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

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# 6

## SUMMARY AND GENERAL DISCUSSION

## SUMMARY

The use of T-cell receptor (TCR) gene transfer for the treatment of both hematological and solid tumors is increasing. Using TCR gene transfer T cells can be redirected to target tumor- or lineage-specific antigens. Especially for poor immunogenic tumors this offers the potential to circumvent limitations of the endogenous T-cell repertoire. Still, the broad use of TCR-based therapy is hampered by a limited number of targeted antigens and HLA class I binding restrictions of TCRs. Furthermore, several of the pioneering T-cell based therapies have demonstrated that the balance between therapeutic efficacy and safety remains a challenge as T-cell mediated toxicities have occurred. In this thesis we identified novel targets, peptides and TCRs in order to treat a broader patient population, among others ovarian and prostate cancer patients. We stringently selected appropriate tumor- and lineage-specific targets using a differential gene expression analysis, and identified naturally expressed peptides from the HLA class I associated ligandome. We isolated peptide-specific T cells, sequenced their TCRs and carefully selected the most promising TCRs. Overall, we selected ten TCRs that demonstrated an effective and safe reactivity pattern based on the performed T-cell reactivity screenings. These TCRs demonstrated reactivity against broad panels of patient-derived tumor samples and/or tumor cell lines, without reactivity against a broad variety of healthy cell subsets or other antigen negative cells. Furthermore, in this thesis we set up human induced pluripotent stem cell (hiPSC)-derived models to additionally examine toxicity risks of T cells against vital organs or specialized cell subsets. We demonstrated added value of these models in determining toxicity risks in the preclinical pipeline of TCRs.

A tumor-associated antigen (TAA) often targeted in clinical trials is transcription factor Wilms' tumor gene 1 (WT1). In current WT1-targeting vaccine and TCR gene therapy studies, T-cell reactivity is hampered by self-tolerance to WT1 and limited numbers of WT1 peptides. In **chapter 2** we identified additional WT1 peptides derived from the HLA class I associated ligandome of primary acute myeloid leukemia (AML) and ovarian carcinoma samples. By searching in the allogeneic-HLA (allo-HLA) T-cell repertoire we circumvented self-tolerance against WT1 and isolated potent and specific T-cell clones reactive against five different WT1 peptides. By gene transfer of four WT1-TCRs into CD8+ T cells, we analyzed the antitumor potential as well as the safety profile of the TCR-engineered T cells (TCR-T cells). These TCRs are restricted to peptides in common HLA class I restriction molecules, namely HLA-A\*01:01, HLA-A\*02:01 or HLA-B\*35:01. The TCR-T cells recognized none of the included healthy cell subsets and induced efficient recognition and killing of primary AML and OVCA patient samples. Our results demonstrated that the identified WT1-TCRs are promising candidates for TCR gene transfer strategies in patients with WT1-expressing tumors, including AML and ovarian carcinoma. In our search for WT1-specific T-cell clones, notably no T-cell clones reactive against tumor cells could be identified for the previously clinically used WT1 peptides. These peptides were previously all identified based on HLA peptide binding prediction

algorithms and the most commonly used WT1 peptide is the RMF peptide presented in HLA-A\*02:01. Although we identified high numbers of T-cell clones recognizing this RMF peptide, no WT1 expressing tumor cells were recognized, indicating that this peptide is not efficiently processed and presented on tumor cells. In summary, our data suggested better clinical results may be achieved with the novel naturally expressed WT1 peptides for which potent TCRs were identified. Overall, our results demonstrated that the set of four WT1 TCRs and naturally expressed WT1 peptides are expected to improve WT1-targeting therapies for patients with *WT1*-expressing tumors, including AML and ovarian carcinoma.

In **chapter 3** we established preclinical models with hiPSC-derived cardiomyocytes, epicardial cells, and kidney organoids to investigate toxicity risks of tumor-specific TCR-T cells with clinical potential. We investigated whether these models can fulfill the need for *in vitro* screening options to examine toxicity risks of T cells directed towards vital organs or specialized cell subsets. CD8+T cells reactive against preferentially expressed antigen of melanoma (PRAME), minor histocompatibility antigen (MiHA) HA-1H, CD20 or WT1, currently used or planned to be used in phase I/II clinical studies, were included. In case of expression of the target gene, as demonstrated for housekeeping gene *USP11* and tumor gene *WT1*, on-target toxicity was observed in these models. Multiple measures of T-cell reactivity demonstrated this toxicity, such as cytokine production, killing potential and phenotypic analysis of T cells and hiPSC-derived target cells. In addition, phenotypic analysis illustrated interaction and crosstalk between infiltrated T cells and kidney organoids. Overall, in this chapter we demonstrated the benefit of hiPSC-derived models in determining toxicity risks of tumor-specific T cells and emphasized the additional value of other measures of T-cell reactivity on top of the commonly used cytokine levels. Furthermore, the reactivity of WT1 T cells towards hiPSC-derived kidney organoids and epicardial cells reflected the immature phenotype of the models, but also indicated possible toxicity risks of WT1-targeting therapies.

We selected PRAME, CCCTC-binding factor (CTCF), and Claudin-6 (CLDN6) as potential targets for the treatment of ovarian cancer patients in **chapter 4**. These genes are classified as cancer-testis antigens (CTA's) or TAA's and they are highly and differentially expressed in ovarian cancer, without expression in healthy tissues of risk. In the HLA class I ligandome of ovarian cancer patient samples and cell lines we identified naturally expressed peptides, and we identified high-avidity T-cell clones recognizing these peptides. In total three PRAME TCRs and one CTCFL TCR of the most promising and safe T-cell clones were sequenced and transferred to CD8+ T cells. The HLA-A\*02:01, HLA-A\*24:02 or HLA-B\*07:02 restricted PRAME TCR-T cells demonstrated potent and specific antitumor reactivity *in vitro* and *in vivo*. The HLA-A\*02:01 restricted CTCFL TCR-T cells efficiently recognized primary patient-derived OVCA cells, and OVCA cell lines treated with demethylating agent 5-aza-2'-deoxycytidine (DAC). In conclusion, we considered the selection of potent PRAME and CTCFL TCRs to be promising candidates for patients suffering from ovarian cancer or other *PRAME* or *CTCF*

expressing cancers. In addition, we expected our selection of differentially expressed genes and naturally expressed peptides to broaden the applicability of TCR therapies in patients with ovarian cancer.

In **chapter 5** we selected kallikrein-related peptidases 2, 3 and 4 (KLK2, KLK3, KLK4) and homeobox B13 (HOXB13) as strictly prostate lineage-specific genes that can be used for the treatment of prostate cancer. From the HLA class I ligandome of tumor cells we identified a library of prostate lineage-specific peptides that are processed and presented in the context of HLA class I. To avoid immunological tolerance towards the prostate lineage, we isolated high-avidity T-cell clones from the allo-HLA T-cell repertoire. Of these T-cell clones, three KLK4 HLA-A\*02:01 specific T-cell clones and two KLK3 HLA-B\*35:01 specific T-cell clones most efficiently and antigen-specifically recognized prostate tumor cell lines, without detectable on-target off-tumor or off-target reactivity. We sequenced their TCRs and upon TCR gene transfer into CD8+ T cells, the TCRs demonstrated potent prostate-specific cytokine production and cytotoxic killing potential *in vitro* as well as *in vivo*. These TCRs are considered promising candidates for the treatment of patients with prostate cancer. Overall, we provided proof-of-concept that KLK3 and KLK4 can effectively be targeted by TCR gene transfer. In addition, our set of naturally processed HLA class I binding KLK2, KLK3, KLK4 and HOXB13 peptides can be used to identify additional prostate-reactive TCRs.

## GENERAL DISCUSSION

### ADVANCES AND LIMITATIONS OF TCR GENE TRANSFER TARGETS

WT1 has frequently been described as an ideal tumor target, based on high expression in tumors and tumor-promoting characteristics. WT1 was even ranked by the US National Cancer Institute as the most promising tumor target for cancer vaccination.<sup>1</sup> In **chapter 2** we indeed quantified high *WT1* expression in patient-derived ovarian cancer, AML and acute lymphoblastic leukemia (ALL) samples. Of all healthy cell subsets included, only limited *WT1* expression was quantified in CD34+ hematopoietic precursor cells, and no recognition by the investigated WT1-reactive TCR-T cells was observed. WT1 is mainly described as an antigen involved during embryonic development. Still, in adults WT1 is involved in homeostasis processes for tissue maintenance and recovery, resulting in continuous *WT1* expression in renal podocytes and temporary *WT1* expression in epicardial cells after a myocardial infarct.<sup>2-4</sup> When only focused on bulk gene expression data, these *WT1* expression levels in specialized cell subsets and temporary expressed genes can be easily missed. Furthermore, the quantification of *WT1* in heart and kidney is complex and among others mouse models have been used to predict human *WT1* expression after myocardial infarct.<sup>4</sup> Most WT1-targeting studies only inform about the *WT1* expression in renal podocytes and CD34+ hematopoietic precursor cells, and state that WT1 is considered a safe target as no toxicities

of WT1-targeting therapies have been reported.<sup>5-7</sup> However, the lack of toxicities may also be related to the limited T-cell reactivity of current WT1-targeting vaccine and TCR therapy studies. The T-cell reactivities in these studies are hampered by relatively low TCR affinities, limited processing and presentation of the targeted WT1 peptides and self-tolerance to WT1 in case of vaccine studies. In **chapter 3** we demonstrated potential on-target off-tumor toxicity risks of WT1-targeting therapies, using a high-avidity T-cell clone identified in **chapter 2**. High *WT1* expression was quantified in hiPSC-derived epicardial cells and kidney organoids. The highly effective WT1-specific T cells were highly reactive against these heart cells and kidney organoids. Based on our results the safety of WT1-targeting therapies has to be reviewed carefully again, especially when improving T-cell reactivities using high-affinity TCRs directed against naturally expressed peptides.

The differentially expressed (DE) target antigens identified in **chapters 4 and 5** are strict tumor-specific target antigens in ovarian cancer and strict lineage-specific target antigens in prostate cancer. According to our criteria for strict DE antigens, most antigens currently targeted in clinical studies with ovarian cancer patients lack specificity. CAR-T cells targeting extracellular proteins CLDN6, mucin16, mesothelin, folate receptor- $\alpha$  and human epidermal growth factor receptor 2, are currently investigated in clinical studies.<sup>8</sup> Of these target antigens, we only considered CLDN6 a strictly tumor-specific target for ovarian cancer patients. For the other targets the difference between expression in ovarian cancer and some of the healthy tissues was smaller. A small difference indicates a narrow therapeutic window and on-target off-tumor toxicity risks in healthy tissues expressing the targets on a detectable level.<sup>9</sup> Moreover, we questioned whether frequently studied New York esophageal squamous cell carcinoma-1 (*NY-ESO-1*) and melanoma-associated antigen 4 (*MAGE-A4*) are optimal targets for TCR therapy since for the majority of ovarian cancer patients the mean expression levels of *NY-ESO-1* and *MAGE-A4* were low in the included ovarian cancer samples. We considered a mean expression below 100 absolute read counts low and most likely biologically irrelevant. For prostate cancer patients, most gene transfer studies investigate CAR-T cells targeting folate hydrolase 1 (*FOLH1*) and acid phosphatase prostate (*ACPP*).<sup>10</sup> According to our criteria, both antigens lack differential expression between healthy and tumor tissues. Our differential gene analysis revealed potential toxicity risks of *FOLH1* in spinal cord and of *ACPP* in CD14+, CD34+, and bronchial epithelial cells. To date, no TCR gene transfer studies are performed in patients with prostate cancer. We identified *KLK2*, *KLK3*, *KLK4* and *HOXB13* as promising prostate-lineage specific target antigens for TCR gene transfer. Of these target antigens, the *KLK* genes are currently targeted using anti-tumor vaccines, bispecific antibodies and engineered *KLK* inhibitors.<sup>11-13</sup>

Unfortunately, our performed DE analysis revealed no new target antigen candidates for ovarian and prostate cancer, although little was known about the in our pipeline identified DE genes *CTCF* and *HOXB13*. In addition, despite several studies already focused on the DE genes *PRAME*, *CLDN6*, *KLK2*, *KLK3* and *KLK4*, no or a limited number of TCRs targeting these

genes were reported thus far. The low number of DE genes identified in our pipeline may be related to our strict DE cut-off and strict minimum expression value in tumors. In particular for high-affinity TCR-T cells, a large therapeutic treatment window is preferred to reduce on-target off-tumor toxicity risks. The TCRs we isolated from the allo-HLA T-cell repertoire are highly potent and capable of recognizing low expression levels. To illustrate, our PRAME TCRs in **chapter 4** even recognized healthy mature DCs, which express PRAME approximately 30 fold lower compared to *PRAME* positive tumor cells. Nevertheless, these mature DCs can be more easily recognized since they are professional antigen presenting cells with high levels of HLA, adhesion and co-stimulatory molecules.<sup>14</sup> Furthermore, also tissue-specific characteristics may be related to the low number of DE genes. The lack of ovarian lineage-specific antigens was most likely due to absence of unique tissue characteristics, based on shared biological similarities with other organs. Since most ovarian tumors originate from the fallopian tube, we focused on lineage-specific DE genes expressed in healthy ovary and/or fallopian tube. Both healthy organs however lack unique tissue characteristics. Fallopian tube shares with lung and brain the motile cilia that facilitates transport of fluids<sup>15,16</sup>, and ovary shares similarities with other endocrine glands like adrenal gland and pancreas<sup>17</sup>.

Despite the low number of suitable target antigens, the selected DE genes that did fulfill our criteria are considered promising therapeutic target antigens for TCR gene transfer. Furthermore, the DE analysis described in this thesis provide a tool for the identification of suitable shared lineage- and tumor-specific antigens for other cancer types as well.

## SELECTION OF NATURALLY PROCESSED AND PRESENTED TARGETABLE PEPTIDES

The majority of targetable peptides used in literature and clinic are selected based on HLA peptide binding prediction algorithms. The limited pool of frequently used peptides is repeatedly used, often without evidence of efficient processing and presentation in tumors. For target gene *WT1* the most commonly used peptide is RMFPNAPYL presented in HLA-A\*02:01 (RMF/A2). Our results in **chapter 2** suggested this peptide is not efficiently processed and presented in *WT1* positive tumors and demonstrated the relevance of establishing the HLA class I associated ligandome of tumors using mass spectrometry. To our knowledge this RMF/A2 peptide has not been found in peptide-elution databases of tumor samples<sup>18</sup> and we also failed to elute this peptide in our large set of *WT1*-expressing HLA-A\*02:01 tumor samples. In chapter 2 we showed that none of the T-cell clones recognizing RMF/A2 were reactive against naturally *WT1*-expressing tumor cells, only artificially overexpressed *WT1* transduced Raji cells were recognized. These results suggest that the RMF/A2 peptide can be processed and presented, but the level of presentation in tumor cells may not be sufficient for T-cell recognition. Based on our data limited antitumor reactivity can be expected of RMF/A2-specific T cells and this corresponds to the limited antitumor effects found in (pre)clinical studies targeting the RMF/A2 peptide.<sup>7,19</sup>



Others also questioned whether RMF/A2 is a suitable peptide for WT1-targeting therapies. In the autologous T-cell repertoire of many healthy individuals RMF/A2-specific T cells were easily found, suggesting the RMF/A2 peptide is indeed not optimally processed and presented, since RMF/A2 reactive T cells would otherwise have been deleted during negative selection.<sup>20</sup> Moreover, none of the identified RMF/A2-specific T cells demonstrated antitumor reactivity.<sup>20</sup> Furthermore, while investigating a high-affinity RMF/A2-specific TCR, no reactivity against *WT1*-expressing tumor cells was observed.<sup>21</sup> By analyzing the immunoproteasome, the authors concluded that the processing and presentation of the RMF/A2 peptide is limited in the absence of the immunoproteasome. The immunoproteasome is involved in the processing of numerous MHC class I-restricted T-cell epitopes.<sup>22</sup> Hematopoietic cells generally express immunoproteasomes and under inflammatory conditions other cell types can express immunoproteasomes as well.<sup>22,23</sup> The proteasome type impacts which peptides are processed and presented, some peptides are only produced by the standard proteasome or the immunoproteasome, whereas other peptides can be produced by both types.<sup>23,24</sup> More recently, another group confirmed in relapsed AML samples that the presentation of RMF/A2 depends on the immunoproteasome.<sup>25,26</sup> The authors described immunoproteasome downregulation as a possible immune evasion mechanism in relapsed AML, which can be circumvented by using other immunoproteasome-independent WT1-derived epitopes.

Likewise, for target gene *PRAME* the VLDGLDVLL peptide presented in HLA-A\*02:01 (VLD/A2) is often used in (pre)clinical studies. We hypothesize also this peptide is not optimally processed and presented in *PRAME* positive tumors. By analyzing the HLA class I associated ligandome of tumor samples in **chapter 4**, we identified 23 naturally expressed *PRAME* peptides, of which 8 peptides were presented in HLA-A\*02:01. We were not able to identify the VLD/A2 peptide and also others have not succeeded. Furthermore, since *PRAME* is also expressed in mature dendritic cells (DCs) and proximal tubular epithelial cells in kidney,<sup>27,28</sup> high-avidity *PRAME*-reactive T cells are expected to be deleted during the negative selection. Still, two groups identified VLD/A2 reactive T cells with high peptide reactivity in the auto-HLA T-cell repertoire.<sup>20,29</sup> This may suggest the VLD/A2 peptide is indeed not optimally processed and presented, since these VLD/A2 reactive T cells would otherwise have been deleted during negative selection. Moreover, although these identified T cells demonstrated high avidity for target cells loaded with the VLD/A2 peptide, the limited antitumor reactivity against *PRAME* expressing tumor cells also suggests limited peptide processing and presentation.<sup>20,29</sup>

A limited number of primary patient-derived tumor samples can hamper the identification of naturally processed and presented peptides, since multiple samples are essential to cover most common HLA-alleles and target gene expression. Still, alternative sources for HLA ligandome analyses are optional when tumor samples are not available, such as cell lines or humanized mice.<sup>30</sup> Despite the lack of prostate patient-derived samples in **chapter 5**, we alternatively identified 14 prostate lineage-specific gene derived peptides from prostate

tumor cell lines and Epstein-Barr virus transformed lymphoblastoid cell lines (EBV-LCLs) transduced with the prostate lineage-specific genes of interest. This alternative demonstrated to be an effective approach, although the differences between transduced EBV-LCLs and prostate tumors should be taken into account. The EBV-LCLs probably expressed the prostate lineage-specific genes on an artificial high level. As observed in **chapter 2** for the RMF/A2 peptide, artificial high expression can result in the processing and presentation of peptides that are not or limited expressed in tumor cells. Furthermore, the proteasome differs between EBV-LCLs and prostate tumor cells, which also impacts peptide presentation. Since EBV-LCLs predominantly express the immunoproteasome, our approach may result in a selection of immunoproteasome-dependent peptides that are limited expressed in prostate tumors. Despite these differences, in **chapter 5** the most potent KLK3, KLK4 or HOXB13-reactive T-cell clones that recognized prostate tumor cell lines were restricted to peptides identified in EBV-LCLs. These data indicated EBV-LCLs transduced with tumor genes can be used for the identification of peptides derived from tumor genes exclusively expressed on solid tumors. Still, since the KLK3, KLK4 or HOXB13-reactive T-cell clones could not be tested against patient-derived prostate tumors, direct evidence of peptide presentation in prostate tumors is missing for the recognized peptides. Overall, alternative HLA ligandome sources can be used, but they should be used with caution since they can result in the identification of artificially expressed peptides.

A large set of peptides increases the chance of identifying peptide-specific T cells. In our large-scale T-cell search in healthy donors, peptide-specific T cells were found for half of the included naturally expressed peptides. In **chapter 2, 4** and **5** we identified T-cell clones for 9 of the 12, 9 of the 17 and 6 of the 14 included peptides, respectively. Following all safety and efficacy screenings, in each chapter only a few T-cell clones appeared to be truly peptide-specific and reactive against tumor cells. Comparable findings were observed by others using different peptide sets.<sup>31-34</sup> There are several reasons for not finding T cells recognizing a specific peptide. Peptides may be limited processed and presented by MHC on the cell surface. Our mass spectrometry analysis provided evidence of processing and presentation of the peptides, although a low peptide-MHC (pMHC) binding affinity<sup>35</sup> or low pMHC stability<sup>36</sup> might still hamper sufficient presentation for T-cell recognition. T cells may also not be able to distinguish a peptide from other look-alike peptides.<sup>37</sup> These look-alike peptides can be a result of sequence or structural homologies that are involved in the TCR interaction, also referred to as molecular mimicry.<sup>38</sup> The TCR repertoire is assumably tolerant for peptides resembling ubiquitously expressed peptides presented in self-HLA. For peptides resembling ubiquitously expressed peptides presented in non-self HLA, we probably excluded T cells recognizing these peptides during our T-cell selection screenings for lack of peptide specificity.

Ultimately, a large set of naturally processed and presented peptides identified in the HLA ligandome of patient-derived tumor samples increases the chance of identifying truly tumor-

specific T cells. Alternative HLA ligandome sources can be used, but should be used with caution since peptides identified in these sources lack direct evidence of processing and presentation in tumors.

### RELEVANCE OF ACCURATE PRECLINICAL EFFICACY, SPECIFICITY AND SAFETY SCREENINGS FOR TCR THERAPIES

In order to estimate preclinical efficacy of TCR therapies, patient-derived samples are preferably included. In **chapter 2** and **4** we tested the reactivity of the new TCR-T cells against primary patient-derived ovarian cancer and AML cells. Based on those results we were able to better estimate the clinical potential of the TCRs. Although there was large overlap between the reactivity against primary patient-derived cells and tumor cell lines, some differences were observed. The WT1-reactive 12.5H9<sup>VLD/A1</sup> TCR exhibited high recognition and killing of most HLA-A\*01:01 and *WT1* positive tumor cell lines, but only one of the three HLA-A\*01:01 and *WT1* positive AML samples was recognized. These data indicate that the 12.5H9<sup>VLD/A1</sup> TCR is not a suitable candidate for treatment of all HLA-A\*01:01 and *WT1* positive AML patients, most likely due to differences in processing and presentation of the WT1 peptide VLD/A1. These data demonstrated the relevance of efficacy screenings with primary patient-derived samples. Unfortunately in **chapter 5** the KLK3- and KLK4-reactive TCRs could not be tested against primary patient-derived prostate cancer samples because of lack of patient materials. Although recognition and killing of the prostate tumor cell lines gave insight in the clinical potential of these TCRs, the lack of patient samples reduces the strength of our conclusions. Before these TCRs may enter a clinical study, the reactivity against preclinical patient-derived models, such as organotypic tissue slices or PDX models,<sup>39</sup> has to be tested first.

Besides patient-derived samples, positive and negative controls are highly relevant for accurate preclinical specificity screenings of TCR-T cells. Without accurate controls, absence or presence of T-cell reactivity may not be related to safety or specificity of the investigated TCR. To illustrate, in a recent publication a WT1-reactive TCR specific for the RMF peptide in HLA\*02:01 was described that induced killing of three HLA-A\*02:01 positive primary AML samples, while sparing an HLA-A\*02:01 negative primary AML sample.<sup>6</sup> Since *WT1* expression was not quantified in these AML samples, it is unsure whether the reactivity of the TCR-T cells was WT1-specific, as reactivity against another antigen in the context of HLA-A\*02:01 could not be excluded. Likewise, no *CLDN6* or *PRAME* negative cells were included in recent studies investigating a *CLDN6*-reactive TCR<sup>40</sup> or *PRAME*-reactive TCR mimicking antibody that targets a pMHC-complex<sup>41</sup>. Besides the need of antigen-negative tumor cells that do express the targeted HLA, it should be tested whether these antigen-negative cells can be recognized by T cells. Optional positive controls are T cells recognizing a housekeeping gene to demonstrate sufficient HLA and adhesion molecules on the included target cells. Alternatively, target cells could be loaded with the peptide of interest to prove appropriate presentation ability of the cells, independent of antigen expression or natural peptide presentation. Accurate efficacy

screenings are also relevant for safety statements related to a TCR or target antigen. When preclinical efficacy of TCR-T cells is limited or when preclinical specificity is inadequately tested, the absence of reactivity against healthy cells may just be a consequence of limited efficacy instead of specificity or safety. This is also valid for clinical studies investigating safety of TCRs or targets. For example, WT1 is often described as a safe target since no toxicities have been reported during clinical studies. However, due to limited clinical reactivities of the current investigated TCRs, these statements lack value.

Even if a broad variety of preclinical safety screenings have been executed, most screenings with TCR-T cells are restricted to a selection of primary healthy cells, cell lines, allo-HLA reactivity panels and peptide libraries. Toxicity risks associated with vital organs or understudied cell subsets are therefore potentially missed. The broad safety screenings in **chapter 2** and **4** were for example limited by lack of heart and kidney tissues, although we reported *WT1* and *PRAME* expression in heart and/or kidney. The hiPSC-derived kidney organoids and heart cells set up in **chapter 3** demonstrated hiPSC-derived preclinical models may be a valuable option to overcome the limitations of current safety screening options for TCR-T cells. The included hiPSC-derived models resembled the different heart cell types and kidney tissue in gene expression, morphology and functional properties.<sup>42-44</sup> In addition, the use of 3D models offered the potential to investigate T-cell infiltration and interaction between T cells and tissues. Unfortunately the models also exhibited immature properties, which might result in an underestimation of toxicity risks for genes exclusively expressed in mature cells and an overestimation of toxicity risks for genes involved in embryonic development. Furthermore, whether these models are predictive for the absence or presence of TCR-related toxicity in patients has not been validated yet. The validation of the utility of these models in predicting TCR-related toxicity is complex and requires a broad selection of TCRs including clinical data. Although the hiPSC-derived preclinical models have not been validated yet, at least the presence of TCR-related toxicity indicates potential toxicity risks of the investigated TCR.

Overall, a broad variety of preclinical efficacy and safety screenings with accurate positive and negative controls is essential in order to select the most effective, specific and safe TCR candidates.

## **FUTURE OF TCR GENE TRANSFER THERAPIES**

The promising TCRs identified in this thesis, reactive against various prostate lineage- and tumor-specific antigens with different HLA restrictions, are a valuable addition to the limited number of available TCRs.<sup>45</sup> Before these TCRs may enter the clinic, additional preclinical screenings are needed to further demonstrate their clinical potential. For example mouse models to investigate whether the TCRs reactive for *PRAME*, *CTCF*, *WT1*, *KLK3* or *KLK4* are capable of reaching solid tumors, and screenings to test whether *KLK3* and *KLK4* TCR-T cells are reactive against prostate tumor samples. The TCRs may also be further screened for

safety using the developed hiPSC-derived preclinical models. This extensive testing is recommended for all novel identified TCRs and will remain a time-consuming, costly and risky hurdle for the application of TCR therapies. In order to make TCR gene transfer available for the majority of patients, ideally a library of TCRs is available that covers most HLA restrictions and tumor antigens. If possible, patients should be even treated with multiple TCRs targeting more antigens in different HLA alleles, to reduce the chances of antigen-loss escape variants.<sup>46,47</sup> Furthermore, in order to cover most patients, the TCRs in the library are preferably reactive against shared antigens such as lineage- or tumor-specific antigens. Although shared neoantigens exist as well, the majority of neoantigens are not shared by a significant number of patients.<sup>48</sup> Despite their high tumor-specificity, most neoantigen-reactive TCRs are therefore less suitable for this library of shared TCRs.<sup>48</sup>

Apart from the number of TCRs and antigens, a major factor limiting the wide application of TCR therapies are the high costs. The high costs are related to the clean rooms, equipment, production materials, personal required for production of the cell product under good manufacturing practices (GMP) condition and the intensive clinical care.<sup>49</sup> Lessons can be learned from the rapidly changing CAR-T cell field, with most of the FDA-approved CAR-T cell products being produced in centralized production centers of pharmaceutical companies. Since most CAR and TCR therapies require the use of autologous T cells, the therapy is personalized and not an 'off-the-shelf' type of treatment. This means for most patients leukapheresis samples and cell products have to be shipped back and forth to production centers. Pharmaceutical companies calculated in 2020 between €300,000 and €350,000 per CAR-T cell product within Europe, excluding costs for clinical care.<sup>50</sup> Apart from the actual high costs, the pharmaceutical industry raises their prices due to high development costs, high risks and small patient population sizes.<sup>51</sup> By decentralizing the production within academic centers, costs may be reduced approximately five times, depending among others on yearly productions and materials used.<sup>49</sup> Besides reducing the costs, in-house production lowers the production time and may enhance quality of the final product by limiting freeze-thaw cycles. To date, most hospitals are not able to produce these complex T-cell products or provide the required specialized clinical care. The further automation of several production steps might enhance the wider application among hospitals.<sup>52</sup> In order to make TCR therapy affordable, including the scenario of targeting a patient with multiple TCRs, costs must be reduced dramatically. Ideally the production of TCR-T cell products becomes less dependent on pharmaceutical companies or pharmaceutical companies lower their prices and cooperate with academia. Among others, innovative technologies reducing production costs and a TCR library increasing patient population sizes may reduce these prices. The feasibility and accessibility of a TCR library will rely heavily on continuous joint efforts to identify, test and share novel TCRs, by academia and pharmaceutical companies.

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