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Next-generation TCR-transgenic T cells for Immunotherapy

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Summary

Adoptive T cell therapy (ACT) provides the means to specifically restore immunity against infections or cancer. ACT with donor-derived physiological T cells has shown its safety and efficacy in multiple different clinical settings. However, the availability, accessibility, and quality of donor-derived T cells intended for ACT are drastically restricting the applicability of this therapy to a few, carefully selected patients.

The genetic introduction of a transgenic T cell receptor (TCR) can reprogram the specificity of a T cell. Therefore, TCR editing enables us to overcome the limitations of conventional ACT with donor-derived T cells since the desired TCR and the human leukocyte antigen (HLA)-matched T cell can be taken from different sources. So far, clinical trials with TCR-edited T cells showed promising results, yet therapy responses were only moderate and temporary.

In this thesis, we elaborate on the complexity of TCR editing and how it might contribute to the observed low clinical response rates. For that, we set out to systematically investigate the two most important aspects of TCR editing: First, the isolation and identification of optimal TCRs for clinical use. Second, the functional consequences introduced by the editing process itself and how it can be improved to produce better TCR-edited T cell products. Particularly the latter aspect was so far little acknowledged by the scientific community.

Here, we developed a platform for reliable isolation of paired TCR α- and β-chains facilitating the generation of a comprehensive TCR library consisting of 261 unique virus-specific TCRαβ sequences. Subsequently, we used this TCR library for the development of a cellular platform for functional testing and identification of suitable TCRs for clinical use, as well as for the systematic investigation of different TCR editing methods.

The current gold standard for investigation of TCR-intrinsic characteristics is transgenic re-expression in primary T cells. However, this is not only a very laborious and costly process but also faces a high degree of variability due to factors such as T cell activation status, phenotype, or donor origin. In contrast, usage of an immortalized cell line offers more standardized conditions in conjunction with a drastically decreased workload. Here, we

developed a Jurkat-based test system for high-throughput and reliable TCR characterization. Most importantly, TCR-intrinsic features such as antigen-HLA specificity and functional avidity determined with this cell line closely paralleled measurements in primary T cells, therefore enabling highly standardized and unbiased assessment of TCR functionality.

Furthermore, we investigated the method of TCR editing as a potential factor introducing bias in transgenic TCR function. By in-depth systematic investigation of conventional TCR editing in comparison to novel clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated orthotopic TCR replacement (OTR), we identified substantial functional differences originating from the different editing methods. First, we highlight the importance of full endogenous TCR α- and β-chain knock-out (KO) for the production of safe and functional T cell products. Second, we show that transgenic TCR placement under the endogenous promoter is feasible and results in more physiological TCR regulation upon antigenic stimulation. Third, we demonstrate that random transgene integration and variable copy numbers introduce functional variability, whereas, targeted TCR integration via OTR results in highly defined, homogenous T cell products. Fourth, by comparing OTR T cell products to clinically relevant low copy number conventionally produced T cells, we observed increased TCR surface expression and functional avidity after OTR. Fifth, we provide evidence that OTR T cell products – through a more homogenous TCR surface expression – are more predictable in terms of *in vivo* functionality.

In summary, we developed a test system that proved to be a powerful tool for high-throughput and reliable screening of TCRs intended for clinical use and – more general – for investigation of TCR function and biology. Furthermore, we demonstrate that TCR editing via OTR facilitates the production of highly defined, homogenous TCR-redirected T cell products with an enhanced safety profile, physiologic TCR regulation as well as increased and more predictable functionality in comparison to conventional editing. These novel tools and findings are of utmost importance for the production of next-generation TCR-transgenic T cells and will probably contribute to better and more prolonged therapy responses.

1 Introduction

1.1 Adoptive cell therapy with physiological T cells

1.1.1 Antigen-independent T cell therapy

The first **hematopoietic stem cell transplantation (HSCT)** in man was performed by Edward Donnall Thomas and colleagues in 1957[1] and marked the beginning of adoptive cellular therapies. The purpose of HSCT is to replace a patient's diseased hematopoietic system with a healthy one and was first successfully performed in mice in the year 1956[2]. The same report suggested that allogeneic HSCT transfers functional immunity that can eradicate the recipient's tumor cells[2] as demonstrated by the first reported patient to be cured of leukemia via HSCT in 1962[3]. In the following decades, a large number of clinical studies helped to understand the anti-tumor effect of allogeneic HSCT, which was later termed graft-versus-leukemia (GvL) or graft-versus-tumor (GvT) reaction. For instance, it was observed that immunosuppressive medication restrains the GvL effect[4], that the risk of leukemic relapse is higher when recipients received syngeneic instead of allogeneic grafts[5,6], and when grafts were depleted of T cells. It was also observed that post-transplant Cytomegalovirus (CMV) infections occur less frequently in patients who received HSCT from a virus-seropositive immunocompetent donor[7,8]. As the T cell compartment is generally expected to be most essential for CMV-control[9,10], this again indicates that T cell-mediated immunity (in this case directed against the virus) was transferred along with the stem cell transplant.

In order to harness the full potential of the GvL reaction, clinicians next started to actively infuse donor-derived immune cells independent of the stem cell graft. Led by Hans-Jochem Kolb in Munich, one of the first studies that performed such a **donor lymphocyte infusion (DLI)** in patients suffering from relapsed chronic myelogenous leukemia reported complete cytogenetic remission in all of the three patients[11]. Subsequently, studies with larger cohorts confirmed these results (76-79% complete remission) but also reported severe (often fatal) adverse events in 40-60% of patients[12,13]. This so-called graft-versus-host disease (GvHD) is caused by immunocompetent donor lymphocytes recognizing and damaging the recipient's tissue[14]

and usually coincides with GvL activity[12,13]. This illustrates a delicate interplay between GvL and GvHD after allogeneic HSCT and DLI since the mechanism by which donor T cells recognize and eliminate recipient-derived tumor cells (GvL) or healthy tissue (GvHD) is essentially the same. In both cases, T cells target immunologically foreign tissue in an antigen-independent manner. Against this background it became apparent that the transfer of tumor antigen-specific T cells would promise to maintain GvL activity without inducing GvHD.

1.1.2 Antigen-specific T cell therapy

The presence of **tumor-infiltrating lymphocytes (TIL)** is reported to be predictive for improved clinical outcomes in various types of cancer (first such observations by Rudolf Virchow in 1863[15] were later confirmed specifically for melanoma[16], ovarian carcinoma[17], and colorectal cancer[18]), indicating the presence of tumor antigen-specific T cells that could be harnessed for therapy. The first proof-of-concept for ACT with TILs, i.e. resection of tumor tissue, isolation of infiltrating T cells, *in vitro* expansion, and re-transfer into the patient, was provided by Rosenberg and colleagues in 1986[19] shortly followed by the first clinical application in 1988[20]. More recent clinical trials reported objective clinical responses in up to 56% of metastatic melanoma patients without any substantial adverse events caused by the T cell infusion[21–23]. While these data are highly promising and provide further evidence for the potential of ACT, TIL treatment faces substantial limitations. In many cases, particularly in other cancer entities than melanoma, difficult accessibility of the tumor site obstructs TIL isolation. In addition to that, tumor-reactive TIL populations can only be generated from about 50% of melanoma resections[24]. Moreover, only about 10% of infiltrating cells are tumor-reactive with some resections containing no tumor-specific T cells at all[25]. Finally, tumor antigen-specific TILs are often found to be dysfunctional. Continuous TCR triggering and immunosuppressive signals within the tumor microenvironment, as well as prolonged *in vitro* cultivation, push TILs into exhaustion and dysfunctionality[26–28].

Another field for application of ACT is the treatment of immunocompromised patients suffering from infections, especially after allogeneic HSCT. Myeloablative conditioning regimen and

subsequent immunosuppressive medication temporarily leave the patient highly susceptible to common viral infections. CMV, Epstein-Barr-Virus (EBV), or Adenovirus (AdV) are highly prevalent viruses and major contributors to morbidity and mortality after allogeneic HSCT[29,30]. Similar to TIL treatment, **ACT with pathogen-specific T cells** enables one to specifically restore antiviral immunity to combat post-transplant infections with a low risk of alloreactivity. This approach was pioneered in 1992 by Stanley R. Riddell, who isolated T cells from the HLA-matched virus-seropositive stem cell donor, performed a five to twelve week long ***in vitro* expansion** of CMV-specific $CD8^+$ T cells, and retransferred large numbers of those cells (up to $1x10^9$ cells/m^2 body surface area) into the HSCT patient for prophylaxis of CMV reactivation[31]. This study and another that shortly followed reported the absence of CMV viremia in all 17 treated patients and observed persistence of CMV-specific T cells until at least four[31] and twelve[32] weeks post transfer. ACT of post-transplant EBV reactivations was first performed by Rooney and colleagues in 1995 who furthermore implemented a genetic marker that facilitated long-term monitoring of the transferred T cell product[33]. Follow up studies showed that none of 101 high-risk patients developed EBV-related disease and that 11 of 13 patients with already established EBV lymphoma achieved complete remission. Most importantly, this coincided with the persistence of transferred T cells up to nine years after treatment[34,35]. Overall, these studies showed very high response rates without any toxicities related to the T cell transfer[31,32,35], therefore highlighting the great potential and safety of antigen-specific ACT. A major disadvantage of these approaches, however, are the long culture times as well as manufacturing costs and logistics that obstruct broader implementation as well as the pre-emptive application of this therapy[29,36]. Furthermore, long-term cultivation is also very likely to be unfavorable for the *in vivo* persistence and functionality of the T cell product as it drives T cell differentiation and exhaustion[27,28,37,38].

In the following years, these problems could be overcome by the application of new techniques for ***ex vivo* isolation** of sufficiently large virus-specific T cell populations from a virus-seropositive stem cell donor. This facilitated the direct transfer of the T cell product into the patient without the need for extensive *in vitro* propagation. In a pioneering study by Cobbold

and colleagues, CMV-specific $CD8^+$ T cells were isolated using peptide-major histocompatibility complex (pMHC)-multimers coupled to magnetic beads[39]. Despite relatively small numbers of transferred T cells ($1.2x10^3$ - $3.3x10^4$ cells per kg body weight), cells were able to expand and persisted at least until day 100 after transfer. No case of CMV disease was reported in a study group of nine patients. Despite these very promising results, there were concerns that this isolation technique might hamper the functionality of the T cell product through prolonged TCR-pMHC-multimer interactions[40,41]. Using reversible pMHC-multimers, so-called streptamers, allows dissolving the TCR-pMHC-multimer complex which resulted in enhanced proliferative and cytotoxic capacity in comparison to conventional multimers[42,43]. Functionality and *in vivo* persistence of streptamer-isolated T cells were later demonstrated in pre-clinical[38,43] and clinical models[44,45]. Yet, streptamer-guided isolation of antigen-specific T cells is limited to MHC class-I restricted $CD8^+$ T cells and some studies suggested that antigen-specific $CD4^+$ T cell help is beneficial for T cell graft persistence[32,46]. Hence, Feuchtinger and colleagues performed interferon gamma (IFNγ) capture for *ex vivo* isolation of either AdV, CMV, or EBV-specific $CD4^+$ and $CD8^+$ T cells[47–49]. While response rates achieved in these studies were overall high, there was also a slightly increased incidence of alloreactivity[49,50]. This might be explained by impure T cell grafts indicating that IFNγ capture is inferior to pMHC-multimer-guided purification methods in terms of cell product purity and thereby functional specificity. However, regardless of the isolation method, all of these approaches have one problem in common: For direct *ex vivo* isolation of virus-specific T cells, a sufficiently large virus-specific T cell population has to be present in the HLA-matched stem cell donor, which is often not the case[7,8,51].

One obvious solution for this problem is to get a **third-party donor** involved who is seropositive for the virus of interest. This approach also allows for generating comprehensive biobanks of virus-specific T cell lines available for off-the-shelf therapy. While this is clearly a large logistic effort, it would facilitate pre-emptive therapy due to the fast availability of ready-to-use T cell grafts. In clinical studies, third-party partially HLA-mismatched T cell grafts were protective and mostly safe but performed slightly worse than HLA-matched stem cell donor-derived T cells[52].

Interestingly, response rates correlated to the grade of HLA-matching[53,54]. A study by Neuenhahn and colleagues directly compared the clinical outcomes after the transfer of HLA-matched and HLA-mismatched virus-specific ACT[45]. Whereas they observed a 62.5% response rate in the HLA-matched setting, partially mismatched third-party donor-derived T cells remained undetectable in seven out of eight patients, indicating that partially HLA-mismatched T cells are frequently rejected.[45]

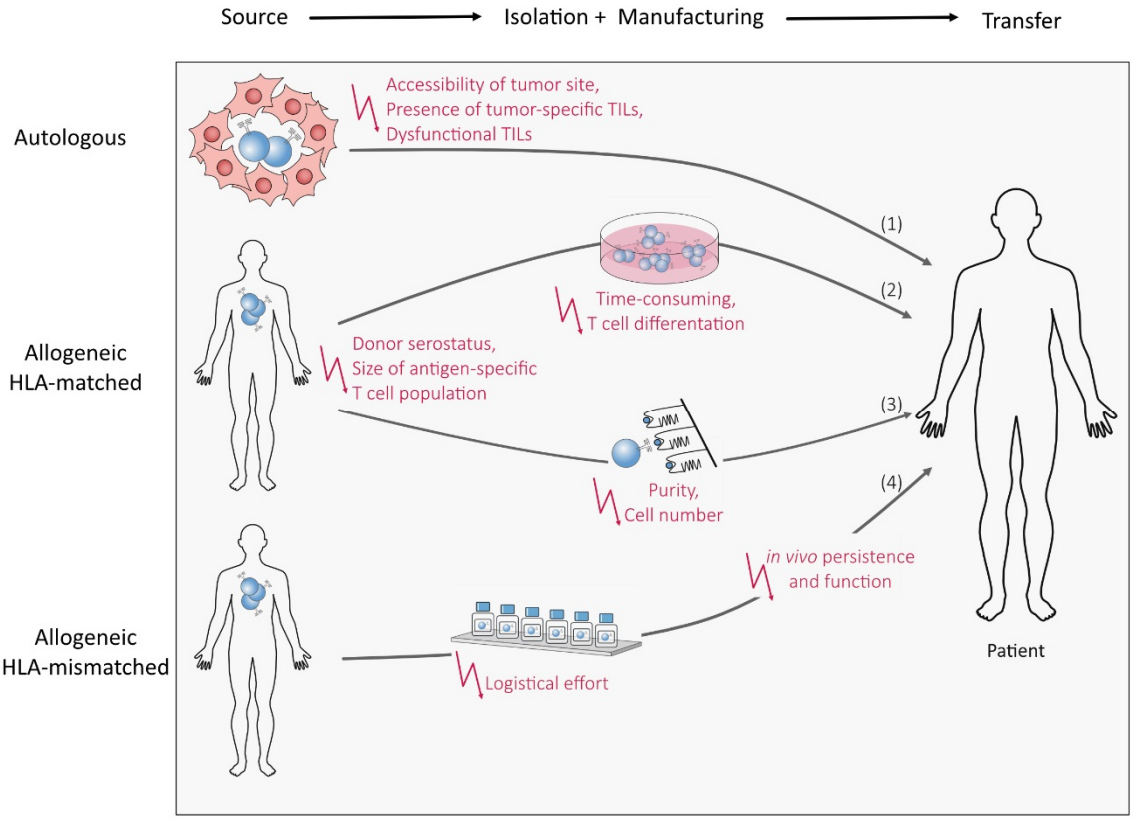

Figure 1: Limitations of antigen-specific donor-derived T cells intended for ACT. From left to right: antigen-specific T cell source, T cell isolation and manufacturing of T cell product, adoptive T cell transfer and *in vivo* function. Limitations of (1) TIL therapy, (2) HLA-matched *in vitro* cultivated pathogen-specific T cells, (3) HLA-matched directly *ex vivo* isolated pathogen-specific T cells, and (4) third party, partially HLA-mismatched, off-the-shelf pathogen-specific T cells are indicated by bolt-symbol and red color.

In summary, ACT with TILs or pathogen-specific T cells has proven to be a highly promising treatment strategy with a drastically increased safety profile in comparison to DLI. However, all of the above-described approaches are also restricted in many ways (see figure 1). Accessibility and availability of TILs (tumor site) and virus-specific T cells (serostatus of stem cell donor and size of the virus-specific population) restrict this treatment option to a relatively

low number of carefully selected patients. Also, banked T cell lines derived from third-party donors do not solve this problem since partial HLA-mismatching results in inferior *in vivo* graft persistence and functionality[45]. Furthermore, extensive cell culture of T cell products is not only hardly compatible with clinical routine but also results in T cell differentiation and exhaustion. Since far differentiated T cell stages show reduced proliferative capacity[37,38,55,56] and limited migration behaviour[57,58], it is today well-established that T cells with stem-cell-like properties would be ideal for ACT. Finally, *ex vivo* isolation and/or *in vitro* cultivation of physiological T cells results in heterogeneous cell products of unknown function, contrasting the high degree of definedness and predictability that is usually required for a medicinal product.

1.2 ACT with genetically engineered T cells

Limitations of ACT with physiological T cells could be overcome by genetic engineering of antigen-specific receptor-transgenic T cells since the desired receptor and the HLA-matched T cell can be taken from different sources. By combining optimal T cell phenotype, HLA haplotype, and receptor-intrinsic features such as antigen specificity and avidity, engineered T cells provide unique therapeutic opportunities for infectious diseases and cancer. Reprogramming of a T cell's specificity can be achieved either via the genetic introduction of a chimeric antigen receptor (CAR) or a TCR.

1.2.1 Clinical application of CAR-T cells

A CAR is a synthetic receptor that contains an antibody-derived single-chain variable fragment that is attached to an extracellular hinge region, a transmembrane domain, and an intracellular T cell signaling domain[59]. Thereby, a CAR is an autonomous functional unit providing both antigen-binding and T cell-activating function. The clinical application of CAR-engineered T cells is an exceptional success story. From the first reported clinical studies in 2010 and 2011 pioneered by the groups of Rosenberg[60] and June[61,62], it took only eight more years until the first US Food & Drug Administration (FDA) approval of CAR-T cells. Currently, the FDA

granted three approvals for the treatment of B cell malignancies with two different CAR constructs.

Tisagenlecleucel/Kymriah (marketed by Novartis) is the first-ever approved genetically modified cell product. These T cells are redirected with an anti-CD19 CAR comprising a 4-1BB co-stimulatory domain and their application is approved for the treatment of relapsed/refractory B-cell acute lymphoblastoid leukemia (B-ALL) in young patients below 25 years of age. In a phase 2 clinical study, the overall remission rate within three months was 81% with an overall survival rate of 76% twelve months after CAR-T cell transfer[63]. Tisagenlecleucel/Kymriah is also approved for the treatment of relapsed/refractory diffuse large B-cell lymphoma (DLBCL) in adults. Here, a clinical study reported complete remission in 43% of patients, and of those, 86% maintained the complete response[64].

The second FDA approved CAR-T cell product is Axicabtagene ciloleucel/Yescarta (marketed by Kite/Gilead). Here, the CAR is also directed against the B cell lineage marker CD19 but it contains a CD28 co-stimulatory domain. This gene-modified T cell product is approved for the treatment of relapsed/refractory DLBCL in adults and a phase 2 trial with 111 patients reported a complete response rate of 54% and an 18-month overall survival rate of 52%[65]. An overview of clinical studies with now FDA-approved CAR-T cell products is presented in table 1.

Table 1: Overview of FDA-approved CAR-T cell product function. CR = complete response, OS = overall survival (*12 months, **median of 28.6 months, ***18 months)

Pathology	Product	Antigen	Patient no.	CR	OS	Reference
B-ALL	Kymriah	CD19	75	81%	76%*	Maude et al. 2018
DLBCL	Kymriah	CD19	28	43%	37%**	Schuster et al. 2017
DLBCL	Yescarta	CD19	111	54%	52%***	Neelapu et al 2017

These results represent substantial progress in the treatment of B-cell malignancies and CAR-T cells already saved many lives. The large potency of these cell products is also reflected in the occurrence of severe adverse events, mostly cytokine release syndrome and neurotoxicity[66,67]. Overall, CAR-T cells currently represent the poster child of immunotherapy and might help to increase the acceptance of antigen-specific receptor gene-modified T cells

for clinical use. However, CARs are restricted to antigens expressed on the cell surface such as the homogenously high expressed B-cell lineage marker CD19. While other surface antigens for CAR treatment are currently being explored[68], CD19 might represent an exceptional target.

1.2.2 Clinical application of TCR-edited T cells

Naturally, T cells recognize infected or cancerous cells via the TCR and subsequently exert immunomodulatory and cytotoxic functions[69,70]. Pathogen-derived or tumor-specific peptides are intracellularly processed and presented on the cell surface in the context of MHC. Recombination of the TCR gene locus generates a mathematical receptor diversity of 10^{20} to 10^{60} and thereby enables T cells to identify an almost indefinite number of epitopes[71,72] with extremely high sensitivity[73], both being unique TCR characteristics in comparison with CARs.

The first clinical trial with TCR-edited T cells in the year 2006 encompassed the treatment of patients suffering from metastatic myeloma with autologous T cells that were retrovirally transduced to express a MART1-specific TCR[74]. Whereas this important pioneering work could find evidence for *in vivo* function of these T cells, overall response rates were low and only temporary. Similar results were obtained in a handful of other clinical studies treating metastatic melanoma, synovial cell carcinoma, or multiple myeloma (see table 2)[74–79].

Table 2: Overview of clinical studies using TCR-edited T cells for ACT. OR = objective response, CR = complete response, *originally reported as nCR = near complete response (no data of %CR available)

Pathology	Antigen	Patient no.	OR	CR	Reference
Metastatic melanoma	MART1	15	13%	0%	Morgan et al. 2006
Metastatic melanoma	MART1	20	30%	0%	Johnson et al. 2009
Metastatic melanoma	gp100	16	19%	6%	
Metastatic melanoma	NYESO-1	11	67%	18%	Robbins et al. 2011
Synovial cell carcinoma	NYESO-1	6	45%	0%	
Metastatic melanoma	MART1	14	69%	0%	Chodon et al. 2014
Multiple myeloma	NYESO-1	20	80%	70%*	Rapoport et al. 2015

Despite relatively low response rates, these studies provided general proof-of-concept and - with two exceptions – the safety of adoptively transferred TCR-edited T cells. One study had to be terminated after the first three treated patients developed transient severe inflammatory colitis which was assumed to be a result of on-target off-site reactivity of the transferred carcinoembryonic antigen-specific T cell graft[76]. Another study adoptively transferred T cells engineered to express an artificially affinity-enhanced MAGE-A3-specific TCR. Here, the first two treated patients rapidly developed a fatal cardiogenic shock that was most probably caused by cross-reactivity directed against the human protein titin that is expressed by actively beating cardiac myocytes[80].

In summary, these studies provided evidence for the promising potential of adoptively transferred TCR-redirected cells, yet overall therapy success was rather low, highly variable, and in most cases only temporary.

1.3 TCR engineering

It is tempting to directly compare the differential clinical successes of TCR-transgenic T cells and CAR-T cells, however, it is important to notice that in contrast to the homogenously expressed B cell lineage marker CD19, targets on solid tumors such as MART1, NYESO-1, or neo-epitopes are most probably more challenging (lower antigen expression levels, simultaneous expression on healthy tissue, immunosuppressive microenvironment of solid tumors). To find TCRs of optimal functionality directed against these targets is therefore of utmost importance but also highly complicated. Furthermore, the generation of TCR-redirected T cell products is highly demanding due to the TCR's complex genomic organization[81,82], its heterodimeric structure[83], and the interaction of the endogenous and the introduced transgenic receptor (see figure 2). It is well-established that interactions between the endogenous and the introduced transgenic receptor can result in T cell products with inferior functionality[84,85]. The transgenic TCR competes with the endogenous TCR for a limited amount of CD3 molecules, which decreases surface expression of both[86]. Furthermore, because of the TCR's

heterodimeric structure, individual chains of the transgenic and the endogenous receptor can form mispaired TCR variants, which again decreases surface expression of the desired transgenic TCR and also poses a safety hazard since mixed TCR dimers can exert off-target toxicity[87–90].

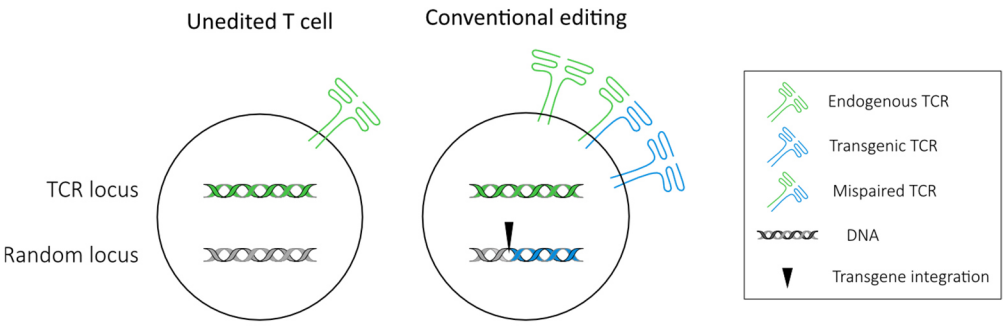

Figure 2: Complexity of TCR engineering. Transgenic TCR expression is complicated by competition for surface expression as well as potential mispairing with the endogenous TCR chains. For the sake of clarity, individual TCR α and β gene loci are not shown.

In summary, the more challenging target epitopes, suboptimal TCR quality as well as the increased complexity of TCR engineering might explain the differential clinical progress of transgenic TCRs and anti-CD19 CARs. In the following, particularly the latter two aspects – TCR quality and TCR engineering - will be discussed.

1.3.1 Identification of suitable TCRs

It is very well-established that a T cell's functional profile is to a large extent determined by its TCR. The strength of a TCR's interaction with the peptide-MHC complex directly affects T cell activation, expansion, as well as differentiation[91–93], and determines its overall protective capacity[94], thereby largely affecting a T cell's long-term fate[95].

Therefore, it is of utmost importance to carefully select optimal TCRs for clinical use. Yet, parameters that clearly define a TCR's therapeutic relevance are not established. A major reason for this is the fact that protocols for isolation and in-depth characterization of TCRs are laborious and costly. Very few studies have therefore investigated more than a handful of TCRs side-by-side[25,96], and many basic questions on TCR biology have not yet been addressed.

Several sources can be used for the isolation of TCRs. The antigen un-experienced, naïve repertoire represents a rich and unbiased source that was so far mostly used for the identification of tumor (neo-)antigen-specific TCRs[97,98]. Isolation of TCRs from naïve repertoires, however, is highly challenging due to low precursor frequencies[99]. Furthermore, since tumor-antigens are usually self-antigens, thymic selection results in a very low number of functional TCRs[100]. Hence, the identification of functional TCRs from naïve repertoires literally represents the search for the needle in a haystack.

By-passing the problem of negative selection of high avidity self-antigen-specific TCRs, the group of Thomas Blankenstein developed a mouse model for isolation of such TCRs. They introduced the whole human TCR gene repertoire and the human HLA-A*0201 into the murine system, which facilitates the generation of high avidity TCRs after immunization with any HLA-A*0201-restricted epitope[101]. Downsides of this approach are the restriction to HLA-A*0201 and that these TCRs are only trained in the context of this one MHC class-I molecule and in the absence of other human peptides. Therefore, the risk of allo- or noncognate-reactivity is supposedly higher[102].

In contrast to that, the antigen-experienced repertoire is generally assumed to be a safer source of functional TCRs. Isolation of TILs from patients represents a very promising option for the identification of functional tumor-specific TCRs with an increased safety profile[25]. A very intriguing source of virus-specific TCRs are patients that received pathogen-specific TCRs via ACT after allogeneic HSCT. In the case of virus reactivation and successful immune control, TCRs derived from this repertoire have proven their *in vivo* efficacy and should be ideal candidates for clinical use. Here, we developed a platform for reliable TCRαβ sequence identification from virus-seropositive healthy donors or ACT patients (see figure 3) and built up a comprehensive library, containing 261 unique TCRαβ sequences covering 13 different virus epitopes and six of the most common MHC-class I molecules (table 3).

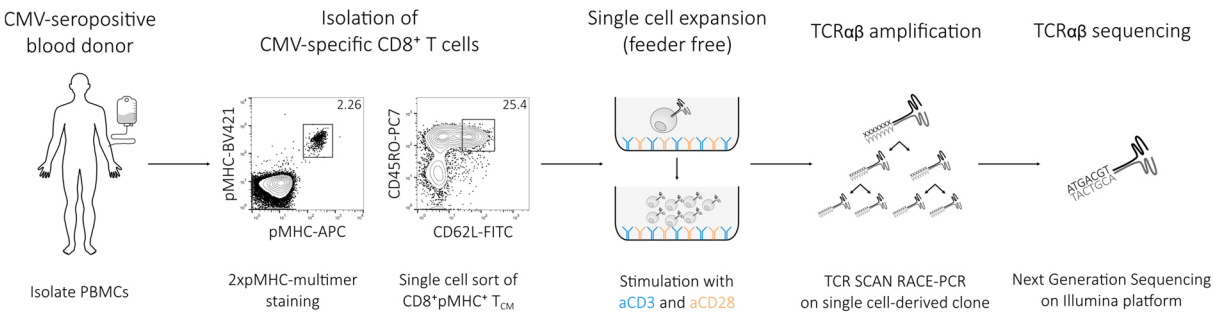

Figure 3: A platform for reliable TCRαβ sequence identification from virus-seropositive donors. A schematic illustration of the TCRαβ sequence identification workflow, from left to right: Isolation of PBMCs from CMV seropositive blood donors. Staining of PBMCs with double pMHC-multimer (same peptide-MHC monomer, but backbones labeled with different fluorochromes) and T cell memory markers (pre-gated on living $CD3^+CD8^+CD19^-$). Sort of 2xpMHC-multimer$^+$ $CD62L^+CD45RO^+$ single cells into individual wells of a CD3/CD28 antibody-coated culture plate. Performance of TCR SCAN RACE-PCR[103] on expanded single cell-derived clones for TCRαβ sequence amplification. Identification of TCRαβ via Next-Generation Sequencing.

Table 3: Overview of the number and specificity of TCRs isolated as part of this work. 'Specificity' as HLA/epitope, 'Full TCRs' indicates paired alpha-beta TCRs, 'Unique TCRs' represents count of unique TCR sequences (i.e. same TCR sequence is not counted twice)

Virus	Specificity	Peptide	Donors	Full TCRs	Unique TCRs
CMV	A1/pp50	VTEHDTLLY	5	127	32
CMV	A2/pp65	NLVPMVATV	17	417	120
CMV	A3/IE1$_{KLG}$	KLGGALQAK	1	41	1
CMV	A3/IE1$_{RIK}$	RIKEHMLKK	1	58	4
CMV	B7/pp65	TPRVTGGGAM	4	56	19
CMV	B8/IE1	QIKVRVDMV	2	60	22
CMV	B8/IE1$_{199K}$	ELKRKMIYM	2	12	4
EBV	A11/EBNA3b	AVFDRKSDAK	2	31	12
EBV	B7/EBNA3a	RPPIFIRRL	1	30	2
EBV	B8/BZLF1	RAKFKQLL	2	43	17
EBV	B8/EBNA3a	FLRGRAYGL	2	47	9
AdV	A1/Hexon5	LTDLGQNLLY	3	32	13
AdV	A3/DNApol	ALYGSFATK	1	8	6
		Total	**43**	**962**	**261**

Today, due to continuous improvements in the field of Next Generation Sequencing (NGS), high-throughput identification of full αβ TCR sequences is no longer a bottleneck[104–107].

Moreover, advanced bioinformatical analytical tools are being developed to gain deep insight into such large TCR repertoire data and – for instance – to predict antigen-HLA specificity from raw TCR sequence information[108,109]. However, a major hurdle that remains is the functional testing of TCR candidates. Earlier studies characterized TCRs by *in vitro* generation and investigation of T cell clones[110–112]. Importantly, TCR function is dependent on its cellular context, so that the phenotype of a T cell clone affects TCR functional avidity or even specificity, as previously demonstrated by investigation of TIL-derived TCRs[25]. Hence, transgenic re-expression of TCRs in a suitable cell line or primary T cells[113] is the most standardized approach to assess TCR functionality. However, TCR testing in primary T cells faces an increased degree of variability due to factors such as T cell activation status, phenotype, or donor origin and is also accompanied by high workloads as well as ethical aspects. Hence, the usage of immortalized T cell clones represents an attractive alternative.

The Jurkat leukemic T cell line is a widely used model system for the study of TCR function[114]. A previously developed triple parameter TCR signaling reporter cell line (TPR) based on the Jurkat line E6.1[115] has proven to be highly suitable for the evaluation of co-stimulatory pathways and the function of CARs[116–118] but its potential to evaluate transgenically expressed TCRs has not been tested yet.

1.3.2 Conventional TCR editing

The first report of a successful redirection of a T cell's antigen-HLA specificity via genetic TCR introduction in 1986[113] was soon followed by many attempts to improve the efficiency of TCR editing. Increased transgenic TCR surface expression and decreased levels of mispairing were achieved through improved vectors for high-level transgene expression[119], RNA-mediated silencing of the endogenous TCR[85], co-delivery of accessory or co-stimulatory molecules[120], introduction of single-chain TCRs[121], TCR constant region domain swapping[122] as well as framework engineering[96]. The current gold standard for TCR editing is lenti-/retroviral transduction of full αβTCR constructs containing murine constant regions[123,124] with an additional disulfide bond[125,126]. This strategy improves transgenic TCR surface expression

through the reduction of mispairing and increased stability of the transgenic TCR heterodimer. However, undisturbed expression of the transgenic TCR could only be achieved through the complete elimination of mispairing via stable knock-out of the endogenous TCR[88,127,128].

In addition to the absence of the endogenous TCR, conventional editing lacks control over the quantity and site of transgenic TCR integration events, both representing crucial aspects for T cell product functionality and safety (see figure 4). Increased transgenic TCR surface expression can be achieved via transduction with high virus loads, presumably resulting in an increased number of integration events and thereby a better transgene expression[85]. Advanced analysis of single-cell vector copy numbers (VCN) in such high virus load transduced cells revealed up to 44 transgene integrations in a primary T cell[129]. Yet, both the FDA and European Medicines Agency (EMA) highlight that the risk of gene-modified cell therapies via insertional oncogenesis[130] should be reduced through the limitation of VCN[131]. Along these lines, clinical studies that reported VCN for antigen-specific receptor transgenic T cells documented an average VCN between 1 and 2[63,78,80]. However, even with low VCN, viral transduction results in close-to-random transgene integration[132]. While a low VCN may be desired for safety reasons, it may also limit TCR transcription levels and protein surface expression which could ultimately compromise the functionality of conventionally engineered T cell products[85,133,134].

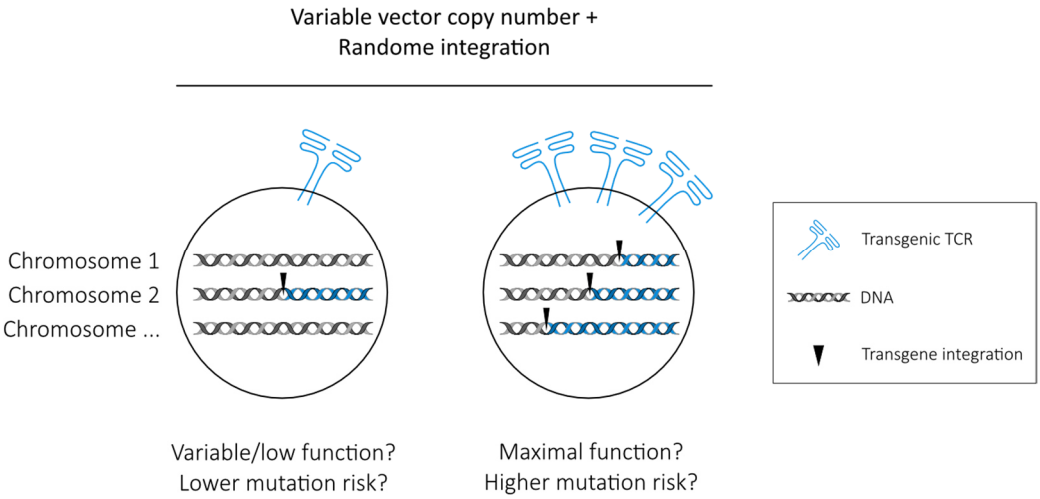

Figure 4: Conventional editing lacks control over quantity and site of transgenic TCR integration. Variable VCN in combination with (semi-) random genomic integration might affect T cell product functionality and safety. For the sake of clarity, individual TCR α and β genes are not shown.

Finally, random TCR transgene integration via conventional editing necessitates the usage of a constitutively active extrinsic gene promoter for transgene expression, which presumably does not reflect the dynamic regulation of the TCR observed after antigenic stimulation in unedited, normal T cells[126,135].

1.3.3 Orthotopic TCR replacement

To achieve physiological TCR regulation, it has recently been shown that CRISPR/Cas9 can be used to knock-in (KI) a transgenic CAR into the endogenous TCR locus (orthotopic placement). This was achieved by targeting the constant region of TCRα (TRAC) with adeno-associated virus 6-mediated delivery of the CAR DNA template[136]. The transgenic receptor is thus expressed under the natural TCR promoter, which presumably enabled more physiological transgene regulation and thereby resulted in better sustained long-term functionality[136]. Recently, another landmark study reported the first true OTR via non-virally delivered TCR DNA template in conjunction with CRISPR/Cas9-mediated homology-directed repair (HDR)[137].

In summary, this new technology offers a high safety profile through targeted genomic transgene insertion and presumably a more physiological way of TCR editing through placement under the endogenous promoter. Simultaneously, endogenous TCR expression and interaction with the transgenic receptor should be eliminated. In comparison to conventional editing via viral transduction, advanced T-cell engineering via OTR (see figure 5) now makes it possible to investigate the distinct contributions of missing competition through the endogenous TCR and orthotopic placement of transgenic TCRs in the physiological gene locus.

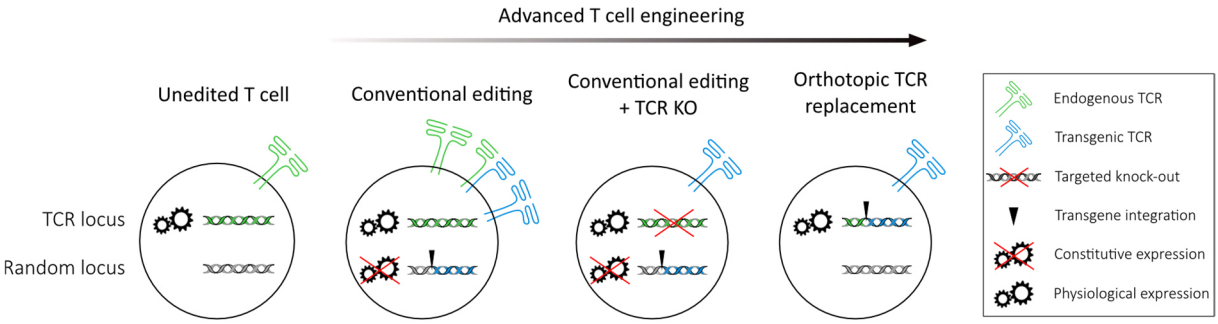

Figure 5: Advanced T cell engineering through CRISPR/Cas9-mediated OTR. In unedited, physiological T cells (left) the endogenous TCR (green) is naturally regulated (illustrated with gear wheels) under the endogenous TCR gene locus. Conventional editing leads to un-targeted (semi) random genomic insertion (black inverted triangle) of the transgenic TCR (blue), which requires an extrinsic promotor for transgene expression from this random gene locus, resulting in unphysiological transcriptional regulation (illustrated by crossed gear wheels). Furthermore, without additional KO of the endogenous TCR, mispairing and competition between endogenous and transgenic TCR can occur. In contrast, upon orthotopic TCR replacement (right), the transgenic TCR is placed in the endogenous TCR gene locus. The TCR is thereby transcriptionally regulated as it would be in physiological T cells. For the sake of clarity, individual TCR α and β gene loci are not shown.

1.4 Overall aims and structure of this thesis

Investigating and improving TCR-redirected T cell products for clinical application was the main goal of this thesis. Two important aspects of TCR editing were identified and systematically investigated: (1) TCR-intrinsic functionality and the selection of optimal TCRs for clinical use and (2) the method of genetic TCR introduction.

The first point (1) was addressed in chapters 1 and 4, in which we isolated 261 virus-specific TCRs from healthy donors and ACT patients. To functionally characterize these TCRs, we established and evaluated the suitability of a TCR signal reporter cell line with regard to:

1. the sensitivity of TCR functional avidity assessment
2. accurate reflection of TCR function and biology in comparison to primary T cells
3. suitability for a high-throughput TCR specificity and functionality screening

To address the second point (2), we investigated the functional consequences of different TCR editing strategies to provide new and important insights relevant to the production of improved TCR-edited T cell grafts. In chapters 2 and 3, we systematically compared conventional TCR editing to novel CRISPR/Cas9-mediated OTR particularly with regard to:

1. Functional consequences of residual expression of endogenous TCR chains
2. Influence of the promoter on TCR transgene expression and regulation
3. Impact of random versus targeted TCR transgene integration as well as …
4. … transgene copy number on T cell product safety, functionality, and predictability

Finally, in chapter 4, we demonstrate clinical translation of our TCR isolation platform as well as non-viral OTR T cell production with a focus on functionality and safety of the engineered T cell product.

2 Methods

This part is divided into two thematic sections. The subsection ‘TCR gene editing’ comprises all methods used for genetic engineering, *in vitro* culture, and preparation of cells for editing, as well as methods for validation of gene editing. The subsection ‘Functional assays’ comprises all methods used for the determination of TCR functionality after gene editing.

2.1 TCR gene editing

2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs) and T cell culture

Whole blood from healthy donors was obtained for isolation of PBMCs via density gradient centrifugation using Ficoll (Biochrome) according to standard protocols. T cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS, 0.025% L-glutamine, 0.1% HEPES, 0.001% gentamycin and 0.002% streptomycin and 180 IU ml^{-1} interleukin-2 (IL-2) ('RPMI' hereafter) unless indicated otherwise. Flow cytometry sorted cells were cultured with $1x10^6$ ml^{-1} irradiated (30 Gy) allogeneic feeder PBMCs, 1 µg ml^{-1} PHA and 180 IU ml^{-1} IL-2.

Written informed consent was obtained from the donors, and usage of the blood samples was approved according to national law by the local Institutional Review Board (Ethikkommission der Medizinischen Fakultät der Technischen Universität München).

2.1.2 TCR identification

PBMCs of virus-seropositive donor PBMCs were stained with respective pMHC-multimer that was individually conjugated with two different fluorophores to achieve reliable double pMHC-multimer staining. Single cells negative for CD19 and positive for CD8, CD62L, CD45RO, and both pMHC-multimers were singularized via fluorescence-activated cell sorting (FACS) into a 384 well plate and stimulated with 10 µg ml^{-1} plate-bound anti-CD3 and anti-CD28 each. RPMI medium was supplemented with 200 IU ml^{-1} IL-2 and 5 ng ml^{-1} IL-15. Single cell-derived clones were harvested between days 7 and 14 after sorting. TCR α- and

β-chains derived from a single clone were amplified via TCR-SCAN RACE polymerase chain reaction (PCR)[103] and subsequently sequenced on the Illumina MiSeq platform. TCR nomenclature represents a consecutive numbering with no meaning for the here presented data.

2.1.3 TCR DNA template design for retroviral transduction

DNA templates were designed *in silico* and synthesized by GeneArt (Life Technologies, Thermo Fisher Scientific) or Twist Bioscience. DNA constructs for retroviral transduction had the following structure: Human Kozac sequence[138] followed by TCR β (including as indicated either human TRBC or murine TRBC with additional cysteine bridge[123,125,126]), followed by a porcine teschovirus-1 2A self-cleaving peptide (P2A), followed by TCR α (including as indicated either human TRAC or murine TRAC with additional cysteine bridge[123,125,126]), cloned into pMP71 vectors (kindly provided by Prof. Dr. Wolfgang Uckert, MDC Berlin).

2.1.4 Retroviral transduction

Retroviral transduction of cell lines and primary human T cells was performed using the RD114 virus packaging cell line. For the production of retroviral particles, RD114 cells were transfected with pMP71 expression vector (containing the TCR-, fluorochrome-, or HLA-construct of interest) by calcium phosphate precipitation. Virus supernatant was harvested after 72 hours and subsequently coated on retronectin (TaKaRa) treated well plates. PBMCs were activated for two days with CD3/CD28 Expamer (Juno therapeutics a Bristol-Myers Squibb Company), 300 IU ml^{-1} IL-2, 5 ng ml^{-1} IL-7, and 5 ng ml^{-1} IL-15 per ml RPMI for $1x10^6$ T cells. Expamer stimulus was removed by incubation with 1 mM D-biotin. For the transduction of cell lines, $1x10^5$ ml^{-1} cells were seeded in a 24 well plate two days before transduction. Activated T cells or cell lines were transduced via spinoculation on virus-coated plates. TCR transduction occurred 15 min after CRISPR/Cas9 mediated TCR KO editing of T cells.

2.1.5 TCR DNA template design for CRISPR/Cas9-mediated KI

DNA constructs for CRISPR/Cas9-mediated HDR had the following structure: 5' homology arm (300-400 bp), P2A, TCR β (including as indicated either human TRBC or murine TRBC with additional cysteine bridge[123,125,126]), thosea asigna virus 2A self-cleaving peptide (T2A), TCR α (including as indicated either human TRBC or murine TRBC with additional cysteine bridge[123,125,126]), bovine growth hormone polyA signal (bGHpA), 3' homology arm (300-400 bp). When the introduced TCR was a fully human construct (i.e. did not contain a murine constant region), the TCR construct only contained the sequence of the first exon of TRAC positioned 5’ of the Cas9 cutting site. This seamless integration facilitated the usage of endogenous 3’ TRAC sequence for the transcription of a full TCR α-chain. Depending on the KI strategy, homology arms were applied that either bind to the TRAC or TRBC locus. The homology arm sequences of the TRBC locus were derived from TRBC1 and are highly homologous to TRBC2.

2.1.6 Cas9 ribonucleoprotein (RNP) production

CRISPR-RNA (crRNA) sequences that were used for assembly of Guide-RNAs (gRNA) were 5'-GGAGAATGACGAGTGGACCC-3' for TRBC[139] (targeting both TRBC1 and TRBC2) and 5'-AGAGTCTCTCAGCTGGTACA-3' for TRAC[139]. gRNA was produced via incubation of 80 µM trans-activating crRNA (tracrRNA) (IDT DNA) together with 80 µM crRNA (IDT DNA) at 95°C for 5 min, then cooled to room temperature (RT) on the benchtop. 20 µM high fidelity Cas9 (IDT DNA) was added slowly to gRNA solution to yield RNPs with 10 µM Cas9 and 20 µM gRNA, as well as 20 µM electroporation enhancer (IDT DNA). RNPs were incubated for 15 min at RT.

2.1.7 Production of double-stranded template DNA

dsDNA PCR products were used for electroporation and HDR. Vector DNA was amplified by PCR and PCR products were purified by Ampure XP beads (Beckman Coulter) at a 1:1 sample

to bead ratio. All TCR DNA templates were titrated but generally, electroporation of 1 µg DNA yielded the best KI efficiencies.

2.1.8 CRISPR/Cas9-mediated KO and KI

PBMCs were activated for two days with CD3/CD28 Expamer (Juno therapeutics a Bristol-Myers Squibb Company), 300 IU ml^{-1} IL-2, 5 ng ml^{-1} IL-7, and 5 ng ml^{-1} IL-15 per ml RPMI for $1x10^6$ T cells. Expamer stimulus was removed by incubation with 1 mM D-biotin. For electroporation of TPR cell lines, $1x10^5$ ml^{-1} cells were seeded in a 24 well plate two days before electroporation. Cells were electroporated (pulse code EH100 for primary T cells and CL-120 for TPR cells) with 1 µl of TRAC Cas9 RNP +/- 1 µl TRBC Cas9 RNP and – in case of KI – 1 µg HDR DNA template in Nucleofector Solution (20 µl per $1x10^6$ cells; Lonza) with a 4D Nucleofector X unit (Lonza). After electroporation, nucleofection solution was diluted 1:5 with RPMI (without IL-2) and cells were rested at 37°C for 15 min. Subsequently, cells were cultured in RPMI with 180 IU ml^{-1} IL-2 (primary T cells) or RPMI without IL-2 (TPR cells) until a first FACS analysis on day five after editing.

2.1.9 Sanger sequencing for KO validation

Genomic DNA was extracted (Wizard SV Genomic DNA Purification System, Promega) from flow-sorted CD3-negative cells according to the manufacturer's instructions. PCRs were performed to amplify the intended CRISPR/Cas9-mediated cutting sites within the first exon of TRAC as well as the first exon of TRBC1/2. Purified PCR products were Sanger sequenced (Eurofins Genomics).

2.1.10 Sanger sequencing for KI validation

Genomic DNA was extracted (Wizard SV Genomic DNA Purification System, Promega) from flow-sorted T cells expressing the transgenic TCR after CRISPR–Cas9-mediated HDR according to the manufacturer's instructions. PCRs were performed from 5′ homology arm to

3′ homology arm to verify full construct integration, as well as from outside the 5′ homology arm to 3′ homology arm to validate seamless integration into the genomic locus. Purified PCR products were Sanger sequenced (Eurofins Genomics)

2.1.11 Targeted locus amplification (TLA)

TLA including sequencing was performed by Cergentis as previously described[140]. Two primer sets were designed to target each transgene. Primer sets were used in individual TLA amplifications. PCR products were purified and library prepped using the Illumina Nextera flex protocol and sequenced on an Illumina sequencer. NGS reads were aligned to the transgene sequence and host genome (human hg19 reference sequence). Bioinformatic analysis of integration sites and detection of hit cancer genes via Enhort (unpublished, https://enhort.mni.thm.de/), Cosmic[141], and Network of Cancer Genes[142].

2.2 Functional assays

2.2.1 pMHC-multimer and antibody staining

pMHC-monomers were generated as previously described[143]. All pMHC-multimer reagents were generated by incubation of 4 µg biotinylated pMHC-monomer with 1 µg streptavidin (coupled to either BV421, PE, or APC) in a total volume of 100 µl FACS buffer for staining of up to $1x10^7$ cells. pMHC-multimer staining and surface antibody staining was usually performed in two consecutive steps. The following antibodies were used: anti-human TCR α/β FITC (BioLegend), anti-human TCR α/β PE (BioLegend), CD3 PC7 (BD Biosciences), CD8α PE (Invitrogen), CD8α APC-eFlour 780 (eBioscience, Thermo Fisher Scientific), CD8β PC5.5 (Beckman Coulter), anti-murine TCR β-chain APC (BioLegend), anti-murine TCR β-chain APC/Fire 750 (BioLegend), CD62L FITC (BD Biosciences) and CD45-RO PC7 (BioLegend), CD45 PerCP (Thermo Fisher Scientific), CD45 ECD (Beckman Coulter), and CD45 PC7 (eBioscience). Live/dead discrimination was performed with propidium iodide (Invitrogen). All stainings were performed on ice.

2.2.2 K_{off} rate measurement

K_{off} rates were determined as dissociation of reversible pMHC-Streptamers upon addition of D-Biotin in a flow cytometry-based assay (CyAn ADP Lx 9 color flow cytometer, Beckman Coulter) as described previously[144]. In brief, pMHC molecules were multimerized with Strep-Tactin APC (IBA lifesciences) according to the manufacturer's instructions and incubated with $5x10^6$ TCR-transgenic T cells for 45 min. After 25 min, cells were additionally stained with CD8α PE (clone OKT8, eBioscience). For life/dead discrimination cells were incubated with propidium iodide (Invitrogen). $1x10^5$ - $1x10^6$ pre-cooled cells were analyzed by flow cytometry. After 30 s, D-Biotin was added to a final concentration of 1 mM while analysis continued. Dissociation kinetics were analyzed using FlowJo (FlowJo, LLC) and GraphPad Prism software (GraphPad Software).

2.2.3 K562 antigen-presenting cell line library

The HLA-deficient K562 cell line was used for the standardized presentation of different antigens in various MHC class-I contexts. For this, we genetically introduced nine of the most common MHC class-I molecules within the European Caucasian population individually into the K562 cell line (HLA-A*0101, HLA-A*0201, HLA-A*0201, HLA-A*1101, HLA-A*2402, HLA-B*0702, HLA-B*0801, HLA-B*4402, and HLA-C*0702; all vectors were kindly provided by Dr. Kevin Dennehy, UKT Tübingen). For simple FACS- or microscope-based identification of K562-HLA cells we further stably introduced individuals or mixes of different fluorochromes (BFP, CFP, GFP, T-Sapphire, and YFP; all vectors were kindly provided by Dr. Simon Grassmann, TUM), thereby generating a matrix of $HLA^+fluorochrome^+$ K562 cell lines. In this thesis, HLA-A*0101-, HLA-A*0201- and HLA-B*0801-positive K562 cell lines were used for antigen-presentation in functional assays.

2.2.4 Antigen-specific activation and intracellular cytokine staining

One day before co-culture with TCR-redirected T cells, K562 cells (retrovirally transduced to express the human MHC class-I molecule of interest) were irradiated (80 Gy) and loaded with peptide (10^{-12} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M) overnight at 37°C. T cells were co-cultured with peptide-loaded K562 cells and Golgi plug (BD Biosciences) in a 1:1 ratio for 4 h at 37°C. PMA (25 ng ml^{-1}) and ionomycin (1µg ml^{-1}) were used for positive control. pMHC-multimer and surface marker antibody staining for CD8α (PE, Invitrogen) and anti-murine TCR β-chain (APC/Fire750, BioLegend) was followed by permeabilization using Cytofix/Cytoperm (BD Biosciences), and staining of IFNγ (FITC, BD Biosciences), TNFα (PC7, eBioscience), and IL-2 (APC, BD Biosciences). Live/dead discrimination was performed with ethidium-monoazide-bromide (Invitrogen).

2.2.5 Antigen-specific activation and TCR signaling

Antigen-specific stimulation of TCR-transgenic TPR^{KO} cells was performed using irradiated (80 Gy) and peptide-pulsed (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M) K562 cells (retrovirally transduced to express the human MHC class-I molecule of interest). Effector and target cells were co-cultured in a 1:5 ratio for 18 h. For multiplexing, cells were stained with combinations of aCD45 antibodies (CD45 PerCP, CD45 ECD, CD45 PC7). Subsequently, NFAT-GFP and NFκB-CFP reporter activity was analyzed on a flow cytometer.

2.2.6 Cytotoxic T lymphocyte assay

For assessment of the cytotoxic capacity of A2/pp65-specific TCRs, HLA-A*0201-positive HepG2 cells (kindly provided by Prof. Dr. Ulrike Protzer, TUM) were loaded with 10^{-10} M of $A2/pp65_{495-503}$ (NLV) peptide. $4x10^4$ peptide-pulsed HepG2 cells were plated per well in a 96 well E-Plate (ACEA Biosciences). The plate was subsequently placed into an xCELLigence RTCA MP Real Time Cell Analyzer (ACEA Biosciences) and HepG2 cell growth was monitored every 15 minutes. After 24 hours, $4x10^4$ rested TCR-transgenic T cells were added per well

containing HepG2 target cells. Mock edited (TCR transgene negative) T cells derived from the same donor were used as a negative control. Effector and target cells were co-incubated for 48 h and target cell growth/death was monitored every 15 minutes.

2.2.7 TCR downregulation assay

Rested T cells were stimulated either using CD3/CD28 or antigen-specific pMHC/CD28. CD3/CD28 TCR stimulus was provided with a pre-mixed CD3/CD28 Expamer solution (Juno Therapeutics a Bristol-Myers Squibb Company) according to the manufacturer's protocol. Antigen-specific stimulation was performed using pMHC/CD28 Expamer. T cells were stained 0 (baseline), 4, 10, 24, 48, and 144 h after the start of stimulation. For staining, samples were co-incubated with 1 mM d-biotin (10 min at RT) for the dissolution of Expamer complexes. Then, cells were stained with respective pMHC-multimers as described above and the following antibodies: human TCR α/β FITC (Biolegend), CD3 PC7 (BD Biosciences), CD8α PE (Invitrogen), anti-murine TCR β-chain APC or APC/Fire750 (Biolegend). Live/dead discrimination was performed with propidium iodide (Invitrogen). Furthermore, RNA was isolated at different time points using the RNeasy isolation kit (Qiagen). Quantitative PCR for mTRBC was performed with Custom Plus TaqMan RNA Assay SM FAM (Life Technologies, Thermo Fisher Scientific) on a QuantStudio 7 Flex cycler (Life Technologies, Thermo Fisher Scientific).

2.2.8 Single-cell RNA sequencing (scRNA seq)

TCRs were introduced either via CRISPR/Cas9-mediated KI or via retroviral transduction into endogenous TCR-KO primary T cells as described. T cells that underwent different TCR editing approaches (OTR, transduction with a low virus load, transduction with a high virus load) were color-barcoded via additional transduction with three different fluorochromes (BFP, CFP, GFP). Five days after editing, FACS sorted $CD8^+TCR^+fluorochrome^+$ cells were used for the determination of transgenic TCR expression and whole transcriptome analysis on the 10x

Genomics platform. The protocol was performed according to the manufacturer's instructions. The only adaptation was the application of custom primer sets for specific amplification of transgenic TCR constructs.

2.2.9 scRNA seq – Data processing

Combined fastq-files (transcriptome and transgenic TCR library) for each TCR were annotated against a custom reference containing all genes of the human genome (GRCh38), the fluorochromes (BFP, CFP, GFP) used for multiplexing, and the respective TCR constructs using Cell Ranger (V3.0.2). Data analysis of the annotated count matrix was performed in SCANPY[145]. Cells were filtered to contain at least 200 genes and genes being present in less than 3 cells were excluded. 20% mitochondrial gene expression was allowed. Counts were normalized to 10.000 counts per cell and expression was log-transformed. The number of counts, percent of mitochondrial genes, S and G2M phase scores were regressed out. To demultiplex subsamples according to fluorochrome expression, cells were filtered to express only one of the three fluorochromes and resulting leiden-clusters[146] of the neighborhood graph were annotated according to fluorochrome expression. Highly variable genes (HVGs) were identified with mean values between 0.0125 and 3 and a minimal dispersion of 0.5. Expression values exceeding a standard deviation of 10 were clipped. The neighborhood graph was calculated for the 10 nearest neighbors and the first 7 components of the principal component analysis for the HVGs. Fluorochromes and constructs have been excluded for the neighborhood embedding. Violin plots show the normalized raw gene expression.

2.2.10 MCMV mutagenesis

MCMV-ie2-ANLV was generated by fusing AANLVPMVATV peptide at the C-terminus of ie2 protein. HCMV $pp65_{495\text{-}503}$ epitope (NLVPMVATV) was preceded by two alanine residues that enhance peptide processing and presentation[147]. The peptide was inserted at the ie2 C-terminus position 187,296 (NCBI accession: NC_004065) using en passant mutagenesis as

described before[147,148]. MCMV mutagenesis was performed by M. Zeeshan Chaudry (Helmholtz Centre for Infection Research, Braunschweig).

2.2.11 *In vivo* assay

In vivo experiments were performed with HLA-A*0201 transgenic NOD/SCID/IL-2rg$^{-/-}$ mice and a chimeric murine CMV engineered to express the human CMV A2/pp65$_{495\text{-}503}$ (NLV) peptide[149]. First, mice were irradiated (2 Gy) and subsequently 1x10^5 CD8$^+$TCR$^+$ cells (per respective TCR) were injected intraperitoneally. About 24 h after infection, mice were infected with 5x10^3 plaque-forming units (PFU) of mCMV-ie-ANLV. Mice were sacrificed on day 9 after T cell transfer and their liver harvested. Intra-hepatic lymphocytes were isolated via density gradient centrifugation using Percoll (GE Healthcare) and subsequently stained with pMHC-multimer, anti-human CD45 PC7 (eBiosciences), anti-human CD8α PE (Invitrogen), anti-murine TCR β-chain APC/Fire750 (Biolegend), and propidium iodide (Invitrogen).

3 Chapter overview

This publication-based thesis contains four published research articles each representing a single chapter. In the following, a summary and description of author contributions will be presented for each chapter.

Chapter 1:

A T-cell reporter platform for high-throughput and reliable investigation of TCR function and biology

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Summary

Reprogramming a T cell's antigen-HLA specificity through TCR gene editing provides exciting new options for ACT and facilitates highly individualized patient care. A major prerequisite for this approach is the isolation and functional characterization of TCR candidates intended for clinical use. Whereas recent advancements in next-generation TCR repertoire sequencing enable isolation of hundreds of thousands of TCR sequences from a single blood sample, functional testing of TCR candidates remains a critical bottleneck.

Usually, TCR function is assessed via transgenic re-expression in primary T cells followed by *in vitro* and *in vivo* studies. This is not only a very laborious process but also faces an increased degree of variability due to factors such as T cell activation status, phenotype, or donor origin. These limitations could be overcome, however, by the usage of an immortalized T cell line.

Our collaboration partners previously developed a triple parameter TCR signaling reporter cell line (TPR) based on the Jurkat line E6.1 (Jutz et al. 2016, *J. Immunol. Methods*) and we now aimed to use this cell line for high-throughput testing of transgenically expressed TCRs. For this, we additionally performed a genetic introduction of the CD8αβ co-receptor as well as a CRISPR/Cas9-mediated KO of the endogenous TCR to facilitate highly sensitive and unbiased TCR functional characterization.

We – to the best of our knowledge for the first time and to an unprecedented extent – performed in-depth functional characterization of 59 TCRs in our newly developed TPR^{KO} cell lines and

– most importantly – also in endogenous TCR-KO primary T cells, facilitating direct comparison of TCR function.

We show that our cell lines can be used for high-throughput screening of antigen-HLA specificity via pMHC-multimer staining. Most importantly, our data clearly demonstrate that TPR^{KO} cell lines can be used as a surrogate for primary human T cells since TCR function in our cell lines is almost identical to TCR function in primary T cells. Furthermore, we validate previous findings regarding the influence of the CD8αβ co-receptor on TCR-pMHC complex stability and TCR function, implicating that our cell line constitutes an attractive tool for the investigation of basic TCR biology. These results highlight the suitability of our TPR^{KO} cell lines for high-throughput and reliable testing of TCR candidates for clinical use as well as for basic research.

Author contributions

T.R.M., K.S., D.H.B., and P.S. conceived the study. T.R.M. and D.H.B. designed and analyzed experiments. T.R.M., M.H., and K.S. developed the TCR library. T.R.M and A.K. generated HLA-transgenic K562 cell lines. J.L., S.J., and P.S. generated and provided TPR and TPR-CD8 reporter cells. T.R.M and C.S. generated TPR^{KO} cell lines, performed TCR editing, and *in vitro* experiments. T.R.M., D.H.B., and P.S. wrote the manuscript. All authors read and reviewed the manuscript.

Chapter 2:

Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function

Kilian Schober*, **Thomas R. Müller***, Füsun Gökmen, Simon Grassmann, Manuel Effenberger, Mateusz Poltorak, Christian Stemberger, Kathrin Schumann, Theodore L. Roth, Alexander Marson & Dirk H. Busch

* These authors contributed equally

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Summary

ACT aims at providing antigen-specific T cell-mediated immunity to fight infectious diseases or cancer. Clinical application of *ex vivo* isolated pathogen-specific or TILs has proven the great potential of this approach. Genetic engineering further provides the means to re-direct T cells to recognize a distinct target antigen, and thereby generate well defined and highly predictable cell products for clinical application. The success of CD19-targeting T cells engineered to express a chimeric CAR has recently culminated in two FDA approvals for Tisagenlecleucel/Kymriah (marketed by Novartis) as well as Axicabtagene ciloleucel/Yescarta (marketed by Kite/Gilead). These cells are the first genetically modified cells ever to be FDA-approved.

In contrast, a vast number of clinically relevant targets are recognized by different natural TCRs, but so far it has remained a challenge to unite the enormous potentials of TCRs and T cell engineering. This can be explained by the TCR's complex genomic organization, its heterodimeric structure, and dynamic transcriptional regulation. Conventional gene transfer by viral transduction of TCRs leads to non-targeted integration, which poses a safety hazard and necessitates unphysiological constitutive transgene expression. Furthermore, transgenic TCR

chains compete and can mispair with endogenous TCR chains, leading to decreased functionality and/or unwanted toxicities like GvHD.

Endogenous TCR chains can be eliminated by advanced genomic engineering tools such as CRISPR/Cas9 and excitingly, non-viral CRISPR/Cas9-mediated knock-in of large DNA sequences now also enables true orthotopic placement of transgenic TCRs into the endogenous TCR gene locus (Roth et al. 2018, *Nature*).

In this study, we for the first time investigated the specific consequences of endogenous TCR elimination and OTR on surface expression and mispairing as well as on antigen-specific functionality and TCR downregulation. We found that while OTR leads to functional T cells, mispairing still occurs when only a single TCR chain is targeted. Editing of both endogenous TCR chains eliminates mispairing and hence the risk of off-target toxicities. Concomitantly, α- and β-chain editing also enhances TCR surface expression of transgenic TCRs placed in the endogenous locus. Moreover, we investigated TCR genomic integration sites after OTR and could confirm targeted transgene integration without off-target events. Most importantly, we could observe that OTR, but not virally transduced T cells dynamically regulate the transgenically expressed TCR highly similar to unedited physiological T cells.

Hence, OTR combined with α- and β-chain editing, enables T cell engineering with a high safety profile, accurate TCR pairing, and near-physiological TCR regulation.

Author contributions

K.Schober and D.H.B. conceived the study. K.Schober, T.R.M., and D.H.B. designed and analyzed experiments. K.Schober, T.R.M., and F.G. performed CRISPR editing and transductions. F.G. performed double-stranded DNA production and gDNA analysis. S.G. performed FACS. T.R.M. and M.E. identified TCRs. M.E. produced pMHC reagents. K.Schober and T.R.M. performed flow cytometric analyses and functional assays. M.P., C.S., K.Schumann, and A.M. advised on CRISPR/Cas9 RNP editing of T cells. T.L.R. and A.M. developed and advised on non-viral CRISPR/Cas9 large gene KI via HDR. K.Schober, T.R.M.,

and T.L.R. designed HDR DNA templates. K.Schober, T.R.M., and D.H.B. wrote the manuscript. All authors read and reviewed the manuscript

Chapter 3:

Targeted T cell receptor gene editing provides predictable T cell product function for immunotherapy

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Summary

Building on the findings from chapter 2, we now set out to systematically investigate the consequences of conventional editing and novel CRISPR/Cas9-mediated OTR on the safety, functionality, and predictability of the transgenic T cell product.

Conventional editing introduces the TCR transgene in a semi-random manner and leaves the endogenous TCR intact. This hinders characterization of TCR-intrinsic features in an unbiased manner, requires careful titration of transduction virus doses for controlled transgene copy numbers, harbors the potential of variable transgene expression efficiency depending on the integration site, and results in an ever-present risk of insertional oncogenesis. Yet, for application as 'living drugs', therapeutic T cell products need to be not only safe but also highly predictable in their functionality. In this line, first clinical trials with conventionally edited TCR-transgenic T cell products showed sometimes promising, yet overall rather variable therapeutic results.

The advent of technologies such as CRISPR/Cas9 has provided the means for highly controlled genetic editing and the first CRISPR/Cas9-edited T cell products have entered the clinics at a surprisingly fast pace. However, so far it has not been demonstrated whether OTR can be used to overcome major engineering hurdles for clinical translation, especially the

generation of cell products – which are often derived from different donors – with predictable *in vivo* functionality. Such studies would require an extensive comparison of many different TCRs and engineering conditions since multiple factors - including TCR-intrinsic factors – can affect T cell functionality.

Given how relevant safe and predictable cell products are for the field of adoptive cell therapy, we here set out to investigate whether OTR enables the generation of T cell products with more predictable functionality than conventional editing. We thereby employed an array of cutting-edge technologies. This included – to the best of our knowledge in each case for the first time or to an unprecedented extent – functional characterization of overall 51 TCRs without bias from the endogenous TCR; scRNA seq of endogenous and transgenic TCRs after OTR or viral transduction; investigation of whole-genome integration sites of CRISPR/Cas9-mediated targeted integration and viral transduction; and an *in vivo* model entailing the adoptive transfer of a polyclonal human T cell repertoire by color barcoding.

Our data clearly demonstrate that targeted TCR integration via OTR results in controlled, near-physiological TCR transgene expression on the single-cell level. This directly translates into a more defined TCR surface expression and a more homogenous TCR-redirected T cell product on the population level in comparison to conventional editing. Finally, we demonstrate that the increased homogeneity of OTR T cell products results in a more predictable *in vivo* T cell response in comparison to conventionally edited T cells. These results are of utmost and quite general importance for the production of genetically modified cell products in the clinics as well as in basic research.

Author contributions

T.R.M., K.S. and D.H.B. conceived the study. T.R.M., K.S. and D.H.B. designed and analysed experiments. T.R.M., M.H. and K.S. developed the TCR library. M.N. provided a donor biobank for TCR isolation. M.E. generated pMHC-multimers. T.R.M performed TCR editing and *in vitro* experiments. B.B., K.Sch., A.M. and U.P. provided and generated lentivirus for transduction.

P.S. developed and advised on experiments with J-TPR cell line. T.R.M, K.S. and J.L. performed *in vivo* experiments. M.Z.C and L.C-S. generated mCMV for *in vivo* experiments. S.G. and I.A. performed and advised on FACS sorting. T.K. and T.F. performed whole genome sequencing. T.R.M., S.J. and M.H. performed single-cell transcriptomics. S.J. performed bioinformatics analysis. T.R.M., K.S. and D.H.B. wrote the manuscript. All authors read and reviewed the manuscript.

Chapter 4:

Protective T-cell receptor identification for orthotopic reprogramming of immunity in refractory virus infections

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Summary

Persistent adenovirus infection (but also CMV or EBV) is under T cell-mediated immune control in healthy individuals. However, such protective T cell responses are absent in immunocompromised patients, often resulting in life-threatening disease. ACT with allogeneic HLA-matched virus-specific T cells has proven to be a highly attractive approach to specifically restore the anti-viral immune response in these patients. Unfortunately, such virus-specific HLA-matched physiological T cells are often not available.

Genetic TCR engineering enables to reprogram a T cell's antigen-HLA specificity and would thereby substantially broaden the applicability of adoptive T cell therapy. However, both the selection criteria for clinically relevant TCRs and the TCR engineering method are still matters of debate. TCR function can be assessed in a plethora of *in vitro* assays and *in vivo* mouse models, but parameters that define clinically protective TCRs are so far not established. Furthermore, as outlined in chapters 2 and 3, currently performed conventional TCR editing faces concerns regarding the function and – most notably – the safety of TCR-redirected T cell products intended for clinical application.

Here, we treated a 17-year old patient, who developed life-threatening AdV viremia upon allogeneic HSCT, with *ex vivo* isolated, purified A1/Hexon5 (LTDL)-specific $CD8^+$ T cells

derived from the haploidentical stem cell donor. Upon adoptive T cell transfer, the sustained presence of LTDL-specific T cells correlated with viral clearance. Importantly, we then isolated these T cells in order to identify LTDL-reactive TCRs. This strategy enables the identification of clinically relevant TCRs with proven *in vivo* protective capacity.

We could identify two highly prevalent T cell clones and re-expressed their TCRs in human primary T cells for in-depth characterization. Both TCRs showed high structural as well as functional avidity measured *in vitro*. These data could help to define parameters of clinically protective TCRs.

Furthermore, we performed CRISPR/Cas9-mediated OTR to reprogram primary human T cells with these LTDL-specific TCRs. Concomitant KO of the endogenous TCR eliminates potential allo- or off-target-reactivity and targeted integration decreases the risk of insertional mutagenesis. Here, in addition to targeted integration site analysis performed in chapters 2 and 3, we performed whole-genome sequencing and evaluated unwanted transgene integrations at predicted off-target sites as well as genome-wide. Importantly, we could not identify any mutations in the exome in two different donors, highlighting the precision and safety of CRISPR/Cas9 editing. Finally, OTR T cell products were highly functional in terms of proliferation, cytokine response, and cytotoxic capacity.

In conclusion, we provide a strategy to use protective T cell immunity in a safe, highly efficient, and flexible procedure for precision adoptive T cell therapy.

Author contributions

TF and DB set up the concept of the approach. The patient was treated by TF, MD, FB, JF, PL and RH. Experiments were conceived and designed by TK, TS, FB, SW, TF and DB. TCR isolation, identification and template design was done by TM & KS. Experiments were performed by TS, TM and KD. Data analysis and manuscript preparation was done by TS, TK and TF. The manuscript was critically reviewed by all authors. The authors declare no competing interests.

4 Discussion

This part is divided into two thematic sections. The section “Identification of TCRs for clinical use” contextualizes the TCR isolation and functional screening described in chapters 1, 3, and 4. Subsequently, the section “TCR engineering” discusses the findings from chapters 2, 3, and 4, with a focus on their relevance for the production of next-generation TCR-transgenic T cell products for clinical use and basic research.

4.1 Identification of TCRs for clinical use

4.1.1 Strategies for TCR isolation

Several TCR repertoire sources can be explored for the identification of clinically relevant TCRs. While the antigen-unexperienced naïve T cell compartment offers an unbiased and diverse T cell repertoire, memory populations comprise TCRs that have already proven their functionality and safety *in vivo*.

Here, we performed efficient identification of virus-specific TCRs from virus-seropositive healthy donors. We used a biobank consisting of CMV-, EBV-, and AdV-seropositive healthy donors (kindly provided by Dr. M. Neuenhahn) and isolated TCRs via highly reliable double pMHC-multimer staining. We further chose to select $CD8^+$ T cells exclusively from the central memory compartment since the TCR repertoire in this population was shown to be more diverse than higher differentiated compartments[150]. While these TCRs derived from the antigen-experienced repertoire should in principle be functional and protective *in vivo*, selection criteria for an optimal TCR for clinical use are so far not established. In this regard, patients that received donor-derived T cells via ACT after allogeneic HSCT are a highly valuable source for TCRs with proven *in vivo* efficacy.

Here, we performed this approach with allogeneic HSCT patients that received either stem cell donor-derived polyclonal central memory T cells (PACT clinical trial, NCT02758223; unpublished) or antigen-specific T cells (see chapter 4). Functional characterization of such

TCRs, which have proven their protective capacity *in vivo*, might help to establish parameters that clearly define a TCR's therapeutic relevance. Such TCR functionality benchmarks would help to evaluate the clinical relevance of TCRs derived from other repertoires. As a potential parameter defining TCR functionality, we determined the K_{off} rate (as a measure of structural avidity) of 45 virus-specific TCRs, either derived from virus-seropositive healthy donors or ACT patients (figure 6). The K_{off} rate is particularly valuable since structural avidity is hardwired in the TCR sequence and independent of T cell physiology, such as activation status or phenotype. The five patient-derived TCRs (marked in color) showed K_{off} rates larger than 100s, which was indeed described in the literature as a minimal threshold for highly functional TCRs[94]. Yet, the broad distribution of these five TCRs along the K_{off} rate spectrum indicates that this one parameter probably cannot serve as a clear TCR functionality benchmark. Furthermore, optimal TCR affinity might be highly dependent on the specific clinical context, which comprises – amongst others – antigen load, the kinetics of antigen presentation, and the T cell microenvironment. In the future, standardized determination of multiple TCR functionality parameters of a substantially larger set of TCRs derived from patient material with different clinical history as well as controlled infection and cancer mouse models will be required for the definition of clear TCR functionality benchmarks.

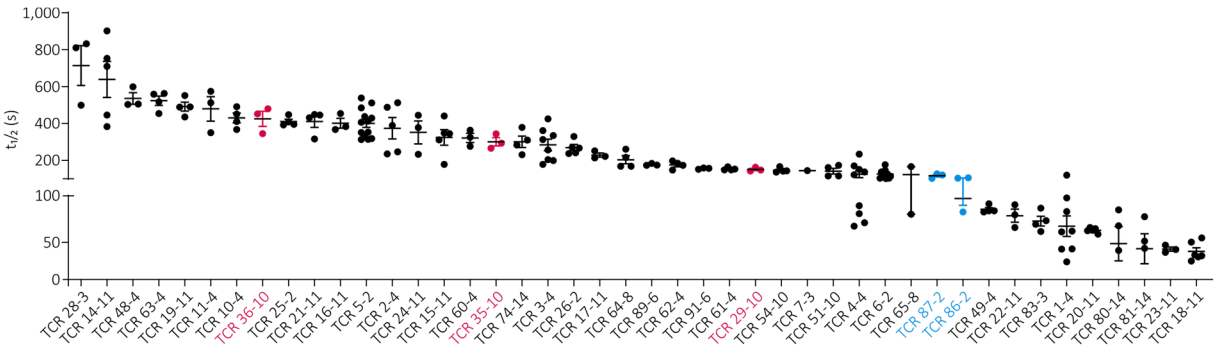

Figure 6: Structural avidity of transgenically expressed virus-specific human T cells. Quantification of K_{off} rate of 45 MHC-class I restricted TCRs transgenically expressed on primary human $CD8^+$ T cells. TCRs isolated from a PACT-patient marked in dark red, TCRs isolated from an antigen-specific ACT receiving patient in blue. Summary of multiple experiments. Data are depicted as mean ± SEM with each dot representing one measurement.

In summary, the antigen-experienced T cell compartment is an attractive source for the isolation of functional T cells. In-depth functional characterization of TCR repertoires from patients receiving ACT might help to define clear TCR functionality benchmarks that can be used as a reference for the educated selection of TCRs (derived from other repertoires/sources) for clinical use. To establish and apply such benchmarks, a standardized test system for the determination of a TCR's antigen-HLA specificity and functionality is required.

4.1.2 A cellular platform for reliable TCR investigation of TCR function

Due to continuously improved large scale sequencing of TCR repertoires[104–107] and bioinformatical tools for data processing[108,109], it is now possible to investigate an almost endless number of TCRs on the sequence level *in silico*. However, large scale functional testing of transgenically expressed TCRs remains a major challenge. In this regard, recently reported protocols for high-throughput direct cloning of TCRs for transgenic re-expression represent major advancements for large-scale TCR re-expression[106,107,151]. What is still missing, however, is a platform that enables high-throughput and reliable investigation of TCR functional avidity and biology.

The functional characterization of TCRs is most widely performed after transgenic re-expression in primary T cells. In contrast to the hardwired K_{off} rate, however, functional avidity is largely affected and biased by phenotype, activation status, or donor origin of primary T cells. Hence, a stable cell line that provides TCR function close to primary T cells would enable a more standardized testing as well as simplify the whole process due to their easy handling and almost unlimited proliferation capacity. The urgent need for such a cell line is highlighted by various publications that proposed different cellular platforms for TCR testing[152-156]. While these publications demonstrated, with only a handful of TCRs in total, that in principle TCR functionality can be assessed in cell lines, they did not validate their findings in primary T cells. It is therefore unclear whether or not functional TCR data assessed in cell lines are at all meaningful.

In this thesis, we propose an advanced Jurkat E6.1-based TCR signal reporter system that is unbiased by endogenous TCR expression (see chapter 1). Our study, which analyzed 59 different human TCRs, is – to our knowledge – the first to comprehensively compare TCR function in a cell line with primary human T cells. As TCR function was closely parallel to primary T cells, our TPR^{KO} cell lines proved highly suitable for functional characterization of individual TCRs and also for the investigation of TCR biology in general.

Using TPR^{KO} cell lines, we validated that pMHC-multimer staining intensity is not predictive for functionality[157,158], highlighting the importance of in-depth functional testing for the identification of suitable TCRs for clinical use. Further, we confirmed previous findings that the CD8αβ co-receptor increases a TCR's peptide sensitivity to a highly differential TCR-dependent extent[159,160] and CD8αβ co-receptor dependency inversely correlates with functional avidity[159-161]. The latter implicates that measured TCR functional avidity in absence of CD8αβ might more directly reflect the structural avidity of a TCR to its cognate pMHC. We further observed a disparity between CD8αβ dependency of pMHC-multimer staining and TCR functional avidity, indicating the presence of two different mechanisms of CD8αβ contribution to pMHC-multimer binding and antigen-specific TCR activation. Our TPR^{KO} cell lines could be used as a tool to investigate this more closely.

Most importantly, we document highly reliable pMHC-multimer staining on our TPR^{KO} cell lines demonstrating their suitability for large-scale antigen-HLA specificity screening approaches. For instance, this enables re-expression of large combinatorial libraries of TCR α- and β-chains in our TPR^{KO} cell lines for high-throughput screening of antigen-HLA specificities of interest. Furthermore, we have demonstrated that TPR^{KO} cell lines facilitate a high-throughput functionality screening of TCRs with high sensitivity and reliability. Hence, TPR^{KO} cell lines facilitate the generation of large datasets connecting TCR sequence, antigen-HLA specificity, and function to an unprecedented extent. This would be a substantial contribution to the development of improved algorithms for antigen-HLA specificity and probably also functionality prediction from raw TCR sequence data[108,109,164]. Fast sequencing of TCR repertoires in

combination with such reliable prediction algorithms has the potential to revolutionize patient-individualized ACT through an educated selection of optimal TCRs.

Determination of TCR functionality in the presence and absence of CD8αβ enables identification of largely CD8αβ co-receptor independent TCRs, which could be of particular interest for clinical application. On the one hand, CD8αβ independent TCRs would maintain their functionality in T cell products despite largely variable CD8αβ expression[162]. On the other hand, it was shown that $CD4^+$ T cells expressing an MHC class-I restricted TCR provide important additional TCR functions, such as increased IL-2 help, and thereby contribute to an increased anti-tumor response[165–167]. Therefore, CD8αβ co-receptor-independent TCRs would represent ideal candidates for such an approach.

In summary, we here propose a Jurkat-based TCR signal reporter cell line for testing of TCR specificity and functionality unbiased by endogenous TCR expression. TCR functional avidity of 30 individual TCRs in our TPR^{KO} cell lines was strongly correlating to primary human T cells, highlighting the suitability of our cell line for highly reliable investigation of TCR function and biology. Hence, this platform represents a valuable tool for the characterization and selection of TCR candidates for clinical use. It facilitates the generation of large TCR functionality datasets for the development of prediction algorithms and the definition of functionality benchmarks based on TCRs derived from clinical settings that indicate high *in vivo* efficacy.

4.2 TCR engineering

4.2.1 Functional consequences of TCR editing

The TCR's complex genomic organization[81,82], its heterodimeric structure[83], and the interaction of the endogenous and the introduced transgenic receptor represent major challenges in the field of TCR engineering. Advanced genomic engineering tools, such as CRISPR/Cas9[168,169], now enable us to address these problems by providing the means to eliminate the endogenous TCR[88,170] and insert a transgenic antigen-specific receptor directly into the endogenous gene locus. Pioneered by the groups of Michel Sadelain (first CAR placement into the TCR locus)[136]

and Alex Marson (first true OTR)[137], we validated and provided technological proof-of-concept of OTR.

Using this novel engineering tool, we further wanted to investigate how OTR T cell products differ from conventionally edited T cells. Considering that conventional editing – e.g. via viral transduction – is currently the most widely used method for the production of clinically applied T cell products but also in basic research, in-depth and systematic comparisons of T cell products which are generated through these different editing methods are of fundamental importance. In chapters 2 and 3, we re-expressed more than 50 virus- and tumor-specific TCRs via OTR and conventional editing and investigated the consequences of differential genetic TCR integration profiles on the magnitude and the variability of transgenic TCR expression, the kinetics of TCR regulation, and the overall functional capacity.

First, we investigated the interactions between endogenous and transgenic TCR upon conventional editing with or without additional KO of an individual or both TCR chains as well as after OTR (see chapter 2). Several studies have shown by the introduction of individual TCR chains[88,171], that mispairing occurs, particularly under these enforced conditions, but mispairing had not been directly observed following KO of an individual TCR chain. Indeed, we observed that individual TCR-chain editing led to a substantial degree of mispairing. Orthotopic TCR placement in the TRAC locus without additional elimination of TRBC even resulted in increased mispairing in comparison to conventional editing, presumably due to a more balanced ratio between transgenic TCR α-chain and the remaining endogenous β-chain.

Furthermore, by studying a comprehensive set of TCRs, we validated a large, yet TCR-intrinsic impact on competition and mispairing between endogenous and transgenic receptors that ultimately affects surface expression (see chapter 3). In this context, the concept of ‘weak’ and ‘strong’ recombinant TCR expression is well-established[171,172]. A recent study showed that a few amino acids in the TCR variable regions have a great impact on TCR surface expression, potentially due to increased protein folding efficiency and/or α-β-chain assembly[96]. However, addressing whether strong TCRs are more capable of outcompeting endogenous TCRs, or whether they are less affected by mispairing of individual TCR chains, is only possible through

genetic KO studies. Our results indicate that TCR competitiveness and mispairing promiscuity are independent and TCR-intrinsic features, which both affect transgenic TCR surface expression. We could further reveal that mispairing promiscuity is not a digital but an analogous event. In the future, it will be interesting to investigate how these two factors are separately coded within the TCR sequence. Overall, our data underline the importance of endogenous TCR α- and β-chain KO to generate safer and more standardized TCR-redirected T cell products.

Second, we investigated the functional consequences of targeted placement under the endogenous promoter versus random integration in conjunction with the effect of different transgene copy numbers. We speculated that random integration of an undefined number of transgene copies might result in substantial variability in terms of editing efficiency, transgenic TCR expression, and function. In fact, it is well-known that VCN affects editing efficiency and transgene expression[85,133]. To systematically investigate this, we measured TCR surface expression, transgene integration site, and TCR transgene transcription on the single-cell level after conventional editing with a low or high virus MOI (multiplicity of infection), as well as after OTR (see chapter 3). Thereby, we could directly relate defined transgene integration via OTR to a more homogenous, physiological TCR transcription, and surface expression. In contrast, conventional editing resulted in largely variable TCR transcription and surface expression, especially after transduction with a low virus MOI. On the one hand, this might be explained by VCN heterogeneity between single cells[129]. On the other hand, differential accessibility of a transgene at a specific genomic locus should also have an impact on transgene transcription[173]. In this regard, variability should be amplified when only a single transgene integration took place (as with low virus MOI), whereas, upon multiple integrations, averaging effects can synchronize TCR expression (as with high virus MOI). This is ultimately reflected in the higher inter-donor variability of TCR surface expression in MOI^{lo} edited cells.

Most importantly, we have also demonstrated that the level of TCR surface expression directly affects functionality, which implicates that conventional editing with a low virus MOI generates T cell products of lower and variable function (see chapter 3). Such variability can be

decreased through transduction with a high virus load. However, the random integration pattern of conventional editing in combination with a high VCN, in turn, increases the risk of insertional oncogenesis as observed for two young patients with retroviral vector integrations in the proximity to the LMO2 proto-oncogene promoter[174]. Consequently, the use of high VCN for the generation of transgenic T cell products is discouraged by FDA and EMA[130].

Hence, we compared the functionality of OTR T cell products to Tx MOI^{lo}, as conventional editing with a copy number of one is generally regarded as being most relevant for clinical application[63,78,80] (see chapter 3). Concomitantly, controlling for a defined copy number standardizes the functional comparison between editing methods. This aspect has not been taken into account by previous studies (including our earlier study, see chapter 2), which reported that OTR T cells exhibit similar functionality *in vitro*[137,175] and similar or enhanced functionality *in vivo*[137,175]. Here, we show that all four tested A2/pp65-specific TCRs showed increased TCR surface expression and functionality when introduced via OTR compared to transduction with a low virus MOI. However, these observations are solely based on snapshot *in vitro* analyses. Therefore, it is of utmost importance that we could also document that TCRs placed under the endogenous promoter showed a more dynamic TCR regulation kinetic upon antigenic stimulation very similar to that of physiological T-cell populations, and distinct from transduced T cells (see chapter 2). This finding is in line with previously described regulation of TRAC-KI CARs which was related to better sustained long-term functionality in a xenograft *in vivo* tumor model[136]. Still, long-term *in vivo* protection experiments are needed to test whether OTR T cells show prolonged maintenance and enhanced protective capacity in comparison to conventionally edited T cells.

Third, we have demonstrated that OTR generates T cell products with defined, homogenous TCR surface expression, in sharp contrast to conventional editing. Furthermore, we could show that average surface expression on the population level affects the overall functionality of a T cell product. Based on these observations, we were wondering if increased cell-to-cell TCR expression variability as observed after conventional editing would also affect the predictability of *in vivo* T cell graft performance. Upon adoptive T cell transfer, not all

transferred T cells are 'recovered' and recruited into the immune response, as reflected by the 'recovery rate' in single-cell transfer experiments[176]. Hence, cell-to-cell variability in combination with a probabilistic recovery process could result in substantial inter-host functional variability of the very same T cell product. Indeed, using a novel polyclonal transfer system to study different color-barcoded TCRs side-by-side *in vivo*, we could show that the observed heterogeneity within conventionally edited T cell products introduces substantial T cell response variability. In contrast, OTR T cell products showed conserved T cell responses in different hosts (see chapter 3).

In summary, OTR facilitates the production of highly defined, homogenous TCR-redirected T cell products with an enhanced safety profile, physiologic TCR regulation as well as increased and more predictable functionality in comparison to conventional editing. Hence, OTR facilitates a more standardized and unbiased investigation of transgenic TCR function in basic research and also represents an improved way for the production of TCR-edited T cell products for clinical application.

4.2.2 Implications for clinical translation

A major challenge for the clinical application of TCR-edited T cells is the development of protocols for T cell graft production compliant with good manufacturing processes. In this regard, the reduced editing efficiency of OTR should not represent a major obstacle for clinical application. OTR T cells can be easily sorted with high purity and expanded. Furthermore, it has been shown extensively in preclinical models and clinical studies that low numbers of T cells can be highly functional when isolated with minimally manipulating reagents[42,43,45] and/or harboring a phenotype with high proliferative capacity[37,38,55]. Moreover, while we have used an electroporation system in a 96-well plate format for our experimental studies, electroporation can easily be scaled up. Lastly, CRISPR/Cas9-mediated HDR efficiencies are continuously being improved[177].

A strength of TCRs (e.g. in comparison to CARs) is a large number of available clinically relevant targets, such as pathogen-derived epitopes or neo-epitopes associated with tumor mutations. Consequently, manufacturing processes need to allow the engineering of an equally large number of different TCRs to exploit them therapeutically in a broad manner. This is not easily achieved with viral vectors but can be accomplished through non-viral CRISPR/Cas9-mediated KI of electroporated DNA templates[137]. A completely non-viral CRISPR/Cas9-mediated editing process is also more likely to be approved by regulatory authorities.

Regarding T cell product functionality, we have shown that OTR resulted in increased *in vitro* functionality compared to low virus dose transduced T cells. However, whether more physiological engineering via OTR also results in T cell products with 'better' *in vivo* protectivity is likely to depend on the clinical context. Testing of orthotopic TCR placement should therefore be performed with several different TCRs and in models that allow controlled investigation of long-term functionality. Such preclinical models are still mostly lacking for the investigation of human TCRs. However, orthotopic TCR α- and β-replacement enables the generation of defined cell products close to physiological T cells, which have already proven their functionality in a large number of clinical studies[21–23,31,39,52].

Therefore, we rather would like to propose viewing physiological T-cell engineering as a process that enables the generation of defined 'baseline' T-cell products, which can be further modified according to the specific clinical need (e.g. incorporation of a safety switch[178] or KO of additional genes[179]). Of note, we independently performed TLA and whole genome sequencing with overall three donors and five samples, and could not find evidence for exonic off-target insertions or translocations following double TRAC/TRBC editing. However, we did find some degree of homology-independent integration into the simultaneously edited TRBC locus, which will be particularly important to consider when alternative gene loci such as PDCD1 are additionally targeted.

Finally, a highly defined and homogenous T cell product that provides predictable *in vivo* function is of utmost importance for clinical application. Conventionally edited TCR-redirected

T cell products showed sometimes promising, yet overall rather variable therapeutic results[74,77,79,80]. This might be – at least to some extent – attributable to uncontrolled TCR transgene integration and variable VCN. It is therefore remarkable how little it has so far been acknowledged that through variable integration events, conventional editing introduces substantial variability both within and between T cell products. In contrast, OTR facilitates the production of highly defined and homogenous TCR-redirected T cell products with predictable *in vivo* function, thereby better conforming to the usual requirements of a medicinal product.

5 Conclusion

In this thesis, we developed a platform for reliable isolation of paired TCR α- and β-chains in conjunction with a high-throughput test system for highly sensitive assessment of TCR antigen-HLA specificity and functional avidity. We successfully utilized this platform for the identification of clinically relevant virus-specific TCRs in preparation for a planned clinical trial, i.e. ACT with TCR-redirected T cells for the treatment of post-transplant infections after allogeneic HSCT. Furthermore, we also demonstrate that this platform can be a powerful tool for high-throughput and reliable investigation of TCR function and biology. Since large-scale measurement of TCR function was so far a critical bottleneck, this platform represents a door opener for the investigation and definition of TCR functionality benchmarks characterizing optimal TCRs for clinical use.

Moreover, we systematically investigated the functional consequences of conventional TCR editing and novel CRISPR/Cas9-mediated OTR. We provide evidence for substantial functional differences originating from these different editing methods and demonstrate that OTR facilitates the production of TCR-redirected T cells with properties that closely resemble physiological T cells. Thereby, OTR enables the production of highly defined, homogenous T cell products resulting in more predictable *in vivo* functional responses.

In conclusion, we provide novel tools and insights for the educated selection of optimal TCRs and the production of safer, more functional, and more predictable TCR-edited T cell products. Together, this paves the way for the next generation of TCR-transgenic T cells for immunotherapy as well as for basic research.

List of abbreviations

AdV	Adenovirus
ACT	Adoptive T Cell Therapy
B-ALL	B-cell Acute Lymphoblastoid Leukemia
BFP	Blue Fluorescent Protein
CAR	Chimeric Antigen Receptor
Cas9	CRISPR-associated protein 9
CFP	Cyan Fluorescent Protein
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR-Ribonucleic Acid
DLBCL	Diffuse Large B-Cell Lymphoma
DLI	Donor Lymphocyte Infusion
EBV	Epstein-Barr Virus
EMA	European Medicines Agency
FACS	Fluorescence-Activated Cell Sorting
FDA	US Food & Drug Administration
GFP	Green Fluorescent Protein
gRNA	Guide-Ribonucleic Acid
GvHD	Graft-versus-Host Disease
GvL	Graft-versus-Leukemia
GvT	Graft-versus-Tumor
HDR	Homology-Directed Repair
HLA	Histocompatibility Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
HVGs	Highly Variable Genes
IFNg	Inteferon gamma
IL-2	Interleukin-2

KI	Knock-in
KO	Knock-out
LTDL	Adenovirus A1/Hexon5-derived peptide fragment LTDLGQNLLY
MOI	Multiplicity Of Infection
NFAT	Nuclear Factor of Activated T-cells
NFkB	Nuclear Factor 'Kappa-light-chain-enhancer' of activated B-cells
NGS	Next Generation Sequencing
NLV	Human Cytomegalovirus A2/pp65-derived peptide fragment NLVPMVATV
OTR	Orthotopic TCR Replacement
P2A	Porcine Teschovirus-1 2A
PACT	Prophylactic Application of Donor-derived TCM After Allogeneic HSCT
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pMHC	peptide-Major Histocompatibility Complex
RNP	Ribonucleoprotein
RT	Room Temperature
scRNA seq	single-cell RNA sequencing
TCR	T Cell Receptor
TIL	Tumor-Infiltrating Lymphocyte
TLA	Targeted Locus Amplification
TNFa	Tumor Necrosis Factor alpha
TPR	Triple Parameter Reporter
TRAC	T cell Receptor Alpha Constant region
TRBC	T cell Receptor Beta Constant region
VCN	Vector Copy Number
YFP	Yellow Fluorescent Protein

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