Chapter 1

General introduction and aim of the thesis

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General introduction

T-cell immunobiology

T cells have diverse functions

T cells are highly specific immune cells that can discern self- from non-self-antigens with their T-cell receptor (TCR). Self-antigens are expressed in healthy cells, whereas pathogens and malignant cells can give rise to non-self or foreign antigens. After their development in the thymus, naive CD8 and CD4 T cells continuously surveil the body for the presence of foreign antigens. In the lymphatic system, naive T cells interact with professional antigen-presenting cells (APCs) that are designed to present foreign antigens to T cells (1). Naive T cells that recognize a foreign antigen on a professional APC are activated (primed) and start to proliferate and differentiate into effector T cells. The main function of effector or cytotoxic CD8 T cells is direct lysis of cells expressing their target antigen. Effector CD4 T cells are more versatile. Helper T cells provide help to other immune cells to initiate and promote immune responses, while regulatory T cells modulate and dampen immune responses by suppressing other immune cells (1). In addition, a small subset of CD4 T cells is cytotoxic and can directly kill target cells (2). After activation, CD8 and CD4 T cells give rise to memory T cells (3). The resulting immunological memory will lead to faster and stronger immune responses upon rechallenge with the same antigen.

The TCR is highly variable and specific

The TCR is a cell surface receptor that binds to peptide ligands presented on cells by membrane-bound proteins called human leukocyte antigen (HLA) molecules (4). The TCRs of CD8 and CD4 T cells are structurally similar and are composed of an α and β chain, each folded into a constant and a variable domain. The variable domains make up the antigen-binding site. Within each variable domain are three hypervariable regions, termed complementarity-determining regions (CDR) 1, 2 and 3 (5). CDR1 and CDR2 mainly interact with the presenting HLA molecule, whereas CDR3 interacts with the peptide ligand in the context of the presenting HLA molecule. Only the combination of a peptide with a specific HLA molecule is recognized by a T cell. Each T cell carries a single unique TCR that is capable of recognizing only one or a limited number of antigens. To cover the vast range of potential antigens, millions of TCRs with different specificities are present within the naive T-cell repertoire (6). This TCR diversity and specificity is created by a stochastic process termed V(D)J recombination, in which functional exons encoding the variable regions of the TCR α and β chain are generated by gene rearrangement of numerous variable (V), diversity (D) and joining (J) segments of non-functional germline genes (7). The variable region of the α chain is encoded by one V and one J segment, whereas the

variable region of the β chain also requires a D segment. The vast number of different V, D and J combinations contributes to TCR diversity, which is further increased by insertion or deletion of random nucleotides at the VDJ junctions. After V(D)J recombination, the genes for the α and β chain can be transcribed and translated. The TCR is assembled in the endoplasmic reticulum (ER) and associates with the CD3 protein complex, which is necessary for expression of the TCR on the cell surface (8) and responsible for intracellular signal transduction after the TCR binds to its ligand (9).

Functional and safe T cells are selected in the thymus

V(D)J recombination takes place during T-cell development in the thymus and creates TCRs specific for a vast number of unknown antigens. These TCRs include non-functional receptors that are unable to recognize peptides in the context of HLA as well as autoreactive receptors that recognize self-peptides in self-HLA. T cells with non-functional or autoreactive TCRs are deleted from the T-cell repertoire by the process of thymic selection (10). The first step is positive selection and leads to selection of T cells that can recognize peptides in the context of HLA molecules. These peptide-HLA complexes are presented on cortical thymic epithelial cells and only T cells with TCRs that bind to these complexes are signaled to survive. The second step is negative selection and results in the elimination of autoreactive T cells. Medullary thymic epithelial cells and other APC subsets present self-peptides in the context of self-HLA molecules and T cells with TCRs that bind to these complexes are triggered to undergo apoptosis, a process called clonal deletion. Only self-tolerant T cells that do not recognize self-antigens in self-HLA survive thymic selection, leading to a state of immunological tolerance.

Two types of HLA molecules present distinct and large sets of peptides

Two classes of HLA molecules exist, HLA class I and HLA class II. HLA class I molecules present peptides derived from intracellular proteins to CD8 T cells, while HLA class II molecules mainly present peptides from extracellular and membrane-bound proteins to CD4 T cells (4). Almost all nucleated cells express HLA class I. Expression of HLA class II is restricted to specialized APCs of hematopoietic origin, but can be induced on non-hematopoietic cells under inflammatory conditions. HLA class I molecules consist of a variable α heavy chain that gives rise to the peptide-binding site and a supporting invariant β_2-microglobulin light chain. The heavy chain also contains a binding site for the CD8 coreceptor. HLA class II molecules are composed of an α and β chain which both constitute the peptide-binding site. The β chain also has a binding site for the CD4 coreceptor. The peptide-binding site in both HLA classes is shaped like a groove and fits one peptide at a time (11). In HLA class I, this groove is closed at both ends, thereby allowing the binding of peptides with a limited length of approximately 8-11 amino acids. The peptide-binding groove in HLA

class II is open-ended and accommodates peptides of 13-25 residues in length. Each HLA molecule is capable of presenting a large repertoire of different peptides (12). The biochemical properties of the amino acids in the peptide-binding groove determine which amino acids are favored at which positions within a peptide in order for it to bind to HLA (12-14). This 'preferred' peptide sequence is called the peptide-binding motif. Not all residues within a peptide contribute equally to the peptide-binding motif. For some positions, the identity of the amino acid has little or no effect on the peptide-HLA interaction. Substitutions of the preferred amino acids at other positions, however, can lead to a substantial reduction or abrogation of peptide-HLA binding, or to a less pronounced but still relevant reduction in peptide-HLA binding. The residues at these positions are called primary and secondary anchor residues, respectively (12-14). For most HLA class I molecules, the second and last residue of a peptide function as primary anchors (12-14), whereas anchor residues in peptides binding to HLA class II molecules are more heterogeneous (12, 13).

HLA molecules are highly polymorphic

HLA class I and II molecules are encoded by a complex of genes on chromosome 6 (15). The heavy chains of HLA class I are encoded by three functional genes, *HLA-A*, *HLA-B* or *HLA-C*, whereas β_2-microglobulin is encoded by a single gene on chromosome 15. The α and β chains of HLA class II are each encoded by three functional genes, *HLA-DRA*, *HLA-DPA1* or *HLA-DQA1*, and *HLA-DRB1*, *HLA-DPB1* or *HLA-DQB1*, respectively. Some individuals also possess a functional *HLA-DRB3*, *-DRB4* or *-DRB5* gene. Classical HLA genes are highly polymorphic, with HLA class I being more polymorphic than HLA class II and, within HLA class II, the β chain being more polymorphic than the α chain (16). The sequence diversity between HLA alleles is mainly located in regions encoding the peptide-binding site, thereby influencing the peptide specificity of the different HLA allotypes (17). As a result, each HLA allotype presents a large and distinct set of peptides. These peptide repertoires can, however, overlap between allotypes (17, 18). For instance, HLA-A*03:01 and HLA-A*11:01 have a similar peptide-binding groove to which similar peptides bind (19). Due to the large diversity of HLA alleles, most individuals are heterozygous for each HLA locus, leading to an HLA type of 6 different HLA class I alleles and 6-8 different HLA class II alleles (dependent on the presence of *HLA-DRB3*, *-DRB4* or *-DRB5*). The chance that two unrelated individuals have the same HLA type is very low. However, HLA allotypes are not evenly distributed within the human population, as specific allotypes can be more common or rare in populations from different geographical regions (20) and certain combinations of allotypes are frequently inherited together (4). The likelihood that two individuals share one or more HLA allotypes is therefore much higher.

Different antigen processing pathways generate HLA-binding peptides

Peptides presented by the two classes of HLA molecules are differentially processed within the cell (21). HLA class I-binding peptides are derived from cytosolic proteins that are cleaved into peptides by a large proteolytic protein complex, the proteasome. The proteasome can be converted into an alternative form, the immunoproteasome, which more efficiently produces peptides that are likely to bind to HLA class I (21, 22). The immunoproteasome is constitutively expressed in hematopoietic cells, in particular in professional APCs, and produced under the influence of IFN-γ in non-hematopoietic cells (22, 23). Peptides formed in the cytosol are translocated into the ER by a heterodimeric membrane transporter termed transporter associated with antigen processing (TAP) (21). TAP facilitates the transport of peptides with appropriate size and biochemical properties for HLA class I binding. Peptides may be further trimmed by ER aminopeptidases ERAP1 and ERAP2. The peptides are then sampled by HLA class I molecules. This process is catalyzed by a protein complex associated with the HLA molecules, the peptide-loading complex. After binding of a suitable peptide, the HLA class I molecule is released from this complex and transported from the ER through the Golgi apparatus to the cell surface. HLA class II-binding peptides are derived from exogenous and membrane-bound proteins that are internalized by cells through endocytosis and phagocytosis. The resulting endosomes are acidified to support protein unfolding. Proteolysis occurs after acidified endosomes fuse with lysosomes and is mediated by lysosomal proteases that become activated in the acidic environment. The resulting peptides remain in the endolysosomal compartment for sampling by HLA class II molecules. HLA class II molecules assemble in the ER and associate with the invariant chain (Ii) protein to prevent premature binding of peptides. The Ii also coordinates the transport of the HLA molecules to the endolysosomal compartment. Within the endolysosomes, the Ii is cleaved into the class II-associated invariant chain peptide (CLIP), a small remaining fragment that occupies the peptide-binding site. CLIP is removed by HLA-DM, a chaperone protein that catalyzes successive loading of peptides onto the HLA class II molecule. When a stable peptide-HLA complex is formed, HLA-DM dissociates and the complex is transported to the cell surface. In addition to peptides from exogenous proteins, HLA class II molecules can also present peptides derived from endogenous proteins (24). This occurs mainly through autophagy, a process in which autophagosomes containing cytosolic and nuclear content are formed. These autophagosomes then fuse with endosomes or lysosomes to deliver their cargo to the HLA class II antigen processing pathway.

Peptide-HLA binding can be measured or predicted

Peptides that are recognized by T cells need to be properly processed and presented on the cell surface by HLA molecules. HLA-binding peptides, also called HLA ligands, can be identified by three approaches, i.e. peptide-HLA binding assays,

mass spectrometry and T-cell assays (25). Peptide-HLA binding assays quantify the ability of peptides to bind to specific HLA alleles. This is often tested by competition with a labelled known ligand of the specific HLA allotype, resulting in IC50 values that denote peptide-HLA binding affinities (26). Peptide-HLA binding assays, however, do not provide information about endogenous processing and immunogenicity. HLA ligands that are naturally processed and presented on cells and tissues can be identified by mass spectrometry. These natural HLA ligands are part of the HLA ligandome, which is the collection of all HLA-bound peptides in a given cell type or tissue. To identify these ligands, peptide-HLA complexes are isolated from cells, and peptides are eluted from HLA molecules and separated by chromatography (27, 28). Peptides are then measured by a mass spectrometer to determine their amino acid sequences. Using this technique, thousands of natural HLA ligands can be identified. Similar to peptide-HLA binding assays, the immunogenicity of HLA ligands needs to be validated. T-cell assays provide the ultimate validation of HLA binding and immunogenicity by testing reactivity of T cells against target cells expressing HLA alleles and peptides of interest. These peptides can be exogenously pulsed onto target cells, or encoded by genes naturally present in the genome or artificially introduced into target cells, thereby revealing whether the peptides are endogenously processed. Application of T-cell assays is, however, hindered by the limited availability of T cells with known specificity for the peptide-HLA complex of interest. In addition to experimental approaches, peptide-HLA binding can be predicted *in silico* (29). These machine learning-based algorithms, which have been trained on large sets of previously identified HLA ligands, can predict binding of peptides to different HLA class I or II allotypes. Algorithms for HLA class I outperform those for HLA class II. Prediction algorithms are useful for selecting potential HLA-binding peptides from large sets of protein sequences or candidate peptides and can be used to predict which HLA alleles bind to ligands identified by mass spectrometry.

T cells in cancer immunotherapy

T cells are key mediators of anti-tumor immunity

T cells play a central role in anti-cancer immunity and can eliminate cancer cells through recognition of tumor-associated and -specific antigens (30, 31). These antigens may be released after cancer cell death, taken up by professional APCs, in particular dendritic cells (DCs), and presented to patrolling naive T cells after DC migration to tumor-draining lymph nodes. Upon recognition of HLA-presented tumor antigens, naive T cells are primed. In addition to the interaction between a TCR and peptide-HLA complex, T-cell priming requires binding of the CD28 costimulatory receptor on T cells with CD80 or CD86 ligands on professional APCs. T-cell priming

results in the formation of effector T cells that migrate via the blood to the tumor site, where they extravasate and infiltrate the tumor. Within the tumor, effector T cells can recognize and kill malignant cells presenting their cognate antigens, resulting in release of more tumor antigens and amplification of the immune response. Since cancer cells primarily express HLA class I, direct elimination of malignant cells is mostly mediated by cytotoxic CD8 T cells. CD4 T cells contribute indirectly by providing help (32-34). For instance, CD4 T cells interact with DCs to induce DC maturation and release cytokines, which promote CD8 T-cell priming and enhance effector and memory functions in anti-cancer immunity. Some cancer types express HLA class II, which can make them susceptible to direct CD4 T-cell-mediated cytotoxicity (2). Malignant cells can exploit intrinsic mechanisms to resist T-cell cytotoxicity (35). For instance, downregulation or loss of HLA or tumor antigen expression prevents recognition and lysis by cytotoxic T cells, leading to tumor immune evasion or escape. Tumor immune evasion can also be facilitated by the tumor microenvironment, which is an ecosystem composed of cancer cells and non-cancerous cells, including immune, stromal and vascular cells (36). For example, tumor cells and other cells within the tumor microenvironment can express immune checkpoint molecules that impede tumor-infiltrating effector T cells by inhibiting TCR signaling and contributing to a dysfunctional state of T-cell exhaustion in which T-cell effector functions are impaired (37). Natural anti-tumor T-cell responses can thus be hampered by tumor cell intrinsic as well as extrinsic resistance mechanisms. T-cell-based cancer immunotherapies aim to overcome these resistance mechanisms by stimulating potent anti-tumor T-cell responses.

Natural anti-tumor T-cell immunity can be enhanced by immunotherapy

Various immunotherapeutic approaches aim to boost naturally occurring anti-tumor CD8 and CD4 T-cell responses against tumor-associated or -specific antigens. These include tumor-infiltrating lymphocyte (TIL) therapy, immune checkpoint therapy (ICT) and various cancer vaccination strategies. The efficacy of TIL therapy depends on tumor-reactive T cells present within the tumor. These TILs are grown *in vitro* from resected tumor tissue, expanded to large numbers and reinfused into the patient after lymphodepleting treatment (38). TIL therapy has shown clinical benefit, particularly in patients with metastatic melanoma (39). In ICT, immune checkpoint molecules that inhibit T-cell function are blocked with monoclonal antibodies to restore impaired anti-tumor immunity (40). The best studied checkpoint molecules are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1), and ICTs targeting these molecules are included in the standard treatment of multiple tumor types. Whereas TIL therapy and ICT boost tumor-reactive T cells with unidentified antigen specificity, most cancer vaccination strategies target preselected tumor-associated or -specific antigens. This selection is complicated by the difficulty of predicting which

antigens will be immunogenic *in vivo* (41). Selected antigens are incorporated into different vaccine strategies as synthetic peptides, mRNA or DNA vectors or plasmids, either alone or loaded onto DCs, which are then administered to the patient to elicit or augment tumor antigen-specific T-cell responses (42). Clinical benefit of cancer vaccines targeting tumor-specific antigens has been observed for some tumor types in trials.

Allogeneic T cells can induce anti-tumor reactivity in patients with hematological malignancies

Allogeneic hematopoietic stem cell transplantation (alloSCT) is a potential curative treatment for patients with hematological malignancies (43). Hematological malignancies are heterogenous tumors originating from diverse hematopoietic cell types. These cell types are produced in the bone marrow by hematopoiesis, a process in which hematopoietic stem cells give rise to myeloid and lymphoid cells through successive steps of differentiation and maturation (44). Following hematopoiesis, lymphoid cells travel to the lymph nodes where they can be activated to differentiate into effector cells. Malignant transformation can occur at different stages during and after hematopoiesis. AlloSCT facilitates the eradication of malignant cells by replacing the diseased hematopoietic system of the patient with a healthy hematopoietic system of a donor. To abrogate patient hematopoiesis, patients are treated with chemotherapy, which can be combined with radiotherapy, prior to alloSCT (43). This conditioning regimen aims to eliminate malignant and healthy hematopoietic cells, leading to tumor eradication and destruction of the functioning hematopoietic system. Elimination of healthy hematopoietic cells by the conditioning regimen as well as by administration of (T) lymphocyte depleting antibodies also reduces the risk of stem cell graft rejection by patient T cells caused by genetic differences between patient and donor (45). To restore hematopoiesis, patients receive donor-derived hematopoietic stem cells that, after successful engraftment, reconstitute a healthy hematopoietic system of donor origin (43). Both healthy and malignant hematopoietic cells of patient origin may survive the conditioning regimen. Following alloSCT, donor T cells can target these patient-derived hematopoietic cells, thereby inducing a desired anti-tumor effect called graft-versus-tumor (GvT) or graft-versus-leukemia (GvL) reactivity (46). Healthy hematopoietic cells of donor origin will be spared, leaving healthy hematopoiesis unaffected. If donor T cells target patient non-hematopoietic tissues, side-effects known as graft-versus-host disease (GvHD) may develop. GvHD can range from mild to severe and even lethal, and represents a major obstacle in alloSCT. T cells can be depleted from the donor stem cell graft to reduce the frequency and severity of GvHD. This, however, also increases the risk of relapse and infections. To re-install GvT reactivity and protection against infections, donor T cells can be administered as donor lymphocyte infusions several months after alloSCT (47).

AlloSCT forms an effective treatment for previously incurable hematological malignancies, but its efficacy can be hampered by opportunistic infections, GvHD and disease relapse (43). However, improved management of GvHD and the use of novel T-cell-engineered immunotherapies have increased the application of alloSCT as treatment for patients with hematological malignancies.

T-cell engineering produces large numbers of tumor-reactive T cells for immunotherapy

Chimeric antigen receptor (CAR) T-cell therapy has transformed cancer immunotherapy in hematological malignancies (48). CAR T-cell therapy redirects the specificity of patient T cells towards malignant cells through introduction of a CAR. A CAR is an artificial antigen receptor that typically contains an antibody-derived single-chain variable fragment as antigen-binding domain linked to a transmembrane domain, costimulatory domain and CD3ζ signaling domain of a TCR (49). As such, CARs bind to extracellular surface antigens in their native form independent of HLA. CAR T cells are produced from patient T cells, which are genetically engineered to express a CAR, expanded and reinfused into the patient (50). CAR T-cell therapies targeting different cell surface molecules have demonstrated clinical benefit in various hematological malignancies, particularly in B cell malignancies (49, 51). The anti-tumor efficacy of CAR T cells can be complicated by substantial toxicities related to T-cell activation, most frequently cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome, or to expression of the target antigen on healthy tissues (50). The identification of target antigens with restricted expression on tumor cells is a fundamental challenge of CAR T-cell therapy and hinders its broad applicability. Most CAR therapies are currently directed against cell surface lineage-restricted antigens (49). Lineage-restricted antigens are antigens that are present in all or most cells within a certain cell lineage and on tumor cells derived from these lineages. Since these antigens are expressed at similar levels on healthy and malignant cells, lineage-restricted antigens can only be targeted if they are presented on healthy tissues performing non-vital or replaceable functions (52). A limited number of such antigens has been identified for certain hematological malignancies, whereas for the majority of cancer types, in particular solid tumors, such antigens have not yet been discovered. To increase the number of targetable tumor antigens, patient T cells can instead be redirected towards malignant cells by the introduction of a transgenic TCR (53). In contrast to CARs, TCRs recognize antigens derived from intracellular proteins that are presented on the cell surface by HLA, thereby limiting their applicability to patients with specific HLA types, yet enabling the targeting of a large repertoire of intracellular antigens. Numerous intracellular tumor antigens presented by different HLA molecules have been targeted by TCR-engineered T-cell therapies in clinical studies and clinical responses have been observed in hematological as well as solid tumors (53). Similar

to CAR T-cell therapies, TCR-engineered T-cell therapies offer the possibility to generate large numbers of tumor-reactive T cells by genetically modifying patient T cells with a transgenic TCR and reinfusing engineered T cells into the patient after expansion (50). An important benefit of TCR T-cell therapy is the ability to select for potent and safe TCRs with predefined anti-tumor specificities that have been extensively tested *in vitro* and *in vivo* in preclinical models (53). Transgenic TCR α and β chain sequences can be integrated into the genome of patient T cells using retro- or lentiviral vectors, or non-viral genome editing. TCR transduction with viral vectors is well-established and relatively efficient, but results in semirandom integration of variable copy numbers of TCR transgenes (53). This can cause heterogenous TCR expression which can result in variable T-cell functionality (54). Insertional mutagenesis forms another risk of using viral vectors (55) and can potentially cause malignant transformation. In contrast to viral vector-based approaches, non-viral genome editing methods are less efficient and carry the risk of introducing off-target double-strand DNA breaks, but they enable targeted insertion of defined copy numbers of TCR transgenes at selected genomic locations (53). Non-viral genome editing methods can also be employed to disrupt endogenous TCR genes, thereby reducing or preventing mispairing between endogenous and introduced TCR α and β chains (56-58). TCR chain mispairing reduces expression of the introduced TCR and generates new TCRs with unknown specificity, which may be autoreactive (59, 60). Other approaches to promote preferential pairing of introduced TCR chains include substitution of transgenic TCR human constant α and β regions with murine regions (61) or introduction of cysteine residues in transgenic TCR constant α and β regions to form a stable interchain disulfide bridge (62).

Tumor antigens targeted by T-cell-based immunotherapies

Tumor antigens can be derived from self-proteins

Although immunotherapies stimulating or initiating natural, allogeneic or engineered T-cell responses each have their specific benefits and limitations, the efficacy and toxicity of each approach is largely determined by the type of tumor antigen that is targeted. Self-proteins encoded by unmutated wild-type (wt) genes can give rise to tumor antigens. These tumor antigens include cancer germline antigens, overexpressed tumor antigens and tissue differentiation antigens (63). Cancer germline antigens are expressed in cancer cells, but their encoding genes are presumed to be epigenetically silenced in all normal tissues, except for germline and trophoblast cells. Overexpressed tumor antigens are overexpressed in tumor cells, but low expression levels are also found in certain healthy tissues. Tissue differentiation antigens are expressed at similar levels on differentiated cell types

within certain healthy tissues and tumor cells originating from these cell types. For tissue differentiation antigens that are expressed at high levels in certain healthy tissues, T cells with high-affinity TCRs are expected to be deleted during thymic selection, as these T cells can mediate substantial on-target off-tumor toxicity against healthy tissues. For overexpressed tumor antigens that are expressed at low levels in certain healthy tissues, T cells with relatively low-affinity TCRs recognizing high antigen expression on tumor cells, but not low antigen expression on healthy cells, may have survived thymic selection and be present in the T-cell repertoire (64). For cancer germline antigens that are not expressed in healthy tissues, T cells with high-affinity TCRs may be present in the T-cell repertoire. Immunotherapies targeting tissue differentiation antigens have a high risk for on-target off-tumor toxicity, due to high expression of the antigen on healthy tissues (65, 66). Immunotherapies targeting overexpressed tumor antigens also carry a substantial risk for on-target off-tumor toxicity, since these antigens can be expressed at low levels on certain healthy tissues (67). Immunotherapies targeting cancer germline antigens have a low risk for on-target off-tumor toxicity, since germline and trophoblast cells lack HLA expression and are therefore protected against T-cell attack.

Minor histocompatibility antigens (MiHAs) arise from polymorphic proteins after alloSCT

After (partially) HLA-mismatched alloSCT, mismatched HLA alleles that are expressed by the patient but not by the donor can induce strong allo-HLA-reactive donor T-cell responses against patient cells. Although this allo-HLA reactivity often leads to a potent GvT effect, it can also elicit life-threatening GvHD. For this reason, patient and donor are preferably matched for all or most HLA alleles (68, 69). Following HLA-matched alloSCT, donor T cells mediate GvT reactivity and GvHD by targeting polymorphic peptides presented in HLA on the surface of patient cells (70). These polymorphic peptides are known as MiHAs and arise as a result of single nucleotide polymorphism (SNP) differences between patient and donor. The majority of MiHA-encoding SNP differences encode single polymorphic amino acids (70, 71). Polymorphic peptides recognized by donor T cells on patient cells are MiHAs, whereas the corresponding peptides on donor cells are called allelic variants (70). In addition to SNP mismatches, genes specific to the Y chromosome can encode MiHAs known as H-Y antigens in female-to-male transplantations (72). The distribution of MiHAs on patient tissues significantly influences the balance between GvT reactivity and GvHD (70). Donor T cells targeting MiHAs on hematopoietic cells are expected to elicit GvT reactivity, whereas donor T cells specific for MiHAs on healthy non-hematopoietic cells are expected to provoke GvHD. Most MiHAs are broadly expressed on hematopoietic and non-hematopoietic tissues and donor T cells targeting these antigens are presumed to contribute to both GvT reactivity and GvHD (71, 73). However, various MiHAs are exclusively presented on hematopoietic

tissues and are of special interest, as donor T cells against these antigens are expected to induce GvT reactivity without GvHD (71, 73, 74).

Mutated proteins give rise to tumor-specific antigens

Acquisition of somatic mutations by healthy cells can result in malignant transformation. After transformation, cancer cells gain additional mutations, some of which contribute to tumor progression. Mutations that are essential for tumor initiation and progression are called driver mutations (75). Driver mutations are, however, rare and most acquired mutations are passenger mutations. In contrast to driver mutations, passenger mutations are not essential for tumor development. Both driver and passenger mutations can encode mutated proteins which, after endogenous processing, can result in presentation of mutated peptides in HLA on the cell surface of malignant cells. HLA-presented mutated peptides on cancer cells that are recognized by T cells are called neoantigens (76, 77). In contrast to cancer germline antigens, overexpressed tumor antigens and tissue differentiation antigens, neoantigens are tumor-specific antigens created by genetic aberrations that are present in cancer cells, but absent in normal cells. As such, T cells targeting neoantigens are unlikely to induce on-target off-tumor toxicity against healthy tissues. In addition, T cells with high-affinity TCRs against neoantigens are expected to survive negative selection in the thymus. For these reasons, neoantigens are promising targets for immunotherapy. Naturally occurring neoantigen-specific CD8 and CD4 T-cell responses have been detected in patient TIL products (77, 78) and in patients treated with ICT (77). After infusion of TIL products, clinical responses have been observed in patients with diverse tumor types, indicating that T-cell reactivity against neoantigens plays a direct role in tumor control (79-81). Although natural T-cell responses against neoantigens are induced, most somatic mutations in cancer cells do not create neoantigens (76, 77). To create neoantigens, somatic mutations need to be present in genes that are expressed. Additionally, the resulting mutated proteins need to be endogenously processed to produce mutated peptides, which need to be presented on the cell surface by HLA molecules that are expressed by the patient. Finally, the mutated peptide-HLA complexes need to be recognized by T cells carrying specific TCRs that are able to bind to these complexes. Most somatic mutations fail to meet all these requirements for neoantigen formation. The probability of neoantigen formation is dependent on the number of nonsynonymous somatic mutations present in the genome of a tumor, known as the tumor mutational burden or load, which differs between tumor types and patients (82). Cancers with a high mutational burden are more likely to present neoantigens than cancers with a low mutational load (76, 77). Tumor mutational burden is associated with clinical benefit from ICT across different tumor types (83), providing indirect evidence that cancers with a high mutational load present more neoantigens and that T-cell responses against neoantigens contribute to tumor control after ICT. In addition to

neoantigen quantity, the type of mutation encoding a neoantigen may influence the anti-tumor efficacy of T-cell-based immunotherapies. T-cell reactivity against neoantigens encoded by passenger mutations can result in immune escape due to loss of these mutations by the tumor (84), whereas loss of driver mutations and their resulting neoantigens is less likely due to their essential role in tumorigenesis (85). The type of mutation encoding a neoantigen is of less importance for tumors with a high mutational burden, in which polyclonal T-cell responses can be induced against numerous neoantigens presented by diverse HLA molecules on different tumor populations, thereby reducing the risk of immune escape by antigen loss. Polyclonal anti-tumor T-cell responses can be boosted by TIL therapy and ICT, and these therapies are most effective in cancers with a high mutational load. In cancers with a low mutational burden that present few neoantigens, induction of polyclonal T-cell responses is not feasible. Instead, these tumors are suitable candidates for TCR-engineered T-cell therapies directed against one or a few carefully selected neoantigens (86). Public neoantigens encoded by driver mutations that are clonally expressed in all tumor cells and are present in multiple patients are especially preferable as target antigens, since TCR-engineered T-cell therapies against these antigens are broadly applicable and expected to induce anti-tumor responses with no or low risk of immune escape by antigen loss (85). Immune evasion can, however, still occur through loss of the HLA allele presenting the targeted neoantigen by tumor cells (35).

Neoantigen identification and isolation of neoantigen-specific T cells

Neopeptides encoded by somatic mutations are candidate neoantigens

Most mutations in a tumor do not encode mutated peptides that fulfill all criteria for neoantigen formation, and predicting which mutations give rise to a neoantigen is difficult (76, 77, 87). The discovery of neoantigens is therefore challenging and different identification strategies can be followed (87). Many strategies start with identifying somatic mutations that are present in the whole tumor genome, exome or in selected genomic regions by whole genome or exome sequencing of tumor and healthy tissue samples from patients. Alternatively, mutations can be identified from public databases, such as Catalogue Of Somatic Mutations In Cancer (COSMIC), that collect and catalogue data on somatic mutations from scientific literature and open access cancer genome data portals (88). The number of identified mutations is often extensive and screening each mutation for neoantigen formation can be laborious and expensive. To filter the number of mutations that need to be screened, mutations can be examined for encoding mutated peptides or neopeptides that are likely to fulfill the requirements of neoantigen formation (87). Candidate neoantigens can be filtered for gene expression using RNA sequencing data, and *in silico*

algorithms can be used to predict immunobiological features of neopeptides, for instance HLA binding affinity, proteasomal processing and/or TAP transport (87, 89). RNA expression data and tools to predict HLA binding, such as NetMHC algorithms (90), are most frequently used in computational pipelines to identify and prioritize candidate neoantigens. Although computational pipelines significantly reduce the number of neopeptides that need to be screened, a major drawback of these pipelines remains that most selected candidate neoantigens are false-positives that cannot be validated as true neoantigens (77, 87, 91). Candidate neoantigens can also be identified using mass spectrometry to measure the HLA ligandome of tumor cells or cells expressing mutations of interest (87, 92). In contrast to *in silico* prediction, mass spectrometry directly identifies neopeptides that are presented by HLA on the cell surface. Mass spectrometry typically requires large cell numbers and identifies peptides that are abundantly present on the cell surface and have strong HLA binding affinity (87). All candidate neoantigens, whether preselected by computational pipelines or mass spectrometry, ultimately need to be screened for T-cell reactivity to be validated as true neoantigens.

T-cell recognition is necessary for validation of neoantigens

To be validated as true neoantigens, candidate neoantigens need to be recognized by a specific T cell within the human T-cell repertoire. Neoantigen-specific T cells can be searched in T-cell repertoires from patients or healthy donors (53, 87). For patients, TILs or peripheral blood mononuclear cells (PBMCs) can be screened (93). TILs can be enriched for tumor-reactive T cells, including neoantigen-specific T cells, but are not available from all patients (53, 87). In contrast, peripheral blood can be easily collected from most patients and permits isolation of large numbers of PBMCs, although the frequency of neoantigen-specific T cells is generally considerably lower than in TILs. Neoantigen-specific T cells can also be searched in PBMCs from healthy donors (94). Whereas neoantigen-specific T cells in patients can be derived from the memory and naive T-cell compartment, neoantigen-specific T cells in healthy donors have not previously encountered their cognate antigen and are therefore expected to be derived from the naive T-cell compartment. Despite their extremely low frequency in peripheral blood, the number of neoantigen-specific T cells that can be isolated from healthy donors is compensated by the vast amount of available donor PBMCs. In addition, the naive T-cell compartment in healthy donors contains T cells with a broad range of specificities that have not been subjected to immunosuppressive mechanisms employed by tumors (53, 95). Generally, the frequency of neoantigen-specific T cells in the human T-cell repertoire is low and the chance of isolating these T cells may be increased by enriching TILs or PBMCs using peptide-HLA multimers or *in vitro* stimulation (53, 87). Peptide-HLA multimers consist of multiple HLA complexes with predefined neopeptides and are typically conjugated with fluorochromes to enable isolation of antigen-specific T cells by flow cytometry.

In vitro stimulation of TILs or PBMCs can be performed by coculturing T cells with autologous tumor cells, or with autologous or HLA-matched allogeneic cells that are exogenously pulsed with neopeptides or modified to express genes encoding candidate neoantigens. T cells isolated from (enriched) TILs or PBMCs can be cultured in bulk or as single T-cell clones. To assess the presence of neoantigen-specific T cells, reactivity of bulk T cells or T-cell clones can be tested, for instance by measuring cytokine secretion or cytotoxicity, against stimulator cells that are exogenously loaded with individual or pooled neopeptides or transduced with gene constructs encoding one or more neopeptides (87). As stimulator cells, autologous APCs can be used or allogeneic cells or cell lines in which the HLA alleles of interest are naturally expressed or artificially introduced. Upon T-cell recognition, neopeptide pools or gene constructs encoding multiple neopeptides are deconvoluted to evaluate T-cell reactivity against individual neopeptides and elucidate which neoantigen is recognized. Neoantigen-specific T cells can be further tested *in vitro* against malignant and healthy cell types that are positive or negative for the neoantigen and/or restricting HLA allele to characterize their specificity, potency and safety (53). Neoantigens are ultimately validated by demonstrating T-cell reactivity against tumor cells naturally expressing the neoantigen-encoding mutation and respective HLA allele. TCRs from T cells specific for identified neoantigens may be promising candidates for TCR-engineered T-cell therapy (53, 87). TCR α and β chains can be sequenced and introduced into CD8 and/or CD4 T cells from patients or healthy donors for extensive preclinical characterization of their safety and potency.

Acute myeloid leukemia (AML)

AML is a hematological malignancy with a poor prognosis

AML is a malignancy of the myeloid lineage that arises during hematopoiesis. Genetic aberrations that accumulate in hematopoietic stem cells and myeloid progenitor cells may lead to malignant transformation into leukemic cells (96). These leukemic cells, or AML blasts, are arrested in differentiation and show uncontrolled clonal expansion. The resulting malignant leukocytosis hinders normal hematopoiesis, causing myelosuppression. Failure of normal hematopoiesis can produce diverse symptoms in patients with AML, such as increased bleeding tendency or susceptibility to infections. Approximately 800 people are diagnosed with AML each year in the Netherlands (97). AML mainly affects older individuals and the median age at diagnosis is 68 years (96). Diagnostic evaluation includes examination of peripheral blood and bone marrow samples by morphology, immunophenotyping, cytogenetics and molecular genetics, and assessment of patient fitness (96, 98). This diagnostic evaluation allows AML classification, informs

prognosis and risk stratification, and guides treatment decisions. AML is classified mainly based on cytogenetic and molecular disease characteristics using the WHO (99) or ICC (100) diagnostic classification systems. Cytogenetic and molecular abnormalities also substantially influence patient outcome and are used to stratify patients into prognostic risk groups with the ELN risk classification (98). The ELN risk stratification and patient fitness play an essential role in therapy selection (96, 98). Treatment of patients with AML consists of induction therapy with the aim to induce complete remission, followed by consolidation therapy to deepen the remission and prevent disease recurrence. Induction therapy can consist of (combinations of) chemotherapy, hypomethylating agents, BCL-2 inhibitor and targeted small molecule inhibitors for selected mutations, while consolidation therapy can also include autologous or allogeneic stem cell transplantation. The choice of consolidation therapy also relies on the persistence of low numbers of leukemic cells during complete remission, called measurable residual disease, which can be assessed by flow cytometry or quantitative PCR for specific genetic aberrations. Most patients achieve complete remission following induction and/or consolidation therapy, but many patients relapse within a few years after diagnosis (101, 102). Disease relapse is a major concern in patients with AML and significantly contributes to its poor prognosis.

AML has a heterogenous clonal architecture

The accumulation of genetic aberrations in hematopoietic and leukemic cells is a stepwise process that is shaped by Darwinian selection (103, 104). Each acquired mutation is expressed by the respective mutated cell as well as its progeny, which in turn can acquire additional mutations. As a result, leukemic cells possess mutational patterns that are formed by the initial and all subsequent genetic aberrations that have been gained. Most mutations are acquired passenger mutations with no individual effect on cell fitness (103). In contrast, a single driver mutation does confer a fitness advantage, leading to clonal outgrowth of cells with this mutation. A single driver mutation can sometimes be sufficient for leukemic transformation, giving rise to an AML founding clone. This founding clone can gain subsequent driver mutations, creating AML subclones with distinct mutational patterns (103, 104). This process of mutation accumulation followed by clonal selection is called clonal evolution. Because of clonal evolution, AML often has a clonal architecture composed of multiple AML subclones at varying frequencies. AML subclones can differ in their susceptibility to therapy (104-106). Subclones with mutations that convey resistance to therapy may survive treatment and cause refractory or relapsed disease. Disease relapse is believed to be driven by therapy-resistant leukemic stem cells, which are stem-cell like cells that are capable of long-term self-renewal and leukemia initiation and maintenance (107).

Recurrent driver mutations are common in AML

The tumor mutational burden in AML is low compared to other cancer types (82, 108). Next-generation sequencing of 200 adult AML cases demonstrated an average of 13 coding somatic mutations per AML genome (108). Almost half of these mutations occur in genes that are mutated in multiple patients. These recurrently mutated genes include genes that have been identified as driver genes in AML (109, 110). Nucleophosmin 1 (*NPM1*) and DNA methyltransferase 3A (*DNMT3A*) are amongst the most frequently mutated driver genes in AML (108, 109). Hotspot mutations in *NPM1* and *DNMT3A* are present in approximately 30-35% (108, 109, 111) and 15% (108, 109, 112) of adult patients with AML, respectively. Hotspot mutations in *NPM1* are 4 base pair insertions in the last exon causing a shift in protein translation (111, 113). These 4 base pair insertions, which can differ in exact sequence, almost all produce the same C-terminal alternative reading frame of 11 amino acids, thereby extending the mutated protein by 4 amino acids compared to the wt protein. *NPM1* mutations are early driver events in leukemogenesis, indicating their importance for tumor initiation and progression (110, 114). *NPM1* mutations are often present in all AML subclones and are infrequently lost by AML cells (115-117). Hotspot mutations in *DNMT3A* are single nucleotide variants in exon 23 creating single amino acid substitutions at position R882 (112). *DNMT3A* mutations can arise in preleukemic hematopoietic stem cells (118) and cause clonal expansion of hematopoietic cells, leading to clonal hematopoiesis of indeterminate potential (CHIP) or clonal cytopenia of undetermined significance (CCUS) (119). CHIP and CCUS mainly affect elderly individuals and convey an increased risk of developing hematological malignancies. The low mutational burden combined with the presence of driver mutations that are shared between patients, like mutations in *NPM1* and *DNMT3A*, makes AML a promising candidate for TCR-engineered T-cell therapy. Therefore, there is a strong need to identify neoantigens that are derived from such common recurrent driver mutations in AML.

Aim of the thesis

Since patients with AML frequently relapse after achieving complete remission with current standard treatments, there is an urgent need for new and effective therapies. Immunotherapy targeting neoantigens is a promising new treatment strategy for patients with AML. In AML, there are a limited number of recurrent driver mutations (109, 110). Neoantigens encoded by these mutations are especially attractive targets for immunotherapy, since they are shared between patients and unlikely to be lost as a form of immune escape (85). The aim of this thesis is to identify neoantigens encoded by recurrent driver mutations that can serve as targets for T-cell-based immunotherapy to treat patients with AML (Figure 1).

Neoantigen identification pipelines often incorporate *in silico* prediction algorithms to filter neopeptides that need to be screened for neoantigen formation (87, 89). The value of these computational tools for neoantigen discovery varies between algorithms and is dependent on their ability to reduce the number of false positives, while retaining true neoantigens. In **chapter 2**, we aim to investigate the potential of various *in silico* prediction algorithms for the discovery of T-cell ligands. Using algorithms that predict different immunobiological features, we will determine *in silico* characteristics for a set of autosomal HLA class I-binding MiHAs that have been identified as true T-cell ligands. MiHAs and neoantigens are conceptually similar, as both are encoded by genetic variants and recognized by T cells (76). The *in silico* characteristics of MiHAs will be compared with a reference set, consisting of peptides with predicted HLA binding that have not been identified as T-cell ligands. Data from MiHAs and reference peptides will be used to determine the specificity and sensitivity of the different algorithms.

One of the most frequently mutated driver genes in patients with AML is *NPM1* (108, 109). The 4 base pair hotspot insertion in *NPM1* is an early driver mutation (110, 114) that is often clonally expressed and rarely lost by AML blasts (115-117). Neoantigens encoded by mutated *NPM1* are therefore desirable targets for T-cell-based immunotherapies. In **chapter 3**, we aim to identify HLA class I-binding neoantigens that are derived from the alternative reading frame of mutated NPM1 as created by hotspot frameshift mutations. First, we aim to identify neopeptides derived from mutated NPM1 that are naturally processed and presented by HLA on AML cells. We will generate and search HLA class I ligandome data from patient-derived *NPM1*-mutated AML cells for neopeptides binding to different HLA molecules. Next, to confirm that these neopeptides are true neoantigens that are targeted by T cells, we will search for T cells specific for one HLA-A*02:01-binding NPM1 neopeptide by screening patient and healthy donor PBMCs using peptide-

HLA tetramers. Reactivity of isolated T-cell clones against HLA-A*02:01 positive patient-derived AML cells with wt or mutated *NPM1* will be assessed in cytokine secretion assays. Finally, we aim to examine the value of TCRs from potent NPM1 neoantigen-specific T-cell clones for TCR-engineered T-cell therapy of AML. TCRs will be sequenced, introduced into CD8 and CD4 T cells from healthy donors and TCR-engineered T cells will be tested for reactivity against HLA-A*02:01 positive patient-derived AML cells with wt or mutated *NPM1* in cytokine secretion and cytotoxicity assays. *In vivo* efficacy of NPM1 neoantigen-specific TCR-engineered T cells will be evaluated in a murine model engrafted with a human *NPM1*-mutated AML cell line.

Identification of neoantigens for different HLA molecules increases the number of patients that can be treated with NPM1 neoantigen-directed T-cell-based immunotherapies. In **chapter 4**, we aim to investigate whether another naturally processed NPM1 neopeptide that binds to both HLA-A*03:01 and HLA-A*11:01 is a neoantigen that can be targeted on AML by T-cell-based immunotherapy. To achieve this aim, similar methods as described for the HLA-A*02:01-binding NPM1 neopeptide will be employed to isolate specific T cells from healthy donors and to generate TCR-engineered T cells that will be evaluated for reactivity against AML cells *in vitro* and *in vivo*.

CD4 T cells are crucial for effective anti-tumor immunity by providing help to other immune cells (32-34). In addition, CD4 T cells can be cytolytic and directly kill tumor cells that express HLA class II (2). Since AML blasts frequently express HLA class II, HLA class II-binding neoantigens may be particularly relevant for T-cell-based immunotherapy of AML. In **chapter 5**, we aim to identify neoantigens encoded by recurrent driver mutations in AML that are presented in HLA class II. Because isolation of neoantigen-specific CD4 T cells by peptide-HLA class II tetramers is challenging, we aim to develop an *in vitro* T-cell stimulation and isolation method. Using public database COSMIC (88), long neopeptides encoded by recurrent genetic aberrations in AML will be synthesized and used to stimulate and isolate CD4 T cells from healthy donors. These peptides will be selected for predicted binding to common HLA class II molecules using an *in silico* algorithm (120). Isolated CD4 T-cell clones will be tested in cytokine secretion and cytotoxicity assays to determine their HLA restriction as well as their potency and specificity. Reactivity will be tested against cells that are exogenously pulsed with the neopeptides of interest and cells expressing the relevant driver mutation artificially after introduction of the mutated gene or naturally as expressed on patient-derived AML blasts.

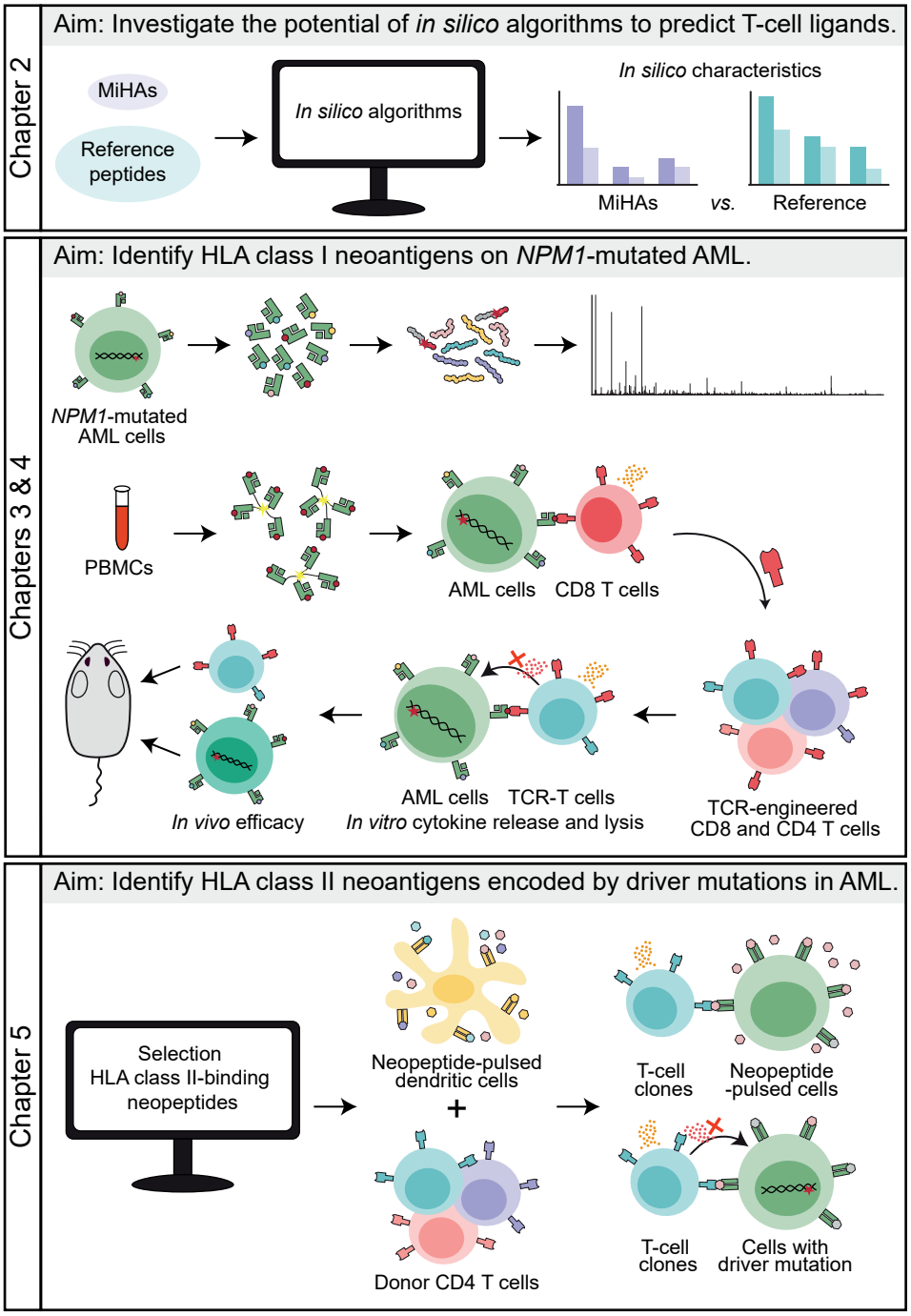

Figure 1. Outline of the thesis.

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