradation into peptide fragments.[18] MHC class II molecules, synthesized within the ER, are released within vesicles into the cytosol where they can fuse with peptide-containing vesicles. Once a stable pMHC complex is formed it can be transported to the cell surface.

In humans, MHC molecules are also called human leukocyte antigen (HLA). There are three HLA class I molecules, HLA-A, HLA-B and HLA-C, and three HLA class II molecules, HLA-DR, HLA-DQ and HLA-DP.[10] The genes encoding for these molecules are located on chromosome 6 and each individual inherits one paternal and one maternal HLA haplotype.[19] This region with HLA genes is highly polymorphic[20] Whereas the frequencies of certain HLA molecules are low, some HLA alleles are highly frequent although they vary between different populations.[21] For example HLA-A*02:01 is expressed in 43% of the European population and 49% of the East Asian population express HLA-A*24:02.[22] Although HLA molecules are structurally highly similar, their peptide-binding grooves differ and can only form stable complexes with certain amino acids on certain positions, also called anchor residues.[23] Due to the peptide-binding specificities of each HLA molecule, many peptides are only able to bind to specific HLA molecules. As a consequence, HLA polymorphism benefits the immune system since it increases the number of peptides and thus antigens that can be presented.[10] HLA polymorphism is important in organ and stem cell transplantations.[24] The immune system may reject transplanted tissues or cells expressing non-self HLA molecules, also named allogeneic HLA molecules. The HLA molecules of the donor should preferably be fully matched with the HLA molecules of the recipient receiving the transplant. Due to the extensive polymorphism of HLA genes, a fully HLA-matched donor is often only possible for related donors.

The final fate of developing T cells is determined during the thymic selection and is based on the interaction of T cells with pMHC complexes on various types of thymic APCs.[25,26] First, T cells expressing TCRs that are capable of binding with MHC complexes are positively selected

for survival by TCR-mediated signals. The majority of T cells die by neglect, as their TCRs are not able to bind the available MHC complexes. Second, T cells expressing TCRs with high affinity for self-antigens are negatively selected and eliminated. Affinity refers to the strength of the pMHC-TCR interaction, while the strength of the overall interaction between T cells and targets cells is referred to as avidity. The pMHC-TCR interaction is essential for the activation of naïve T cells during thymic selection, but binding of co-receptors and costimulatory molecules are required as well.[11,27] The thymic selection will result in a functional and self-tolerant T-cell repertoire, that is capable of recognizing foreign peptides presented in self HLA. Since T cells do not encounter pMHC complexes in the context of non-self HLA during the thymic selection, T cells expressing TCRs with high affinity for self-antigens presented in non-self HLA are left unharmed. As a consequence, a large repertoire of non-tolerized T cells recognizing peptides presented in non-self HLA is still present.[28]

TCR GENE TRANSFER

The first evidence of T cells eradicating tumor cells was observed in leukemia patients following alloSCT.[29] After engraftment of donor hematopoietic stem cells, donor T cells may recognize tumor cells and mediate antitumor effects, referred to as a graft-versus-leukemia (GvL) effect. Nowadays this therapy is still broadly applied to treat various hematopoietic malignancies. The first adoptive cell therapy transferring naturally occurring tumor-specific T cells was applied in 1988. Patients with metastatic melanoma were successfully treated with TILs from the patient, referred to as autologous TILs.[30] For each patient, lymphocytes were isolated from freshly resected melanoma and following sufficient *ex vivo* expansion these autologous lymphocytes were infused back into the patient followed by high-doses of interleukin-2 (IL2). For patients lacking tumor-reactive T cells, autologous T cells may be redirected towards a tumor-associated antigen. Using viral vectors, autologous T cells can be genetically engineered to express a tumor-reactive TCR or CAR and following sufficient *ex vivo* expansion these T cells can be infused back into the patient. In 2006 the first melanoma patients were treated with genetically engineered T cells, whereby the patient's own T cells were engineered to express a tumor-reactive TCR. This TCR was reactive against 'melanoma antigen recognized by T cells 1' (MART-1) and the TCR-engineered T cells induced specific killing of MART-1 expressing melanoma cells.[31] Following the first clinical trial, the T-cell gene transfer field increased and promising clinical results have been achieved with both TCR- and CAR-engineered T cells. TCRs and CARs are both reactive for a single target, but exhibit several mechanistic and functional differences.[32,33] CARs are synthetic antibody-based receptor proteins that typically consist of an extracellular target-binding domain, a transmembrane domain and one or more intracellular signaling domains that can induce T-cell activation.[34] Since CARs recognize target cells via an antibody-based targeting moiety, they are restricted to epitopes of proteins located at the cell membrane. TCRs can target different types of antigens, since peptides derived from both intra- and extracellular proteins can be recognized, including those involved in essential intracellular processes. Given the

HLA restriction of most peptides and the high number of different HLA molecules among the population, multiple TCRs targeting different peptide-HLA complexes are required to allow TCR gene therapy for the majority of patients.

The TCR gene transfer approach starts with the isolation of T cells, either patient-derived (autologous T cells) or donor-derived (allogeneic T cells). Following *ex vivo* activation, often by crosslinking anti-CD3 and anti-CD28 antibodies, these T cells can be engineered to express the TCR of interest. To date, mainly conventional TCR editing with retroviral or lentiviral vectors is used. These vectors deliver and integrate DNA sequences of the TCRs into the host genome of the T cells.[35,36] More recently, the feasibility and safety of the CRISPR-CAS9 technology was investigated for targeted TCR editing.[37,38] This gene-editing technology enables targeted TCR transgene insertion directly into the TCR gene locus, resulting in replacement of the endogenous TCR and near physiological regulation via the original TCR promotor. After sufficient expansion of the TCR-engineered T cells (TCR-T cells) with growth factors such as IL7 and IL15 or IL2, the TCR-T cells can be infused back into the patient. Patients require a preconditioning therapy with chemotherapy to increase the engraftment and expansion of the infused T cells. Most patients receive a lymphodepleting chemotherapy, leading to inhibition of host immune cells and accumulation of homeostatic cytokines.[39]

Most clinical successes with engineered T cells have been achieved with CD19-reactive CAR-T cells in patients suffering from B-cell acute lymphoid leukemia (B-ALL) and a variety of B-cell lymphoma.[40] Also patients suffering from multiple myeloma (MM) have been successfully treated with B-cell maturation antigen (BCMA)-reactive CAR-T cells.[41] These successes resulted in the market authorization of several CARs, all targeting B-cell antigens CD19 or BCMA.[42] TCR-T cells are explored for safety and efficacy as well. TCR-T cells targeting tumor antigen NY-ESO-1–LAGE-1 induced objective clinical responses in 80% of treated MM patients.[43] TCR-T cells are also clinically tested in patients suffering from solid cancers. NY-ESO-1-reactive TCR-T cells demonstrated objective clinical responses in 55% of melanoma patients and 61% of the synovial sarcoma patients.[44] PRAME-reactive TCR-T cells induced clinical responses across multiple solid cancers in an ongoing clinical trial.[45] Recently, also TCR-T cells reactive against a mutation in oncogene KRAS mediated objective regression in a patient with metastatic pancreatic cancer.[46]

TARGET ANTIGEN TYPES FOR TCR GENE TRANSFER

Several of the pioneering T-cell based therapies have demonstrated that the balance between therapeutic efficacy and safety remains a challenge as T-cell mediated toxicities have occurred.[47] These therapies demonstrated that on-target off-tumor toxicities can occur when T cells target an antigen also expressed on healthy cells. The stringent selection of appropriate tumor-specific targets is essential for a safe and effective TCR gene transfer therapy. An ideal therapeutic target is highly and homogeneously expressed in tumors and is

not or very limited expressed in healthy tissues. Publicly available gene expression databases can be used to select appropriate targets. A broad variety of antigen types can be targeted using TCR gene transfer. These antigens can be specific for an individual patient, patient group, cancer type or lineage.[48,49]

Minor histocompatibility antigens (MiHA) are polymorphic peptides derived from single nucleotide polymorphisms (SNPs). MiHAs play an important role during an alloSCT, as MiHAs can differ between patient and donor. Following HLA-matched alloSCT, an induced graft-versus-leukemia (GvL) or graft-versus-host (GvH) reactivity is mainly mediated by donor T cells recognizing MiHAs.[50] Reactivity against hematopoietic-restricted MiHAs will mainly induce GvL, while reactivity against MiHAs expressed on all tissues can cause GvH disease. A TCR reactive against hematopoietic-restricted MiHA HA-1H was originally isolated from a leukemia patient presenting GvL after a HLA-matched allo-SCT.[51] In a phase 1 clinical trial, this HA-1H reactive TCR has been investigated using TCR gene transfer in leukemia patients after an allo-SCT.[52]

Neoantigens are a group of antigens that are specific to the genome of the cancer cell. They are encoded by somatic mutations and can be specific for one patient or shared between cancer patients within or between different cancer types. Especially neoantigens encoded by shared driver mutations are attractive targets, since they are tumor-specific, involved in tumor progression and assumably expressed by all tumor cells.[53] Examples are mutated KRAS, frequently observed in various gastrointestinal cancers,[46] and mutated nucleophosmin 1 (NPM1), mutated in 30-35% of AML patients.[54] The highest number of neoantigens is expected in tumors with a high mutational load, such as melanoma.[8]

Cancer-testis antigens (CTAs) are a group of antigens predominantly expressed in testis and cancer cells. As testis is an immune-privileged site, with absence of HLA class I expression and presence of Sertoli cells forming a blood-testis barrier, the restricted expression profile suggests CTAs can be a potential target for TCR gene transfer. Potential roles in tumorigenesis and prognostic values have been described for several CTAs.[55] Over 200 genes have been classified as CTA, but not all CTAs are a potential target.[56] Some CTAs are also limited expressed in other healthy tissues and not all CTAs are highly expressed in tumor cells. Using TCR gene transfer, melanoma-associated antigen 3 (MAGE-A3) and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) are most often targeted in clinical trials.[57] More recently, also CTA gene preferentially expressed antigen of melanoma (PRAME) has been targeted.[45] Given the broad and high PRAME expression in many cancer types, PRAME-reactive TCRs are suggested for a variety of cancer types.[58-62]

Tumor-associated antigens (TAAs) are a group of antigens often overexpressed in tumor cells as compared with healthy cells. Some of them are frequently overexpressed in various

cancer types. Amongst the numerous TAAs, an example is transcription factor Wilms' tumor gene 1 (WT1). WT1 is an ideal tumor target based on its expression in a wide range of hematological malignancies and solid tumors but low-level expression in normal tissues, as well as tumor promoting characteristics through induction of tumor angiogenesis and metastasis formation.[63-65] WT1 was even ranked by the US National Cancer Institute as the most promising tumor target for cancer vaccination.[66] WT1 is involved during embryonic development and WT1 is still expressed in some healthy tissues at lower levels. Another TAA that is involved during embryonic development is Claudin-6 (CLDN6). As CLDN6 is a cell surface membrane protein, both CLDN6-reactive TCRs and CARs are being developed for the treatment of various CLDN6-positive tumor types.[67,68]

Lineage-specific antigens that are highly expressed on tumors and not expressed on essential cells or tissues are potential attractive targets for TCR gene transfer. For instance, targeting B-cell lineage-restricted antigens has demonstrated to be safe and effective.[40,41] Although B cells are important immune cells exhibiting specific functions, patients can live without B cells as long as they receive immunoglobulin replacement therapy.[69] In addition to the CD19- and BCMA- reactive CARs, other B-cell lineage-restricted antigens along with reactive CARs or TCRs have been described, among others targeting CD20 or BOB1.[70-72] Furthermore, targeting reproductive lineage-specific antigens with T-cell therapy could potentially be allowed as well, as most reproductive organs are dispensable in later stages of adult life. This offers the potential to target prostate or ovarian lineage-specific antigens in patients with prostate or ovarian cancer. These organs are often radically resected in patients with advanced disease. CAR-T cells targeting extracellular prostate lineage-specific antigen folate hydrolase 1 (FOLH1), also known as prostate-specific membrane antigen (PSMA), demonstrate this prostate lineage may be targeted.[73] No TCRs targeting the prostate lineage or other reproductive lineages are known yet. Targeting lineage-specific antigens is not for all non-essential lineages without risks, as demonstrated for TCRs targeting the melanocyte lineage. In melanoma patients treated with TCRs reactive against melanocyte-specific antigens MART-1 or gp100, severe toxicity in eyes, ears and skin was observed.[74]

IDENTIFICATION OF TUMOR-REACTIVE TCRS

Once a suitable antigen for TCR gene therapy is defined, tumor-reactive T cells must be identified. Since TCRs recognize peptides presented by HLA, peptides derived from the target antigens of interest are required for the isolation of antigen-reactive T cells. Which peptides are presented in HLA depends on processing of peptides and binding to HLA molecules.[75] Algorithms are available for the prediction of presented peptides and they are widely used in research. These algorithms search in protein sequences to predict processed peptides by integrating protease cleavage sites combined with TAP transport and processing characteristics.[76] Furthermore, peptide-binding preferences of the different HLA molecules are used to predict which HLA molecule is capable of binding and presenting a particular

peptide.[77] As HLA molecules share peptide-binding preferences, some peptides are able to bind multiple HLA molecules. Although these algorithms advanced over the years, predicting whether these peptides are indeed naturally processed and presented on HLA complexes remains challenging. For the direct identification of naturally processed and presented peptides in the HLA ligandome of tumor cells, mass spectrometry can be used.[78-80] Both patient-derived tumor cells or tumor cell lines can be used, preferably expressing HLA alleles of interest. This approach starts with the isolation of peptide-HLA complexes from lysed cells using an immunoaffinity column, followed by the separation of peptides using acidic elution and ultrafiltration. Using a tandem mass spectrometer equipped with a high-performance liquid chromatography (HPLC) system, these eluted peptides can be fractionated and characterized. By matching the tandem mass spectra of the eluted peptides with a database of the entire human proteome, peptides of specific antigens can be identified with high confidence.

Given the HLA restriction of peptides and TCRs, the HLA typing of the patient or donor that is used for the isolation of tumor-reactive T cells is highly relevant. T-cell repertoires that can be used for the isolation of tumor-reactive T cells are patients' TILs, patients' T cells, donor T cells or T cells from humanized mice.[81] The humanized mice must be transgenic for an HLA molecule and possible even for the entire TCR gene loci. Following immunization with target antigens of interest, T cells with human TCRs can be identified in these mice.[82] For target antigens exclusively expressed in the tumor such as neoantigens, high-avidity T cells recognizing the tumor as foreign can be detected in the tumor infiltrating T-cell repertoire of patients. If target antigens are also expressed in healthy tissues, referred to as self-antigens, the likelihood of finding high-avidity T cells in the autologous T-cell repertoire is low due to tolerance induction during thymic development. The T-cell repertoire of an HLA-mismatched donor or humanized mice offers the opportunity to search for non-tolerized T cells in the allogeneic T-cell repertoire. During thymic development, T cells capable of recognizing self-antigens in the context of self HLA are centrally deleted in order to prevent autoimmunity. The lack of high-avidity T cells recognizing self-antigens is evident from the low clinical successes obtained with vaccines targeting self-antigens.[81] In contrast, during thymic development T cells do not encounter peptides in the context of non-self HLA. As a consequence, high-avidity T cells recognizing self-antigens in the context of non-self HLA are left unharmed and a large repertoire of non-tolerized T cells recognizing non-self HLA is still present.[28] For several self-antigens, high-affinity TCRs with clinical reactivity have been isolated from HLA-mismatched donors[83-86] and humanized mice[74,87].

The isolation of tumor-reactive T cells can be performed using different approaches, starting with either bulk T cells or antigen-stimulated T cells. The latter are for example TILs or T cells cocultured with irradiated APCs exogenously loaded with peptides or transfected with mRNA coding for peptides or antigens.[88,89] Using peptides derived from the target antigen of interest,

pMHC-multimer complexes labeled with a fluorescent marker can be used to directly single-cell isolate T cells via fluorescence-activated cell sorting (FACS sort).[86,90] Tumor-reactive T cells can also be isolated based on reactivity upon coculture with autologous tumor sample, target cells genetically engineered to express tumor-specific antigens, or target cells loaded with peptides.[91-93] T cells demonstrating upregulation of activation markers or cytokine production can be selected by FACS sort. Subsequently, tumor-reactive T cells can either be expanded to confirm their tumor-reactivity or the TCRs can be directly sequenced. More recently, high-throughput single cell sequencing has been used for the direct isolation of tumor-reactive TCRs from TILs.[94-96] This approach enables the isolation of TCRs without a selection of target antigens, but is in general only feasible for TILs derived from hot tumors. For cold tumors or the isolation of T cells reactive against shared antigens, preferably donor T cells are used.

PRECLINICAL EFFICACY AND SAFETY SCREENINGS

Following the isolation of tumor-reactive T cells, a broad variety of preclinical screenings are essential for the selection of TCRs with most therapeutic potential in mediating effective and safe clinical effects. The safety screenings must focus on T-cell mediated on-target off-tumor toxicities and off-target toxicities. The occurrence of TCR-mediated toxicities in clinical trials demonstrate the estimation of therapeutic efficacy and safety remains challenging. Both T-cell clones and TCR-T cells can be used for preliminary screenings. Since TCR-T cells are the final product, using TCR-T cells in the final screenings is most appropriate.

Efficacy screenings are used to select the most potent TCRs. Functional efficacy can be investigated *in vitro* by coculturing T cells and target cells. Preferably patient-derived tumor cells are used as target, but tumor cell lines, target cells transduced with target antigens or target cells loaded with peptides are valuable targets as well. By including panels of target cells expressing varying levels of the targeted antigen or including target cells loaded with titrated peptide concentrations, T-cell reactivity can be investigated. Various efficacy read-outs can be analyzed within hours after start of the coculture, as CD8+ T cells are able to rapidly induce cell death of antigen-expressing target cells.[97] Using an enzyme-linked immunosorbent assay (ELISA), cytokine production can be measured in the supernatant. Pro-inflammatory cytokine interferon (IFN)-γ levels are often measured, given its crucial roles in activation of immune cells and induction of antigen-presenting molecules.[98,99] Using flow cytometry, antigen-induced activation can be measured on the T cells, such as expression of intracellular cytokines, inducible costimulatory receptor CD137 (4-1BB) and degranulation marker CD107a.[100,101] Flow cytometry can also be used to estimate T-cell mediated cytotoxicity based on survival or disappearance of target cells after coculture. Using a 6-hour ^{51}chromium release assay direct T-cell mediated cytotoxicity can be quantified as well. Besides *in vitro* reactivity, killing potential of TCR-T cells can be assessed using *in vivo* mouse models. For most efficacy screenings immunodeficient mice are injected with luciferase-expressing

tumor cell lines or patient-derived tumor cells, and after sufficient engraftment these mice can be treated with TCR-T cells.[102] Important information can be obtained from these mouse models concerning effectivity. However, toxicity measurements are limited since the human proteome as well as the HLA class I and II molecules are not expressed.[81] Furthermore, many other factors differ between human and mice, such as cytokine and growth factor levels, and expression of molecules involved in T-cell homing, migration and function.[103,104]

On-target off-tumor toxicity may occur when the target antigen is also expressed in healthy tissues, which may lead to severe toxicity. Selection of tumor targets with highly tumor-restricted expression patterns is essential in preventing this toxicity. These expression patterns can be deduced from publicly available gene expression databases. Although databases with thousands of samples across a wide variety of healthy tissues are available, gene expression data of specialized tissues and cell subsets remain limited.[81,105] For instance, unknown MAGE-A12 expression in a cell subset present in brain tissue resulted in severe neurological on-target off-tumor toxicity following anti-MAGE-A3/A9/A12 TCR gene therapy.[106] In addition, even if gene expressions are known, estimating cut-off gene expression values resulting in absence of T-cell reactivity is difficult and very complex to predict, since reactivity is dependent on affinity of the TCR and avidity of the interaction between engineered T cells and target tissues.[107] For example, although expression of carcinoembryonic antigen (CEA) in colonic crypts was known but estimated to be too low to induce severe toxicity, a CEA-reactive TCR resulted in unwanted severe toxicity.[108] These clinical examples illustrate the need for thorough toxicity screenings in the preclinical pipeline of T-cell based therapies. T-cell reactivity screenings against vital organs would preferably be included in these pipelines. Unfortunately, *in vitro* screening options using vital organs are still limited. This also means that the toxicity risks associated with specialized or understudied cell subsets in these vital organs may potentially be missed.

Off-target toxicity can be caused by cross-reactivity of the TCR for a different peptide in the context of the target HLA or allogeneic HLA. To investigate off-target toxicity in the context of the target HLA, various studies demonstrated the relevance of assessing TCR-reactivity against peptide libraries loaded on cells expressing the target HLA.[109-111] These peptide libraries can represent the entire peptide universe of a given length, a combinatorial peptide library (CPL), or the target peptide can be adjusted using single amino acid substitutions, named altered peptide ligands (APLs).[112] A large panel of various healthy cell subsets, all expressing the target HLA, can also be used for the detection and prevention of off-target toxicity. In retrospect, both approaches may have prevented the fatal off-target cardiac toxicity caused by an affinity-enhanced MAGE-A3 TCR. This TCR reacted against a lookalike peptide expressed in cardiomyocytes.[113] Furthermore, TCRs can also be cross-reactive against a peptide presented in allogeneic HLA, also referred to as alloreactivity. This type of off-target reactivity can be investigated by screening TCR-T cells against panels of cell lines expressing various HLA alleles.[72]

RATIONALE FOR IMMUNOTHERAPY AGAINST OVARIAN AND PROSTATE CANCER

Patients with metastasized ovarian or prostate cancer lack curative treatment options, but may benefit from TCR gene transfer. In most ovarian and prostate tumors the mutation burden (TMB) is low, resulting in limited T-cell infiltration and lack of tumor-reactive T cells. Consequently, effects of immunotherapies such as infusion of TILs and treatment with immune checkpoint inhibitors are expected to be limited. To circumvent the limitations of the endogenous T-cell repertoire, redirecting T cells to target other non-mutated antigens may offer a solution for the treatment of ovarian and prostate cancer. Interestingly, not only CTAs and TAAs are optional non-mutated targets, also lineage-specific antigens can potentially be targeted in ovarian and prostate cancer patients. In patients with advanced prostate or ovarian cancer, the healthy counterpart is often resected since these reproductive organs are dispensable in later stages of adult life.

Ovarian cancer is the fifth most lethal cancer type among women.[114] Due to lack of specific symptoms, 58% of the ovarian cancer patients are diagnosed at an advanced or metastatic stage. These advanced stages have 5-year survival rates of only 30%, compared to about 80% for earlier stages.[115] Although late-stage patients initially respond well to standard treatments like debulking surgery, platinum- and taxane-based chemotherapy, or more recently poly (ADP-ribose) polymerase inhibitors, recurrent disease emerges in the majority of patients.[116-118] In clinical trials with ovarian cancer patients, TCRs targeting CTAs NY-ESO-1, MAGE-A4 and more recently PRAME have been investigated.[119] Yet, targeting more antigens is desired and target antigens restricted by more HLA alleles are essential, as most of the investigated TCRs are HLA-A*02:01 restricted.

Prostate cancer is well treatable when localized, but patients with metastasized prostate cancer have a 5-year survival of only 31%.[114,120] Despite high response rates of hormone-sensitive metastasized prostate tumors to androgen deprivation therapy, eventually all metastasized prostate tumors become resistant, without curative treatment options.[120] In addition, most immunotherapies currently developed are constrained due to poor immunogenicity of prostate cancer.[121,122] Multiple extracellular prostate lineage-specific antigens have been targeted by CAR-T cells, of which folate hydrolase 1 (FOLH1), also known as prostate-specific membrane antigen (PSMA), is most frequently investigated in clinical trials.[73,123,124] No TCRs targeting the prostate lineage have been described yet. In conclusion, patients with ovarian or prostate cancer may benefit from TCR gene transfer. To date, a limited number of antigens have been described and no or a limited number of TCRs are available for these patients. Given the HLA restriction of peptides and the high number of different HLA molecules among the population, multiple TCRs targeting different peptide-HLA complexes are required to allow TCR gene therapy for the majority of patients.

AIM OF THE THESIS

The use of TCR gene transfer therapies for the treatment of hematological and solid tumors is increasing. In particular for patients lacking tumor-reactive T cells, this therapy may offer a solution as T cells can be redirected to target tumor- or lineage-restricted antigens. However, the number of known TCRs, target antigens and HLA restrictions are limited for the majority of patients. Furthermore, the occurrence of TCR-mediated toxicities demonstrate the need of preclinical screening options investigating therapeutic efficacy and safety. The research described in this thesis focusses on the selection of strict tumor- or lineage-specific antigens as well as the identification and preclinical screening of potent and safe TCRs recognizing these antigens.

In **chapter 2** we aim to identify high-avidity WT1-specific CD8+ T cells targeting newly identified peptides. Transcription factor WT1 is an ideal tumor target based on its expression in a wide range of tumors, low-level expression in normal tissues and promoting role in cancer progression. In current WT1-targeting vaccine and TCR gene therapy studies, T-cell reactivity is hampered by self-tolerance to WT1 and limited number of WT1 peptides. With newly identified WT1 peptides, derived from the HLA class I associated ligandome of primary acute myeloid leukemia (AML) and ovarian carcinoma samples, we aim to find potent and specific WT1 TCRs from the allo-HLA T-cell repertoire. Once found we will sequence their TCRs and upon retroviral TCR gene transfer into CD8+ T cells we will investigate antitumor reactivity against WT1-expressing solid tumor cell lines, primary AML blasts, and ovarian carcinoma patient samples. Additionally, we aim to compare immunogenicity of our new set of WT1 peptides with previously identified WT1 peptides. We will finally discuss whether our set of TCRs and naturally expressed WT1 peptides are expected to improve WT1-targeting therapies.

In **chapter 3** we aim to overcome the lack of toxicity screening options against vital organs or specialized cell subsets in the preclinical pipelines of T-cell based therapies. In this work we aim to set up preclinical models with hiPSC-derived heart cell subsets and kidney organoids to investigate toxicity risks of tumor-specific T cells. We will include CD8+ T cells reactive against PRAME, HA-1H, CD20 or WT1 (identified in **chapter 2**), all currently used or planned to be used in phase I/II clinical studies. By analyzing cytokine production, cytotoxicity, and phenotypic analysis of T cells and target cells we aim to assess T-cell reactivity of the tumor-specific T cells. In addition, we will focus on possible interaction and crosstalk between infiltrated T cells and hiPSC-derived organoids. Overall, we aim to investigate whether our hiPSC-derived models of specialized cell subsets or complete organoids can predict toxicity risks of tumor-specific T cells and broaden the preclinical pipeline of T-cell based therapies. In addition, we will discuss whether the included tumor-specific T cells or target genes can be considered safe based on our findings.

In **chapter 4** we aim to broaden the population of ovarian cancer patients that can be treated with TCR gene transfer, by identifying additional TAAs, peptide derived from these TAAs and TCRs recognizing them. The number of TCRs with potential for the treatment of ovarian cancer patients are limited and most are restricted to a peptide presented in HLA-A*02:01. We plan to perform a differential gene expression analysis using mRNA-seq datasets to select strictly TAAs with high expression in ovarian cancer, without expression in healthy tissues of risk. In the HLA class I ligandome of ovarian cancer patient samples we aim to identify naturally expressed peptides and identify high-avidity T-cell clones recognizing these peptides. Once identified, we will sequence the TCRs of the most potent and safe T-cell clones and retrovirally transfer them into CD8+ T cells. Using panels of ovarian cancer patient samples, cell lines, healthy cell subsets, and an *in vivo* model, we intend to assess the clinical potential of the identified TCRs.

In **chapter 5** we aim to investigate whether TCR-based therapies targeting prostate lineage-specific antigens may be used for the treatment of prostate cancer. As most reproductive organs are dispensable in later stages of adult life and often radically resected in patients with advanced disease, targeting the prostate lineage could potentially be allowed. We plan to perform a differential gene expression analysis using mRNA-seq datasets to select genes that are highly expressed in prostate cancer and not expressed in healthy tissues, with the exception of healthy prostate tissue. In the HLA class I ligandome of prostate tumor cell lines and prostate gene transduced cells we plan to search for naturally processed peptides derived from the selected antigens and perform T-cell enrichments in the allo-HLA T-cell repertoire. We will screen the isolated T-cell clones, sequence the TCRs of the most potent and antigen-specific T-cell clones and assess their prostate-specific recognition and cytotoxic killing potential *in vitro* as well as *in vivo*. Finally, we aim to discuss whether these TCRs have the potential to be effective as immunotherapy for the treatment of prostate cancer.

In **chapter 6** the results of this thesis are summarized and the results and future perspectives are discussed.

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