Chapter 6

Summary and general discussion

Summary

Acute myeloid leukemia (AML) is an aggressive disease that, despite initial complete responses to current standard therapies in most patients, frequently relapses (1, 2). Relapsed AML is difficult to treat and has a very poor prognosis (3). To improve the outcome of patients with AML, novel potent and safe therapies are required to treat and prevent relapsed disease.

Immunotherapy directed against neoantigens forms a promising new treatment option for patients with AML. Neoantigens are created by tumor-specific mutations and their expression is thus restricted to malignant cells (4, 5). Neoantigens are not presented during negative selection in the thymus and, therefore, T cells with high-affinity T-cell receptors (TCRs) for these antigens are expected to be present in the T-cell repertoire. As such, T-cell-based immunotherapies targeting neoantigens could potentially induce effective anti-leukemic responses without on-target off-tumor toxicity against healthy tissues.

Although AML generally contains few somatic mutations (6, 7), many patients carry driver mutations that are recurrently mutated in AML (8, 9). AML may therefore be particularly suitable for TCR-engineered T-cell therapy in which one or a few neoantigens are targeted. Especially neoantigens encoded by common recurrent driver mutations are relevant targets for TCR-engineered T-cell therapy, since these antigens are shared between patients and carry a low risk of being lost during immune escape (10).

In this thesis, we aimed to identify human leukocyte antigen (HLA) class I- as well as HLA class II-binding neoantigens encoded by frequent recurrent driver mutations in AML. We employed different strategies to select candidate neoantigens and isolate neoantigen-specific CD8 or CD4 T cells. For selected T cells that demonstrated superior reactivity against leukemic cells *in vitro*, TCRs were sequenced. After introduction into T cells from healthy individuals, anti-leukemic reactivity of these TCRs was evaluated *in vitro* and in a preclinical mouse model to assess their suitability for TCR-engineered T-cell therapy to treat patients with AML.

Most strategies that are followed for neoantigen discovery start with large sets of candidate neoantigens that all need to be screened for T-cell recognition. To improve the efficiency of neoantigen discovery, candidate neoantigens can be filtered based on immunobiological features as predicted by *in silico* algorithms (11, 12). In **chapter 2**, we explored the potential of various online available prediction tools to identify T-cell antigens, such as neoantigens and minor histocompatibility antigens (MiHAs).

Using these prediction tools, we determined the *in silico* characteristics for 68 autosomal HLA class I-binding MiHAs that have been identified as T-cell targets in patients who developed immune responses after allogeneic hematopoietic stem cell transplantation. These MiHAs by definition fulfill all requirements for endogenous processing, HLA presentation and T-cell recognition. To allow accurate evaluation of the predictive performance of the online algorithms, we also composed a set of reference peptides that have not been identified as T-cell antigens. The reference set consisted of peptides with predicted binding to HLA-A*02:01 or HLA-B*07:02 that are encoded by the same genes for which MiHAs in these HLA alleles have been identified. By comparing the *in silico* characteristics of MiHAs and reference peptides, we found that only the prediction algorithm for HLA class I binding substantially contributed to MiHA characterization (Figure 1). Using this algorithm, 87% of MiHAs were correctly predicted as HLA class I ligands, with most MiHAs being predicted as strong HLA binders. We observed that the default threshold for strong HLA binding has a high specificity, but low sensitivity, whereas the default threshold for weak HLA binding has a low specificity, but high sensitivity. This implies that applying the strong binding threshold will exclude many true ligands from the list of candidate T-cell antigens, whereas the weak binding threshold will result in many false-positives. We also compared and demonstrated similar *in silico* characteristics for most MiHAs and their allelic variants, supporting the suggestion that allelic variants can also be endogenously processed, presented by HLA and recognized by T cells. The reason why many allelic variants have not (yet) been identified as MiHAs may be their low mismatch frequencies in patient-donor pairs.

Amongst the most frequent mutations in AML are 4 base pair insertions in nucleophosmin 1 (*NPM1*), which occur in 30-35% of adult patients with AML (6, 8, 13). The mutations produce an 11 amino acid alternative reading frame (most often CLAVEEVSLRK) at the C-terminus of the mutated protein, resulting in elongation of the mutated protein by 4 amino acids compared to the wild-type (wt) protein (13, 14). The alternative reading frame could potentially give rise to neoantigens. These neoantigens are desirable targets for T-cell-based immunotherapies, since mutated NPM1 is an early driver mutation that is essential for AML development (9, 15). The mutation is also clonally expressed and retained upon relapse in most patients (16-18). In **chapter 3**, we identified a neoantigen that could efficiently be targeted on *NPM1*-mutated AML *in vitro* and *in vivo* by TCR-engineered T cells. We first searched for NPM1 neopeptides in the HLA class I ligandome of patient-derived AML samples and identified 5 neopeptides in the ligandome of *NPM1*-mutated AML, confirming that peptides derived from the alternative reading frame of mutated NPM1 are naturally processed and presented by HLA on AML. For one of these neopeptides, HLA-A*02:01-binding CLAVEEVSL, we searched for specific CD8 T cells using peptide-HLA class I tetramers. We first screened HLA-A*02:01 positive

patients with *NPM1*-mutated AML who were in remission after chemotherapy, but were unable to detect CLAVEEVSL-specific CD8 T cells. We then screened HLA-A*02:01 positive healthy individuals and isolated multiple tetramer positive CD8 T-cell clones. Two of these clones showed specific recognition of *NPM1*-mutated AML. The TCR from the T-cell clone with superior anti-leukemic reactivity was sequenced and introduced into CD8 and CD4 T cells from HLA-A*02:01 positive healthy individuals. NPM1-A2-specific TCR-transduced CD8 as well as CD4 T cells showed clear and specific recognition and killing of *NPM1*-mutated AML, while no reactivity against AML with wt *NPM1* was seen. TCR-transduced T cells also reduced tumor growth and improved survival in immunodeficient mice engrafted with the HLA-A*02:01 positive *NPM1*-mutated OCI-AML3 cell line. We concluded that CLAVEEVSL is an HLA-A*02:01-binding neoantigen on *NPM1*-mutated AML that could be a relevant target for T-cell-based immunotherapy and demonstrated that our NPM1-A2-specific TCR is a promising candidate for TCR-engineered T-cell therapy to treat patients with *NPM1*-mutated AML.

To broaden the applicability of T-cell-based immunotherapies for patients with *NPM1*-mutated AML, we examined in **chapter 4** whether AVEEVSLRK, another neopeptide detected in the HLA class I ligandome of *NPM1*-mutated AML, is a neoantigen on AML that can be targeted by TCR-engineered T-cell therapy. Predicted binding of AVEEVSLRK to HLA-A*03:01 and HLA-A*11:01 was confirmed in a competition-based peptide-HLA class I binding assay. Using peptide-HLA class I tetramers, we screened HLA-A*03:01 or HLA-A*11:01 positive healthy individuals for specific CD8 T cells. Three HLA-A*11:01-restricted CD8 T-cell clones were isolated that reacted against the AVEEVSLRK peptide and one of these clones selectively recognized *NPM1*-mutated AML. The TCR from this T cell was cloned and tested after transfer into CD8 and CD4 T cells from HLA-A*11:01 positive healthy individuals. The results showed that this TCR conferred peptide reactivity in CD8 T cells, but not in CD4 T cells, indicating that CD8 is required as coreceptor for the function of the NPM1-A11-specific TCR. NPM1-A11-specific TCR-transduced CD8 T cells demonstrated recognition and lysis of *NPM1*-mutated AML. In contrast to the NPM1-A2-specific TCR, CD8 T cells transduced with the NPM1-A11-specific TCR did not induce an anti-tumor effect in immunodeficient mice engrafted with HLA-A*11:01-transduced *NPM1*-mutated OCI-AML3 cells. These *in vivo* results were supported by lower levels of IFN-γ released by NPM1-A11-specific TCR-transduced T cells as compared to NPM1-A2-specific TCR-transduced T cells upon incubation with HLA-A*11:01-transduced OCI-AML3 cells *in vitro*. We concluded that AVEEVSLRK is an HLA-A*11:01-binding neoantigen on *NPM1*-mutated AML. Whether this neoantigen can be efficiently targeted in patients treated with our NPM1-A11-specific TCR after transfer to autologous T cells is unclear and requires additional preclinical testing.

CD4 T cells are often essential as helper T cells in efficient anti-tumor immune responses (19-21) and can also have the potential to directly kill HLA class II positive tumor cells (22). Since AML blasts frequently express HLA class II, we investigated in **chapter 5** whether neoantigens are presented by HLA class II and recognized by specific CD4 T cells. We first used the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (23) to select 26 recurrent genetic aberrations in AML, which were subsequently analyzed for encoding neopeptides with predicted binding to common HLA class II molecules. A total of 24 long neopeptides derived from 10 recurrent genetic aberrations were synthesized. These neopeptides included 8 overlapping peptides spanning the entire alternative reading frame of mutated NPM1, which were added to the set of neopeptides irrespective of predicted HLA class II binding. Mixes of the long neopeptides were loaded onto dendritic cells (DCs), which were used to stimulate autologous CD4 T cells from healthy individuals. Numerous neopeptide-specific CD4 T-cell clones were isolated and tested for their potency and specificity. For two neopeptides, one created by the *DNMT3A-R882H* hotspot mutation and one derived from a long alternative reading frame created by *RUNX1* frameshift mutations, T-cell clones were isolated with reactivity against cell lines that were transduced or CRISPR-Cas9-edited with the relevant driver mutation, indicating that these neopeptides can be endogenously processed and presented by HLA class II on the cell surface. The DNMT3A neopeptide was also recognized by a CD4 T-cell clone on *DNMT3A*-mutated AML cells that were positive for HLA-DQB1*06:02 or HLA-DQB1*06:03. Despite clear recognition in IFN-γ ELISA, the CD4 T-cell clone failed to directly kill AML cells, demonstrating that the CD4 T-cell clone lacked cytotoxic capacity. We concluded that the *DNMT3A-R882H* hotspot mutation encodes an HLA class II-binding neoantigen on AML that could potentially become a relevant target for CD4 T-cell-based immunotherapy.

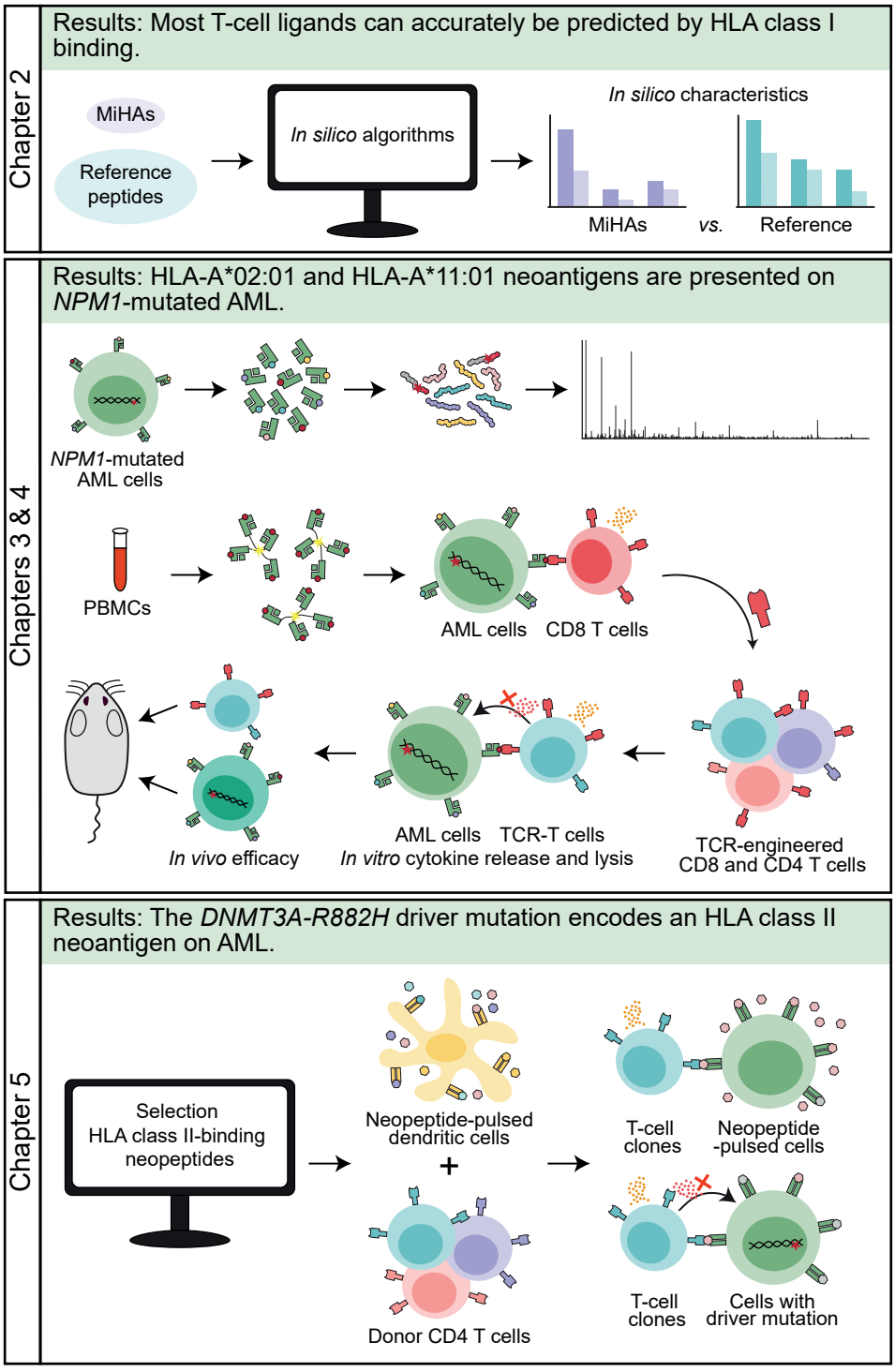

Figure 1. Summary of the thesis.

General discussion

Mutated NPM1 as target for TCR-engineered T-cell therapy

The frequent occurrence of *NPM1* hotspot mutations in adult patients with AML points to an important role of these mutations as drivers in leukemogenesis. Wt *NPM1* encodes a protein that functions as nucleocytoplasmic shuttle and nucleolar chaperone (15, 24). Although wt NPM1 shuttles between the nucleolus and cytoplasm, it primarily resides in the nucleolus. Wt NPM1 plays a role in various cellular processes, including nucleolus formation, ribosome biogenesis and transport, inhibition of centrosome duplication and DNA repair (24). It is also involved in the tumor suppressive p14ARF-MDM2-p53 pathway. The preferential nucleolar localization of wt NPM1 is promoted by a nucleolar localization signal at the C-terminus of the protein (15, 24). In mutated NPM1, the new alternative reading frame disrupts this nucleolar localization signal and creates a novel nuclear export signal. As a result, mutated NPM1 is delocalized from the nucleus to the cytoplasm. This cytoplasmic relocation seems to be required for tumorigenesis, since all hotspot mutations in exon 12 of *NPM1*, as well as rare mutations outside the hotspot region and rare *NPM1* gene fusions, result in displacement of the mutated protein to the cytoplasm. How cytoplasmic displacement of mutated NPM1 leads to leukemia is not yet understood (24). Disruption of essential processes in the nucleus due to haploinsufficiency of wt NPM1 or new interactions of mutated NPM1 with cytoplasmic proteins or, as a small fraction of the mutated protein is not exported to the cytoplasm, new interactions with nuclear proteins are all possible explanations. Mutated NPM1 may also associate with nuclear proteins and disrupt their function by transporting these proteins from the nucleus into the cytoplasm. Besides the observation that all *NPM1* mutations share a final common outcome of cytoplasmic delocalization of the mutated protein (15, 24), the importance of *NPM1* hotspot mutations for the development of AML is further supported by their early acquisition during malignant transformation (9, 15) and the finding that these mutations are only occasionally lost upon relapse (16-18). Since *NPM1* mutations are essential for leukemogenesis, AML blasts are not expected to escape from NPM1 neoantigen-targeted TCR-engineered T-cell therapy by losing these essential driver mutations.

We successfully isolated a CD8 T-cell clone that is specific for the HLA-A*02:01-binding neoantigen CLAVEEVSL on *NPM1*-mutated AML in **chapter 3**. Analysis of the HLA class I ligandome of 8 *NPM1*-mutated AML samples revealed that neopeptides derived from mutated NPM1 could be detected in 7 out of 8 samples, with CLAVEEVSL in two out of three HLA-A*02:01 positive samples, indicating that mutated NPM1 is processed and presented by AML cells. Despite surface presentation of NPM1 neopeptides, no CLAVEEVSL-specific CD8 T cells could be

isolated from a total number of 80 x 10^6 peripheral blood mononuclear cells (PBMCs) from 6 patients with *NPM1*-mutated AML who were in remission after chemotherapy. The failure to isolate CLAVEEVSL-specific CD8 T cells from patients with *NPM1*-mutated AML indicates that these cells were not present at high frequencies in the memory T-cell repertoire, and were either completely absent or present at frequencies in the naive T-cell repertoire that are too low to detect in limited numbers of available PBMCs from these patients. We therefore searched for CLAVEEVSL-specific CD8 T cells in 6 healthy individuals. By screening a total number of 5 x 10^9 PBMCs, two T-cell clones were isolated that were reactive against patient-derived *NPM1*-mutated AML. Of these two clones, one T-cell clone demonstrated reactivity against all *NPM1*-mutated AML samples tested. Two other research groups have also searched for CLAVEEVSL-specific CD8 T cells in patients with *NPM1*-mutated AML or healthy individuals. Forghieri et al. (25) analyzed PBMCs or bone marrow mononuclear cells from patients in IFN-γ ELISPOT assays after stimulation with NPM1 neopeptides, while Greiner et al. (26) stimulated CD8 T cells from patients or healthy individuals *in vitro* with NPM1 neopeptide-pulsed autologous PBMCs for 8 days before analyzing their reactivity against neopeptide-pulsed T2 cells in IFN-γ and granzyme B ELISPOT assays. Both groups failed to detect CD8 T cells specific for CLAVEEVSL, but successfully identified T cells for other NPM1 neopeptides. These results collectively suggest that CLAVEEVSL-specific CD8 T cells are rare and that these T cells are absent in the T-cell repertoire of most HLA-A*02:01 positive patients and healthy individuals.

The neoantigen CLAVEEVSL is not expected to be presented on healthy cells, since the peptide is derived from an alternative reading frame that is created by a tumor-specific frameshift mutation. However, it is known that out-of-frame translation can also occur in healthy cells. This has, for instance, been demonstrated for MiHAs that are translated from wt genes in alternative open reading frames (27, 28). It can therefore be hypothesized that wt *NPM1* may also produce peptides in alternative reading frames. If alternative translation occurs, wt *NPM1* encodes SLAVEEVSL, which differs from CLAVEEVSL by only one amino acid. Although SLAVEEVSL is predicted to bind to HLA-A*02:01, the peptide could not be identified in the HLA class I ligandome of 7 HLA-A*02:01 positive AML samples carrying no (*n* = 4) or heterozygous (*n* = 3) *NPM1* mutations. Furthermore, as demonstrated in **chapter 3**, T cells expressing the NPM1-A2-specific TCR showed no reactivity against HLA-A*02:01 positive autologous mature monocyte-derived DCs and allogeneic Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL), suggesting that SLAVEEVSL is not produced in these cells. Restricted expression of SLAVEEVSL as a result of alternative translation of wt *NPM1* in specific healthy tissues, however, cannot be entirely excluded. It is interesting to speculate that SLAVEEVSL may be produced in medullary thymic epithelial cells or other antigen-presenting cell (APC)

subsets in the thymus during negative selection. Since SLAVEEVSL is highly similar to CLAVEEVSL, presentation of SLAVEEVSL as self-peptide during negative selection may result in clonal deletion of T cells with TCRs that can interact with both peptides. This could explain the low frequency of CLAVEEVSL-specific T cells in the T-cell repertoire, since only T cells expressing a TCR that interacts with abundant expression of CLAVEEVSL on *NPM1*-mutated AML, but not with low expression of SLAVEEVSL on healthy tissues would survive negative selection.

Besides CLAVEEVSL, mutated *NPM1* also encodes the neoantigen AVEEVSLRK. In **chapter 3**, CD8 as well as CD4 T cells transduced with the NPM1-A2-specific TCR for CLAVEEVSL were shown to bind to peptide-HLA class I tetramers and strongly react against patient-derived *NPM1*-mutated AML. In **chapter 4**, we isolated an HLA-A*11:01-restricted T-cell clone specific for AVEEVSLRK and tested the TCR from this clone similarly after introduction into CD8 and CD4 T cells. In contrast to the NPM1-A2-specific TCR, CD4 T cells transduced with the NPM1-A11-specific TCR were poorly stained with tetramers and failed to recognize neoantigen peptide-pulsed T2 cells. Furthermore, when introduced into CD8 T cells from the same individual, T cells with the NPM1-A2-specific TCR induced an anti-tumor effect in immunodeficient mice engrafted with an *NPM1*-mutated AML cell line, whereas no effect was observed for T cells with the NPM1-A11-specific TCR. Altogether, these results indicate that the NPM1-A11-specific TCR has a lower avidity for *NPM1*-mutated AML than the NPM1-A2-specific TCR. *In vitro*, however, CD8 T cells transduced with the NPM1-A11-specific TCR showed clear recognition and killing of patient-derived *NPM1*-mutated AML. This raises the question whether the NPM1-A11-specific TCR may still be useful for clinical application. T cells with high-avidity TCRs can achieve potent tumor control (29-33) and are generally favored for clinical application. Furthermore, T cells with high-avidity TCRs are expected to mediate anti-tumor reactivity even in the context of low antigen expression on tumor cells. However, this can also increase the risk of substantial on-target off-tumor toxicity if other antigens than neoantigens are targeted that are not exclusively presented on malignant cells, but also expressed at low levels on healthy cells (34-36). Besides high-avidity T cells, T cells with lower avidity can also play an important role in anti-tumor immunity (37-39). For instance, a recent study in mice demonstrated that adoptive transfer of relatively low-avidity T cells specific for a neoepitope controlled tumor growth and improved survival in mice bearing palpable tumors of a murine sarcoma cell line, whereas high-avidity T cells failed to induce tumor control (39). The lack of anti-tumor immunity by high-avidity T cells could be explained by their increased expression of exhaustion markers. In addition to reduced susceptibility to T-cell exhaustion (32, 37-39), T cells with relatively low avidity may also carry a decreased risk of on-target off-tumor toxicity, as low antigen expression on healthy cells is insufficient to activate these T cells (40). T cells with high- and relatively low-

avidity TCRs may thus both contribute to anti-tumor immune responses. Although high-avidity TCRs specific for AVEEVSLRK in HLA-A*11:01 are still preferable for clinical application, a relatively low-avidity NPM1-A11-specific TCR may also be used for immunotherapy, particularly considering that high-avidity TCRs for AVEEVSLRK are absent or extremely rare in the T-cell repertoire, as evidenced by our failure to isolate any high-avidity TCRs from 7 x 10^9 screened PBMCs. An optimal immunotherapeutic strategy may be to treat patients with *NPM1*-mutated AML with a mixture of T cells in which high- and relatively low-avidity TCRs have been introduced (40). In **chapter 3**, we also isolated a CLAVEEVSL-specific T-cell clone with weak reactivity against AML that may express a relatively low-avidity TCR. Further preclinical characterization and clinical development of the TCR from this T-cell clone would allow targeting of HLA-A*02:01-binding CLAVEEVSL on *NPM1*-mutated AML with this relatively low-avidity TCR as well as the high-avidity NPM1-A2-specific TCR.

The possible roles of neoantigen-specific CD4 T cells in anti-leukemic immunity

In **chapters 3**, **4** and **5** of this thesis, we have identified two HLA class I-binding neoantigens derived from the hotspot frameshift mutation in *NPM1* as well as one HLA class II-binding neoantigen derived from the *R882H* hotspot mutation in *DNMT3A*. Interestingly, *DNMT3A-R882H* mutations are frequently found in patients with *NPM1*-mutated AML (8), thereby potentially allowing concurrent targeting of AML blasts using neoantigen-specific CD8 and CD4 T cells. Many studies have reported that effective anti-tumor immunity relies on CD8 as well as CD4 T-cell responses (19-21). CD8 T cells are the main mediators of direct tumor cell killing. CD4 T cells can also induce direct cytolysis (22), but most often promote anti-tumor immunity by helping other immune cells (19-21). In contrast to aiding anti-tumor immunity, CD4 T cells can also have pro-tumorigenic functions that enable tumor progression (20, 21). Understanding the different roles that CD4 T cells can play in cancer immunity is crucial for employing their functions to stimulate anti-tumor immunity while suppressing their pro-tumorigenic functions in T-cell-based immunotherapies.

Direct lysis of tumor cells by CD4 T cells requires efficient processing of neoantigens from intracellular mutated proteins encoded by somatic mutations in tumor cells and presentation by HLA class II on the tumor cell surface. In **chapter 5**, we confirmed that AML blasts can process and present the HLA class II-binding DNMT3A neoantigen by showing that HLA-DQB1*06:02 or HLA-DQB1*06:03 positive patient-derived AML cells that naturally carry the *DNMT3A-R882H* mutation are recognized

by a CD4 T-cell clone. However, despite efficient recognition, the isolated CD4 T-cell clone did not kill AML cells. It is unknown why this T-cell clone lacks cytolytic capacity. Although we observed intra- and inter-patient heterogeneity in HLA class II expression, AML cells with high HLA class II expression could also not be killed by the T-cell clone. The observed heterogeneity in HLA class II expression also suggests that AML cells with no or low HLA class II expression may resist and escape direct cytolysis by CD4 T cells. Since AML cells were not killed despite high HLA class II expression, the lack of direct cytolytic capacity of the CD4 T-cell clone may be attributable to the T-cell phenotype or to TCR avidity (22). For example, the T-cell clone may lack granules with cytotoxic mediators, such as granzyme B and perforins, or the avidity of the HLA class II-restricted TCR may be sufficiently high to induce cytokine release, but too low for release of cytotoxic mediators. Nevertheless, our data showed that CD4 T cells specific for the HLA class II-binding DNMT3A neoantigen exist in the T-cell repertoire, offering the possibility to leverage their role as helper T cells to induce indirect anti-tumor effects, for instance by promoting tumor-specific CD8 T-cell responses.

CD4 T cells can stimulate CD8 T-cell responses in the priming phase as well as in the effector phase (19-21). During priming, CD4 T cells provide help to CD8 T cells via licensing of DCs (19). DC licensing requires direct CD4 T-cell-DC interaction that is mediated by binding of the TCR and CD40 ligand on CD4 T cells to peptide-HLA class II complexes and CD40 on DCs, respectively. This interaction stimulates DCs to upregulate costimulatory receptors and release cytokines, such as IL-12 and IL-15. In a mouse model of therapeutic cancer vaccination, effector CD8 T cells that were primed by licensed DCs showed enhanced clonal expansion, higher expression of cytotoxic effector molecules, including TNF, IFN-γ and granzyme B, and lower expression of coinhibitory receptors as compared to CD8 T cells primed without CD4 T-cell help (41). Furthermore, helped CD8 T cells demonstrated increased migratory and invasive potential and superior cytotoxicity. CD8 T-cell priming by licensed DCs is also essential for the differentiation, survival and function of memory CD8 T cells (19, 42-45). The value of CD4 help during T-cell priming is particularly important when designing cancer vaccines, since vaccination is often used for inducing *de novo* CD8 and CD4 T-cell responses. Neoantigen vaccines should therefore preferably include HLA class I as well as HLA class II neoantigens (46).

The NPM1 and DNMT3A neoantigens could potentially be used in neoantigen vaccination to stimulate combined DNMT3A neoantigen-specific CD4 T-cell responses and NPM1 neoantigen-specific CD8 T-cell responses *in vivo*. However, as evidenced by our results in **chapter 3** and **chapter 4**, CD8 T cells with high-avidity TCRs specific for HLA-A*02:01-binding CLAVEEVSL and HLA-A*11:01-binding

AVEEVSLRK NPM1 neoantigens are rare or even absent in most individuals. Vaccination with these NPM1 neoantigens to induce *de novo* CD8 T-cell responses is therefore not expected to be successful. Vaccination with NPM1 and DNMT3A neoantigens may, however, be used to boost TCR-engineered CD8 and CD4 T-cell responses (47). In particular in the setting of low tumor burden, for instance in patients who are in disease remission with measurable residual disease (MRD), vaccination may support robust activation and persistence of TCR-engineered T cells, thereby reducing the risk of relapse. When, for instance, synthetic peptide vaccines are used, the DNMT3A as well as NPM1 neoantigen peptides need to be presented by DCs. For the HLA-A*11:01-binding AVEEVSLRK neoantigen, we demonstrated in **chapter 4** that mature monocyte-derived DCs exogenously loaded with the synthetic neoantigen peptide were not recognized by NPM1-A11-specific TCR-transduced CD8 T cells. However, exogenous loading of the neoantigen peptide on other cell types, including the OCI-AML2 cell line, PBMCs and patient-derived AML blasts also resulted in low to no T-cell recognition. In a peptide-HLA class I binding assay, the affinity of the neoantigen peptide for HLA-A*11:01 was similar to a positive control peptide. These results suggest that the HLA-A*11:01-binding NPM1 neoantigen peptide is poorly presented upon exogenous loading, suggesting that neoantigen vaccination should not be performed with synthetic peptides, but rather with mRNA vaccines, for example. As described in **chapter 5**, the CD4 T-cell clone specific for the HLA-DQ-binding DNMT3A neoantigen has been isolated by stimulating CD4 T cells *in vitro* with mature monocyte-derived DCs that were exogenously pulsed with synthetic long peptides, indicating that the HLA class II-binding DNMT3A neoantigen peptide can be efficiently processed and presented by DCs. Whether the results as observed for monocyte-derived DCs are also applicable to DCs involved in CD4 T-cell help, which are primarily conventional type 1 DCs, still remains unclear (19, 48).

Besides help in the priming phase, CD4 T cells can also provide help to CD8 T cells in the effector phase (20, 21). In the tumor microenvironment (TME), intra-tumoral CD4 T cells may secrete cytokines upon recognition of their cognate antigen on APCs or tumor cells. Tumor cells can be positive for HLA class II or become positive in the presence of cytokines released by innate immune cells or CD4 T cells interacting with APCs. These cytokines may also create an inflammatory TME, thereby promoting recruitment, infiltration, local expansion and effector function of anti-tumor CD8 T cells (20, 21). For instance, in a mouse model, IFN-γ secretion by intra-tumoral CD4 T cells resulted in the production of chemokines within the TME, which led to improved recruitment of tumor-specific cytotoxic T cells, while IL-2 production by CD4 T cells enhanced cytotoxic T cells by stimulating their proliferation and granzyme B production (49). The inflammatory environment created by CD4 T cells may also affect antigen processing and presentation by tumor cells and other

cells in the TME. IFN-γ is known to increase expression of HLA, costimulatory and adhesion molecules (50). Furthermore, IFN-γ enhances intracellular antigen processing, for instance by converting the constitutive proteasome into the immunoproteasome or by stimulating transport of peptides into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Due to improved antigen processing and presentation, tumor cells in an inflammatory environment can become more susceptible to CD8 T-cell attack.

Cellular therapy with CD8 and CD4 T cells engineered with the HLA class I-restricted NPM1 TCR and HLA class II-restricted DNMT3A TCR, respectively, in combination with cancer vaccination with HLA class I-binding NPM1 and HLA class II-binding DNMT3A neoantigens may induce effective anti-tumor immunity by eliciting cytotoxic CD8 as well as helper CD4 T-cell responses. It is appealing to speculate about the added value of combining TCR-engineered T-cell therapy and cancer vaccination directed against HLA class I and class II neoantigens in general. CD4 T-cell help during priming can be delivered by endogenous CD4 T cells, which can be induced by vaccination, but perhaps also by TCR-engineered CD4 T cells. In the effector phase, help in the TME can also be provided by vaccine-stimulated endogenous CD4 T cells or by TCR-engineered CD4 T cells. In both situations, help can boost cytotoxic T-cell responses either by stimulating endogenous CD8 T cells, provided that these T cells are naturally present in the T-cell repertoire, or TCR-engineered CD8 T cells. Especially in tumors with an immunosuppressive environment in which endogenous T cells may fail to infiltrate the tumor in sufficient numbers, administration of large numbers of TCR-engineered CD4 T cells may convert the cold TME into a hot inflammatory TME, thereby promoting the accumulation and effector function of tumor-specific endogenous and TCR-engineered CD8 and CD4 T cells within the tumor, ultimately leading to efficient tumor destruction.

Equipping CD4 and CD8 T cells with identical neoantigen-specific TCRs

Identifying HLA class II neoantigens and their cognate TCRs for TCR-engineered T-cell therapy requires considerable effort and time. To bypass this laborious process while retaining the beneficial anti-tumor effects of CD4 T cells, CD4 T cells can be redirected towards tumor cells by introducing an HLA class I-restricted TCR derived from neoantigen-specific CD8 T cells. This is supported by our own observations in **chapter 3**, in which we demonstrated that, similar to CD8 T cells, CD4 T cells transduced with the HLA class I-restricted NPM1-A2-specific TCR were capable of recognizing and killing *NPM1*-mutated AML. Whereas CD4 T cells redirected to HLA class I-binding neoantigens have not yet been administered in clinical trials, the

administration of CD8 and CD4 T cells engineered to express identical HLA class I-restricted TCRs specific for a tissue differentiation antigen, cancer germline antigen, viral antigen or MiHA has been investigated in patients with hematological or solid tumors (51-58). Clinical responses have been observed in these trials and several trials also reported persistence of TCR-engineered CD8 as well as CD4 T cells in the peripheral blood (51-55, 58). Apart from eliminating the need for HLA class II neoantigen discovery and TCR identification, another benefit of HLA class I TCR-redirected CD4 T cells is the more ubiquitous expression of HLA class I on tumors (59), which is expected to stimulate CD4 T-cell-mediated cytotoxicity and help. While many solid tumors do not express HLA class II (59, 60), HLA class I is present on most hematological and solid tumor types and is often more uniformly expressed on malignant cells within the tumor as compared to HLA class II (60). Another advantage of introducing the same HLA class I-restricted TCR into CD8 and CD4 T cells is that only one viral vector or non-viral delivery system needs to be produced under Good Manufacturing Practice conditions, and that CD8 and CD4 T cells do not need to be separated and processed individually, as would be necessary when introducing different TCRs (61). Despite these advantages, some disadvantages can also be envisioned. CD8 and CD4 T cells may differ in their cytolytic potential, expansion rate and capacity to integrate the transgenic TCR into their genome. This increases the risk that suboptimal T-cell products will be generated in which strongly cytolytic CD8 T cells are outcompeted by CD4 T cells with no or inferior cytolytic function (61). CD4 T cells could also potentially possess higher copy number integrations of the introduced TCR (61), which may increase the risk of insertional mutagenesis and could possibly result in malignant transformation. It may therefore still be preferred to process CD8 and CD4 T cells individually to create well-defined T-cell products.

In most clinical trials, HLA class I-restricted tumor-reactive TCRs have been introduced into CD8 and CD4 T cells without the CD8 coreceptor (51-57), except for one trial in which a MiHA-specific TCR was co-transferred with CD8 (58). For the NPM1-A2-specific TCR, we demonstrated peptide-HLA class I tetramer binding as well as anti-AML efficacy for CD4 T cells transduced with the TCR in the absence of CD8. CD8 interacts with the non-polymorphic region of the HLA class I heavy chain, a site that is distinct from the peptide-binding groove that interacts with the TCR (62). Binding of CD8 to HLA class I stabilizes the TCR:peptide-HLA interaction and promotes TCR signaling, thereby increasing antigen sensitivity of the T cell and enhancing T-cell activation. The importance of the CD8:HLA interaction for T-cell activation and function is dependent on the affinity of the TCR for its cognate peptide-HLA ligand (63, 64). While T cells with relatively low- or intermediate-affinity TCRs require CD8 for their activation, T cells with high-affinity TCRs can function in a CD8 independent manner. The (partial) CD8 coreceptor independence of the NPM1-A2-specific TCR may indicate that the TCR has a relatively high affinity for its peptide-

HLA ligand. In contrast, the NPM1-A11-specific TCR has a lower affinity and CD4 T cells transduced with this TCR showed poor binding to tetramers and failed to recognize cells exogenously pulsed with the NPM1 neoantigen peptide. It may be interesting to investigate whether the efficacy of CD4 T cells transduced with the NPM1-A11-specific TCR can be stimulated by introducing CD8, either the wt coreceptor or a modified version with enhanced affinity for HLA, as recently demonstrated *in vitro* for CD4 T cells engineered with other HLA class I-restricted tumor-targeting TCRs (65).

Analogous to HLA class I TCR-redirected CD4 T cells, CD8 T cells can be engineered to express a tumor-reactive HLA class II-restricted TCR. These TCR-redirected CD8 T cells can potentially be used to target HLA class II positive tumor cells, including AML blasts. HLA class II TCR-redirected CD8 T cells may also be safe, since HLA class II expression on healthy cells is mainly restricted to specialized APCs of hematopoietic origin. However, HLA class II can be broadly induced on hematopoietic and non-hematopoietic healthy cells under inflammatory conditions, for example during infection. This may increase the risk of toxicity, especially when tumor antigens are targeted that are not exclusively expressed on malignant cells (66). The anti-tumor potential of HLA class II TCR-redirected CD8 T cells has been reported in a preclinical study in which an HLA-DP-restricted TCR against an overexpressed tumor antigen was introduced into CD4 and CD8 T cells (67). The authors investigated combined TCR-engineered CD4 and CD8 T cells, and demonstrated killing of melanoma cell lines endogenously expressing the tumor antigen as well as autologous malignant ascites cells, while no reactivity was observed against healthy hematopoietic cells. The TCR-engineered CD4 and CD8 T cells also reduced tumor growth and improved survival in a xenograft mouse model. The TCR from the CD4 T-cell clone specific for the HLA-DQ-binding DNMT3A neoantigen as isolated in **chapter 5**, may, in future studies, be sequenced, cloned and used to redirect CD8 T cells in a similar way as described above. However, as discussed previously, it should be noted that HLA class II expression on AML is often heterogeneous, suggesting that complete clearance of tumor cells may not be possible. Furthermore, no clinical studies have been reported yet that demonstrate that CD8 T cells can acquire strong antigen-specific cytolytic potential upon transfer of an HLA class II-restricted TCR.

Future perspective on treatment of patients with the NPM1-A2-specific TCR

The safety and efficacy of the NPM1-A2-specific TCR are currently being investigated in a phase I/II clinical trial (registered at https://clinicaltrials.gov/ as

NCT06424340). In this trial, HLA-A*02:01 positive patients with *NPM1*-mutated AML who have refractory, relapsed, or MRD positive disease are treated with autologous T cells that are engineered to express the NPM1-A2-specific TCR. If proven safe and effective in clinical studies, NPM1-A2 TCR-engineered T-cell therapy could become available as a highly needed new treatment option for these patients. Ultimately, NPM1-A2 TCR-engineered T-cell therapy may be approved as treatment for patients with newly diagnosed *NPM1*-mutated AML and, if effective, it may even eliminate the need for allogeneic hematopoietic stem cell transplantation. Following induction therapy, patients could be treated preemptively or prophylactically with NPM1-A2 TCR-engineered T-cell therapy as consolidation treatment. Preemptive treatment may be guided by detection of mutated *NPM1* in MRD analyses (68) for optimal timing of NPM1-A2 TCR-engineered T-cell therapy and prevent unnecessary intervention in patients who have achieved durable complete remissions. Since AML is an aggressive disease and leukemic blasts can rapidly expand to life-threatening numbers, MRD analyses should be regularly performed at short time intervals to ensure timely administration of NPM1-A2-specific TCR-engineered T cells. However, frequent MRD evaluations can place a substantial psychological and logistical burden on patients and the risk remains that leukemic progression outpaces production of the T-cell product. Prophylactic administration of NPM1-A2 TCR-engineered T-cell therapy provides an attractive alternative, but is presently hindered by its feasibility. Since *ex vivo* production of TCR-engineered T cells is a laborious and logistically complex procedure, expanding the application of NPM1-A2 TCR-engineered T-cell therapy to large patient numbers will be challenging (69). However, novel cell engineering methods are currently being explored, which could potentially overcome these capacity issues in the future (70, 71). For example, various strategies are under development to selectively deliver an antigen receptor into autologous T cells and produce receptor-engineered T cells *in vivo* (70). If these novel methods prove safe, effective and feasible, they could make NPM1-A2 TCR-engineered T-cell therapy more widely available as treatment option for HLA-A*02:01 positive patients with *NPM1*-mutated AML.

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