TCR-engineered T cells in the era of CAR T cells: seeking the best of both worlds

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CHAPTER 6

Summary and general discussion

SUMMARY

Cellular therapy using chimeric antigen receptor (CAR-) engineered T cells can be effective in the treatment of refractory B-cell and plasma cell malignancies. Despite high initial response rates, a majority of patients will eventually experience relapse from CAR T-cell therapy. Part of relapses from CAR T-cell therapy can be ascribed to antigen escape, indicating that targeting of a single antigen may not always be sufficient to induce curative responses. On top of that, patients may experience antigen-positive relapse, indicating insufficient CAR T-cell performance to completely clear tumor cells. Both observations warrant that further improvements are needed, but also that there is room for alternative or complementary approaches. Alternatively to CAR engineering, T cells can be engineered to express a tumor-targeting T cell receptor (TCR). Opposed to CARs, TCRs recognize protein-derived peptides that are bound to human leukocyte antigen (HLA). While this HLA-restriction limits the patient pool that can be treated with a certain TCR, this also accesses a broad spectrum of potential antigens that can be targeted.

With the emergence of CAR T cells, the role of TCR-engineered T cells (eTCR T cells) needs to be reevaluated. The work presented within this thesis aims at putting CAR- and TCR- based strategies in context to each other, helping to define unique features of both approaches, and ultimately providing perspective on how to potentially improve current receptor-engineered cellular therapy approaches.

CAR and eTCR T cells are different in their mode of antigen recognition, but also how a signal is transduced into the T cell upon antigen binding. It remains unclear how eTCR T cells and CAR T cells perform in a side-by-side comparison when targeting tumor cells that can be targeted by either. In **chapter 2**, we compared the activity of TCR- and CAR-engineered T cells in targeting B-cell malignancies expressing the B-cell-associated antigen CD20. We generated T cells expressing different CD20-targeting CARs, and compared those to eTCR T cells recognizing a CD20-derived peptide in the context of HLA-A*02. While both reacted in an antigen-specific manner, we observed that the functionality of eTCR and CAR T cells was contextual. Generally, CD20-targeting CAR T cells produced higher amounts of IFNγ, and short term killing was more effective. Interestingly, eTCR and CAR T cells reacted differently towards different levels of target cell abundance. While CAR T cells proliferated vigorously after exposure to a small amount of target cells, CAR T-cell expansion correlated negatively with an increase in target cell exposure. In contrast, eTCR T expansion was rather moderate when targeting a low amount of target cells, but correlated positively with increasing amounts of target cells. At the highest level of target cell exposure, antigen-specific eTCR T-cell expansion was superior to that of CAR T cells. This observation paired with changes in the phenotypes of CAR and eTCR T cells. Increase in antigen exposure drove effector memory cell differentiation, upregulation of coinhibitory

molecules and sensitivity towards activation-induced cell death in CAR T cells. eTCR T cells on the other hand maintained a central memory phenotype, limitedly upregulated coinhibitory molecules and were more resistant towards activation-induced cell death. We conclude that target cell abundance differentially affects CAR and eTCR T-cell functionality, indicating that CAR and eTCR T-cell performance is contextual.

While CAR-mediated recognition is co-receptor independent, TCRs are dependent on coreceptors to function optimally. In **chapter 3**, we explored whether manipulations of the CD8 coreceptor could be exploited to functionally tune the performance of eTCR T cells. We designed and characterized variants of CD8 that displayed an increased affinity for HLA. We hypothesized that these CD8 variants might increase the therapeutic efficacy of eTCR CD4 and CD8 T cells. We characterized two CD8a mutants, S53G and S53N, that both displayed increased affinity for HLA class I in cell-free biophysical measurements. Using cellular model systems, we showed that overexpression of those variants indeed could increase the functional sensitivity of T cell lines towards cognate antigen without inducing non-specific recognition. Functional sensitivity was only increased when the respective TCR-peptide-HLA (pHLA) interaction was of low affinity, while affinity-enhanced CD8 variants did not affect intermediate or high affinity TCR-pHLA interactions. When introduced into primary CD8 T cells transduced to express tumor-targeting TCRs, overexpression of the CD8 variants led to an increase in antigen-specific cytokine secretion. However, the same was observed when overexpressing CD8 wildtype (WT) in CD8 T cells, indicating that overall increased availability of surface CD8 may be beneficial for eTCR T-cell function. We also introduced the CD8 variants into CD4 T cells transduced to express HLA class I restricted tumor-targeting TCRs. The tumor-targeting TCRs were dependent on CD8, indicated by a lack of reactivity of CD8-negative CD4 T cells towards endogenously processed and presented peptide on target cells. When additionally equipped with CD8, those eTCR CD4 T cells could react towards antigen expressing target cells. In this setting, the CD8-S53N mutant could provide an additional benefit in antigen-specific cytokine secretion without inducing unwanted reactivity. Manipulation of CD8 either by overexpression of CD8 WT or through the use of increased affinity variants may therefore prove useful for improving eTCR T-cell function.

One of the major reasons of relapse from CAR T-cell therapy is antigen escape. In **chapter 4**, we explored the potential complementary use of eTCR T cells and CAR T cells to prevent tumor escape. Our rationale was to harness both HLA-dependent and HLA-independent targeting to access a broader range of targetable antigens, thereby enabling an effective multi-antigen targeting strategy for the treatment of multiple myeloma. Initially, we compared the activity of BOB1-targeting eTCR and BCMA-targeting CAR T cells in lysing primary patient derived myeloma cells. Both eTCR and CAR T cells efficiently lysed primary myeloma cells. Following exposure to eTCR or CAR T cells, primary myeloma cells upregulated HLA,

while BCMA expression was downmodulated. Subsequently, we investigated whether a multiple myeloma cell line resistant to BCMA CAR-mediated target recognition would still be susceptible to TCR-mediated antigen recognition. eTCR T cells effectively cleared tumor cells lacking expressing of BCMA, both *in vitro* as well as in a murine xenograft model *in vivo*. Similarly, we examined whether myeloma cells that are resistant to TCR-mediated target recognition would remain susceptible to CAR-mediated targeting. To explore this, we generated myeloma cells with impaired HLA class I surface expression. These cells were efficiently cleared by BCMA-targeting CAR T cells both *in vitro* and *in vivo*. Finally, combining BCMA-targeting CAR T cells with BOB1 eTCR T cells demonstrated efficient eradication of established heterogenous myeloma in the bone marrow of immunocompromised mice, whereas single-antigen targeting led to progressive tumor growth as a consequence of antigen escape. Combination of HLA-dependent targeting by CAR T cells with HLA-dependent targeting of eTCR T cells therefore presents an interesting to strategy to enable multi-antigen targeting of heterogenous tumors to prevent antigen escape.

Following up on this strategy, we explored in **chapter 5** whether the concept of dual-antigen targeting utilizing a transgenic TCR alongside a CAR could be extended to the development of a unified, single product. From a manufacturing standpoint, the generation of a single product is potentially advantageous as it aligns with existing manufacturing approaches when expressed from a single-expression vector. We cloned a single expression vector encoding for both a BCMA-targeting CAR alongside the BOB1-targeting TCR. These cells, referred to as TRaCR (TCR and CAR) cells, underwent testing for their antigen-targeting capabilities, both when encountering either the CAR antigen or the TCR antigen alone as well as in settings of mixed antigen exposure. TRaCR T cells demonstrated antigen-recognition of tumor cells expressing either the CAR antigen, the TCR antigen, or both antigens, therefore highlighting general dual-antigen specificity. Target cell recognition though the TCR was however less effective as compared to T cells expressing the BOB1 TCR only, likely as a consequence of lower expression levels of the TCR in the single-expression vector design. Furthermore, we observed that CAR-mediated target recognition was dominant when TRaCR T cells were exposed simultaneously to target cells expressing either the CAR or the TCR antigen alone. This resulted in TRaCR T cells preferentially lysing target cells expressing the CAR antigen, whereas target cells only expressing the TCR antigen experienced a survival benefit in the presence of CAR-antigen expressing target cells. We hypothesized that this phenomenon could be explained by reduced expression levels of the TCR in the single-expression vector TRaCR format. We therefore generated TRaCR T cells following an alternative protocol where CD8 T cells were first transduced to express the BOB1 TCR, followed by restimulation and transduction with the aBCMA CAR, thereby ensuring comparable eTCR expression between BOB1 eTCR T cells and TRaCR T cells. TRaCR T cells generated this way displayed improved expression of the BOB1 eTCR. However, TRaCR T cells still displayed preferential target cell recognition through the

aBCMA CAR. Furthermore, preferential target recognition via the CAR could be replicated in TRaCR designs utilizing other TCRs, including a clinically validated TCR specific for NY-ESO-1 and a virus-specific TCR targeting a cytomegalovirus-derived epitope. Using dual-luminescence imaging in an *in vivo* model of heterogenous multiple myeloma, we validated that TRaCR T cells preferentially lysed BCMA-expressing target cells *in vivo*, suggesting translational relevance of this phenomenon. In conclusion, while TRaCR T cells display general dual-antigen specificity, a combination of separate products with single-antigen specificities might prove more useful for multi-antigen targeting strategies.

GENERAL DISCUSSION

Chimeric antigen receptor (CAR-) engineered T-cell therapy has demonstrated great potential in the treatment of certain malignancies. While CAR T cells can induce deep and sustained responses in refractory patients, the fraction of truly curative responses remains however limited, leaving room for alternative or complementary approaches. Conceptually older, TCR-engineering can similarly redirect T cells towards cancer cells. With the emergence of CAR T cells, it is crucial to reassess the role of eTCR T cells. The work presented in this thesis aims to put TCR therapy into context within the framework of CAR T-cell therapy. Based on the findings in this thesis, we identify multiple anchor points for complementarity rather than competition of both approaches. The experience gained from CAR T cells can guide improvements in TCR-based strategies, and *vice versa*, improvements in CAR T-cell designs can be achieved by aligning them more closer with normal T-cell physiology. We further develop a rationale that both modalities should be utilized together, as each contributes to generating a product that is not only diverse in the antigens it can target, but also diverse in functionality.

Comparing contextual performance can provide a rationale for designing better T-cell products

In **chapter 2**, the sensitivity of CAR T cells to overactivation became evident through a direct comparison with eTCR T cells and highlights a potential window of improvement by making CAR T cells more resilient towards overactivation, or to “make CARs more TCR-like”. This is congruent with several examples of preclinical CAR designs shifting more towards normalization of CAR signaling, such as the expression of a CAR through the endogenous TCR constant alpha (TRAC) locus via CRISPR/Cas9 mediated homology directed repair [1]. CARs expressed that way showed a dynamic and reduced expression that followed the pattern of natural TCR expression directly regulated by activation. Activation of TRAC-CAR T cells through the CAR led to receptor-internalization and temporary downmodulation of the activity of the TRAC locus, allowing for the establishment of a refractory period where CAR T cells could not be triggered again, before reestablishing CAR expression to a default

levels. This mode of expression also reduced tonic signaling, and protected CAR T cells from overactivation and rapid 'exhaustion', indicating that restriction of activation may be beneficial for CAR T-cell performance. Similarly, temporary signaling cessation through pharmacological inhibition of T-cell signaling could improve CAR T-cell function [2]. Treatment of CAR T cells with the kinase inhibitor dasatinib during manufacturing ameliorated the effects of tonic signaling, while a single time injection of dasatinib into tumor-bearing mice improved the efficacy of CAR T-cell therapy via the establishment of a rest period of CAR T-cell activation *in vivo* [2]. Nevertheless, even dynamically activated CAR T cells remain sensitive towards (initial) overactivation. Additional improvements of CAR performance can be achieved through more subtle changes in the signaling domains of CARs, including modifications of the CD3 as well as costimulatory domains. Commonly, clinically applied CARs use CD3ζ, which intuitively seemed the best choice as CD3ζ contains 3 activating ITAM motifs, whereas other CD3 motifs only contain 1 ITAM motif. Interestingly, more ITAMs does not necessarily equal better performance. Instead, CAR T-cell performance could be tuned through inactivation of individual ITAM motifs within the CD3ζ domain, resulting in CAR T cells with improved population maintenance after antigen encounter [3]. Similarly, a recent publication demonstrated that incorporating CD3δ instead of CD3ζ can improve CAR T-cell performance [4]. CD3δ only contains one ITAM motif, but on top of that also includes an inhibitory domain that can recruit the inhibitory phosphatase SHP-1. This inhibitory domain presumably protected CD3δ-CAR T cells from excessive stimulation, resulting in improved *in vivo* performance.

Conversely, eTCR T-cell performance may be improved through incorporation of concepts of the CAR field such as T-cell autonomous costimulation. In **chapter 2**, eTCR T cells produced rather moderate effector functions when encountering target cells with a low stimulatory capacity. The target cells that were used for those experiments, acute lymphoblastic leukemia (ALL) cells, are usually inefficient antigen-presenting cells (APC) characterized by low expression of adhesion molecules and costimulatory ligands, although some APC features may be inducible [5]. CAR T cells on the other hand, providing their own costimulatory signaling, elicited strong effector functions despite the poor stimulatory phenotype of the target cells. For eTCR T cells, it could therefore be beneficial to engage costimulatory pathways to improve the response towards poorly stimulating target cells. One way to engage costimulatory signals on T cells would be through artificial costimulatory receptors that bind a tumor associated antigen (e.g. CD19) with an extracellular domain while transducing a costimulatory signal through the intracellular portion of common costimulatory receptors such as CD28 or 4-1BB [6]. This type chimeric costimulatory molecule resembles a CAR, but does not induce TCR-like T-cell activation due to the absence of CD3 signaling domains. Similarly, chimeric switch receptors that bind an inhibitory ligand such as PD-L1 or TIGIT can be used to translate an inhibitory stimulus into a costimulatory signal [7-10]. In both cases, selection of a suitable TAA or coinhibitory ligand will be necessary for

different tumor types that will likely require further optimization. Alternative ways to engage costimulatory signals could be through engineering of T-cell autonomous costimulation, whereby costimulatory signals are provided independently of tumor factors. One way to achieve that would be through expression of the costimulatory ligands on the engineered T cells themselves, providing trans-costimulation to bystander T cells or even auto-costimulation [11-13]. Alternatively, costimulatory motifs can be introduced into endogenous CD3ζ, thereby transducing costimulatory signals following TCR-mediated activation [14]. That way, costimulatory pathways could be engaged regardless whether a costimulatory molecule is expressed on target cells or not, potentially improving effector functions, proliferation and persistence of eTCR T cells in sub-optimally stimulating environments.

TCR-mediated antigen recognition is generally perceived as more sensitive than CAR-mediated antigen recognition, whereby sensitivity is defined as the amount of stimulatory molecules that are needed to evoke T-cell activation [15-17]. When targeting ALL-RL that expressed low levels of CD20 in **chapter 2**, CD20-directed CAR T cells displayed stronger effector functions and better long term tumor clearance as compared to CD20 eTCR T cells. While this may seemingly contradict the notion that TCR-mediated antigen recognition is generally more sensitive, one has to consider different levels of abundance of a full length surface protein versus a protein-derived peptide complexed in HLA. It is likely that the number of CD20 molecules is magnitudes higher than compared to $CD20_{SLF}$-peptide presented in HLA-A*02. The amount of stimulatory CD20-pHLA present on ALL-RL may therefore be suboptimal for TCR-mediated recognition, whereas the amount of full-length CD20 may still be sufficient for CAR activation. This observation highlights that sensitivity as a response function to antigen density should only be compared within the same entity, and not between e.g. a full-length protein and protein-derived pHLA. The question of differential sensitivity of eTCR or CAR T cells becomes then more relevant when targeting the exact same epitope as is the case for TCR-like-CARs, i.e. CARs that are directed to pHLA complex rather than a full-length membrane protein [15,18].

How the functional differences that we found during *in vitro* cultures in **chapter 2** would clinically translate remains to be elucidated but allow for some speculation. Regarding toxicity, in the ZUMA-1 trial, a total of 93% of patients treated with axicabtagene ciloleucel (Yescarta) experienced cytokine release syndrome (CRS), with 13% of all patients experiencing high grade (>= 3) CRS [19]. In **chapter 2**, CD20-directed CAR T cells secreted high amounts of IFNγ, even when stimulated by low amounts of target cells with a low antigen expression level. Notably, we used CAR designs for both the CD20-directed CAR as well as the CD19-directed control CAR that followed the design of Yescarta, i.e. a retrovirally expressed CAR design incorporating the costimulatory domain CD28. It is likely that the overall cytokine secretion by CAR T cells generally is much higher as compared to eTCR T cells, although we did not assess secretion of other pro-inflammatory cytokines such

as IL-2, GM-CSF or TNFα. While the main driver of clinical CRS is IL-6, a cytokine mostly derived from macrophages and monocytes rather than T cells, secretion of proinflammatory cytokines by CAR T cells is considered a critical event in the pathophysiology of CRS [20]. In the *in vitro* studies in **chapter 2**, the long term clearance of $CD20^{high}$ ALL-CM was actually comparable between eTCR T cells and CAR T cells despite lower IFNγ secretion by eTCR T cells, indicating that high cytokine secretion might not necessarily be linked to better long-term tumor clearance. Therefore, reduction of cytokine production by CAR T cells could potentially lead to comparable clinical responses while limiting toxicity due to CRS. Preclinically, neutralization of IFNγ has been demonstrated not to compromise CAR T-cell efficacy[21]. Clinically, this notion is at least partially supported by the findings of a trial utilizing a CD19 directed CAR based on a humanized antigen recognition domain and different hinge and transmembrane domains [22]. During this academia led trial, patients achieved response rates comparable to those treated with a commercial product. The incidence of CRS however was significantly lower, correlating with lower cytokine secretion *in vitro* and lower levels of IFNγ and other cytokines in the serum of treated patients. Nevertheless, eTCR T cells remain to be tested clinically for the treatment of B-cell malignancies before definitive statements on the correlation of *in vitro* cytokine release and *in vivo* efficacy of eTCR T cells and incidence of CRS can be made.

Our findings in **chapter 2** showed that high tumor cell exposure may drive CAR T-cell dysfunction characterized by impaired CAR T-cell expansion. This is line with the observation that high disease burden is a negative predictor of response to CAR T-cell therapy in patients with lymphoma or leukemia [23-26]. In turn, *in vivo* CAR T-cell expansion is an indicator of response [27]. However, the clinical evidence for a direct negative correlation between tumor burden and expansion of CAR T cells is less clear. In one report the authors noted that disease burden was not a predictor for impaired CAR T-cell expansion, although disease burden was associated with worse response rates as in line with other reports [28]. It hast to be noted though that the authors used a binary cutoff for high tumor burden (>40% blasts in bone marrow) that might occlude subtleties in the dose-response function of tumor burden and CAR T-cell expansion. Another study actually reported a positive correlation of tumor burden with CAR T-cell expansion in ALL patients [29]. Nevertheless, the use of binary cutoff, in this case >5% blasts in bone marrow, again may result in an oversimplification. A more nuanced view on tumor burden, CAR T-cell expansion and response rates is presented by Locke et. al in CAR T-cell-treated patients with large B cell lymphoma. Here, CAR T-cell expansion first correlated positively with tumor burden, whereas patients with the highest tumor burden actually displayed impaired CAR T-cell expansion [25,30]. To more accurately explain response to CAR T-cell therapy as a function of tumor burden and CAR T-cell expansion, multiple authors turn to an '*in vivo*' E:T ratio instead, i.e. the ratio of CAR T cells at peak expansion normalized to baseline tumor burden as measured by metabolic volume or blast counts [25,26,30]. This view would be compatible with our finding in **chapter 2,** where

CAR T-cell expansion first correlates positively with antigen exposure before eventually declining upon stimulation with a larger excess of target cells. For eTCR T cells, there is insufficient clinical data available to correlate tumor burden with response and eTCR T-cell expansion. Our data is suggestive though that eTCR T cell could potentially expand better in settings of high tumor burdens than do CAR T cells, which could make eTCR an interesting addition to the cellular therapy portfolio for the treatment of B cell malignancies.

Combination therapies

In **chapter 4**, we advocated to combine CAR T-cell therapy with eTCR T-cell therapy for multi-antigen targeting strategies. Antigen escape remains one of the major reasons of relapse from receptor-engineered cellular therapy, and combinatorial targeting of multiple antigens will likely reduce the occurrence of antigen escape [31-41]. The rationale of combining both eTCR and CAR strategies revolved around their different modes of antigen recognition. CAR T-cell recognition is HLA-independent, whereas eTCR T-cell-mediated recognition is dependent on antigen processing and presentation within HLA. The mode of antigen recognition affects the type of antigens that can be targeted, but also the way how tumor cells may evade CAR- or eTCR-mediated targeting. CAR T cells typically bind surface proteins, and loss or reduction of surface expression of the respective antigen can therefore result in evasion from CAR T-cell therapy [37]. Furthermore, soluble antigen can bind to CAR T cells and thereby reduce CAR T-cell sensitivity [42]. On the other hand, eTCR T cells recognize protein derived peptides in the context of HLA. Tumor cells can similarly evade TCR-mediated antigen recognition through loss of expression of the respective target antigen [43], but also through defects in antigen processing and presentation. This may include loss or mutation of the respectively targeted HLA-molecule, but can extend to more general features of antigen presentation such as loss of beta-2-microglobulin (B2M) [44]. The benefit of combining CAR and eTCR mediated targeting would therefore be two-sided: TCRs can access antigens that are not amenable for CAR-mediated targeting, while conversely CARs can target tumor cells independently of defects in the general antigen processing and presentation machinery.

The type of antigens that can be targeted by CARs or TCRs are different, and the likelihood of antigen escape will vary between individual antigens. Targeting antigens that are relevant for tumor cell biology will less likely display complete loss of expression, whereas loss of antigens that are not immediately relevant for tumor cell biology will be more frequently observed. For example, CD19 is commonly targeted using CAR T cells for the treatment of several B cell malignancies [45]. While CD19 is a coreceptor of B cell receptor signaling and important for B-cell differentiation, CD19 is not essential for B-cell survival and also not considered to be directly involved in the etiology of most B cell malignancies [46]. Complete loss of CD19 surface expression could therefore be more probable, and, in line with that, up to 25% of patients treated with CD19-targeting CAR T cells relapse with complete loss of CD19

surface expression on malignant cells [37]. In contrast, BCMA is thought to be essential for the long term survival of bone marrow resident plasma cells [47], and has also been suggested to be involved in myeloma biology [48,49]. A role in myeloma biology would overlap with the observation that true escape variants displaying complete lack of BCMA surface expression are relatively rare events after BCMA targeting CAR T-cell therapy. Instead, BCMA is more often down-modulated rather than completely lost [32,50-56]. Moreover, point mutations in the CAR binding site of BCMA have been observed, thereby potentially preserving BCMA surface expression and function while escaping CAR-mediated recognition [31]. Nevertheless, BCMA appears not to be essential for survival in multiple myeloma cell lines. In **chapter 4**, BCMA knockout did not affect the growth of myeloma cell lines both *in vitro* as well as *in vivo*, suggesting that BCMA may become redundant for myeloma survival and proliferation at some point during myeloma pathogenesis. While there are CAR T-cell therapies available targeting other antigens, such as GPRC5D or SLAMF7 for myeloma, there are no CAR T-cell therapies that target truly essential myeloma antigens. In contrast, eTCR T cells can target peptides derived from intracellular proteins that may be essential for the survival of malignant cells, a feature that could make antigen escape less likely. In **chapter 4**, we used a TCR targeting a peptide derived from BOB1. BOB1, a transcriptional coactivator, is considered an essential protein for the survival of multiple myeloma, making BOB1 an attractive target [57-60]. This is confirmed by unpublished data not presented in this thesis showing that knockout of *POU2AF1*, the gene encoding BOB1, results in impaired survival in several multiple myeloma cell lines, including U266 and UM9 that were unaffected by loss of BCMA expression. Nevertheless, tumor cells that display general defects in antigen presentation, such as loss of B2M, can still evade eTCR T-cell therapy even if a truly essential antigen is targeted. In multiple myeloma, defects in the antigen presentation machinery have been described and are associated with disease progression from monoclonal gammopathy of undetermined significance to multiple myeloma [61]. As CAR-mediated target recognition is independent of antigen processing and presentation, potentially immune escaped tumor cells may still be targeted by CAR T-cell therapy. In conclusion, we argue that eTCR T cells and CAR T cells might be complementary to each other in their targeted antigens and recognition modes that together could reduce antigen escape of multiple myeloma after cellular therapy.

Another consideration is whether eTCR and CAR T cells would also exhibit functional complementarity when used together. In **chapter 2**, we highlighted functional differences between eTCR and CAR T cells, demonstrating contextually different performance in terms of effector functions and T-cell expansion. Furthermore, in **chapter 4**, we observed that BCMA CAR T cells induced upregulation of HLA class I expression on primary myeloma cells derived from patient bone marrow aspirates. Conceivably, CAR T-cell activation might support the establishment of a proinflammatory milieu that could be beneficial for eTCR function. This could occur either through direct effects on tumor cells via the induction of antigen presentation pathways, or indirectly on the microenvironment. Furthermore,

CAR T-cell-derived cytokines such as IL-2 could directly support eTCR T-cell function. Additionally, when considering that CAR T-cell products usually consist of both CD8 as well as CD4 T cells, CD4 CAR T cells potentially could exert a help function for eTCR CD8 T cells, although CD4 T cell help for eTCR T cells could also be provided by the introduction of both TCR as well as CD8 into CD4 T cells as described in **chapter 3**. Conversely, incorporation of eTCR T cells might increase the therapeutic breadth of a combined cellular therapy product by sustaining effector functions and T-cell expansion in settings of high antigen exposure. A combination regimen consisting of eTCR and CAR T cells could therefore not only be attractive from an antigen-targeting perspective, but also functionally.

Dual-specificity of T cells

In **chapter 5**, we explored whether T cells could be engineered to express a transgenic TCR alongside a CAR at the same time. We hypothesized that those T cells, termed TRaCR (TCR and CAR) T cells, could exhibit dual-antigen specificity. A single, dual-antigen targeting product could be interesting from a manufacturing point of view, as currently used protocols for commercial production of receptor-engineered T cells usually only allow for the manufacturing of a single product. Dual-specific T cells might therefore increase the accessibility to products that target more than one antigen. While we found that TRaCR T cells indeed could react to both the TCR antigen as well as the CAR antigen, we also observed that antigen recognition by the TCR was inferior in the TRaCR design when expressed from a single vector as compared to T cells only expressing the transgenic TCR. Furthermore, in the presence of both antigens, antigen recognition was preferentially conferred by the CAR, resulting in suboptimal clearance of tumor cells by TRaCR cells that only expressed the TCR antigen. Importantly, this could not be rescued by increased expression of the transgenic TCR. Moreover, TRaCR T cells appeared to be phenotypically more comparable to CAR T cells than to eTCR T cells in terms of expression of memory and coinhibitory markers. Translationally, especially the preferential antigen recognition phenotype could be relevant in the context of antigen escape.

Although the field of engineering T cells with dual- or even multi-antigen specificity is growing, many reports mostly focus on demonstrating general dual-antigen targeting capabilities, while aspects of preferential target recognition are often not or only indirectly assessed. Multiple designs have been described, from dual-antigen specific CAR T cell products, to dual-specific eTCR products, and to mixed eTCR-CAR T cells as presented within this thesis. For dual-antigen-targeting CAR T cells, most approaches follow either a dual-receptor design where two separate CAR constructs are expressed, called “dual CAR” design, or a pool of CAR T cells with separate specificities, or a so called ”tandem CAR” design, whereby the antigen recognition domains of two separate scFvs are fused together and linked to shared T-cell activation domains (figure 1) [33,34,36,39,41,62,63]. Dual CARs can be generated from one expression vector or from two vectors encoding the two CARs

separately. In **chapter 5**, we generated TRaCR T cells both from a single expression vector as well as using two separate vectors.

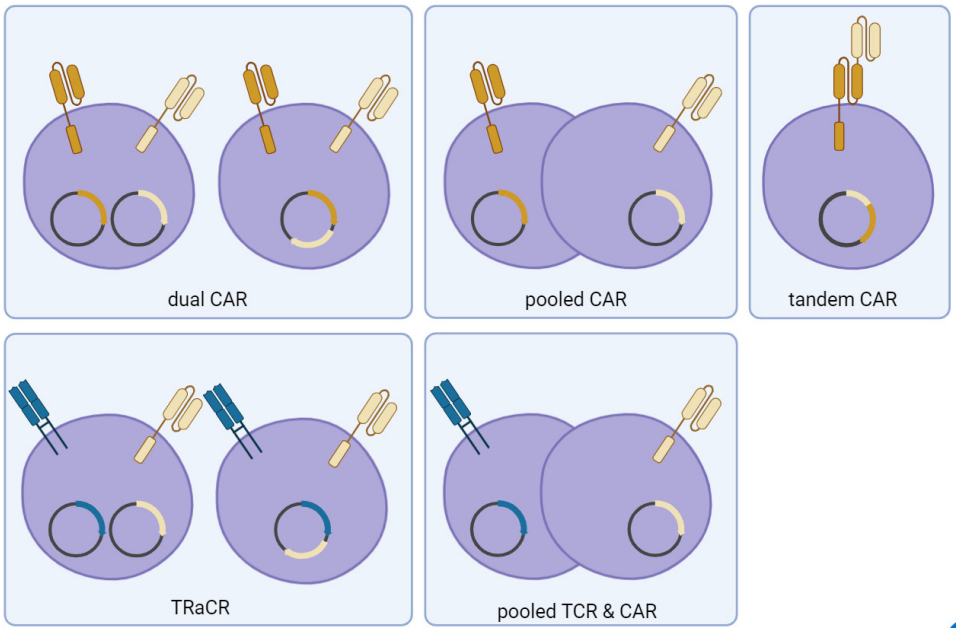

Figure 1 Different dual-specific T-cell product designs using combinations of two CARs or a combination of a TCR and a CAR

For the treatment of B cell malignancies, several dual-antigen targeting CAR based strategies have already been tested clinically. Most strategies relied on simultaneous targeting of CD19 and CD22, although other combinations such as CD19 and CD20 have also been explored for the treatment of B cell malignancies [64,65]. Clinical success varied considerably between studies, and antigen escape as mechanism of relapse was not always assessed. One phase I study testing tandem CARs that simultaneously targeted CD19 and CD22 did systemically evaluate expression of CD19 and CD22 before treatment and after relapse [62]. Interestingly, all patients that progressed due to antigen escape displayed a CD19-negative but CD22-positive relapse, both in ALL as well as in large B cell lymphoma patients. Furthermore, CD22 antigen density appeared not to be changed in relapsed tumor cells compared to pretreatment material. This observation suggests that the product induced sufficient selection pressure versus CD19, whereas selection pressure was insufficient versus CD22. This is in contrast with the experience using CD22 single-targeting CAR T cells, where CD22 antigen density was significantly reduced at the time of relapse [66]. In another phase II study, CD19 and CD22 directed CAR T cells were co-administered (pooled CAR) instead of

using dual-specific CAR T cells for the treatment of 225 ALL patients [67]. Similarly, out of 43 relapsing patients, 17 displayed loss of CD19 expression, whereas only one patient displayed loss of CD22 expression. On the one hand, this observation suggests that targeting CD19 might generally inflict higher selection pressure than does targeting CD22, irrespective of the design of the dual-antigen-targeting approach. On the other hand, relapse from CD22-targeting CAR T-cell therapy is generally associated with a decrease in expression rather than complete loss of CD22 expression [68,69]. As changes in CD22 expression level were not systematically assessed in this phase II study by Wang et. al, it remains difficult to conclude from the available clinical data whether a single dual-specific product or a combination of mono-specific products would be better suited in preventing antigen escape.

For the treatment of multiple myeloma, several CAR-based multi-antigen targeting strategies are being developed targeting different combinations of BCMA, CD38, CD19, SLAMF7 and/or GPRC5D. Clinical data are however limitedly available, and data on antigen escape as potential cause of relapse is equally limited [70]. One preclinical study assessed tandem CARs targeting BCMA and SLAMF7 (alternative name CS1) and assessed the phenotype of outgrowing tumor cells in an in vivo xenograft model of heterogenous myeloma [71]. BCMA-SLAMF7 dual-specific tandem CAR T cells prolonged survival of mice bearing heterogenous tumors compared to mice treated with single-antigen targeting CAR T cells. However, surviving tumor cells were mostly of a BCMA-negative and SLAMF7-positive phenotype, suggesting preferential target antigen recognition for BCMA. Whether the tandem CAR design would be inferior as compared to a combination of separately generated single-antigen targeting CARs was not assessed. Another study did address an analogous question comparing different configurations of CAR T cells specific for GPRC5D and BCMA [34]. They found that both a combination of GPRC5D and BCMA single-antigen-targeting (pooled CAR) as well as dual CARs were superior in an antigen escape rechallenge model in vivo as compared to tandem CARs. However, in a long term tumor-control model using low CAR T-cell doses against tumor cells that all expressed both GPRC5 and BCMA, the dual CAR T-cell design appeared superior as compared to pooled single-specific CAR T cells. The long term tumor control in an antigen escape model was however not assessed. Taken together, there is circumstantial evidence of preferential antigen recognition in different dual-specific CAR T cell designs that might be relevant clinically. The multiparametric nature of the question makes it however difficult to conclude superiority or inferiority of certain dual-specific CAR designs without systematic assessment.

Dual-antigen specific T cells based on TCR-mediated recognition are explored to a lesser extent than are dual-specific CAR T cells. There have been two reports exploring expression of both a transgenic TCR alongside a CAR by mRNA electroporation or in combination with lentiviral transduction [72,73]. In both reports, the authors demonstrated dual-antigen specificity of both introduced receptors, but deeper functional characterizations were limited, and

preferential target antigen recognition was not assessed. Strategies aiming to exploit dual-antigen specificity based on the expression of multiple TCRs have also been described and demonstrated antigen recognition through both receptors [74]. However, this approach comes with additional challenges. Competition between endogenous and introduced TCRs can lead to disbalanced expression between different TCRs, and the formation of mixed dimers can result in the development of novel unwanted reactivities [75,76].

While dual-antigen receptor engineered T cells could increase the accessibility to dual-antigen targeting strategies, the question remains whether this presents a suitable strategy in the future, even if challenges such as preferential target recognition as observed in **chapter 5** due to receptor dominance will be addressed. Multi-antigen-targeting strategies will likely not be limited to two antigens, and especially in heterogenous tumors such as multiple myeloma targeting of more than two antigens will be desirable. Furthermore, the incorporation of TCRs into those strategies warrant additional considerations. Depending on patient HLA, one would need to design different CAR-TCR combinations. Instead, realizing a multi-compound approach combining multiple single-specific T cells may be more desirable. That way, one could also realize a polyfunctional T-cell product leveraging diverse functional properties of different types of engineered immune cells.

CONCLUDING REMARKS

Receptor-edited cellular therapy of cancer has yet to fulfill its full potential. To increase the fraction of curative responses it will be crucial to increase the therapeutic breadth of cellular therapy products, both in the antigens that are targeted as well as in functionality. The ideal cellular therapeutic will target multiple antigens at a time, both HLA-dependently as well as independently of HLA. Functionally, cellular therapy needs to be efficient enough to induce sufficiently deep responses in diverse microenvironments, lesion sites and patients, while at the same time maintaining a manageable toxicity profile. The results presented within this thesis support the complementary use of eTCR T-cell therapy together with CAR T cells to realize a multi-targeting, multi-functional product. We found that eTCR T cells are functionally different from CAR T cells, performing differently in different contexts. Furthermore, HLA-dependent and HLA-independent targeting could be leveraged to enable a broader coverage of tumor antigens to prevent antigen escape. Multi-antigen-targeting, single T-cell products using TCRs and CARs can be utilized to achieve that, but pose additional caveats. Further advancements may include additional target antigens and may extend to the incorporation of other cell types, such as CD4 T cells, γδ-T cells or NK cells. Another layer of complexity can be added by recent advances in genetic and epigenetic T-cell engineering. Defining an optimal combination regimen will be a challenging task, and clinical testing of eTCR products and combination therapies is warranted.

REFERENCES

1 Eyquem, J. *et al.* Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543**, 113-117, doi:10.1038/nature21405 (2017).

2 Weber, E. W. *et al.* Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science* **372**, doi:10.1126/science.aba1786 (2021).

3 Feucht, J. *et al.* Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. *Nature Medicine* **25**, 82-88, doi:10.1038/s41591-018-0290-5 (2019).

4 Velasco Cárdenas, R. M. H. *et al.* Harnessing CD3 diversity to optimize CAR T cells. *Nature Immunology* **24**, 2135-2149, doi:10.1038/s41590-023-01658-z (2023).

5 Stevanović, S. *et al.* Human allo-reactive CD4+ T cells as strong mediators of anti-tumor immunity in NOD/scid mice engrafted with human acute lymphoblastic leukemia. *Leukemia* **26**, 312-322, doi:10.1038/leu.2011.222 (2012).

6 Omer, B. *et al.* A Costimulatory CAR Improves TCR-based Cancer Immunotherapy. *Cancer Immunol Res* **10**, 512-524, doi:10.1158/2326-6066.Cir-21-0307 (2022).

7 Liu, X. *et al.* A Chimeric Switch-Receptor Targeting PD1 Augments the Efficacy of Second-Generation CAR T Cells in Advanced Solid Tumors. *Cancer Res* **76**, 1578-1590, doi:10.1158/0008-5472.Can-15-2524 (2016).

8 Qin, L. *et al.* Co-expression of a PD-L1-specific chimeric switch receptor augments the efficacy and persistence of CAR T cells via the CD70-CD27 axis. *Nature Communications* **13**, 6051, doi:10.1038/s41467-022-33793-w (2022).

9 Hoogi, S. *et al.* A TIGIT-based chimeric co-stimulatory switch receptor improves T-cell anti-tumor function. **7**, 243, doi:10.1186/s40425-019-0721-y %J Journal for ImmunoTherapy of Cancer (2019).

10 Sailer, N. *et al.* T-Cells Expressing a Highly Potent PRAME-Specific T-Cell Receptor in Combination with a Chimeric PD1-41BB Co-Stimulatory Receptor Show a Favorable Preclinical Safety Profile and Strong Anti-Tumor Reactivity. *Cancers (Basel)* **14**, doi:10.3390/cancers14081998 (2022).

11 Stephan, M. T. *et al.* T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection. *Nat Med* **13**, 1440-1449, doi:10.1038/nm1676 (2007).

12 Dobrin, A. *et al.* Synthetic dual co-stimulation increases the potency of HIT and TCR-targeted cell therapies. *Nature cancer*, doi:10.1038/s43018-024-00744-x (2024).

13 Mansilla-Soto, J. *et al.* HLA-independent T cell receptors for targeting tumors with low antigen density. *Nature Medicine* **28**, 345-352, doi:10.1038/s41591-021-01621-1 (2022).

14 Lah, S. *et al.* Engineering second-generation TCR-T cells by site-specific integration of TRAF-binding motifs into the CD247 locus. **11**, e005519, doi:10.1136/jitc-2022-005519 %J Journal for ImmunoTherapy of Cancer (2023).

15 Harris, D. T. *et al.* Comparison of T Cell Activities Mediated by Human TCRs and CARs That Use the Same Recognition Domains. *Journal of immunology (Baltimore, Md. : 1950)* **200**, 1088-1100, doi:10.4049/jimmunol.1700236 (2018).

16 Salter, A. I. *et al.* Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Science Signaling* **11**, doi:10.1126/scisignal.aat6753

(2018).

17 Gudipati, V. *et al.* Inefficient CAR-proximal signaling blunts antigen sensitivity. *Nat Immunol* **21**, 848-856, doi:10.1038/s41590-020-0719-0 (2020).

18 Poorebrahim, M. *et al.* TCR-like CARs and TCR-CARs targeting neoepitopes: an emerging potential. *Cancer Gene Therapy* **28**, 581-589, doi:10.1038/s41417-021-00307-7 (2021).

19 Neelapu, S. S. *et al.* Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med* **377**, 2531-2544, doi:10.1056/NEJMoa1707447 (2017).

20 Morris, E. C., Neelapu, S. S., Giavridis, T. & Sadelain, M. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. *Nat Rev Immunol* **22**, 85-96, doi:10.1038/s41577-021-00547-6 (2022).

21 Manni, S. *et al.* Neutralizing IFNγ improves safety without compromising efficacy of CAR-T cell therapy in B-cell malignancies. *Nature Communications* **14**, 3423, doi:10.1038/s41467-023-38723-y (2023).

22 Brudno, J. N. *et al.* Safety and feasibility of anti-CD19 CAR T cells with fully human binding domains in patients with B-cell lymphoma. *Nat Med* **26**, 270-280, doi:10.1038/s41591-019-0737-3 (2020).

23 Dean, E. A. *et al.* High metabolic tumor volume is associated with decreased efficacy of axicabtagene ciloleucel in large B-cell lymphoma. *Blood Advances* **4**, 3268-3276, doi:10.1182/bloodadvances.2020001900 %J Blood Advances (2020).

24 Jain, M. D. *et al.* Tumor interferon signaling and suppressive myeloid cells are associated with CAR T-cell failure in large B-cell lymphoma. *Blood* **137**, 2621-2633, doi:10.1182/blood.2020007445 %J Blood (2021).

25 Locke, F. L. *et al.* Tumor burden, inflammation, and product attributes determine outcomes of axicabtagene ciloleucel in large B-cell lymphoma. *Blood Advances* **4**, 4898-4911, doi:10.1182/bloodadvances.2020002394 %J Blood Advances (2020).

26 Park, J. H. *et al.* Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med* **378**, 449-459, doi:10.1056/NEJMoa1709919 (2018).

27 Cappell, K. M. & Kochenderfer, J. N. Long-term outcomes following CAR T cell therapy: what we know so far. *Nature Reviews Clinical Oncology* **20**, 359-371, doi:10.1038/s41571-023-00754-1 (2023).

28 Talleur, A. C. *et al.* Preferential expansion of CD8+ CD19-CAR T cells postinfusion and the role of disease burden on outcome in pediatric B-ALL. *Blood Advances* **6**, 5737-5749, doi:10.1182/bloodadvances.2021006293 %J Blood Advances (2022).

29 Turtle, C. J. *et al.* CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J Clin Invest* **126**, 2123-2138, doi:10.1172/JCI85309 (2016).

30 Jain, M. D. & Spiegel, J. Y. Imagining the cell therapist: Future CAR T cell monitoring and intervention strategies to improve patient outcomes. *EJHaem* **3**, 46-53, doi:10.1002/jha2.357 (2022).

31 Lee, H. *et al.* Mechanisms of antigen escape from BCMA- or GPRC5D-targeted immunotherapies in multiple myeloma. *Nat Med* **29**, 2295-2306, doi:10.1038/s41591-023-02491-5 (2023).

32 van de Donk, N., Themeli, M. & Usmani, S. Z. Determinants of response and mechanisms of resistance of CAR T-cell therapy in multiple myeloma. *Blood Cancer Discov* **2**, 302-318, doi:10.1158/2643-3230.BCD-20-0227 (2021).

33 van der Schans, J. J., van de Donk, N. W. C. J. & Mutis, T. Dual Targeting to Overcome Current Challenges in Multiple Myeloma CAR T-Cell Treatment. **10**, doi:10.3389/fonc.2020.01362 (2020).

34 Fernandez de Larrea, C. *et al.* Defining an Optimal Dual-Targeted CAR T-cell Therapy Approach Simultaneously Targeting BCMA and GPRC5D to Prevent BCMA Escape-Driven Relapse in Multiple Myeloma. *Blood Cancer Discov* **1**, 146-154, doi:10.1158/2643-3230.BCD-20-0020 (2020).

35 Zhang, Z. *et al.* Point mutation in CD19 facilitates immune escape of B cell lymphoma from CAR-T cell therapy. *J Immunother Cancer* **8**, doi:10.1136/jitc-2020-001150 (2020).

36 Qin, H. *et al.* Preclinical Development of Bivalent Chimeric Antigen Receptors Targeting Both CD19 and CD22. *Mol Ther Oncolytics* **11**, 127-137, doi:10.1016/j.omto.2018.10.006 (2018).

37 Majzner, R. G. & Mackall, C. L. Tumor Antigen Escape from CAR T-cell Therapy. *Cancer discovery* **8**, 1219-1226, doi:10.1158/2159-8290.Cd-18-0442 (2018).

38 Fry, T. J. *et al.* CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med* **24**, 20-28, doi:10.1038/nm.4441 (2018).

39 Schneider, D. *et al.* A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. *J Immunother Cancer* **5**, 42, doi:10.1186/s40425-017-0246-1 (2017).

40 Zah, E., Lin, M. Y., Silva-Benedict, A., Jensen, M. C. & Chen, Y. Y. T Cells Expressing CD19/CD20 Bispecific Chimeric Antigen Receptors Prevent Antigen Escape by Malignant B Cells. *Cancer Immunol Res* **4**, 498-508, doi:10.1158/2326-6066.CIR-15-0231 (2016).

41 Qin, H. Y., Haso, W., Nguyen, S. M. & Fry, T. J. Preclinical Development of Bispecific Chimeric Antigen Receptor Targeting Both CD19 and CD22. *Blood* **126**, 4427-4427 (2015).

42 Pont, M. J. *et al.* gamma-Secretase inhibition increases efficacy of BCMA-specific chimeric antigen receptor T cells in multiple myeloma. *Blood* **134**, 1585-1597, doi:10.1182/blood.2019000050 (2019).

43 Rapoport, A. P. *et al.* NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* **21**, 914-921, doi:10.1038/nm.3910 (2015).

44 Nagarsheth, N. B. *et al.* TCR-engineered T cells targeting E7 for patients with metastatic HPV-associated epithelial cancers. *Nat Med* **27**, 419-425, doi:10.1038/s41591-020-01225-1 (2021).

45 Mohty, M. *et al.* CD19 chimeric antigen receptor-T cells in B-cell leukemia and lymphoma: current status and perspectives. *Leukemia* **33**, 2767-2778, doi:10.1038/s41375-019-0615-5 (2019).

46 Wang, K., Wei, G. & Liu, D. CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Experimental Hematology & Oncology* **1**, 36, doi:10.1186/2162-3619-1-36 (2012).

47 O'Connor, B. P. *et al.* BCMA is essential for the survival of long-lived bone marrow plasma cells. *The Journal of experimental medicine* **199**, 91-98, doi:10.1084/jem.20031330 (2004).

48 Sanchez, E. *et al.* The Role of B-Cell Maturation Antigen in the Biology and Management of, and as a Potential Therapeutic Target in, Multiple Myeloma. *Targeted Oncology* **13**, 39-47, doi:10.1007/s11523-017-0538-x (2018).

49 Tai, Y. T. *et al.* APRIL and BCMA promote human multiple myeloma growth and immunosuppression in the bone marrow microenvironment. *Blood* **127**, 3225-3236, doi:10.1182/blood-2016-01-691162 (2016).

50 Brudno, J. N. *et al.* T Cells Genetically Modified to Express an Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor Cause Remissions of Poor-Prognosis Relapsed Multiple Myeloma. *J Clin Oncol* **36**, 2267-2280, doi:10.1200/JCO.2018.77.8084 (2018).

51 Samur, M. K. *et al.* Biallelic loss of BCMA as a resistance mechanism to CAR T cell therapy in a patient with multiple myeloma. *Nat Commun* **12**, 868, doi:10.1038/s41467-021-21177-5 (2021).

52 Teoh, P. J. & Chng, W. J. CAR T-cell therapy in multiple myeloma: more room for improvement. *Blood Cancer J* **11**, 84, doi:10.1038/s41408-021-00469-5 (2021).

53 Da Via, M. C. *et al.* Homozygous BCMA gene deletion in response to anti-BCMA CAR T cells in a patient with multiple myeloma. *Nat Med* **27**, 616-619, doi:10.1038/s41591-021-01245-5 (2021).

54 D'Agostino, M. & Raje, N. Anti-BCMA CAR T-cell therapy in multiple myeloma: can we do better? *Leukemia* **34**, 21-34, doi:10.1038/s41375-019-0669-4 (2020).

55 Raje, N. *et al.* Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma. *N Engl J Med* **380**, 1726-1737, doi:10.1056/NEJMoa1817226 (2019).

56 Cohen, A. D. *et al.* B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. *J Clin Invest* **129**, 2210-2221, doi:10.1172/JCI126397 (2019).

57 de Matos Simoes, R. *et al.* Genome-scale functional genomics identify genes preferentially essential for multiple myeloma cells compared to other neoplasias. *Nature cancer* **4**, 754-773, doi:10.1038/s43018-023-00550-x (2023).

58 De Matos Simoes, R. *et al.* POU2AF1 As a Master Regulator of Oncogenic Transcription Factor Networks in Myeloma. *Blood* **136**, 18-19, doi:10.1182/blood-2020-142580 %J Blood (2020).

59 Jahn, L. *et al.* TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. *Blood* **129**, 1284-1295, doi:10.1182/blood-2016-09-737536 (2017).

60 Meeuwsen, M. H. *et al.* A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy. *Mol Ther* **30**, 564-578, doi:10.1016/j.ymthe.2021.08.010 (2022).

61 Racanelli, V. *et al.* Alterations in the antigen processing-presenting machinery of transformed plasma cells are associated with reduced recognition by CD8+ T cells and characterize the progression of MGUS to multiple myeloma. *Blood* **115**, 1185-1193, doi:10.1182/blood-2009-06-228676 (2010).

62 Spiegel, J. Y. *et al.* CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory B cell malignancies: a phase 1 trial. *Nat Med* **27**, 1419-1431, doi:10.1038/s41591-021-01436-0 (2021).

63 Wang, N. *et al.* Efficacy and safety of CAR19/22 T-cell cocktail therapy in patients with refractory/relapsed B-cell malignancies. *Blood* **135**, 17-27, doi:10.1182/blood.2019000017 %J Blood (2020).

64 Larson, S. M. *et al.* CD19/CD20 Bispecific Chimeric Antigen Receptor (CAR) in Naive/Memory T Cells for the Treatment of Relapsed or Refractory Non-Hodgkin Lymphoma. *Cancer discovery* **13**, 580-597, doi:10.1158/2159-8290.CD-22-0964 %J Cancer Discovery (2023).

65 Shah, N. N. *et al.* Bispecific anti-CD20, anti-CD19 CAR T cells for relapsed B cell malignancies: a phase 1 dose escalation and expansion trial. *Nature Medicine* **26**, 1569-1575, doi:10.1038/s41591-020-1081-3 (2020).

66 Shah, N. N. *et al.* CD4/CD8 T-Cell Selection Affects Chimeric Antigen Receptor (CAR) T-Cell Potency and Toxicity: Updated Results From a Phase I Anti-CD22 CAR T-Cell Trial. *Journal of Clinical Oncology* **38**, 1938-1950, doi:10.1200/JCO.19.03279 (2020).

67 Wang, T. *et al.* Coadministration of CD19- and CD22-Directed Chimeric Antigen Receptor T-Cell Therapy in Childhood B-Cell Acute Lymphoblastic Leukemia: A Single-Arm, Multicenter, Phase II Trial. *Journal of Clinical Oncology* **41**, 1670-1683, doi:10.1200/JCO.22.01214 (2022).

68 Ramakrishna, S. *et al.* Modulation of Target Antigen Density Improves CAR T-cell Functionality and

Persistence. *Clinical Cancer Research* **25**, 5329-5341, doi:10.1158/1078-0432.CCR-18-3784 %J Clinical Cancer Research (2019).

69 Fry, T. J. *et al.* CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nature Medicine* **24**, 20-28, doi:10.1038/nm.4441 (2018).

70 Mei, H. *et al.* A bispecific CAR-T cell therapy targeting BCMA and CD38 in relapsed or refractory multiple myeloma. *Journal of Hematology & Oncology* **14**, 161, doi:10.1186/s13045-021-01170-7 (2021).

71 Zah, E. *et al.* Systematically optimized BCMA/CS1 bispecific CAR-T cells robustly control heterogeneous multiple myeloma. *Nat Commun* **11**, 2283, doi:10.1038/s41467-020-16160-5 (2020).

72 Simon, B., Harrer, D. C., Schuler-Thurner, B., Schuler, G. & Uslu, U. Arming T Cells with a gp100-Specific TCR and a CSPG4-Specific CAR Using Combined DNA- and RNA-Based Receptor Transfer. **11**, 696 (2019).

73 Uslu, U., Schuler, G., Dörrie, J. & Schaft, N. Combining a chimeric antigen receptor and a conventional T-cell receptor to generate T cells expressing two additional receptors (TETARs) for a multi-hit immunotherapy of melanoma. *Experimental dermatology* **25**, 872-879, doi:10.1111/exd.13095 (2016).

74 Gladow, M., Uckert, W. & Blankenstein, T. Dual T cell receptor T cells with two defined specificities mediate tumor suppression via both receptors. *European Journal of Immunology* **34**, 1882-1891, doi:https://doi.org/10.1002/eji.200425041 (2004).

75 van Loenen, M. M. *et al.* Mixed T cell receptor dimers harbor potentially harmful neoreactivity. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 10972-10977, doi:10.1073/pnas.1005802107 (2010).

76 Heemskerk, M. H. *et al.* Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. *Blood* **109**, 235-243, doi:10.1182/blood-2006-03-013318 (2007).