TCRs as precision tools against B-cell and plasma cell malignancies

Meeuwsen, M.H.

CHAPTER 1

General introduction and aim of this thesis

GENERAL INTRODUCTION

The immune system and hematopoiesis

The immune system is our body's defense mechanism against pathogens. It comprises two primary arms: the innate and adaptive immune systems. The innate immune system serves as the first line of defense, providing rapid, non-specific protection against pathogens like bacteria and viruses. The adaptive immune system is highly specific and has a memory component. It recognizes and targets pathogens, allowing the body to remember and respond more effectively upon re-exposure. Both innate and adaptive immune cells originate from hematopoietic stem cells located in the bone marrow, a process known as hematopoiesis. This ensures the continuous renewal of immune cell populations, sustaining the body's safeguard against pathogens.

B cells

B-cell immunity

B cells play a crucial role in the adaptive immune response against pathogens by producing and releasing immunoglobulins. These immunoglobulins exist in two forms: as the B-cell receptor (BCR), acting as a membrane-bound antigen receptor for B cells, and as a secreted form, where they serve as the primary effector proteins released by mature B cells and plasma cells.

Immunoglobulins consist of two identical heavy and light chains, each containing constant and variable domains(1). The variable domain is responsible for binding to antigens, while the constant Fc domain facilitates effector functions. When antibodies bind to pathogens, they can coat the pathogen's surface, preventing them from binding and infecting host cells. The Fc tail-mediated effector mechanisms involve three main processes. Firstly, the Fc domains are recognized by innate immune cells through Fc receptors, resulting in the uptake and destruction of antibody-coated pathogens by phagocytic cells(2). Secondly, antibody binding via the Fc domain can trigger the activation of the classical complement cascade, leading to the recruitment of phagocytes or direct destruction of pathogens(3). Finally, Fc domains are bound by receptors that mediate active transport of immunoglobulins across mucosal membranes(4).

B-cell development

The initial phases of B-cell development occur independent of antigen and unfold within the bone marrow. This is where common lymphoid progenitor cells emerge from hematopoietic stem cells. Subsequently, these progenitors differentiate through stages, progressing from common lymphoid progenitors to pro-B cells, then pre-B cells, and eventually immature B cells. This differentiation process is linked to the stepwise formation of a functional B-cell receptor(5).

In the pro-B-cell phase, the initiation of BCR formation begins with VDJ recombination of the heavy chain variable domain. Following successful rearrangement, pro-B cells progress into pre-B cells. During the pre-B-cell stage, a surrogate light chain, comprising lambda5 and VPREB3, is expressed before the successful rearrangement of light chains(6). This surrogate light chain facilitates signaling through the pre-BCR, a necessity for proliferation and further differentiation. Subsequently, in the pre-B-cell stage, VJ recombination of the light chain transpires, leading to the expression of a functional IgM isotype BCR on the surface of immature B cells. These immature B cells then migrate to secondary lymphoid organs such as the lymph nodes and spleen, where they undergo further differentiation into mature B cells. Within secondary lymphoid organs, several developmental pathways are possible. In this context, the route that leads to formation of class-switched memory B cells or plasma cells from mature follicular B cells will be explained. This process is initiated upon native antigen encounter and relies on the assistance by helper T cells(7). Upon activation, follicular B cells progress into an activated state, leading to their differentiation into short-lived plasma blasts that produce IgM immunoglobulins or entry into germinal center reactions. Within these germinal center reactions, somatic hypermutation occurs, resulting in the production of antibodies with enhanced affinities for the specific antigen(8). This is followed by class switching to IgA, IgG, or IgE constant domains, broadening the effector functions of immunoglobulins. Within this context, B cells differentiate into memory B cells or plasma cells. Plasma cells secrete high levels of immunoglobulins, thereby providing direct protection against pathogens. While memory B cells establish immunological memory and can rapidly transform into plasma cells upon reinfection. Long-lived plasma cells return to and reside in the bone marrow.

Throughout each developmental stage, a distinct set of B-cell restricted genes is expressed. Some genes are only transiently expressed during specific stages, such as lambda5 and VPREB3 at the pre-B-cell stage(9, 10). Other genes, like CD19 and CD20, are more broadly expressed throughout the developmental process, but many of these genes are downregulated upon differentiation into plasma cells(11). In contrast, transcription factor POU2AF1 is expressed throughout the complete developmental process and also at the differentiated stage of plasma cell(12).

IgM, IgG and IgA functions and the role of the Jchain

Immunoglobulins come in a variety of isotypes, each with its unique function and specific distribution within the body. Among these isotypes, the most prevalent ones are IgM, IgG, and IgA.

IgM is predominantly secreted in a pentameric form by non-class-switched plasma cells. During an immune response, IgM antibodies are the initial responders, characterized by

their relatively lower affinity(7). Beyond their role in neutralization, IgM molecules serve as initiators of complement activation(13). The pentameric structure of IgM enables it to bind to multiple pathogens, inducing clumping, a process known as agglutination, which aids in their capture and destruction. Notably, IgM antibodies occur in the bloodstream as well as mucosal sites such as the gastrointestinal tract and lungs. In these locations, they can traverse epithelial barriers, providing protection against invading pathogens.

IgG antibodies are the predominant class found in both the blood and extracellular fluids. IgG plays a central role in pathogen neutralization and opsonization, rendering pathogens more susceptible to phagocytosis by immune cells, and initiation of the complement cascade.

IgA antibodies are encountered in both monomeric and dimeric forms, primarily functioning through pathogen neutralization. Consequently, IgA serves as a critical component of mucosal immunity in the gut and lungs.

Multimerization of IgM and IgA is facilitated by the joining chain (Jchain), a small polypeptide with binding sites for IgM and IgA monomers. Additionally, the Jchain acts as a ligand for the polymeric immunoglobulin receptor (pIgR) situated on the epithelium of mucosal tissues(14). Upon binding with the Jchain, the immunoglobulin complex is internalized and transported to the lumen, where IgA and IgM complexes exert their protective functions.

T cells

T-cell function

T cells play an essential role in the adaptive immune response by protecting against intracellular pathogens. They rely on a specific receptor called the T-cell receptor (TCR), which is essential to identify infected cells. Via the TCR, T cells can recognize foreign peptides that are presented in human leukocyte antigen (HLA) molecules on the surface of target cells(15). The TCR forms a complex together with various CD3 molecules. When the TCR binds to its peptide-HLA ligand, the CD3 complex functions as a signal transmitter triggering T-cell activation(16). Furthermore, CD3 plays an essential role in ensuring the TCR is expressed on the cell surface(17). In addition to the TCR and CD3, T cells express a CD4 or CD8 co-receptor. These co-receptors interact with HLA on the target cell and stabilize the interaction between the TCR on the T cell and the peptide-HLA complex on the target cell(7). This stabilization is often crucial for T-cell activation. The expression of either CD4 or CD8 divides T cells into two groups. CD4 positive T cells, known as helper T cells, assist in the activation and differentiation of other immune cell types like B cells and CD8 T cells by producing helper cytokines. CD4 T cells become activated when they recognize peptides presented by HLA class-II molecules. CD8

positive T cells have a cytotoxic function, causing lysis of target cells when their TCR binds to peptides displayed by HLA class-I molecules. When the TCR engages, CD8 T cells release perforin and granzyme from cytotoxic granules. Perforin creates pores in the target cell's membrane, allowing granzyme to enter and initiate a cascade that leads to cell death. In addition to foreign peptides presented by infected cells, T cells can recognize mutated peptides as non-self(18, 19). Mutated peptides can arise from genetic mutations that occur in tumor cells. Consequently, T cells can recognize and clear tumor cells that present such mutated peptides and contribute to tumor control(19).

T-Cell development and selection

Lymphoid progenitor cells undergo development and maturation within the thymus, resulting in the formation of mature naïve T cells. Within the thymus, developing T cells go through various developmental stages, each marked by the rearrangement of their TCR genes. Initially, the TCR beta chain undergoes rearrangement, followed by the alpha chain. During the TCR rearrangement process, an astounding degree of diversity in TCRs is achieved through the stochastic recombination of single V (D) and J gene segments drawn from a vast array of germline-encoded variants(20). Additionally, the rearrangement process allows for the introduction or removal of nucleotides at the V (D) J junctions, thereby generating what is termed "junctional diversity." This process significantly expands the repertoire of unique TCRs, theoretically allowing for a total number of approximately 1x10^18 distinct TCRs(7). In practical terms, the actual number of distinct TCRs is substantially more limited since the entire human T-cell repertoire encompasses approximately 10^12 T cells. In addition, some specific combinations occur more frequently at the population levels than would be expected if TCRs were rearranged randomly(21). These TCRs are referred to as public TCRs and demonstrate that total TCR repertoires are much more limited than theoretical calculations suggest. Studies have estimated that the pool of naïve T cells comprises approximately 1x10^8 unique TCRs(22).

Upon the completion of TCR rearrangement, T cells undergo a critical phase known as positive selection. This process assesses the ability of TCRs to interact with self-HLA molecules. HLA is a highly polymorphic gene for which thousands different genetic subtypes have been identified across the global population. Self-HLA refers to HLA-alleles expressed by an individual while HLA-alleles not expressed by an individual are foreign or non-self HLA-alleles. T cells that fail to bind to self-HLA molecules with sufficient affinity undergo programmed cell death (apoptosis), while those with TCRs proficient in binding to self-HLA molecules persist(23). Subsequently, following positive selection, T cells differentiate into either CD4 or CD8 single-positive subsets. The development continues with another crucial step: negative selection. During negative selection, T cells encounter thymic antigen-presenting cells (APCs) presenting self-peptides. T cells that

exhibit affinity for these self-peptides presented by APCs are removed from the T-cell repertoire(24). While T cells with a high affinity for self-peptides in self-HLA are deleted, no selection is made for T cells that recognize self-peptides in foreign/allogeneic HLA. As a result, many T cells in the T-cell repertoire will have the ability to recognize self-peptides in allogeneic (allo-) HLA molecules(25). T cells with allo-HLA reactive TCRs can mediate graft rejection after solid organ transplantation that occurs in absence of immunosuppressive therapies, as well as graft versus host or graft versus leukemia responses after HLA-mismatched allogeneic stem cell transplantation(26-28).

Positive and negative selection combined yield a finely tuned T-cell repertoire that possesses the capability to recognize foreign peptides presented within self-HLA molecules while maintaining tolerance toward self-peptides presented in self-HLA, thereby ensuring immune competence without autoimmunity.

Antigen presentation

Goal of antigen presentation

Within tissues, intracellular and extracellular contents are consistently processed and presented to T cells as a fundamental part of protection against pathogens. To this end, proteins are continuously degraded inside the cell, resulting in small protein fragments called peptides. These peptides are loaded in HLA molecules and transported to the cell membrane where peptide-HLA complexes can be recognized by T cells. This presentation serves the purpose of revealing intra- and extra-cellular contents to T cells, allowing them to initiate an immune response when foreign peptides are encountered. Two distinct categories of antigens exist, which are each primarily associated with a different type of HLA molecule. Peptides from intracellular proteins are presented in HLA class-I molecules, while extracellular proteins are taken up, processed, and presented in HLA class-II molecules. Beyond their mechanistic differences, HLA class-I and class-II molecules are expressed by different cell types in the body. HLA class-I molecules are found on the surfaces of most nucleated cells under normal conditions, HLA class-II molecules are primarily expressed by antigen-presenting cells (APCs) and may be upregulated in other cell types during inflammation(29).

Within the scope of this thesis, further focus will be on mechanisms underlying antigen presentation in HLA class-I molecules. Presentation of peptides by HLA class-I leads to the recognition of antigens by CD8 T cells and subsequent initiation of an immune reaction that contributes to the body's defense.

Processing and presentation

Synthesis of HLA class-I molecules takes place in the endoplasmic reticulum (ER), where HLA class-I is subsequently loaded with peptides localized in the ER and transported

to the cell surface. The peptide repertoire in the ER is established via the classical and alternative pathways of peptide processing(30). In the classical pathway, proteins are degraded in the cytosol by proteasomes, after which they are transported into the ER by transported associated with antigen processing (TAP). TAP preferentially transports peptides between 8-16 amino acids in length, skewing the ER peptide repertoire to relatively short peptides(31). In the ER trimming of the N-terminus is mediated by ERAP1, generating peptides of length compatible with binding to HLA class-I. Studying cells with defects in components of the classical pathways has revealed various other mechanisms of peptide processing, together referred to as alternative pathways. One example of alternative peptide processing are type II proteins that are part of secretory pathways, these peptides are located in the cell membrane of the ER with their N-terminal side in the cytosol and their C-terminal side in the ER lumen(32). The C-terminal fraction of these proteins can be cleaved by signal peptide peptidases (SPP) releasing them into the ER independent of TAP and enabling their binding to HLA class-I and subsequent presentation to T cells on the cell membrane.

HLA variants and allele frequencies

HLA class-I molecules are composed of an HLA heavy chain and a light chain called beta 2-microglobulin (B2M). HLA-I heavy chains are encoded by three different genes, HLA-A, HLA-B and HLA-C. Each individual carries two variants of each gene resulting in a total of six HLA class-I alleles per person. At a population level, the diversity in peptide repertoires that arises from the variety in HLA alleles provides protection to mutated and newly arising pathogens(33). While the total number of HLA polymorphisms is huge, some alleles are frequently expressed. For example, HLA-A*02:01, HLA-A*24:01, HLA-B*35:01, and HLA-B*07:02 are expressed in 39%, 21%, 13%, and 8% of the world population, respectively(34). The frequency of each allele can be higher or lower within populations of a specific ethnic background.

HLA binding motifs

The peptide binding groove of HLA is flanked by two alpha helixes while the bottom of the groove is formed by beta pleated sheets. The N- and C-terminal sides of HLA class-I are closed restricting the length of peptides that can fit into the groove in a conventional manner to a size of 8-11 amino acids(35). In the peptide binding groove six pockets, termed A-F, can be identified. Pocket B and pocket F are the most dominant determinants of HLA specific peptide binding preferences(36). Pocket B binds peptides at position 2 (p2) and pocket F binds the C-terminal amino acid of peptides (pΩ). Amino acid residues at these positions of peptides are therefore most crucial for binding to specific HLA molecules. Different HLA molecules have amino acids variations in the peptide binding pockets, generating different motifs for preferred peptide binding. Over the past decades, data on peptide binding motifs of HLA alleles has become increasingly

available from mass spectrometry studies as well as peptide-HLA crystallography. These data underly peptide binding prediction algorithms and have increased the reliability of such predictions.

B-cell and plasma cell malignancies

Origin and treatment of B-cell and plasma cell malignancies

During B-cell development, malignancies can arise at any stage and the stage at which a malignancy develops determines the specific type of B-cell malignancy. B-cell malignancies form a heterogenous group of cancers that can be categorized in three main groups: leukemias, lymphomas and plasma cell malignancies. The development of B-cell malignancies is linked to B-cell intrinsic processes like V(D)J recombination, somatic hypermutation (SHM), and class-switch recombination (CSR)(37). These processes are critical to B-cell development and antibody diversification but carry risks due to the genetic alterations they induce. V(D)J recombination, the initial step in creating the diverse repertoire of B-cell receptors, involves the rearrangement of variable (V), diversity (D), and joining (J) gene segments. While essential for generating a wide array of antigen-specific receptors, this process can lead to chromosomal translocations or insertions/deletions if errors occur. Such genetic aberrations can disrupt the regulation of genes governing cell growth and survival, potentially leading to uncontrolled cellular proliferation and malignancy. Additionally, SHM and CSR can add to the development of B-cell malignancies, these processes occur in activated B cells within the germinal centers of lymphoid organs. SHM introduces point mutations in the variable region of the immunoglobulin genes to increase antibody affinity, while CSR alters the constant region of the heavy chain to change the antibody class. Both processes are mediated by the enzyme activation-induced cytidine deaminase (AID) and involve DNA modifications. While these modifications are crucial for critical for developing potent B-cell responses, they also harbor risks. The activity of AID, which is focused on immunoglobulin genes, can sometimes lead to off-target effects, causing mutations or DNA breaks in other genomic regions. This genomic instability, especially when coupled with a genetic predisposition of some individuals that may affect DNA repair mechanisms or the regulation of oncogenes and tumor suppressor genes, significantly elevates the risk of developing B-cell malignancies.

When pro- and pre-B cells undergo malignant transformation, this leads to development of acute lymphoblastic leukemia (ALL)(38). ALL is characterized by rapid proliferation of ALL cells in the bone marrow. The malignant growth leads to a reduction in healthy hematopoietic cell generation causing symptoms such as fatigue, increased susceptibility to infections, and bleeding. The treatment of ALL often involves intensive chemotherapy, which may be followed by stem cell transplantation, especially in high-risk cases or if the leukemia returns after initial treatment. Targeted therapies have also emerged

as effective options for certain subtypes of ALL. The prognosis for patients with ALL varies based on factors such as age, the presence of specific genetic aberrations, and the response to initial treatment(39). While advancements in treatment have improved outcomes, achieving a cure remains challenging and often requires aggressive treatment. Chronic Lymphocytic Leukemia (CLL) arises when mature B lymphocytes undergo malignant transformation(40). This type of leukemia is characterized by the slow accumulation of these malignant B cells in the bone marrow, blood, and lymphoid tissues. Unlike ALL, CLL typically has a more indolent course, but it can lead to a decrease in the production of healthy blood cells over time, resulting in symptoms like fatigue, increased susceptibility to infections, and anemia. The treatment of CLL in adults is less intensive than that for ALL and may not be immediately required in early stages. Treatment strategies include targeted therapies, such as monoclonal antibodies and kinase inhibitors, and may involve chemotherapy or immunotherapy, particularly in more advanced or aggressive cases. The prognosis for patients with CLL varies, influenced by factors such as the stage of the disease, the presence of specific genetic mutations, and the individual's overall health. Recent advancements in targeted treatments have significantly improved the management and outcomes for CLL patients(41). The disease is generally considered chronic and may require ongoing management rather than aiming for a cure.

Lymphomas are cancers that arise in the lymphatic system, containing various subtypes with different characteristics and treatment approaches(42). Lymphomas most frequently develop from B cells, but lymphomas of T-cell origin also exist. Indolent forms of B-cell Lymphoma, like Follicular Lymphoma, may not require immediate treatment and can be closely monitored, a strategy known as "watchful waiting." When treatment is necessary, options include chemotherapy, immunotherapy such as monoclonal antibodies, targeted therapy, and radiation therapy. Aggressive lymphomas like Diffuse Large B-Cell Lymphoma are usually treated with a combination of chemotherapy and immunotherapy. In some cases, especially for relapsed or refractory lymphomas, stem cell transplantation may be considered. The development of targeted therapies, which specifically target cancer cells based on their genetic characteristics, has significantly improved treatment outcomes and reduced side effects for many lymphoma patients(43). Personalized medicine, considering the specific characteristics of the lymphoma and the patient's overall health, is increasingly becoming the norm in lymphoma treatment strategies.

Multiple myeloma (MM) is the malignant counterpart of plasma cells. In this disease, a (precursor) plasma cell undergoes malignant transformation and clonally proliferates in the bone marrow(44). The uncontrolled growth interferes with production of healthy blood cells, leading to fatigue, anemia, and increased susceptibility to infections, but also results in the overproduction of a monoclonal antibody, known as paraprotein or

M-protein(44). The high levels of this abnormal protein can cause various complications, including kidney damage and impaired immunity. Additionally, the myeloma cells disrupt normal bone remodeling, leading to painful bone lesions and an increased risk of fractures. Treatment for MM typically involves chemotherapy, targeted therapy such as proteasome inhibitors and immunomodulatory agents, and in some cases high dose chemotherapy and autologous stem cell transplantation. The introduction of novel therapeutic agents has markedly improved patient outcomes, yet MM is still generally incurable, with treatment aimed at controlling disease progression and managing symptoms(45).

CAR T cell therapy for B-cell malignancies

In recent years innovative cellular therapies have emerged as treatment for patients with relapsed/refractory malignancies of B-cell origin. These new treatments use T cells genetically modified to express a receptor directing them to target the tumor. The first FDA approved therapies use chimeric antigen receptor (CAR) T cells. CARs are based on the structure of antibodies, they contain a single chain variable fragment (scFv) that recognizes a specific protein(46). The scFv is combined with co-stimulatory and signaling domains to induce T-cell activation after antigen binding. CAR T cells are designed to target specific surface proteins expressed on malignant cells. For malignancies of B-cell origin, broadly expressed tumor specific antigens are not available and therefore B-cell lineage antigens are targeted. B-cell lineage antigens are antigens that are exclusively expressed by cells of the B-cell lineage for which expression was maintained upon malignant transformation. An example is CD19, which has been used as a CAR target for various B-cell malignancies. CD19 CAR T-cell response rates are between 53% and 74% for patients with B-cell lymphoma(47). 39% to 55% of the patients reached complete responses, of which 49.5%- 80% were still complete responders after 24 months. For ALL initial complete response rates of CD19 CAR T cells are between 62% and 86%(48, 49). Many patients treated with CD19 CAR T cells received consolidative allogeneic stem cell transplantation, which hampers the interpretation of long-term complete responders. Overall, in ALL <50% of patients achieved long term event-free survival. Plasma cell malignancies cannot be treated with CD19 CAR because CD19 expression is lost upon B-cell differentiation to plasma cells. MM cells highly express B-cell maturation antigen (BCMA) and the expression of BCMA is restricted to B/plasma cells(50). BCMA-targeting CAR T cells induced complete response rates in 33-83% of patients, but despite high initial responses most patients eventually relapse(51). The studies on CAR T cells have highlighted the potential of T-cell based therapy for treatment of B-cell malignancies. Additionally, CAR T-cell therapy targeting B-cell lineage antigens resulted in B-cell aplasia as a side effect of therapy(48). While this scenario is not ideal due to subsequently compromised B-cell immunity, B-cell aplasia can be managed by treatment with soluble immunoglobulins. Overall, CAR T cells demonstrate that B-cell lineage antigens are good targets when treating B-cell malignancies using T-cell therapy.

Despite the successes, CAR T-cell therapy has revealed new challenges ahead as many patients relapse after therapy.

Challenges of CAR T-cell therapy

One prominent escape mechanism that has been observed after CAR T-cell therapy is antigen escape, where reduced or loss of antigen expression results in evasion from CAR T cells and subsequent tumor outgrowth(52). The most straight forward mechanism behind antigen escape is heterogenous antigen expression at the start of therapy, which leads to skewing and outgrowth of the antigen negative or low population(53, 54). Furthermore, complete loss of antigen can occur by genetic mutations(55). Besides complete antigen loss, various mechanisms can cause a reduction in antigen expression resulting in an expression level too low to be detected by CAR T cells. Reduced surface antigen expression can result from trogocytosis, a process in which the CAR T cells 'nibble' membrane fragments from the target cell. In the case of BCMA, active cleavage from the cell membrane mediated by gamma-secretase can also reduce BCMA surface expression(56). While patients can relapse due to antigen escape, a substantial fraction of patients display antigen-positive relapse from CAR T-cell therapy. Antigen-positive relapse is the result of incomplete tumor clearance potentially resulting from the inability of CAR T cells to reach all tumor cells or due to suboptimal CAR T-cell performance (52, 57). CAR T cells utilize artificial signaling that may render CAR T cells sensitive to dysfunction, particularly through tonic signaling or activation induced dysfunction(58). Tonic signaling is activation of CAR T cells that occurs in the absence of stimulatory antigen. This antigen-independent activation can result in progressive differentiation, upregulation of coinhibitory molecules, and antigen-independent proliferation, compromising CAR T-cell performance(59, 60). At the same time, CAR T cells have been described to be sensitive to antigen-dependent overactivation(60-62). Depending on the respective target antigen and CAR design, CAR T-cell exposure to antigen expressing target cells can lead to rapid exhaustion and antigen induced cell death, that may especially be at play in settings of high tumor burdens or solid tumors(60). Another challenge of CAR T cells is treatment related toxicity, particularly cytokine release syndrome. Upon activation, CAR T cells release significant amounts of proinflammatory cytokines that can trigger cytokine release syndrome in patients, a condition that can be life-threatening and frequently requires intensive care intervention(63). Furthermore, severe cases of neurotoxicity have been associated with CAR T-cell therapy, termed immune effector cell-associated neurotoxicity syndrome.

TCR T-cell therapy for B-cell malignancies

Rationale for TCR-therapy

While CAR T cells have shown clinical success for therapy of B-cell malignancies, they also revealed challenges that remain to be addressed. Two of these challenges are antigen

escape and CAR T-cell dysfunctionality. To combat antigen escape, combination therapies are likely needed, but the requirement for antigen surface expression limits possibilities of targetable CAR antigens(64). As a complementary modality to CAR T cells, transgenic TCR T cells could be of benefit. TCR T cells could be of unique value owing to their ability to recognize peptides from intracellular located proteins. This allows hypothetical targeting of any protein as long as the criteria for a favorable expression profile is met. This also means that proteins essential for survival of malignant cells, such as certain transcription factors that are located inside the cell can be targeted using TCR T cells(65-67). Furthermore, TCRs can be used to target peptides from proteins that are secreted by malignant cells, such as immunoglobulins. CAR T cells targeting these proteins are coated by target protein present in the circulation, which can render them insensitive(56, 68). Another potential benefit of TCR T cells over CAR T cells, is that TCRs are the natural receptors of T cells and TCR T cells might therefore be less sensitive to overstimulation, potentially leading to increased persistence and functionality compared to CAR T cells(58, 60).

While the recognition of peptides in the context of a specific HLA molecule provides advantages for TCR T-cell therapies, it should also be noted that it limits the applicability of a single receptor to patients expressing the targeted HLA allele.

Proposed antigens for TCR T-cell therapy

In the context of B-cell malignancies, CAR T-cell therapies have focused on targeting antigens specific to the B-cell lineage. As previously mentioned, targeting B-cell antigens induces B-cell aplasia, but this is generally considered an acceptable side effect. Consequently, B-cell lineage antigens present an interesting target for TCR T-cell therapies. A distinct advantage of TCR T cells lies in their capacity to target peptides derived from any protein, irrespective of its cellular localization. To increase the likelihood of successful TCR identification and to allow broad application of TCR therapy across a diverse patient group, identification of as many potential target proteins as possible is important. Gene expression datasets can be analyzed to identify proteins exclusively expressed in both malignant and healthy B-cell lineages(69). Ideal target proteins are characterized by high expression in tumors, coupled with homogeneous expression both within individual tumors and across patients. This homogeneity would ensure that a single receptor could potentially be used to treat a wide array of patients. After the identification of target proteins, it is critical to determine which peptides from these proteins are presented by specific HLA molecules on the surface of malignant B cells. Such peptides are potential candidates for recognition by B-cell antigen targeting TCRs.

Epitope identification

The identification of peptides derived from target proteins for which specific T cells can be identified is a crucial step in the development of TCR-based therapies for B-cell

malignancies. Online prediction algorithms can be used to identify which peptides derived from a protein are predicted to bind to an HLA-allele of interest(70). Identification of T cells which recognize peptides identified using prediction algorithms has been performed in the past. These studies observed that these T cells, despite having a high affinity for the target peptide, are often not able to recognize tumor cells expressing the respective target protein(71). This discrepancy demonstrates that predicted binding to HLA does not necessarily imply effective processing and presentation of the peptide in the respective HLA on the surface of malignant cells. To identified T-cell clones that could have the ability to recognize malignant cells expressing a specific target protein, it is therefore important to establish which peptides derived from a target protein are processed and presented in specific HLA molecules on the surface of malignant cells. To determine this, the HLA peptidome of B-cell malignancies can be analyzed(72). The HLA peptidome refers to the collection of peptides that are presented in HLA on the cell surface. To determine the HLA peptidome of B cell malignancies, peptide-HLA complexes can be isolated from malignant B cells, the peptides can then be eluted from HLA and peptide sequences determined by mass spectrometry. The peptidome can then be searched for peptides derived from a target protein of interest. Finally, the HLA origin of these peptides needs to be determined, this can be done by using HLA binding prediction tools together with the HLA typing of the cells used for peptide elution(73). In this manner peptides derived from target proteins of interest that are presented in a specific HLA molecule of choice can be identified. Using this method ensures that T cells with sufficiently affinity for the targeted peptide should be able to recognize malignant cells expressing the respective protein and HLA molecule. This approach has previously resulted in identification of T cells with TCRs valuable for TCR T-cell therapy of hematologic malignancies and will be applied in this study to identify promising TCRs(65, 74).

Identification of TCRs recognizing self-antigens

In the case of B-cell malignancies, many target antigens are non-mutated self-antigens. For these antigens immunological tolerance exists in the HLA-matched setting, and TCR identification from an HLA-matched individual could at best result in identification of intermediate affinity TCRs (27, 75). To circumvent this tolerance various methods can be applied. For example, the identification of TCRs recognizing human peptide-HLA complexes from mice(76). Alternatively, *ex vivo* affinity maturation can be performed to enhance the affinity of a TCR isolated from the autologous repertoire to ensure sufficient sensitivity to self-antigens(77). Furthermore, T cells can be isolated from HLA-mismatched healthy donors, which is based on the concept that the naïve T-cell repertoire hypothetically contains T cells specific for any foreign peptide-HLA complex(78, 79). While the frequencies of such T cells are extremely low, T-cell isolations over an HLA-barrier have resulted in identification of various tumor targeting TCRs in the past.

For example, identification of TCRs from HLA-mismatched healthy donors resulted in identification of potent TCRs with specificity for B-cell antigens in various HLA alleles (65, 80). To identify T cells which can recognize peptide-HLA complexes of choice, peptide-HLA (pHLA-) multimers can be used. pHLA-multimers contain multiple peptide-HLA complexes linked to beads. pHLA-multimers mimic natural peptide-HLA complexes as present on the surface of cells, and therefore pHLA-multimers can be used to bind T cells with TCRs able to bind the respective peptide-HLA complexes. pHLA-multimers coupled to a fluorochrome allow flow cytometry-based single-cell isolation of pHLA-multimer binding T cells. Sorted T cells can be clonally expanded, and T-cell clones can then be analyzed to determine recognition profiles, including on-target specificity and potency.

Safety of TCRs

As previously mentioned, the number of unique TCRs in the naïve repertoire has been estimated to be around 1x10^8. On the antigen side, calculations suggest that, considering the existence of 20 amino acids, the potential number of unique peptides that could be generated and presented within an individual's HLA molecules stands at approximately 12x10^11, focusing solely on 10-mer peptides (81). This vast surplus of potentially foreign peptides in comparison to the number of unique TCRs within the naïve T-cell repertoire reveals the necessity for TCRs to possess intrinsic cross-reactivity toward various peptides. This aspect should be considered when analyzing the 'specificity' of an individual TCR.

Since all TCR identification methods in the context of non-mutated peptides are based on avoidance of central tolerance or mutations to alter affinity, any of these approaches is inevitably linked with a risk of cross-reactivity with other peptides presented in the HLA allele of interest. In addition, cross-reactivity with any peptide in any other HLA allele could occur highlighting that extensive safety screening should always be performed. Even when safety screenings are performed thoroughly a risk for unidentified cross-reactivity will remain and should be considered. In the past unidentified cross-reactivities have had detrimental effects when such TCRs were used to treat patients, resulting in lethal toxicity(82, 83). Safety screenings to assess cross-reactivity with other peptides within the target HLA, can include extensive cell panels positive for target HLA but negative for antigen of interest and healthy cell subsets negative for the antigen of interest(84). An alternative strategy to assess safety is to first determine the peptide recognition motif of a specific TCR using alanine/serine substitutions or a peptide library scan(84, 85). This motif can then be used to perform a targeted search for peptides to which cross-reactivity might exist. Cross-reactivity with other HLA alleles can be assessed using an Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCL) panel(86, 87). EBV-LCL panels contain EBV immortalized B cells generated from individuals with diverse HLA backgrounds. EBV-LCL panels can be designed in such a way that all common HLA-I alleles are included.

TCR T-cell therapy for B-cell malignancies

To generate multi-antigen-targeting T-cell therapy, TCR T cells can complement CAR T cells to prevent antigen negative immune escape(88). Additionally, the combination of CARs and TCRs can combine the advantages of both therapies and improve the overall efficacy. Multiple TCRs will be needed to allow therapy of patients with divers HLA typings and different types of B-cell or plasma cell malignancies. To identify as many TCRs as possible, all possible target proteins need to be identified and the HLA peptidome of B-cell malignancies can be screened for peptides derived from these proteins that are presented in HLA molecules of interest. T cells with specificity for B-cell lineage protein derived self-peptides can be identified from HLA-mismatched healthy donor PBMCs using pHLA-multimers(65, 80). The cross-reactive nature of TCRs means that thorough safety screenings need to be performed to prevent toxicity(83). Various efficacy screenings should be performed, ultimately including lysis of patient-derived malignant cells as well as *in vivo* anti-tumor efficacy experiments, to identify TCRs that would ultimately be able to induce potent anti-tumor responses in patients. Combining safety and efficacy profiles can reveal which TCRs have value to be further developed for treatment of B-cell and plasma cell malignancies.

AIM OF THIS THESIS

Over the past decades CAR T-cell therapies have been extensively studied and have even become part of standard healthcare in certain parts of the world. However, these advances have also highlighted the heterogeneity and plasticity of B-cell malignancies and demonstrated that multiple antigens should be targeted simultaneously to eliminate antigen escape as a potential way to evade T-cell therapy. TCR T cells could be a useful addition to CAR T cells based on their ability to recognize peptides originating from antigens located on the cell surface as well as inside the cell, allowing them to target antigens that cannot be targeted by CAR T cells. The HLA restricted recognition of TCRs implies that many TCRs need to be identified to have TCR availability for a large population. The aim of this thesis is to identify a large set of TCRs that can be used to treat patients suffering from B-cell malignancies.

In **chapter 2** we address this general aim using various sub-aims, the first aim is the identification of proteins that are exclusively expressed in the B-cell lineage and for which expression is maintained upon malignant information. To identify these genes, a previously established microarray database will be used. Next, we aim to identify which peptides derived from these genes are presented in HLA complexes of interest and can serve as targets for TCR T-cell therapy of B-cell and/or plasma cell malignancies. From a feasibility perspective, a selection of HLA alleles is made that includes HLA-A*01:01,

HLA-A*24:02, HLA-B*08:01 and HLA-B*35:01. Finally, we aim to identify T-cell clones from HLA-mismatched healthy donors that can recognize peptides derived from the selected genes that are presented in these HLA molecules using pHLA-multimer technology. Safety and efficacy screening will be performed to identify T-cell clones with promising recognition profiles. TCRs of these clones will be sequenced and transferred to CD8 T cells to investigate the potential of these TCRs in TCR T-cell therapy of B-cell malignancies.

In **chapter 3** we aim to identify TCRs that can recognize a protein that was identified in **chapter 2** as a promising target protein for treatment of MM, the joining chain (Jchain). The Jchain normally functions as a linker protein between monomer of IgA and IgM when secreted by plasma cells. The Jchain is highly expressed in most MM patient samples independent of the immunoglobulin isotype that is produced and therefore poses an attractive target for TCR T-cell therapy of MM. Next, we aim to identify peptides derived from the Jchain that are presented in HLA-A*01:01, HLA-A*03:01, HLA-A*11:01 and HLA-A*24:02 on the surface of malignant B-cell and/or plasma cells. Using peptide-HLA multimers, T cells will be isolated from PBMCs of HLA-mismatched healthy donors. Isolated T-cell clones will be analyzed for their ability to recognize the targeted peptide-HLA combinations. T-cell clones that demonstrate potent reactivity and high on-target specificity are candidates for TCR sequencing. After TCR sequencing and transfer to CD8 T cells, Jchain-TCR T cells will be screened for their lytic potential to patient derived MM samples as well as *in vivo* targeting potential of MM tumors to establish which Jchain-specific TCRs hold promise for TCR T-cell therapy of MM.

In **chapter 4** we aim to increase tumor specificity of MM-targeting TCRs. MM cells often produce high amounts of immunoglobulins either of IgG or IgA isotypes. In this chapter we therefore want to target IgG or IgA heavy chain constant domain derived peptides, since targeting these isotypes specifically will increase the tumor specificity of TCR T-cell therapy by preventing the depletion of healthy B cells expressing other immunoglobulin isotypes. Epitope and TCR identification will be performed using the same approach as used in **chapter 2** and **chapter 3**. After identification of immunoglobulin-specific T-cell clones, TCR sequencing and transfer, isotype dependent targeting of patient derived MM samples will be tested. Additionally, we will test if IgG- or IgA-targeting TCR T cells can specifically deplete B cells expressing the respective isotype while leaving B cells of other isotypes untouched.

In **chapter 5**, we aim to investigate the possibility that HLA class-I molecules present peptides that are of unconventional length. When selecting epitopes to target with TCR-based therapy from the peptidome of B-cell malignancies, we assume that peptides presented in HLA class-I are relatively short with typical lengths between 8-11 amino

acids. However, peptides with an unconventional length might also be potential targets. To investigate the presentation and subsequent T-cell recognition of unconventionally long peptides we will use a previously identified peptide that is derived from the IL27 receptor alfa. This is a 20-mer peptide that contains multiple amino acids that could be potential anker residues for binding to HLA-A*02:01. Peptide-HLA monomers will be generated and used to create crystal structures that will reveal the conformation of this specific peptide-HLA combination. T cells that recognize this peptide-HLA complex will be identified and used to explore how T cells can recognize such an unconventionally long peptide presented in HLA class-I.

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