Aus dem Institut

Berliner Institut für Gesundheitsforschung
- Zentrum für Regenerative Therapien

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Characteristics of Rapamycin-treated T cell products for advanced adoptive T cell therapy and evaluation of clinical feasibility

zur Erlangung des akademischen Grades

Doctor of Philosophy (PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Leila Amini

aus Heide

Datum der Promotion: 18.09.2020

This thesis summarizes the background, principles of the applied techniques, main results, potential of clinical application and further scientific questions raised based on the publication "Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients" published in Frontiers in Immunology (2019).

1. Abstracts (Zusammenfassung)

1.1 Zusammenfassung

In der soliden Organtransplantation ist die Langzeitimmunsuppression eine Voraussetzung, um Abstoßungsreaktionen zu verhindern, prädisponiert allerdings für schwer oder tödlich verlaufende Viruserkrankungen z.B. hervorgerufen durch das Cytomegalievirus (CMV). Die klassische antivirale Medikation ist oftmals problematisch bzw. ineffektiv. Die virusspezifische adoptive T-Zell-Therapie hat sich daher als eine attraktive therapeutische Option zur Behandlung viraler Erkrankungen nach Transplantation herausgestellt. Trotz der klinischen Sicherheit der antiviralen T-Zell-Produkte und einer kurzzeitig beeindruckenden Wirksamkeit, ist die Langzeiteffektivität in Organtransplantierten häufig eingeschränkt. Dies könnte seine Ursache in der kurzzeitigen Persistenz der transferierten T-Zellen haben, was eventuell mit deren späten Differenzierungsstatus assoziiert sein könnte. Eine Inhibition des *mechanistic-Target-of-Rapamycin*-(mTOR)-Signalwegs mittels Rapamycin reguliert die Gedächtnis-T-Zell-Differenzierung. Diese Strategie wurde durch unsere Arbeitsgruppe zur Verbesserung der Zusammensetzung von T-Zell-Produkten durch Anreicherung frühdifferenzierter zentraler Gedächtnis-T-Zellen sowie CD4$^+$ T-Zellen in unser klinisches Protokoll zur Herstellung antiviraler T-Zell-Produkte integriert. Präklinische und klinische Daten implizieren, dass dies die Langzeitwirkung adoptiver T-Zell-Therapie verstärken kann.

In der vorliegenden Arbeit war es das Ziel, in *in-vitro*-Versuchen Hinweise für eine Bestätigung dieser Hypothese mittels molekularer Charakterisierung von CMV-spezifischen Rapamycin-behandelten (Rapa-)T-Zell-Produkten zu finden, den zu Grunde liegenden Mechanismus genauer zu beschreiben, sowie die Übertragbarkeit des Produktionsprozesses auf Patientenmaterial zu untersuchen.

Rapamycin-Behandlung induzierte eine bevorzugte Expansion und verminderte Differenzierung virusspezifischer zentraler Gedächtnis-T-Zellen sowie eine vermehrte virusspezifische Zytokinproduktion weiter differenzierter CD8$^+$ Gedächtnis-T-Zellen. Darüber hinaus führte die Rapamycin-Behandlung zu einer verbesserten Vitalität von T-Zellen u.a. nach Induktion von Apoptose und Einfrier-/Auftauprozessen, welche für eine klinische Applikation nötig sind. Dies könnte durch eine erhöhte Proteinmenge von Bcl-2 vermittelt sein. Bei der Sequenzierung der Ribonukleinsäure aus Rapa-T-Zell-Produkten wurde ein erfolgsversprechendes Transkriptom identifiziert. Des Weiteren zeigten Rapa-T-Zell-Produkte bei CMV-spezifischer Aktivierung einen stabileren Metabolismus. Außerdem verfügten Rapa-T-Zell-Produkte über ein diverseres Repertoire an T-Zell-Rezeptoren, was eine Immunevasion minimieren könnte.

Darüber hinaus konnten unbehandelte und Rapa-T-Zell-Produkte aus Proben von Patienten im Endstadium renaler Erkrankungen vor Lebendspende/Transplantation einer Niere sowie

derselben Patienten nach Nierentransplantation (unter Immunsuppression) generiert werden. Die Rapa-T-Zell-Produkte, die aus Proben vor und nach Transplantation generiert wurden, zeigten untereinander vergleichbare vorteilhafte Ergebnisse. Die Komposition dieser Produkte ähnelte derer von Rapa-T-Zellprodukten gesunder Spender. Des Weiteren wurden erfolgreich Rapa-T-Zell-Produkte von Patienten mit unterschiedlichem CMV-Reaktivierungsstatus generiert, wobei sich die Gruppe mit kurz zurückliegender CMV-Reaktivierung als sensitive Zielpopulation herausstellte und weitergehende Untersuchungen benötigt.

Die Ergebnisse implizieren, dass Rapamycin die Langlebigkeit und Funktionalität von antiviralen T-Zell-Produkten verbessert. Dies muss nun ultimativ in klinischen Prüfungen bestätigt werden. Eine Übertragung auf andere klinische Effektor-T-Zell-Produkte könnte insbesondere auch bei Anwendungen in der Onkologie eine Optimierung der Langzeitwirkung erzielen.

1.2 Abstract

The requirement for long-term immunosuppression to prevent rejections predisposes solid organ transplant recipients to severe or fatal viral complications, *e.g.* caused by cytomegalovirus (CMV). Classical antiviral medication is often problematic or ineffective. Hence, virus-specific adoptive T cell therapy emerged as attractive therapeutic option for viral diseases occurring after transplantation. Despite clinical safety of antiviral T cell products (TCPs) and impressive initial effectiveness, long-term efficacy is frequently limited in SOT recipients. This might originate in the short-term persistence of transferred T cells, which may be associated with a late differentiation state. Inhibition of the mechanistic-Target-of-Rapamycin-(mTOR)-pathway by Rapamycin regulates memory T cell differentiation and was integrated into our clinical protocol for the manufacture of virus-specific TCPs. Thereby, we optimized the T cell subset composition, yielding enriched proportions of early differentiated central-memory (T_{CM}) and CD4$^+$ T cells. Pre-clinical and clinical data imply this to enhance long-term efficacy of adoptive T cell therapy.

The aim of the present study was to find evidence for this hypothesis in *in vitro* experiments by detailed molecular characterization of CMV-specific Rapamycin-treated (Rapa-)TCPs to thoroughly describe the underlying mechanism and to investigate transferability of the manufacturing process to patient samples.

Rapamycin-treatment induced preferential expansion and reduced differentiation of virus-specific T_{CM} as well as increasing virus-specific cytokine production of further differentiated CD8$^+$ T cells. Moreover, Rapamycin-treatment resulted in enhanced T cell vitality *inter alia* in apoptosis-inducing conditions and even after freezing/thawing processes, which are required for clinical application. This may be mediated by increased levels of Bcl-2 protein. RNA sequencing revealed a beneficial transcriptome of Rapa-TCPs. Furthermore, metabolic analysis disclosed Rapa-TCPs to display a more stable metabolism upon CMV-specific activation. Moreover, Rapa-TCPs exhibited a more diverse T cell receptor repertoire, which minimizes potential viral antigen escape.

In addition, Rapa-TCP protocol applicability to paired samples from end-stage renal disease patients awaiting living-donor kidney transplantation (KTx) and the same patients after KTx and initiation of immunosuppression was illustrated. These Rapa-TCPs (before/after KTx) showed comparable characteristics, which were similar to Rapa-TCPs from healthy donors. Moreover, Rapa-TCPs were successfully generated from KTx patients with different states of CMV reactivation, identifying patients with recent CMV DNAemia as sensitive target population needing further investigations.

The results imply Rapamycin to improve longevity and performance of antiviral TCPs and now await ultimate clinical proof in trials. Translation to other clinically used effector TCPs, may be extremely useful and achieve optimization of long-term efficacy especially for applications in oncology.

2. Current state of research

2.1 Transplantation immunology and prevention of rejections

Chronic organ dysfunction can lead to irreversible end-stage organ failure. In the past century, allogenic solid organ transplantation (SOT) was implemented to replace failed organs and indeed could save millions of lives. However, many limitations including lifelong immunosuppression of the patients and its associated complications, *e.g.* viral disease, could still not be overcome.

Georg Schöne was the first transplantation immunologist, who observed rejection and even accelerated rejection of repeated skin allografts[1], thereby discovering immunological memory in the context of allogenic transplantation. Only a few years later, the lymphoid system was associated with the rejection of allogenic transplants.[2] The first successful human organ transplantation took place between genetically identical twins in 1954[3]. In 1958, the underlying reason for allogenic rejections, constituted by the inter-individually distinct composition of highly variable human leukocyte antigens (HLAs), also named major histocompatibility complexes (MHCs), was discovered[4]. First approaches to target the immune system to prevent allograft rejection were total body irradiations, some of which truly resulted in organ function of transplants from related donors[5], yet only the use of a combination of azathioprine and prednisone meant a breakthrough and significantly decreased loss of organs by rejection and improved organ functionality[6]. Indeed, until today, a similar drug combination is used as the classical immunosuppressive regimen of triple immunosuppression, which includes corticosteroids, mycophenolic acid and Calcineurin inhibitors, in the SOT setting. Mycophenolic acid is a proliferation inhibitor, which inhibits DNA synthesis. Calcineurin inhibitors (*e.g.* Tacrolimus [FK506]) prevent dephosphorylation of nuclear factor of activated T cells (NFAT) by Calcineurin *via* binding to their respective cell intrinsic immunophilin. Thereby, they inhibit T cell activation and function. Glucocorticoids induce among other effects lymphocyte apoptosis, diminish pro-inflammatory cytokines and enhance anti-inflammatory cytokines *via* their respective receptor. The resulting immunosuppression leads to systemic inhibition of the entire adaptive immune system, not specifically targeting alloreactive cells, which are responsible for organ rejection. Hence, complications with normally harmless chronic infections such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) or BK virus, whose control requires the adaptive immune system, can occur in some patients, despite a carefully balanced dosing. In principle, there are three therapeutic options in the case of viral complications: 1) to reduce immunosuppression, which is frequently associated with graft rejection or loss, 2) to target the viral replication machinery, which is often toxic and only partially successful (classical antiviral medication) and 3) to selectively reconstitute the immune response, *e.g.* by adoptive T cell therapy.

2.2 Cytomegalovirus in kidney transplantation

In the following, I will focus on CMV, because this virus was used as a model for the present investigation. CMV disease is a life-threatening complication in SOT recipients. In addition to direct morbidity, inducing colitis, pneumonia and retinitis, CMV can trigger indirect morbidity such as rejection of the transplanted organ or chronic allograft nephropathy in the kidney transplant (KTx) setting[7]. Before development of potent anti-viral medication, up to 1/3 of seronegative KTx recipients (R$^-$) receiving a seropositive graft (D$^+$) were affected by CMV disease[8]. Today, anti-viral therapy of these high-risk patients involves prophylaxis or preemptive therapy, most commonly with intravenous Ganciclovir or oral Valganciclovir[9, 10], though a delicate dosing is crucial to prevent toxicity, *e.g.* neutropenia, and the development of viral resistance[11]. If resistance is acquired, a variety of other drugs is available[12], however many of these are nephrotoxic[13, 14] and cross-resistance between different drugs is common[11]. Despite prophylaxis, a high frequency of late-onset CMV disease (19.2% in D$^+$R$^-$ constellations, 21.5% in D$^+$R$^+$, 7% in D$^-$R$^+$ and 2.5% D$^-$R$^-$) within the first year after discontinuation of prophylaxis was observed in a cohort of 206 KTx recipients[15]. With 2.2% total mortality one year after transplantation, the mortality is strikingly 10-fold higher in KTx recipients with CMV infection compared to uninfected KTx recipients[15], demonstrating the need for improvement of CMV management in the KTx setting.

The T cell-mediated anti-CMV immune response was reported to be predictive for late-onset CMV disease after discontinuation of prophylaxis[16] and monitoring of CMV-specific T cells allows patient stratification[17]. Thus, regeneration of the T cell response as aspired by adoptive anti-viral T cell therapy approaches may prevent or diminish CMV disease, associated indirect morbidities and mortality in the SOT setting.

2.3 Challenges and progress of adoptive T cell therapy in solid organ transplantation

Anti-viral T cell therapy requires the enrichment or isolation of virus-specific T cells from peripheral blood, followed by their activation and expansion and finally the infusion into a patient. Initially, this approach was established in the hematologic stem cell transplant (HSCT) setting in the early 90s[18], which stimulated the development of diverse protocols and clinical investigations in HSCT recipients[19, 20, 21]. Certainly, the SOT setting is much more challenging than HSCT, among other reasons, due to the absence of the availability of the healthy HSCT donor as a cell source. This means that at least in an autologous setting, T cells need to be isolated from an immunosuppressed patient. Furthermore, in HSCT, complete lymphodepletion is a standard therapy, which leads to a completely new establishment of the entire lymphatic system, leaving space for the expansion and engraftment of the transferred T cells. Nevertheless, there are successful approaches for anti-viral T cell therapy in the SOT

setting, mainly concentrating on the therapy of post transplantation lymphoproliferative disease (PTLD) caused by EBV and achieving up to 52% responsiveness at 6 months after T cell infusion[22, 23]. Adoptive T cell therapy was comparably effective in treatment of chronically active EBV disease patients[24]. Indeed, there are few successful case reports for treatment of CMV disease with adoptive T cell transfer[25, 26, 27, 28]. However, especially in CMV disease, limited persistence and longevity of the TCP are challenging issues, although an initially dramatic reduction of symptoms and viral load can be achieved[27]. Therefore, the existing clinical protocol for the manufacture of autologous anti-viral TCPs from our institute[27] was optimized regarding the proportion of long-lived central memory T cells (T_{CM}) by low-dose inhibition of the mechanistic target of Rapamycin (mTOR)-pathway[29] based on findings from a murine study[30].

2.4   T cell memory subsets and their importance for T cell product composition

The T cell memory subset composition of the TCP is supposedly of major importance for efficacy, due to the distinct properties of the individual subsets (summarized in Figure 1). The different human T cell memory subsets are characterized by their unique expression patterns of CCR7, CD62L, CD45RA, CD45RO and CD95. The terminally differentiated and short-lived CD45RA-expressing effector memory T cells (T_{EMRA}) are characterized by immense cytokine and effector molecule production[31]. Effector memory T cells (T_{EM}), characterized by being CCR7$^-$ CD62L$^-$ CD45RA$^-$ CD45RO$^+$, exert massive immediate effector function, but have limited proliferative potential and polyfunctional capacity[32, 33]. CCR7$^+$ CD62L$^+$ CD45RO$^+$ CD45RA$^-$ T_{CM} possess high proliferative potential, co-express multiple cytokines and effector molecules, retain immediate effector function and maintain the capability to self-renew[32, 33, 34]. The recently described memory stem T cells (T_{SCM}) are rare, CCR7$^+$ CD62L$^+$ CD45RO$^-$ CD45RA$^+$ CD95$^+$, but have the highest polyfunctional, proliferative and self-renewal capacity[35, 36]. Phenotypically, T_{SCM} only deviate from antigen-inexperienced naïve T cells (T_N) regarding the expression of CD95. T_{CM}, which are enriched in the next generation TCP[29] by low dose Rapamycin-treatment, were reported to show better engraftment and survival compared to further differentiated T cell memory subsets in preclinical murine and primate animal models[34, 37, 38]. Moreover, the reconstitution of T_{CM}-mediated anti-CMV immune responses in HSCT recipients was associated with the prevention of CMV reactivation[39]. According to clinical studies, the proportion of T_{CM} in TCPs correlated with persistence[40] and T_{CM}-derived cells self-renewed in human patients[41]. Importantly, the Rapamycin-treated (Rapa-)TCP contains a higher proportion of CD4$^+$ T cells[29], which are important in control of chronic viral infections[42]. CD4$^+$ T cells were shown to clinically improve efficacy of anti-viral T cell therapy[43] and are

associated with longer persistence and increased expansion of adoptively transferred T cells in patients[40, 44].

	T_{SCM}	T_{CM}	T_{EM}	T_{EMRA}
phenotype	CCR7$^+$ CD45RA$^+$ CD95$^+$ CD62L$^+$ CD45RO$^-$	CCR7$^+$ CD45RA$^-$ CD95$^+$ CD62L$^+$ CD45RO$^+$	CCR7$^-$ CD45RA$^-$ CD95$^+$ CD62L$^-$ CD45RO$^+$	CCR7$^-$ CD45RA$^+$ CD95$^+$ CD62L$^-$ CD45RO$^-$
differentiation state	+	++	+++	++++
cytokines & effector molecules	+	++	+++	++++
polyfunctionality (multiple cytokines/ effector molecules)	++++	+++	++	+
immediate effector function	++	++	+++	++++
proliferative potential	++++	+++	+/-	-
self-renewal capacity	++++	++	-	-
engraftment/ persistence *in vivo*	++++	+++	+/-	-

Figure 1: Characteristics of different T cell memory subsets.

2.5   Hypothesis, aims and the present study

These studies imply the hypothesis that Rapa-TCPs may confer enhanced long-term efficacy *in vivo*. The aim of the present study was to find evidence for this hypothesis by detailed molecular characterization of the properties of Rapa-TCPs *in vitro*, to comprehensively describe the underlying mechanism and to transfer the manufacturing process to samples from KTx patients for investigation of feasibility and potential identification of risk factors.

The present study deeply characterizes the Rapa-TCP derived from healthy donors on molecular level revealing enhanced survival and partial resistance to apoptosis, beneficial gene expression, a more stable metabolism upon specific activation, increased clonal diversity and preferred phenotype. Additionally, the manufacturing process is applied to samples from 19 KTx patients and applicability is demonstrated reflected by similar properties of the resulting TCPs compared to TCPs derived from healthy donors expanded in parallel. Moreover, comparative analysis of samples collected before and after KTx shows no advantage of blood collection before KTx despite the ongoing immunosuppression after KTx.

3. In-depth methods

In the following, the underlying principles of the methods used for the present study are explained. For detailed explanation of materials, exact procedures and analyses, please refer to the methods section of the attached publication (see → *10. Publication "Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients"*).

3.1 Isolation of peripheral blood mononuclear cells

The starting material for all assays presented were peripheral blood mononuclear cells (PBMCs), which were isolated from heparinized venous blood using Biocoll separating solution density gradient centrifugation (Biochrom). This method relies on the different densities of specific blood cell populations, which are separated by a polymer with distinct density, and the aggregation of erythrocytes leading to pellet formation upon centrifugation. Right above this pellet granulocytes accumulate, which are adjacent to the Biocoll polymer. The PBMCs, *i.e.* lymphocytes and monocytes/macrophages, form a thin layer at the interface of the polymer and the blood plasma, which can be carefully harvested.

3.2 Generation of virus-specific T cell products

TCPs and T cell cultures presented in the publication were all based on isolation of CMV-specific T cells using the IFNγ secretion assay – cell enrichment and detection kit (Miltenyi Biotec). This relies on specific stimulation of T cells by antigen presenting cells (APCs) from the PBMCs, which are mainly represented by monocytes/macrophages and B cells. These present peptides, which are added to the PBMCs after resting overnight, on their MHC molecules. Overlapping peptide pools (JPT) spanning the whole immunodominant antigens CMV_{IE-1} and CMV_{pp65} were used for CMV-specific stimulation. In turn, memory T cells with specific T cell receptors (TCRs) were activated within a few hours and produced cytokines, *e.g.* IFNγ, which is important for the immune response to viruses. After 6 h of stimulation in a humidified incubator at 37 °C and 5% CO_2, the PBMCs were subsequently cooled and coated with bispecific antibodies binding CD45 and IFNγ for the IFNγ secretion assay. Then, these cells were re-transferred into an incubator to allow the IFNγ secretion of activated T cells. This cytokine was captured on the respective cell surface directly after secretion by the bispecific antibody and thereby antigen-specific T cells were identified using a further PE-bound antibody specific to a different epitope of IFNγ. For the isolation of these cells, antibodies specific to PE coupled to magnetic beads were used in a magnetically activated cell sorting (MACS) procedure, capturing the labelled cells in columns placed into a magnetic field and releasing them after several washing steps by removing the column from the magnetic field. This positive

fraction was the starting population for all CMV-specific T cell cultures and was cultured together with 1/5 of the irradiated negative fraction including the CMV-peptide loaded APCs as feeder cells for a prolonged stimulation of the CMV-specific T cells during the first days of culture. Within a few days, the irradiated feeder cells were overgrown by CMV-specific T cells.

3.3 **CMV-specific stimulations of PBMCs** *ex vivo*

The principle explained in 3.2 regarding antigen-specific stimulation with peptide pools presented on MHC molecules of APCs abundant in PBMCs was also applied for the *ex vivo* stimulation of PBMCs to determine the memory phenotype of the starting material derived from end-stage renal disease patients awaiting KTx, KTx recipients after transplantation and healthy donors. In the case of *ex vivo* stimulation, the Golgi apparatus inhibitor Brefeldin A (BFA) was added after the first hour of stimulation, to capture the cytokines produced upon the activation of memory T cells inside the cells, preventing their secretion and allowing for intracellular staining with fluorescently labelled antibodies and their detection by flow cytometry.

3.4 **CMV-specific stimulation of T cell products and cultures**

CMV-specific stimulation of cultured T cells was achieved with the help of lymphoblastoid cell lines (LCLs) derived from B cells immortalized with EBV serving as antigen presenting cells, which were loaded with CMV-specific peptide pools and added to the cultured T cells. Unloaded LCLs served as internal control to exclude T cell activation due to presentation of EBV epitopes or unspecific activation. Like for *ex vivo* stimulations, BFA was added after the first hour of stimulation to allow for intracellular staining of accumulating cytokines.

3.5 **Determination of vital T cells and their sensitivity to Fas-induced apoptosis**

Dead cells lose membrane integrity and thus can be stained by fluorescent dyes, which can only penetrate the cell surface upon cellular death (Live/dead discriminating dyes). Apoptotic cells expose phosphatidylserine, which is an 'eat-me' signal for phagocytes, on the outer leaflet of their membrane, which can be stained by fluorescently labeled Annexin V. Hence, vital cells could be identified in flow cytometry as T cells being double negative for live/dead cell stain and Annexin V staining.

Activation of Fas, which is also called death receptor, by an activating antibody induces the extrinsic pathway of apoptosis activating Caspase 8 leading to programmed cell death and consequently resulting in the self-destruction of the cell and consequential apoptotic body formation. This method was employed for defining T cell sensitivity to apoptosis by determining the number of vital T cells after incubation with Fas-activating antibody.

3.6   Killing assays

Killing capacity of TCPs was analyzed based on the specific killing of CMV peptide-loaded autologous LCLs as opposed to leaving allogenic LCLs untouched. CMV peptide pool-loaded autologous LCLs (targets) and allogenic LCLs (non-targets) were differentially labelled with fluorescent dyes detectable by flow cytometry and mixed at a fixed ratio. Distinct amounts of T cells were added and killing was calculated from the reduced ratio of target to non-target LCLs. Conditions excluding T cells served as an internal reference.

3.7   Flow cytometry

The majority of parameters presented, including T cell subsets, CMV-specific cytokine producers, living T cells, Bcl-2 protein levels and target cell killing were determined by fluorescent dyes or fluorescently-labelled antibodies detected by flow cytometry using a LSR II Fortessa flow cytometer (BD). Within the flow cytometer, cells are singularized and pass by different lasers, which exert the fluorescent dyes in turn emitting electromagnetic waves. These are then filtered or reflected by a sophisticated filter system letting specific ranges of wavelengths pass and allowing the respective detectors to detect the specific emissions. The signal is amplified by photomultiplier tubes (PMTs), whose intensity can be modified by adjustment of the voltage. Potential spectral overlap was reduced by mathematically subtracting the overlapping signal from a different fluorophore (compensation).

3.8   Fluorescently activated cell sorting

Fluorescently activated cell sorting also uses a flow cytometer, here the Aria II Calliope (BD), which contains electrodes in addition to the components described above. The electrodes can polarize single droplets of cells based on the obtained emission signals from the fluorescence detectors. Depending on the polarization, the droplet containing the cell is sorted in an electromagnetic field into the respective fraction indicated by the operator.

3.9   Metabolic analyses

For metabolic analyses, the Seahorse technology (Agilent) was applied, which is based on fluorophore sensors detecting H^+ ions, indicating the extracellular acidification rate and another set of fluorophore sensors detecting O_2, from which the oxygen consumption rate can be calculated. Changes in the respective concentrations of H^+ and O_2 influence the light emitted by fluorophore sensors upon their excitation by the Seahorse device. The extracellular acidification rate reflects the conversion of glucose to lactate$^-$ and H^+, which happens *e.g.* during glycolysis. The oxygen consumption rate mirrors the rate of oxidative phosphorylation. The sensors must be placed in close proximity to the seeded cells. To make the measurement

possible for non-adherent cells, *i.e.* T cells, the cells were fixed at the bottom of the well using Cell-Tak (Corning).

3.10   RNA sequencing

RNA sequencing reveals the whole transcriptome of the cells giving clues about ongoing processes and active signaling pathways. To this end, whole RNA was extracted from cell lysates. After removal of ribosomal RNA, the remaining RNA was reversely transcribed to cDNA using random primers and the RNA strand replaced by DNA. Then adapters, indexes and primer binding sites were ligated to the cDNA, which was in turn amplified in bridge amplification cycles. The next generation sequencing was performed using Illumina technology: Each nucleotide was labelled in a specific fluorescent color preventing addition of further nucleotides. Thus, in each round one single nucleotide was added and the fluorescent color was determined at every position. In the following step, the label was cleaved by an enzyme allowing for addition of the next nucleotide, which in turn was analyzed by detection of fluorescence, *etc*.

The obtained sequences underwent quality control to exclude biased results. Then the sequence bits were annotated to a human genome. Here, differentially expressed genes between Rapa- and untreated TCPs were determined, samples were clustered and principle component (PC) analysis was performed by algorithmic analysis in R (R Core Team).

3.11   TCRβ sequencing

The T cell receptor (TCR) is the tool allowing T cells to mount immune reactions in response to specific peptide antigens presented on MHC molecules. The diversity of TCRs is created by somatic V(D)J recombination of the TCR gene. In classical ($\alpha\beta$) T cells, the TCR consists of an α and a β chain, which are generated by V-J and V-D-J recombination, respectively. Upon joining of the different elements, nucleotides are removed or inserted randomly, resulting in a unique genetic sequence of every T cell clone. The complementary determining region (CDR) 3 of the TCR β chain is spanning recombination sites of V, D and J elements, thereby constituting the most variable part of the TCR. Thus, sequencing of this specific part reflects the clonal composition of a T cell sample. For TCRβ sequencing, DNA was extracted from T cell samples and processed using an immunoSEQ TCR β kit (Adaptive Biotechnologies), which specifically amplifies the CDR3 of the TCR β chain. These fragments were then ligated to adapters and sequenced as described for the RNA sequencing using the Illumina technology. The clonal composition of TCPs was analyzed based on the numbers and frequencies of CDR3 sequences resulting in expression of functional TCR (productive sequences).

4. Essential results

During my PhD project, I focused on the characterization of a newly developed TCP for antiviral T cell therapy in solid organ transplant patients, which employs low dose mTOR inhibition to rejuvenate the differentiation state of TCPs[29]. The present experiments confirm the hypothesis that Rapa-TCPs are long-lived and the *in vitro* data suggest sustained function and beneficial composition as well as applicability to different KTx patient groups. Furthermore, risk factors which require consideration in clinical trials and further investigations were identified. The experimental setup including all analyses is illustrated in Figure 2. First, a detailed and extensive molecular characterization of the TCP generated from healthy donors was in focus. In the second part, the clinical applicability was emphasized by the use of patient material and comparison to TCPs of healthy donors generated in parallel.

healthy donors

(pre-/post-)KTx patients + healthy controls

Ex vivo d0:
  ➢ T_{CM}/T_{EM} sorts for transcriptome analysis

Ex vivo d0:
  ➢ phenotype
  ➢ cytokine production

TCP d14:
  ➢ phenotype
  ➢ cytokine production
  ➢ restimulation
  ➢ Rapamycin withdrawal
  ➢ different cytokine media

TCP d14:
  ➢ phenotype
  ➢ cytokine production

TCP d18:
  ➢ T_{CM} sort for transcriptome analysis
  ➢ transcriptome analysis

TCP d21:
  ➢ phenotype
  ➢ cytokine production
  ➢ different cytokine media
  ➢ Survival
  ➢ sensitivity to apoptosis
  ➢ Bcl-2 protein level
  ➢ transcriptome analysis
  ➢ clonal composition analysis
  ➢ metabolic analysis
  ➢ isolated memory T cell subset analysis

TCP d21:
  ➢ phenotype
  ➢ cytokine production
  ➢ killing
  ➢ survival
  ➢ freezing/thawing
  ➢ Bcl-2
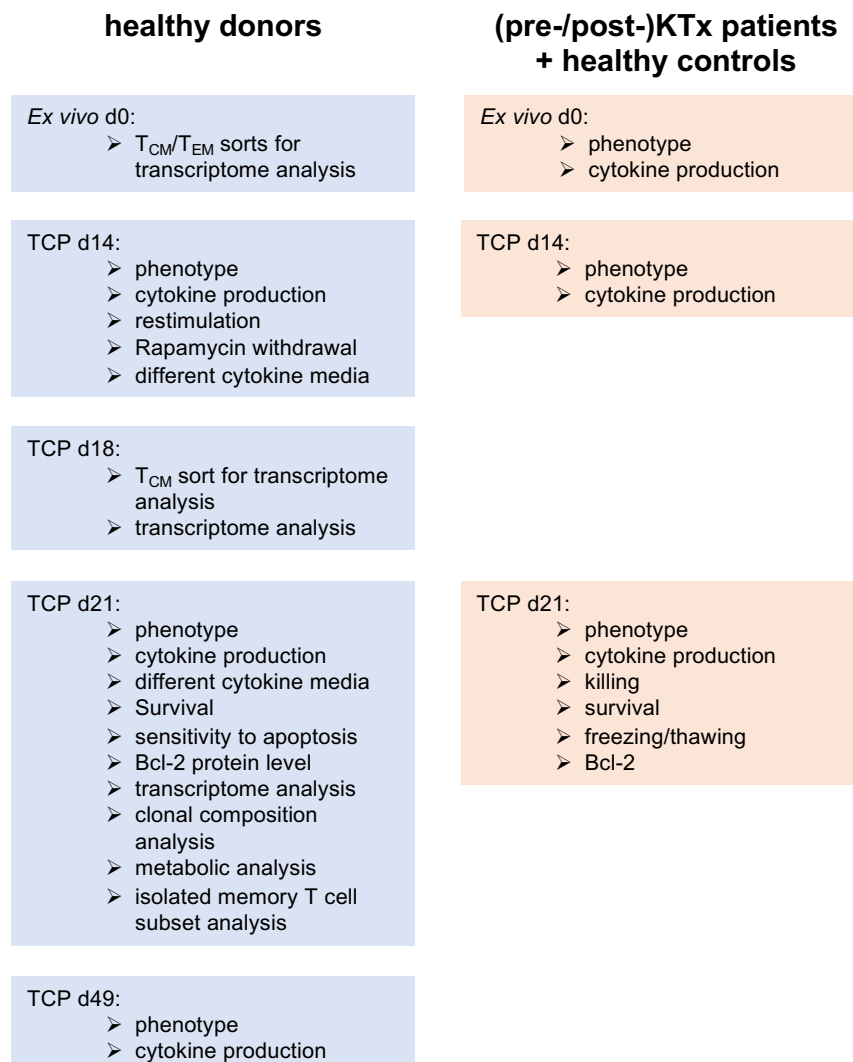
TCP d49:
  ➢ phenotype
  ➢ cytokine production

Figure 2: Experimental setup and analyses.

4.1 Sustained expansion and cytokine production in long-term cultures, enhanced vitality and stabilized metabolism conferred by Rapamycin

The main advantages of the next generation virus-specific Rapa-TCP were described as an increased proportion of CD4$^+$ T cells and CD4$^+$ and CD8$^+$ T$_{CM}$[29], which was confirmed using different cytokine combinations. Thereby, it was demonstrated, that the effect of Rapamycin on the TCPs was not dependent on a certain cytokine combination.

Prolonged culture until d21 revealed recovery of expansion rates of Rapa-TCPs in the 3rd week, which were significantly lower than in conventionally expanded TCPs until d14. Interestingly, once treated with Rapamycin during the first two weeks of expansion, TCPs contained more CMV-specific cytokine producers even after 7 weeks of expansion and withdrawal of Rapamycin since d14.

Strikingly, the next generation Rapa-TCP showed significantly increased survival rates and partial resistance to Fas-induced apoptosis, probably mediated by the increased protein levels of the anti-apoptotic protein Bcl-2.

Metabolic analysis revealed significantly lower OCR/ECAR ratios in Rapa-TCPs, however, upon CMV-specific activation, the metabolism was more stable in Rapa- compared to untreated TCPs.

4.2 Enhancement of T$_{CM}$ and effector function in later differentiated memory T cell subsets by Rapamycin

Isolated cultivation of different virus-specific memory T cell subsets, *i.e.* T$_{CM}$, T$_{EM}$ and T$_{EMRA}$, revealed preferential expansion and significantly reduced differentiation of T$_{CM}$. Rapamycin treatment increased Bcl-2 protein levels in virus-specific T$_{CM}$- and T$_{EM}$-derived cultures and increased the proportion of CMV-specific IFNγ-producers among CD8$^+$ T cells in T$_{EM}$- and T$_{EMRA}$-derived cultures. These findings imply distinct effects on the different memory T cell subsets, which suggest proliferative advantages, long-lasting memory and increased functionality of Rapa-TCPs.

4.3 Beneficial transcriptome and enhanced clonal diversity in Rapamycin-treated T cell products

Transcriptome analysis by RNA sequencing confirmed the results regarding phenotype, increased survival and enhanced functionality on RNA expression level. A majority of the differentially expressed genes identified upon comparison of Rapa- and untreated TCPs could be annotated to processes relevant for TCP performance, of which 84% were actually regulated in a beneficial manner in Rapa-TCPs. Rapa-TCPs clustered with T$_{CM}$-like cells isolated from Rapa-TCPs and untreated TCPs on d18 of culture, confirming a state of

comparatively early differentiation. In line with this, PC analysis showed Rapa-TCPs to cluster with *ex vivo* isolated T_{CM} rather than T_{EM} regarding PC2.

Analysis of clonal composition by TCR sequencing disclosed that Rapa-TCPs comprised a more diverse and balanced TCR repertoire. This may improve viral control by prevention of viral immune escape, which can occur by mutating relevant epitopes if the immune response is exclusive to very specific epitopes.

4.4   Applicability of Rapamycin-treatment to patient samples and identification of risk factors

To investigate applicability to patient samples and determine whether sample collection before organ transplantation and establishment of immunosuppression would be favorable, untreated and Rapa-TCPs from paired samples of seven patients in end-stage renal disease before and a few weeks after KTx were successfully manufactured. The data demonstrate that antiviral (Rapa-)TCP manufacture is feasible for both conditions despite the substantial differences, *e.g.* medication with immunosuppressive drugs and dialysis. Manufacture before KTx and onset of immunosuppression did not reveal any substantial advantages and TCPs generated before and after KTx showed comparable features.

Investigations of untreated and Rapa-TCPs generated from 19 KTx recipients with different states of CMV reactivations identified patients with a record of CMV viremia to be a sensitive group, whose TCPs yielded lower cell numbers than TCPs from healthy donors. Especially the group with recent CMV DNAemia requires further investigations including more patients or requires precautious measures such as adaptation of the amount of the starting material. This is underlined by the negative correlation between the number of CMV DNAemia records and the yield of the TCP. In line with this, correlation analysis showed a negative correlation between the age of the donor and yield of the Rapa-TCPs.

Furthermore, data from one patient suggest that the application of anti-thymocyte globulin (ATG) is problematic and as ATG predisposes for viral complications, blood collection for TCP generation should be recommended before ATG application. Strikingly, TCPs generated from patients with a high degree of terminal differentiation among their CMV-specific T cells did still show substantial amounts of T_{CM} in their Rapa-TCPs, suggesting partial reprogramming of further differentiated memory T cell subsets and applicability even to this specific condition. The hypothesis of partial reprogramming of late differentiated cells is also supported by the isolated cultures of virus-specific memory T cell subsets, in which this phenomenon was also observed for some donors.

Taken together, the data regarding the Rapa-TCP reveal promising advantages (Table 1) and now request ultimate clinical confirmation. Importantly, certain subgroups of patients should be investigated more closely or treated with caution in a clinical setting.

Table 1: Main results of the comparative analysis of conventional and Rapa-TCPs.

parameters	Rapa-TCPs	conventional TCPs
expansion (until week 2)	reduced	increased
expansion (after week 2)	recovered	slows down
proportion of T_{CM}	increased	very low
proportion of CD4$^+$ T cells	enhanced	very low
cytokine production	increased	satisfactory
vitality	increased	satisfactory
freezing/thawing	increased viability	lower viability
Fas-induced apoptosis	partial resistance	sensitive
Bcl-2 protein levels	elevated	reduced
metabolism	stable	switches upon activation
diversity of TCR repertoire	increased	more clonal
transcriptome	longevity, functionality	short lived, less functional

5. Clinical applications and outlook

The *in vitro* data presented confirm the hypothesis that low doses of Rapamycin added during culture induce a long-lived antiviral TCP with numerous beneficial characteristics and encourage a contemporary clinical translation.

5.1   GMP compatibility and clinical translation

Clinical translation of Rapa-TCPs is feasible considering a good manufacturing practice (GMP)-compatible production process. The GMP facility of the Berlin Center for Advanced Therapies (BeCAT) already holds a manufacturing authorization for the conventional production process producing untreated antiviral TCPs. The use of Rapamycin, which is an approved drug and commercially available as Rapamune (Pfizer), is a minor adaptation improving the production process. Currently, we are in the process of discussing this issue with the Paul-Ehrlich-Institut to amend the manufacturing authorization. In parallel, we are transferring the protocol for Rapa-TCP generation to the GMP facility, which will then validate the production process. Basically, the only difference of a GMP-conform protocol to the presented protocol is the use of the CliniMACS system (Miltenyi) for isolation of virus-specific T cells and the use of GMP-approved materials. Once we get the approval from the Paul-Ehrlich-Institut and validation is completed, we aim to start a clinical Phase I/IIa trial in collaboration with the BeCAT to investigate the application of antiviral Rapa-TCPs in transplant recipients with viral complications and resistance to classical antiviral medication. We believe that the risk of antiviral Rapa-TCP application is comparably low, because clinical safety of the application of conventional antiviral TCPs was already demonstrated[27].

5.2   Identified risk factors and their significance

Data from the investigated KTx patients point out risk factors, which may require additional measures such as adaptation of the amount of starting material to the lymphocyte count or preventive blood sampling for TCP generation before administration of ATG. Further potential risk factors included long-term medication with antiviral drugs, which can diminish the CMV-specific IFNγ production of T cells[45], high age, which showed a negative correlation with the yield of Rapa-TCPs and chronic infections *e.g.* by hepatitis B virus. These factors should definitely be further investigated and carefully considered for the study design of a clinical trial. A conceivable solution for patients with risk factors having a substantial impact on TCP yield may be the generation of conventional antiviral TCPs to achieve higher yields for the initial control of the virus followed by administration of the Rapa-TCP to achieve long-lasting immunity and prevent relapses[27]. Of note, the required dosing of adoptive T cell therapy is debatable, however, our data suggest that with Rapa-TCPs probably lower amounts of cells would be sufficient to achieve a long-lasting effect due to the improved survival, less

differentiated state and other beneficial properties of the Rapa-TCPs. Eventually, the actual requirements for dosing have to be determined in patients in a clinical trial setting.

5.3 Transferability to other T cell products

Certainly, the beneficial effects conferred by low dose Rapamycin treatment could also be transferred to other clinically investigated TCPs including effector T cells for adoptive T cell therapy, such as chimeric antigen receptor T cells, TCR transgenic T cells or tumor infiltrating lymphocytes to achieve a more sustained and long-lasting effect. The evident advantage of this strategy is the exclusive application during culture, which appears to have an impact on T cell function even after longer time periods, at least shown for up to 5 weeks after treatment in *in vitro* experiments. This is easily applicable and safe, *e.g.* opposed to genetic modifications[46]. However, behavior of Rapa-TCPs in tumor microenvironments needs further investigations. Definitely, multiple approaches to render TCPs more efficient are published, some of which also induce less differentiated memory T cells. However, the advantage of our approach is the immediate transferability to GMP conditions and the availability of clinical data regarding application of conventional untreated TCPs generated under almost the same circumstances as Rapa-TCPs[27].

5.4 Further new optimization approaches

Despite the less advanced state of differentiation and beneficial properties of the Rapa-TCP, once transferred, the effect of long-term immunosuppression might still intervene with a long-lasting protective immune response conferred by the TCP. Thus, in a further project, I genetically engineered antiviral TCPs to be resistant against one of the most commonly used immunosuppressants in transplantation, Tacrolimus, using CRISPR/Cas9 technology. Therefore, the immunophilin required for Tacrolimus to exert its immunosuppressive function, FKBP12, was knocked out by electroporating the TCP with ribonucleoprotein complexes of Cas9 and guide RNA directed at FKBP12. One very efficient guide RNA was identified, which rendered T cells remarkably resistant to clinically used doses of Tacrolimus, determined by their sustained production of cytokines upon virus-specific stimulation in the presence of clinical doses of Tacrolimus. Even in the presence of classical triple immunosuppression at doses used in KTx patients, these cells showed improved functionality in terms of cytokine production. Currently, we are evaluating the safety of this minimally manipulative genetic engineering approach, which may be a potent alternative optimization strategy for clinically used TCPs for immunosuppressed transplant patients in the future. First clinical investigational application of this approach may be expected in a severe life-threatening setting such as PTLD caused by EBV considering the increased safety risk compared to Rapa-TCPs.

In general, gene engineering allows a variety of optimization strategies, including checkpoint inhibitor pathways or introduction of transgenes to acquire superior function, however is riskier compared to the Rapa-TCP approach.

In the future, allogeneic off-the-shelf T cell products probably will gain more and more importance, due to better standardization and the possibility of detailed characterization before the administration of the same batch probably to many different patients. However, here human lymphocyte antigen matching just as in transplantation is of major importance to prevent rejection of the T cell product and to achieve a sustained regeneration of the immune system.

In summary, optimization strategies for adoptive T cell therapies are of interest to large patient cohorts as advanced therapeutic medicinal products gain more and more importance. This is reflected by the approval of the first chimeric antigen receptor T cell therapies, Kymriah (Novartis) for the use in diffuse large B cell lymphoma and B cell progenitor derived acute lymphoblastic lymphoma and Yescarta (Gilead) for diffuse large B cell lymphoma and primary mediastinal large B cell lymphoma by the European Medicines Agency in 2018. These approvals may pave the way for a broad acceptance of adoptive T cell therapies and facilitate clinical application of future adoptive T cell therapy approaches.

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7. Detailed description of own achievements

Publication: Leila Amini, Tino Vollmer, Desiree J. Wendering, Anke Jurisch, Sybille Landwehr-Kenzel, Natalie M. Otto, Karsten Jürchott, Hans-Dieter Volk, Petra Reinke and Michael Schmueck-Henneresse, Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients, Front. Immunol., May 2019.

My contributions to the publication "Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients" included study conceptualization, planning and exertion of experiments, data analysis, data presentation in figures, data interpretations and composition of the manuscript.

I performed extensive literature search to identify new hypotheses and appropriate techniques and parameters for the characterization of TCPs in addition to those routinely used by our group such as flow cytometric analysis of memory subset defining markers, intracellular cytokine staining and killing assays. In accordance with Dr. Michael Schmueck-Henneresse, Prof. Dr. Petra Reinke and Prof. Dr. Hans-Dieter Volk, I set up the main hypotheses for the study and determined the study design.

Based on the findings in literature, I established the setups for the apoptosis assays and metabolic analyses. For the establishment of metabolic analyses for T cells and handling of the Seahorse device, I benefitted from the knowledge of Dr. Nancy Schanze, who had experience with the device and analytic media, however, worked with adherent cells.

I organized healthy donors, collected venous blood and ordered buffy coats from the DRK. I selected the patients with different CMV reactivation states from a patient database except for the patients for whom samples were collected before and after transplantation, which were selected by Anett Sefrin. She also organized sampling from all other patients who were informed about the study by Prof. Dr. Reinke and Dr. Natalie Maureen Otto. I screened the patient database for relevant information regarding CMV reactivations, immunosuppressive medication and additional disease-related events, which are presented in a supplementary table of the publication. After thorough instruction by Dr. Michael Schmueck-Henneresse and Anke Jurisch, I isolated peripheral blood monocytes (PBMCs) from venous blood of healthy donors and patients, which were used for the experiments. I manufactured and cultivated all TCPs after detailed instruction by Dr. Schmueck-Henneresse, who established the respective standard protocols in our laboratory. I took samples from the TCP cultures and resuspended them in lysing buffer for the following extraction of DNA/RNA performed by Sarina Richter. I performed data analysis of TCR sequencing data based on the data provided by Adaptive Biotechnologies. RNA sequencing data were generated at the Microarray and Deep-

Sequencing Core Facility of the University Medical Center Göttingen. After quality control performed by Marten Jäger and data analysis and graph generation for RNA sequencing data by Dr. Karsten Jürchott, I annotated differentially expressed genes to relevant processes for TCP function, performance and longevity by intense literature research.

I prepared the samples for fluorescence activated cell sorting, which was exerted by the Core Facility for Cell Sorting of the Berlin-Brandenburg Center for Regenerative Therapies. Occasionally, Anke Jurisch, Desiree J. Wendering, Tino Vollmer and Dr. Schmueck-Henneresse helped me with PBMC isolation, cell counting or cultivation of TCPs.

For flow cytometric experiments, I established and compensated panels together with Dr. Schmueck-Henneresse. I performed all treatments, fluorescent (antibody) stainings, data acquisition and data analysis. For flow cytometric data, I discussed gating strategies with Dr. Schmueck-Henneresse. All graphs were designed in collaboration of Dr. Schmueck-Henneresse and me and discussed with all co-authors for optimal data presentation. I interpreted and reviewed all findings, which I discussed with the co-authors, mainly with Dr. Schmueck-Henneresse, Dr. Sybille Landwehr-Kenzel, Prof. Dr. Volk and Prof. Dr. Reinke.

I composed and formulated the entire initial version of the manuscript, which was proofread and optimized by all co-authors, but primarily by Dr. Schmueck-Henneresse. I further optimized the manuscript mainly together with Dr. Schmueck-Henneresse based on the comments we received during the review process at Frontiers in Immunology, which was proofread and adjusted by the other co-authors.

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Signature

8. Eidesstattliche Versicherung

„Ich, Leila Amini, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Characteristics of Rapamycin-treated T cell products for advanced adoptive T cell therapy and evaluation of clinical feasibilty" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

_____   _____
Datum   Unterschrift

9. Journal Summary List "Immunology"

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"Immunology"** Selected Category Scheme: WoS

Gesamtanzahl: 155 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	39,215	41.982	0.085360
2	Annual Review of Immunology	17,086	22.714	0.028800
3	NATURE IMMUNOLOGY	41,410	21.809	0.102290
4	IMMUNITY	46,541	19.734	0.136360
5	TRENDS IN IMMUNOLOGY	11,204	14.188	0.026850
6	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	49,229	13.258	0.083800
7	Lancet HIV	1,476	11.355	0.007950
8	JOURNAL OF EXPERIMENTAL MEDICINE	62,537	10.790	0.078310
9	IMMUNOLOGICAL REVIEWS	14,555	9.217	0.028540
10	Cancer Immunology Research	4,361	9.188	0.021180
11	CLINICAL INFECTIOUS DISEASES	61,618	9.117	0.120010
12	AUTOIMMUNITY REVIEWS	8,956	8.745	0.020990
13	Journal for ImmunoTherapy of Cancer	1,675	8.374	0.007130
14	CURRENT OPINION IN IMMUNOLOGY	9,275	7.932	0.020120
15	JOURNAL OF AUTOIMMUNITY	6,400	7.607	0.015400
16	Cellular & Molecular Immunology	3,633	7.551	0.008300
17	EMERGING INFECTIOUS DISEASES	29,657	7.422	0.057980
18	Mucosal Immunology	6,105	7.360	0.021860
19	SEMINARS IN IMMUNOLOGY	4,552	7.206	0.010950
20	EXERCISE IMMUNOLOGY REVIEW	740	7.105	0.001110
21	Journal of Allergy and Clinical Immunology-In Practice	3,802	6.966	0.009870
22	CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY	2,741	6.442	0.005880
23	Seminars in Immunopathology	2,967	6.437	0.009290
24	BRAIN BEHAVIOR AND IMMUNITY	12,583	6.306	0.026850
25	ALLERGY	16,476	6.048	0.025790
26	Emerging Microbes & Infections	1,318	6.032	0.005910
27	Advances in Immunology	2,423	5.935	0.004250
28	Current Topics in Microbiology and Immunology	5,633	5.829	0.011740
29	World Allergy Organization Journal	1,352	5.676	0.003800
30	Frontiers in Immunology	16,999	5.511	0.067470

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Selected JCR Year: 2017; Selected Categories: "IMMUNOLOGY"

10. Publication "Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients"

Comprehensive Characterization of a Next-Generation Antiviral T-Cell Product and Feasibility for Application in Immunosuppressed Transplant Patients

Leila Amini [1,2,3,4,5], Tino Vollmer [1,3,5], Desiree J. Wendering [1,3,4,5], Anke Jurisch [1,3], Sybille Landwehr-Kenzel [3,5,6], Natalie Maureen Otto [2,5], Karsten Jürchott [1,3], Hans-Dieter Volk [1,3,5], Petra Reinke [2,3,5] and Michael Schmueck-Henneresse [1,3,5*]

[1] Institute for Medical Immunology, Charité University Medicine Berlin, Berlin, Germany, [2] Renal and Transplant Research Unit, Department of Nephrology and Internal Intensive Care, Charité University Medicine Berlin, Berlin, Germany, [3] Berlin Institute of Health Center for Regenerative Therapies (BCRT), Charité University Medicine Berlin, Berlin, Germany, [4] Berlin-Brandenburg School for Regenerative Therapies, Charité University Medicine Berlin, Berlin, Germany, [5] Berlin Center for Advanced Therapies, Charité University Medicine Berlin, Berlin, Germany, [6] Department for Pediatric Pulmonology, Immunology and Intensive Care Medicine, Charité University Medicine Berlin, Berlin, Germany

Viral infections have a major impact on morbidity and mortality of immunosuppressed solid organ transplant (SOT) patients because of missing or failure of adequate pharmacologic antiviral treatment. Adoptive antiviral T-cell therapy (AVTT), regenerating disturbed endogenous T-cell immunity, emerged as an attractive alternative approach to combat severe viral complications in immunocompromised patients. AVTT is successful in patients after hematopoietic stem cell transplantation where T-cell products (TCPs) are manufactured from healthy donors. In contrast, in the SOT setting TCPs are derived from/applied back to immunosuppressed patients. We and others demonstrated feasibility of TCP generation from SOT patients and first clinical proof-of-concept trials revealing promising data. However, the initial efficacy is frequently lost long-term, because of limited survival of transferred short-lived T-cells indicating a need for next-generation TCPs. Our recent data suggest that Rapamycin treatment during TCP manufacture, conferring partial inhibition of mTOR, might improve its composition. The aim of this study was to confirm these promising observations in a setting closer to clinical challenges and to deeply characterize the next-generation TCPs. Using cytomegalovirus (CMV) as model, our next-generation Rapamycin-treated (Rapa-)TCP showed consistently increased proportions of CD4$^+$ T-cells as well as CD4$^+$ and CD8$^+$ central-memory T-cells (T$_{CM}$). In addition, Rapamycin sustained T-cell function despite withdrawal of Rapamycin, showed superior T-cell viability and resistance to apoptosis, stable metabolism upon activation, preferential expansion of T$_{CM}$, partial conversion of other memory T-cell subsets to T$_{CM}$ and increased clonal diversity. On transcriptome level, we observed a gene expression profile denoting long-lived early memory T-cells with potent effector functions. Furthermore, we successfully applied the novel protocol for the generation of Rapa-TCPs to 19/19 SOT patients in a comparative study, irrespective

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of their history of CMV reactivation. Moreover, comparison of paired TCPs generated before/after transplantation did not reveal inferiority of the latter despite exposition to maintenance immunosuppression *post*-SOT. Our data imply that the Rapa-TCPs, exhibiting longevity and sustained T-cell memory, are a reasonable treatment option for SOT patients. Based on our success to manufacture Rapa-TCPs from SOT patients under maintenance immunosuppression, now, we seek ultimate clinical proof of efficacy in a clinical study.

Keywords: cytomegalovirus, adoptive T-cell therapy, solid organ transplantation, Rapamycin, mTOR, immune regeneration

INTRODUCTION

Severe viral infections have a major impact on the clinical course of immunocompromised patients. Despite availability of powerful antiviral medication, cytomegalovirus (CMV) still accounts for significant morbidity and mortality in solid organ transplant (SOT) recipients (1). CMV can trigger direct and indirect morbidities such as chronic allograft rejection or in the case of kidney transplantation (KTx) chronic nephropathy (2, 3). Therapeutic control of CMV may be hampered by the development of anti-viral drug resistance (4). Moreover, after discontinuation of anti-viral prophylaxis, late-onset CMV disease frequently occurs and overall mortality is significantly higher in CMV-infected compared to uninfected KTx patients (1). Of note, T-cell-mediated anti-CMV immunity was reported to be predictive for the development of late-onset disease (5) and anti-CMV_{IE-1}-specific $CD8^+$ T-cell responses stratify risk of CMV disease in heart and lung transplant as well as KTx patients (6, 7). In addition, the magnitude of the CMV_{pp65}- and CMV_{IE-1}-specific T-cell responses turned out to be protective against complications with CMV in hematopoietic stem cell transplantation (HSCT) (8, 9). Consequently, regeneration of the endogenous T-cell response against these antigens, as aspired by AVTT, may prevent and reduce virus-associated morbidities/mortality in the SOT setting. Other viruses with impact on SOT outcomes are Epstein-Barr-virus and BK-virus, for which less efficient or no antiviral drugs are available. T-cells play a key role in protection from severe viral infections (7, 10, 11). Thus, adoptive T-cell therapy (AVTT) is a potent novel treatment strategy to tackle fatal viral complications in immunosuppressed transplant patients. Mechanisms of success or failure of new AVTT approaches need to be thoroughly understood and specific characteristics of patient cohorts have to be considered for successful translation of AVTT.

For clinical application of AVTT, *ex vivo* enrichment and expansion of virus-specific T-cells under GMP conditions are crucial and thus various protocols have been developed for CMV-specific AVTT after HSCT (12–17). However, the success of these approaches is limited in SOT patients due to the T-cell products (TCPs) being derived from patients instead of healthy HSCT donors, the lack of lymphodepletive preconditioning and the need for concomitant immunosuppression. Nevertheless, we and other groups demonstrated not only safety of AVTT, but also significant reduction of viral load and control of

clinical symptoms of CMV disease in SOT recipients under maintenance immunosuppression in proof-of-concept studies (18–21). These observations are in line with positive results of AVTT for treatment of patients with EBV-related *post*-transplant lymphoproliferative disease (22–24). Yet, long-term efficacy failed in some patients, who experienced recurrence of CMV or EBV load and symptoms (18–25). To adapt AVTT to combat these clinical challenges, it is crucial to consider the respective patient cohort and the TCPs' characteristics. Specifically, our aim was to increase longevity of transferred T-cells to improve sustainability of clinical efficacy of AVTT in SOT patients. Failure of long-term control of CMV/EBV infections may be due to limited persistence of adoptively transferred T-cells *in vivo*, which might occur due to the late differentiation state implying limited longevity of infused T-cells. Therefore, advancing the quality of adoptively transferred TCPs with defined compositions by the enrichment for distinct T-cell memory subsets may improve therapeutic outcome. In particular, central-memory T-cells (T_{CM}; $CCR7^+$ $CD62L^+$ $CD45RO^+$ $CD45RA^-$) and memory-stem T-cells (T_{SCM}; $CCR7^+$ $CD62L^+$ $CD45RO^-$ $CD45RA^+$ $CD95^+$) have high proliferative potential, self-renewal capacity and are reported to show superior engraftment, persistence, and survival compared to more differentiated memory T-cells (26–33). Conversely, late-differentiated short-lived effector-memory T-cells (T_{EM}; $CCR7^-$ $CD62L^-$ $CD45RA^-CD45RO^+$) and terminally-differentiated effector T-cells (T_{EMRA}; $CCR7^-$ $CD62L^-$ $CD45RA^+CD45RO^-$) exert immediate effector function, but fail to establish long-lasting protective memory, because of poor proliferative potential and limited survival following antigenic rechallenge (26, 27, 34). Remarkably, these observations match clinical data demonstrating T-cell reconstitution after HSCT and prevention of CMV disease related to T_{CM} proportions in peripheral blood (35). Direct sorting strategies to isolate only CMV-specific long-lived T-cells are barely feasible under GMP conditions and would yield very small cell numbers likely not sufficient for successful AVTT in immunosuppressed SOT recipients. To ensure applicability in a clinical setting, we recently optimized our GMP-conform manufacturing process for autologous virus-specific TCPs and succeeded in attenuating T-cell differentiation by treatment with low doses of Rapamycin (inhibits the *mechanistic-target-of-rapamycin-complex-1*: mTOR-C1, favorable results with 20 nM) during expansion cultures (18, 36, 37). This next-generation antiviral TCP comprises enriched proportions

of early-differentiated T_{CM} being superior for AVTT (37–39). Furthermore, next-generation Rapamycin-treated (Rapa-)TCPs contain higher proportions of CD4$^+$ T-cells (37) reported to improve clinical efficacy (29, 40, 41).

Detailed knowledge regarding the characteristics of Rapa-TCPs is a prerequisite for realization of clinical translation. Thus, we closely investigated the molecular properties of this Rapa-TCP regarding dependence on cytokine supplementation regiments during *in vitro* expansion, long-term stability, survival/sensitivity to apoptosis, metabolism, transcriptome, clonal composition, the role of the different memory T-cell subsets and applicability to SOT patient samples. Our data reveal a beneficial early differentiated phenotype, profound function, elevated clonal diversity, and superior survival of Rapa-TCPs compared to first-generation TCPs, which is further underlined and confirmed by a distinct gene expression signature revealed by mRNA sequencing.

We used *in vitro* models to mimic the situation of TCPs once injected into a patient coping with CMV disease, *i.e.*, massive antigen exposure and withdrawal of Rapamycin. Here, we observed a preserved capacity for CMV-specific production of effector cytokines. Moreover, we tested manufacture of CMV-specific TCPs from material of patients with chronic end-stage renal disease (ESRD) before and after KTx to investigate the impact of chronic immunosuppression, showing no benefits of TCP generation before KTx. We further investigated the influence of CMV-reactivation history after KTx on the differentiation of virus-reactive memory T-cells and the resulting composition of untreated and Rapa-TCPs, implying feasibility of TCP generation from all groups investigated. This next-generation AVTT approach may also be applied to other viral specificities, such as EBV and BKV or even cancer-specific T-cells. Prospectively, implementation of next-generation AVTT may allow for reduction or complete ablation of toxic anti-viral medication and minimize the risk for virus-associated complications in the SOT setting.

METHODS

Patients' and Healthy Donors' Blood Samples

Venous blood samples were collected from 19 healthy donors (HDs) (10 m/9 f; 25–81 years) and 19 KTx patients (11 m/8 f; 34–78 years; **Table S2**) of the Kidney Transplant Ambulance, Charité Virchow Klinikum, Berlin. We worked with buffy coats from 3 of the 19 different HDs to have sufficient cells for different cell sorting steps. Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll Separating Solution density gradient centrifugation (Biochrom). The Charité Ethics Committee (IRB) approved the study protocol and all blood donors provided written informed consent. Detailed characteristics of each patient are presented in **Table S2**.

Enrichment and Expansion of CMV-Specific T-Cells

CMV-specific TCPs were generated using a previously described technique (18, 36, 37, 42). Briefly, PBMCs were stimulated for 6 h with overlapping CMV$_{pp65/IE-1}$ peptide pools (JPT Peptide Technologies; 0.5 μg/ml each). IFNγ-producing cells underwent positive selection using the IFNγ Secretion Assay—Cell Enrichment and Detection Kit according to the manufacturer's instructions (Miltenyi Biotec). Enriched IFNγ^+ cells were cultured for 21 days in 96- or 24-well-plates with irradiated (30 Gy using a GSR D1 [Gamma-Service Medical GmbH]) autologous feeder cells (derived from 1/5 of the capture assays' negative fraction) added only at d0 in complete media (VLE RPMI 1640 supplemented with penicillin (100 IU/ml) and streptomycin [all from Biochrom] and 10% fetal calf serum [FCS, PAA]), supplemented with 10 ng/ml recombinant human (rh) IL-7 and rh IL-15 (CellGenix) or 50 U/ml IL-2 in humidified incubators at 37°C and 5% CO$_2$. Cells were split 1:1 when 100% confluence was reached. For Rapa-TCPs, 20 nM of Rapamycin (Rapamune, Pfizer Pharma GmbH) were added every 2nd day or upon splitting starting from day 1 (37).

For restimulation during culture (only where indicated, **Figure 1**, **Figure S1**), we depleted the donor's PBMCs of CD3$^+$ cells using magnetically activated cell sorting (MACS) with anti-CD3 beads (Miltenyi Biotec) following the manufacturer's instructions. These cells were frozen in FCS with 10% cell culture grade dimethyl-sulfoxide (Sigma-Aldrich) until restimulation at d14. Thawed autologous CD3$^-$ PBMCs were washed twice and pulsed with overlapping CMV$_{pp65/IE-1}$ peptide pools at concentrations of 2 μg/ml each peptide for 2 h and added at a 1:5 ratio to the T-cells in culture.

Functional Tests, Phenotyping, Flow Cytometry, and Sorting

Expanded T-cells were analyzed for effector functions by their ability to recognize antigen-loaded target cells, which consisted of autologous lymphoblastoid B-cell lines (LCLs), transformed with B95-8 EBV and by specific production of cytokines. LCLs were generated as described previously (43).

For CMV-specific stimulation of expanded TCPs for detecting intracellular effector cytokine production, CMV$_{pp65/IE-1}$ peptide pool-loaded LCLs were added to cultured T-cells at a ratio of 1:10 and incubated for 6 h. Un-pulsed LCLs served as unstimulated control. For effector cytokine detection *ex vivo*, PBMCs were stimulated with 1 μg/ml overlapping CMV$_{pp65/IE1}$ peptide pools *ex vivo* for 14 h. After 1 h, 2 μg/ml Brefeldin A (BFA, Sigma-Aldrich) were added to the stimulation to allow for intracellular capture of cytokines.

To induce apoptosis, 1 μg/ml of LEAF-purified Fas-activating antibody (EOS9.1; BioLegend) was added to cultures for 16 h. To determine survival, LIVE/DEAD$^®$ Fixable Blue Dead Cell Stain (Invitrogen) and Annexin V (BioLegend) were added.

For determination of killing capacity, autologous LCLs were pulsed with 2 μg/ml CMV$_{pp65/IE-1}$ peptide pools, whereas unpulsed allogenic LCLs were used as non-target controls. Targets were labeled with 10 mM Carboxyfluorescein-diacetate-succinimidyl-ester (Sigma-Aldrich) and non-target controls with 5 mM CellTrace^{TM} Far Red (Invitrogen). Cells were co-cultured at a T-cell/target-cell ratio of 10:1 for 14 h. Samples were analyzed using a LSR II Fortessa flow cytometer. Samples without T-cells, containing only LCLs, served as an internal control and reference for calculation of the killing capacity. For analysis, we gated

FIGURE 1 | Effects of Rapamycin on T-cell products: Expansion, phenotype and function. **(A)** Schematic overview of experiments: T-cell products (TCPs) were generated from PBMCs isolated from venous blood of healthy donors (HDs) by magnetically activated cell sorting (MACS) of T-cells producing IFNγ in response to
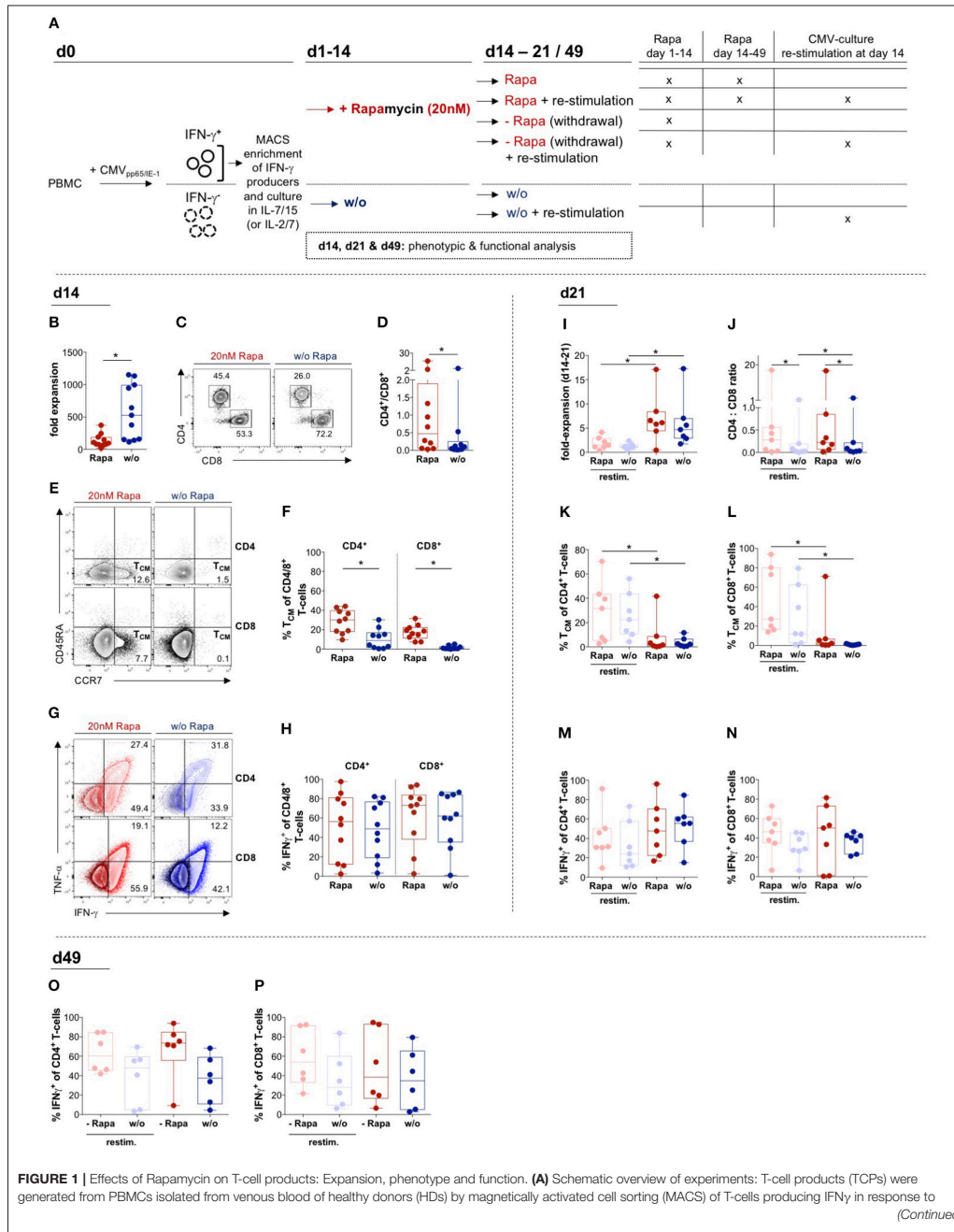*(Continued)*

FIGURE 1 | stimulation with $CMV_{IE-1/pp65}$ peptide pools and expanded in the presence of either IL-2/IL-7 (**Figure S1**) or IL-7/IL-15 without (w/o; blue) or with addition of 20 nMof Rapamycin (Rapa; red) **(B–P)**. Parts of the culture were re-stimulated using thawed CD3$^-$ PBMCs loaded with $CMV_{IE-1/pp65}$ peptide pools, deprived of Rapamycin or a combination of both on d14. **(B)** Expansion rates of IL-7/15-expanded Rapa-treated (Rapa-)TCPs (red) and untreated TCPs (blue) of $n = 10$ healthy donors (HDs) calculated from yield at d14 divided by the number of seeded cells at d0. We gated flow cytometric data on lymphocytes singlets living CD3$^+$ T-cells. **(C)** Exemplary flow cytometry plots of CD4$^+$ and CD8$^+$ populations among living CD3$^+$ T-cells in the Rapa-TCP (left plot) and untreated TCP (w/o, right plot) of one HD. **(D)** CD4/CD8 ratios in Rapa- (red) and untreated TCPs (blue) of $n = 10$ HDs calculated from flow cytometry data as presented in **(C)**. **(E)** Gating strategy for CD45RA$^-$ CCR7$^+$ central memory T-cells (T_{CM}) among CD4$^+$ (upper panel) and CD8$^+$ (lower panel) in Rapa- (left panel) and untreated TCPs (right panel) of one exemplary HD. **(F)** Proportions of CD4$^+$ and CD8$^+$ T_{CM} among Rapa- (red) and untreated TCPs (blue) of $n = 10$ HDs determined from flow cytometric data as shown in **(E)** at d14. **(G,H)** To detect CMV-specific cytokine producers, TCPs were stimulated with $CMV_{IE-1/pp65}$ peptide-loaded autologous lymphoblastic cell lines (LCLs) at a ratio of 1:10 for 6 h and Brefeldin A (BFA) was added after 1 h. **(G)** Representative flow cytometric plots of IFNγ- and TNFα-producers in Rapa- (left panel, red) and untreated TCPs (right panel, blue) of one HD. The dark population represents unstimulated and the light population illustrates $CMV_{IE-1/pp65}$-stimulated CD4$^+$ (upper panel) and CD8$^+$ T-cells (lower panel). **(H)** Proportions of CMV-specific IFNγ-producers among CD4$^+$ and CD8$^+$ T-cells in Rapa- (red) and untreated TCPs (blue) of $n = 10$ HDs determined from flow cytometric data as shown in **(G)** at d14. **(I–N)**: For re-stimulation on d14 of culture, thawed CD3$^-$ autologous PBMCs were loaded with $CMV_{IE-1/pp65}$ peptide pools and added at 1:5 ratio to T-cells. **(I)** Expansion rates of IL-7/15-expanded re-stimulated (pastel colors) or non-re-stimulated (dark colors) Rapa- (red) and untreated TCPs (blue) of $n = 7$ HDs calculated from yield at d21 divided by the number of cells at d14. **(J)** CD4/CD8 ratios in Rapa- (red) and untreated TCPs (blue) of $n = 7$ HDs calculated from flow cytometric data as presented in **(C)** at d21. **(K,L)**: Proportions of CD4$^+$ **(K)** and CD8$^+$ T_{CM} **(L)** among Rapa- (red) and untreated TCPs (blue) of $n = 7$ HDs determined from flow cytometric data as shown in **(E)** at d21. **(M–N)** To detect CMV-specific cytokine producers, TCPs were stimulated with $CMV_{IE-1/pp65}$ peptide-loaded autologous LCLs for 6 h and BFA was added after 1 h. **(M–N)** Proportions of CMV-specific IFNγ-producers among CD4$^+$ **(M)** and CD8$^+$ T-cells **(N)** in Rapa- (red) and untreated TCPs (blue) of $n = 7$ HDs determined from flow cytometric data as shown in **(G)** at d21. **(O,P)** To mimic the situation after infusion, Rapa was withdrawn and TCPs were cultivated long-term until d49. Proportions of CMV-specific IFNγ-producers among CD4$^+$ **(O)** and CD8$^+$ T-cells **(P)** in TCPs withdrawn from Rapa (red) and untreated TCPs (blue) of $n = 6$ HDs determined from flow cytometric data as shown in **(G)** at d49. For all graphs normal distribution of data points was tested with Kolmogorov-Smirnov test and paired t-test was used to determine significance in normally distributed samples or Wilcoxon's matched-pairs signed rank test in not normally distributed samples, respectively. P-values below 0.05 are indicated by * and defined to be significant.

on LIVE/DEAD$^®$ Fixable Blue Dead Cell Stain-negative cells and calculated ratios of target to non-target cells as described previously (44, 45).

To define memory subsets, T-cells were stained extracellularly for surface markers CCR7 (G043H7), CD45RA (HI100), CD45RO (UCHL1), CD62L (DREG-56; eBioscience), and CD95 (DX2). Subsequently, cells were permeabilized and fixed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained intracellularly for CD3 (OKT3), CD4 (SK3), and CD8 (RPA-T8), IFNγ (4S.B3, eBioscience), TNFα (MAb11), and Granzyme B (GZB) (GB11, BD Pharmingen). Cells were analyzed on a LSR II Fortessa flow cytometer using FlowJo Version 10 software (Tree Star). Lymphocytes were gated based on the FSC *vs.* SSC profile and subsequently gated on FSC (height) *vs.* FSC to exclude doublets.

For evaluating of T-cell subsets on transcriptome level, T-cell subsets were sorted from PBMCs from $n = 3$ HDs' buffy coats (DRK) at d0 or derived TCPs at d18 based on the expression of CD3, CD45RA, and CCR7 by the Core Facility Flow Cytometry of the BCRT using a FACS Aria II Calliope (BD).

All antibodies were purchased from BioLegend, unless indicated otherwise.

Metabolic Analysis

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were analyzed using a Seahorse-XFe96-Analyzer following the manufacturer's instructions for non-adherent cells including immobilization of cells with Cell-Tak (Corning). Assay medium consisted of Dulbecco's Modified Eagle's Medium D5030 (Sigma) supplemented with 3 g/l D-glucose (Roth) and 300 mg/ml L-glutamine (Gibco) and was sterile-filtered. For T-cell activation, 0.5 μg/ml of CMV $_{pp65/IE-1}$ peptide pools were added to the microwells relying on reciprocal antigen-presentation of T-cells 0.5 h before the measurement.

RNA Sequencing and Bioinformatics Analysis

RNA was isolated using an All-Prep DNA/RNA Kit (Qiagen) following the manufacturer's instructions. RNA samples were sent to the Deep Sequencing Core Facility in Göttingen, where samples were prepared using TrueSeq Kits (Illumina) and HiSeq_4000 performing 50 million reads/sample.

Fastq-files were quality checked with FastQC (Babraham Bioinformatics) and trimmed for residual adapter sequences. Reads were aligned to the GRCh38 human genome using TopHat^R (2.1.0–Johns Hopkins University, Center for Computational Biology) and Bowtie2 (46). Counts per gene were determined as sum of all reads mapped within a gene region. Principal component (PC) analysis was performed in R (47) using the 1,000 top-variable genes within the data set. Differentially expressed genes were identified using negative binomial distributions as implemented in the DESeq2 package (48) in R. False discovery rates (FDR) were calculated to adjust p-values for multiple testing and FDR-values below 0.05 were considered as significant. Expression data for differentially expressed genes were variance-stabilized transformed and scaled prior to visualization in heat maps. RNA sequencing data are available at the GEO platform with the accession number GSE129196.

T-Cell Receptor Sequencing

For sequencing of T-cell receptors (TCRs) to determine the clonality of TCPs, DNA was isolated using an All-Prep DNA/RNA Kit (Qiagen) following the manufacturer's instructions. TCRβ sequencing was performed using a hsTCRb Kit (Adaptive Biotechnologies) following the manufacturer's instructions and analyzed with the corresponding ImmunoSEQ-Analyzer 3.0 software. Briefly, the most variable complementary-determining region 3 (CDR3), spanning the recombination

site of V-D-J recombinations of TCR β-chains was sequenced. Productive rearrangements were regarded as unique in-frame nucleotide sequences without stop codon, leading to a functional TCR. Productive frequency means the individual frequency of a specific productive rearrangement (clone) among all productive rearrangements. Clonality was calculated based on productive entropy normalized to the total number of productive rearrangements. Sample overlap was investigated using the Morisita index considering unique clones, individual frequencies of clones and the probability of a common origin of two samples. TCR sequencing data is accessible at the ImmuneACCESS platform http://adaptivebiotech.com/pub/amini-2019-frontimmunol (Adaptive Biotechnologies).

Statistical Analysis and Calculations

Graph Pad Prism version 7 was used for graph generation. To test for normal Gaussian distribution Kolmogorov-Smirnov test was performed. If data were normally distributed, Student's paired or unpaired t-test were employed for analysis. If data were not normally distributed, Wilcoxon's matched pairs test was applied to paired samples and Man-Whitney's test to unpaired samples. All tests were two-tailed. Probability (p) values of ≤ 0.05 were considered statistically significant and significance is denoted as follows: $* = p < 0.05$. Correlation analysis was assessed by Pearson's correlation coefficients for normally distributed data or non-parametric Spearman's rank correlation. Fold expansion expresses the manually counted cell count (Neubauer's counting chamber) excluding dead cells by Trypan blue staining (Sigma-Aldrich) at the day indicated divided by the initially seeded cell amount from the positive fraction of the IFNγ Secretion Cell Enrichment Assay.

All datasets are available upon reasonable request.

RESULTS

In order to prepare our approach for clinical translation, we deeply characterized functionality, stability and distinct molecular, metabolic and transcriptional properties of our next-generation Rapa-TCP, for which we applied mTOR inhibition by Rapamycin to enrich for CD4$^+$ T-cells and CD4$^+$/CD8$^+$ T$_{CM}$ (37). First, we addressed the question whether we can reproduce our findings and properties published for supplementation of a certain cytokine regimen, IL-2/IL-7, with a regimen commonly used for GMP applications by many groups, IL-7/IL-15, which was previously shown to support generation of T$_{CM}$ (49).

Supplementation of IL-7/IL-15 Does Not Alter Rapamycin-Mediated Effects in TCPs

To investigate potential differences in the effects of Rapamycin administration dependent on the cytokine regimen supplemented, we expanded CMV-specific T-cells in the presence of different cytokine combinations, namely IL-7/IL-15 (**Figure 1A**) and IL-2/IL-7 (37) (**Figure S1A**). The expansion rates of antigen-reactive T-cells were sufficient considering cell numbers used in a pilot study (18), although Rapamycin significantly reduced expansion in both IL-7/IL-15- (**Figure 1B**) and IL-2/IL-7-expanded TCPs (**Figure S1B**). Overall, different

cytokines did not alter the beneficial effects of Rapamycin treatment (37): Rapamycin significantly increased CD4/CD8 ratio in both IL-7/IL-15- (**Figures 1C,D**) and IL-2/IL-7-expanded TCPs (**Figure S1C**) and significantly increased proportions of CD4$^+$ and CD8$^+$ T$_{CM}$ in both IL-7/IL-15- (**Figures 1E,F**) and IL-2/IL-7-expanded TCPs (**Figures S1D,E**). Furthermore, Rapamycin increased proportions of Interferon-γ (IFNγ)-producing CD4$^+$ and CD8$^+$ T-cells upon exposure to CMV-specific peptides loaded onto autologous lymphoblastic cell lines (LCLs) in both IL-7/IL-15- (**Figures 1G,H**) and IL-2/IL-7-expanded TCPs (**Figures S1F,G**). These data confirm the robustness of beneficial effects of mTOR inhibition using Rapamycin for TCP composition in the case of supplementing commonly used IL-7/IL-15 for expansion of TCPs.

Expansion Rates of Rapa-TCPs Recover Later During Culture

SOT patients often suffer from lymphopenia, which reduces the amount of PBMC, *i.e.*, the starting material, for TCP generation and their medication can impact the functionality of T-cells (50). Thus, TCP manufacture from patient material may require longer *in vitro* expansion periods of up to 21 days to achieve sufficient cell numbers for successful AVTT. To assess the stability of TCPs after a longer period of expansion, we determined phenotype and functionality of TCPs after extended expansion on d21 in IL-7/IL-15- (**Figures 1I,N**) and IL-2/IL-7-expanded TCPs (**Figures S1H,M**). Interestingly, Rapa-TCPs recovered, yet even exceeded expansion of untreated TCPs in the 3rd week of expansion (d14–d21) (**Figure 1I**), which was significant in IL-2/IL-7-expanded TCPs (**Figure S1H**). CD4/CD8 ratios remained significantly higher in Rapa-TCPs at d21 (**Figure 1J**), but IL-2/IL-7-expanded TCPs showed significantly higher CD4/CD8 ratios than IL-7/IL-15-expanded TCPs at d21 (**Figure 1J** *vs.* **Figure S1I**). During expansion, T$_{CM}$ differentiated and the enrichment of T$_{CM}$ proportions upon Rapamycin-treatment lost significance in both IL-7/IL-15- (**Figures 1K,L**) and IL-2/IL-7-expanded TCPs (**Figures S1J,K**).

Antigen Encounter Decreases Expansion Rates, but Promotes Less Differentiated Cells

We further mimicked the scenario happening once the TCPs are injected into a patient coping with CMV viremia in an *in vitro* model. Therefore, we modeled the situation of high antigen load by re-stimulation with CD3-depleted PBMCs pulsed with CMV-specific peptides: CMV-specific re-stimulation significantly reduced expansion rates in both IL-7/IL-15- (**Figure 1I**) and IL-2/IL-7-expanded TCPs (**Figure S1H**). Re-stimulation did not influence CD4/CD8 ratios in IL-7/IL-15- (**Figure 1J**) neither IL-2/IL-7-expanded Rapa-TCPs (**Figure S1I**), but significantly decreased CD4/CD8 ratios in IL-7/IL-15-expanded untreated TCPs (**Figure 1J**). Remarkably, re-stimulation significantly augmented the proportions of CD4$^+$ and CD8$^+$ T$_{CM}$ in both IL-7/IL-15- (**Figures 1K,L**) and IL-2/IL-7-expanded TCPs (**Figures S1J,K**). However, re-stimulation decreased the proportion of CD4$^+$ and CD8$^+$ IFNγ-producers

(**Figures 1M,N**), which was statistically significant in IL-2/IL-7-expanded Rapa-TCPs (**Figures S1L–M**).

Rapamycin Preserves Superior Capacity for IFNγ Production

Importantly, IL-2/IL-7-expanded Rapa-TCPs showed significantly higher proportions of IFNγ-producers among CD4$^+$ T-cells at d14 and d21 (**Figures S1F,L**) and CD8$^+$ T-cells at d21 (**Figure S1M**) compared to untreated TCPs illustrating improved functionality. Because TCPs are deprived of Rapamycin and exposed to antigen once injected, we analyzed samples in which we withdrew Rapamycin and re-stimulated with CMV peptide-loaded CD3-depleted PBMCs on d14 (**Figure 1A**, **Figure S1A**). Interestingly, once treated with Rapamycin during the first 2 weeks of culture, both IL-7/IL-15- (**Figures 1O,P**) and IL-2/IL-7-expanded TCPs (**Figures S1N,O**) comprised more CD4$^+$ and CD8$^+$ IFNγ-producers continuously until d49 of culture.

Rapamycin Enhances Survival of T-Cells

Longevity is a crucial prerequisite for long-term efficacy of adoptively transferred TCPs in patients. Based on findings in B-cell lymphoma cell lines, we hypothesized that Rapamycin treatment may increase viability of T-cells (51). Thus, we analyzed overall survival of T-cells in TCPs. Strikingly, we found significantly higher proportions of living T-cells in TCPs treated with Rapamycin compared to untreated TCPs (**Figures 2A,B**).

Immunosuppressant regiments including Tacrolimus, which are commonly used in SOT, are reported to sensitize T-cells to programmed cell death (52). Hence, we investigated the TCPs' sensitivity to apoptosis employing induction of the death receptor pathway by agonistic Fas-specific antibody to identify differences between untreated and Rapa-TCPs. We recorded partial resistance to Fas-induced apoptosis in Rapa-TCPs, while untreated TCPs were more sensitive to Fas-induced apoptosis (**Figures 2A,C**). The anti-apoptotic effect of Rapamycin observed in B-cell lymphoma lines is reported to depend on upregulation of Bcl-2 on protein level (51). Hence, we assessed (**Figure 2D**) the mean fluorescence intensity (MFI) of Bcl-2 in TCPs and found significantly higher MFIs in CD4$^+$ and CD8$^+$ T-cells of Rapa-TCPs compared to untreated TCPs (**Figures 2E,F**). The findings regarding viability and resistance to apoptosis suggest an increased fitness of T-cells in Rapa-TCPs implying improved long-term survival *in vivo*.

Rapamycin Stabilizes T-Cell Metabolism Upon Activation

Characteristically, memory and effector T-cells are distinguished by differences in metabolic activities (53). Thus, we investigated the ratio of fatty acid oxidation to glycolysis defined by the ratio of oxygen consumption to ECAR (OCR/ECAR). In fact, we detected significantly lower OCR/ECAR ratios in Rapa-TCPs compared to untreated TCPs (**Figure 2G**). Moreover, upon activation with CMV-specific peptides, the metabolism of Rapa-TCPs proofed to be more stable, whereas the OCR/ECAR ratio was significantly decreased in untreated TCPs (**Figure 2G**).

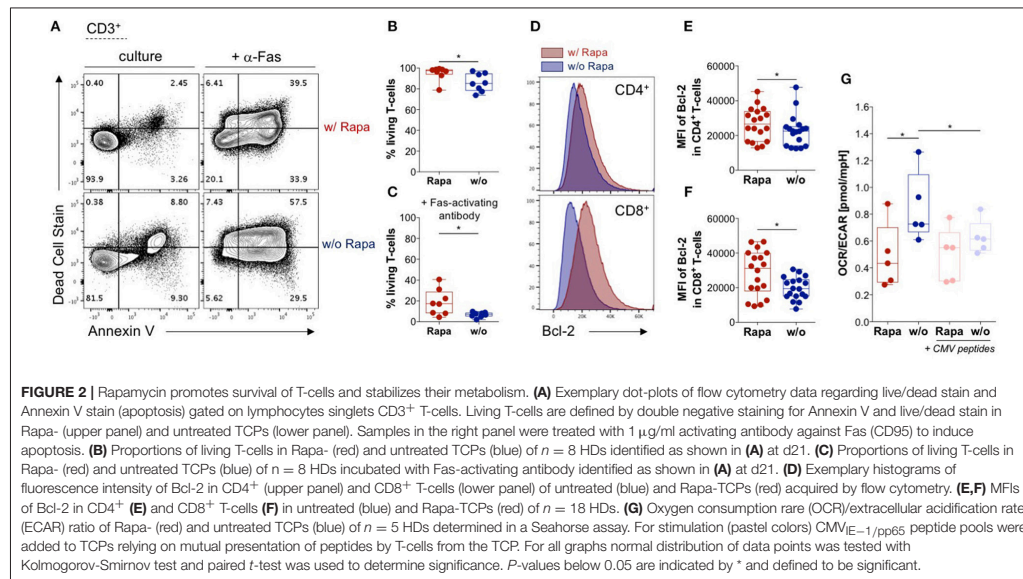## Distinct Effects of Rapamycin on Isolated T-Cell Memory Subsets

Distinct memory T-cell subsets were reported to have defined properties and are not equally suited for long-term regeneration of T-cell immunity (26–33). To assess the effect of Rapamycin on distinct CMV-specific memory T-cell subsets, we performed fluorescently activated cell sorting (FACS) for CCR7$^+$CD45RA$^-$ T$_{CM}$, CCR7$^-$CD45RA$^-$ T$_{EM}$, and CCR7$^-$CD45RA$^+$ T$_{EMRA}$ based on their differential expression of CD45RA and CCR7 *ex vivo* and subsequently performed IFNγ-secretion assays of sorted subsets to isolate CMV-specific T-cells of these particular subsets (**Figure 3A**). The CMV-reactive T-cells of each memory T-cell subset were expanded in the presence or absence of Rapamycin and analyzed after 3 weeks of culture. Interestingly, Rapamycin significantly reduced expansion in T$_{EM}$- and T$_{EMRA}$-derived cultures, which was not significant in T$_{CM}$-derived cultures (**Figure 3B**), indicating preferential expansion of T$_{CM}$ and implying reduced sensitivity to anti-proliferative effects of Rapamycin.

Moreover, Rapamycin prevented a significant proportion of CD4$^+$ and CD8$^+$ T$_{CM}$ from differentiation into late-stage memory/effector T-cells compared to control cultures (**Figures 3C,D**). Remarkably, Rapamycin treatment even induced some cells with a T$_{CM}$-like phenotype in cultures derived from T$_{EM}$ and T$_{EMRA}$ subsets suggesting some "rejuvenation" of late-stage memory cells (**Figures 3C,D**).

Notably, Rapamycin increased the MFI of Bcl-2 in CD4$^+$ and CD8$^+$ T-cells from T$_{CM}$- and T$_{EM}$-, but not T$_{EMRA}$-derived cultures (**Figures 3E,F**). Interestingly, Rapamycin-treated T$_{EM}$- and T$_{EMRA}$-derived cultures contained significantly higher proportions of CD8$^+$ IFNγ-producers than untreated cultures upon CMV-specific re-stimulation (**Figure 3H**), whereas there were no significant differences in CD4$^+$ IFNγ-producers (**Figure 3G**). Overall, Rapamycin conferred distinct effects on different T-cell memory subsets, sustaining T$_{CM}$ features and counteracting differentiation into late-stage memory/effector T-cells.

Rapamycin-Treated T-Cell Products Have a Unique Transcriptome Resembling T$_{CM}$

To confirm that expansion of antigen-reactive T-cells under Rapamycin treatment "freezes" an early memory T-cell stage, we tried to extend our analysis on transcriptome level by RNA-sequencing using next-generation sequencing (NGS) of untreated and Rapa-TCPs at d21 (**Figure 4A**). The RNA expression data revealed a total of 146 differentially expressed genes between Rapa-TCPs and untreated TCPs (**Figure 4A**). Many of these relate to TCP performance (**Figure 4B**, **Table S1**). We reviewed the literature and various databases to identify T-cell associated processes (**Figure 4B**) and to estimate the relevance of the differentially regulated genes (**Table S1**). With reference to previously published data, 84% of the genes identified as potentially relevant for TCP potency and longevity *in vivo* were regulated in a beneficial manner in Rapa-TCPs. Among these differentially expressed genes, we identified increased expression of T$_{CM}$ markers such as *CCR7* and *PIM2*

FIGURE 2 | Rapamycin promotes survival of T-cells and stabilizes their metabolism. **(A)** Exemplary dot-plots of flow cytometry data regarding live/dead stain and Annexin V stain (apoptosis) gated on lymphocytes singlets CD3$^+$ T-cells. Living T-cells are defined by double negative staining for Annexin V and live/dead stain in Rapa- (upper panel) and untreated TCPs (lower panel). Samples in the right panel were treated with 1 μg/ml activating antibody against Fas (CD95) to induce apoptosis. **(B)** Proportions of living T-cells in Rapa- (red) and untreated TCPs (blue) of $n = 8$ HDs identified as shown in **(A)** at d21. **(C)** Proportions of living T-cells in Rapa- (red) and untreated TCPs (blue) of $n = 8$ HDs incubated with Fas-activating antibody identified as shown in **(A)** at d21. **(D)** Exemplary histograms of fluorescence intensity of Bcl-2 in CD4$^+$ (upper panel) and CD8$^+$ T-cells (lower panel) of untreated (blue) and Rapa-TCPs (red) acquired by flow cytometry. **(E,F)** MFIs of Bcl-2 in CD4$^+$ **(E)** and CD8$^+$ T-cells **(F)** in untreated (blue) and Rapa-TCPs (red) of $n = 18$ HDs. **(G)** Oxygen consumption rate (OCR)/extracellular acidification rate (ECAR) ratio of Rapa- (red) and untreated TCPs (blue) of $n = 5$ HDs determined in a Seahorse assay. For stimulation (pastel colors) CMV$_{IE-1/pp65}$ peptide pools were added to TCPs relying on mutual presentation of peptides by T-cells from the TCP. For all graphs normal distribution of data points was tested with Kolmogorov-Smirnov test and paired t-test was used to determine significance. P-values below 0.05 are indicated by * and defined to be significant.
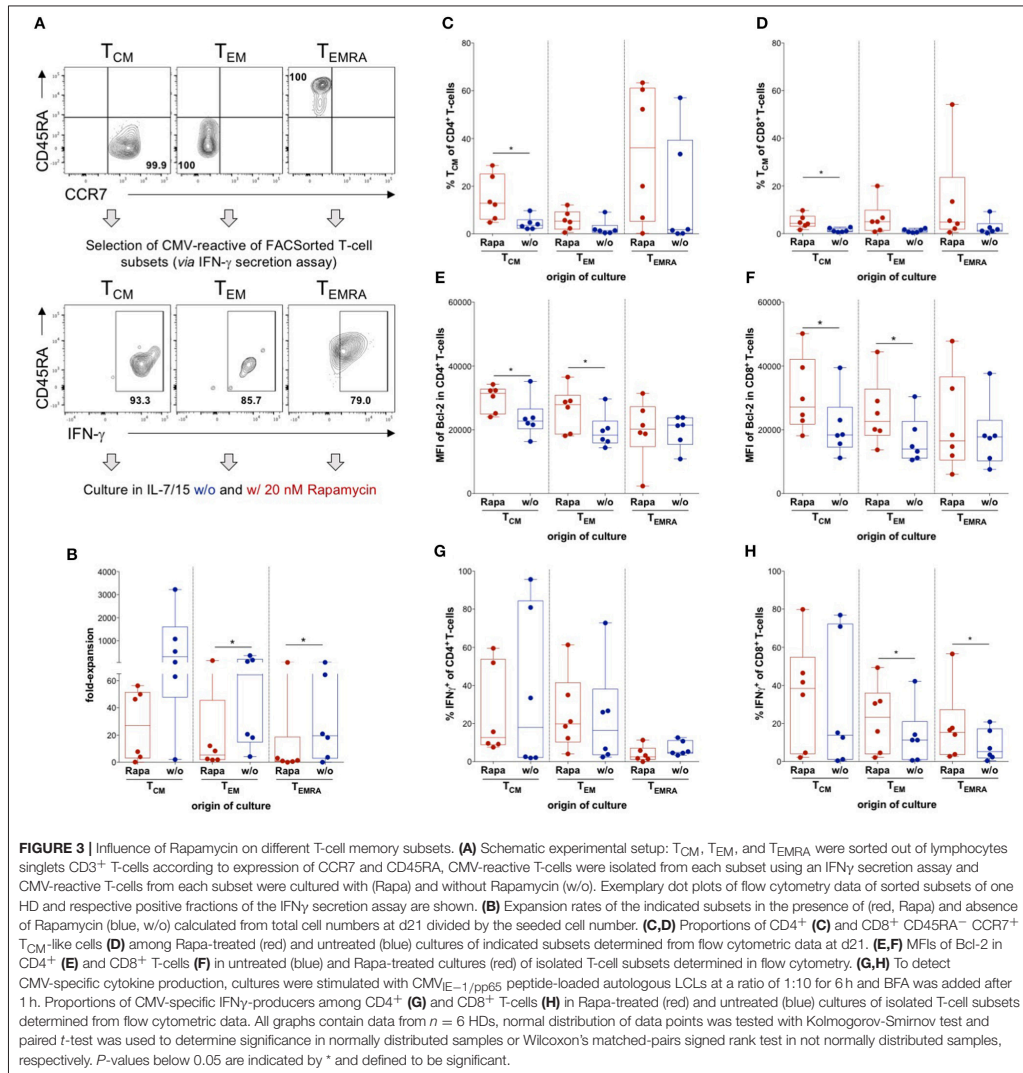
(54), increased expression of *TERT*, which induces self-renewal capacity and increases the proliferative potential of human T-cells (55), and *IL7R*, which is reported to be a marker for persisting and protective CD8$^+$ memory T-cells (56) in Rapa-TCPs (**Table S1**). Furthermore, our observation of sustained IFNγ production in Rapa-TCPs is in line with the findings of increased expression of *IL-13* regulating IFNγ synthesis (57), *DRD2* inducing IFNγ production (58) and *TNFRSF11A*, which increases IFNγ secretion upon binding its ligand (59). In addition, these data are underlined by increased expression of activation enhancing genes, including *e.g.*, *KLF7* (60)*, RGMB* (61), and *TNFRSF19* (62) in Rapa-TCPs. Moreover, the fact that anti-apoptotic Bcl-2 is upregulated on protein level may be supported by increased expression of *MYB*, which exerts its anti-apoptotic activity *via* Bcl-2 (63). However, also many other genes inhibiting apoptosis, such as *e.g.*, *BEX2* (64) and *SIX1* (65), show significantly higher expression in Rapa-TCPs compared to untreated TCPs. The metabolic data of increased glycolysis in Rapa-TCPs are in line with increased expression of *EPAS1* (66), however, also *CHDH*, a gene involved in fatty acid oxidation, (67) is higher expressed in Rapa-TCPs than in untreated TCPs. See **Table S1** for a complete view and annotation of the genes differentially expressed in untreated and Rapa-TCPs and their functions potentially relevant for TCP efficacy *in vivo*. Of note, the T$_{CM}$-like cells sorted from untreated TCPs on day 18 of culture (**Figure 4C**) clustered with Rapa-TCPs (**Figure 4D**) regarding the differentially expressed genes identified in **Figure 4A**. Interestingly, principle component (PC) analysis revealed some components of the transcriptome of Rapa-TCPs to be more similar to *ex vivo*-sorted T$_{CM}$ than T$_{EM}$ (dimension PC2; **Figure S2**).

Rapamycin-Treated T-Cell Products Show Less Clonal and More Diverse TCR Repertoires

To estimate the TCR repertoire of our TCPs, we performed TCRβ NGS. Notably, TCRβ sequencing showed a more diverse clonal composition of Rapa-TCPs compared to untreated TCPs (**Figure 4E**). Venn diagrams of the total numbers of clones and overlap between Rapa- and untreated TCPs are shown in **Figure S3A** and the distribution of the top 100 clones is shown in **Figure S3B**. Correspondingly, the top 10 clones covered around 70 and 90% of the whole TCRβ repertoire of Rapa-TCPs and untreated TCPs, respectively (**Figure 4F**) and Rapa-TCPs contained more different clones than untreated TCPs (**Figure 4G**). The top 10 shared clones and their respective frequencies in Rapa- and untreated TCPs are shown in **Figure S3C**. Comparison of unique nucleotide sequences revealed a high clonal overlap between the distinct Rapa- and untreated TCPs generated from the same donor (**Figure S3D**). In contrast, comparison of clonal repertoires between different individuals showed no overlap, confirming the specificity of the findings (**Figure S3D**).
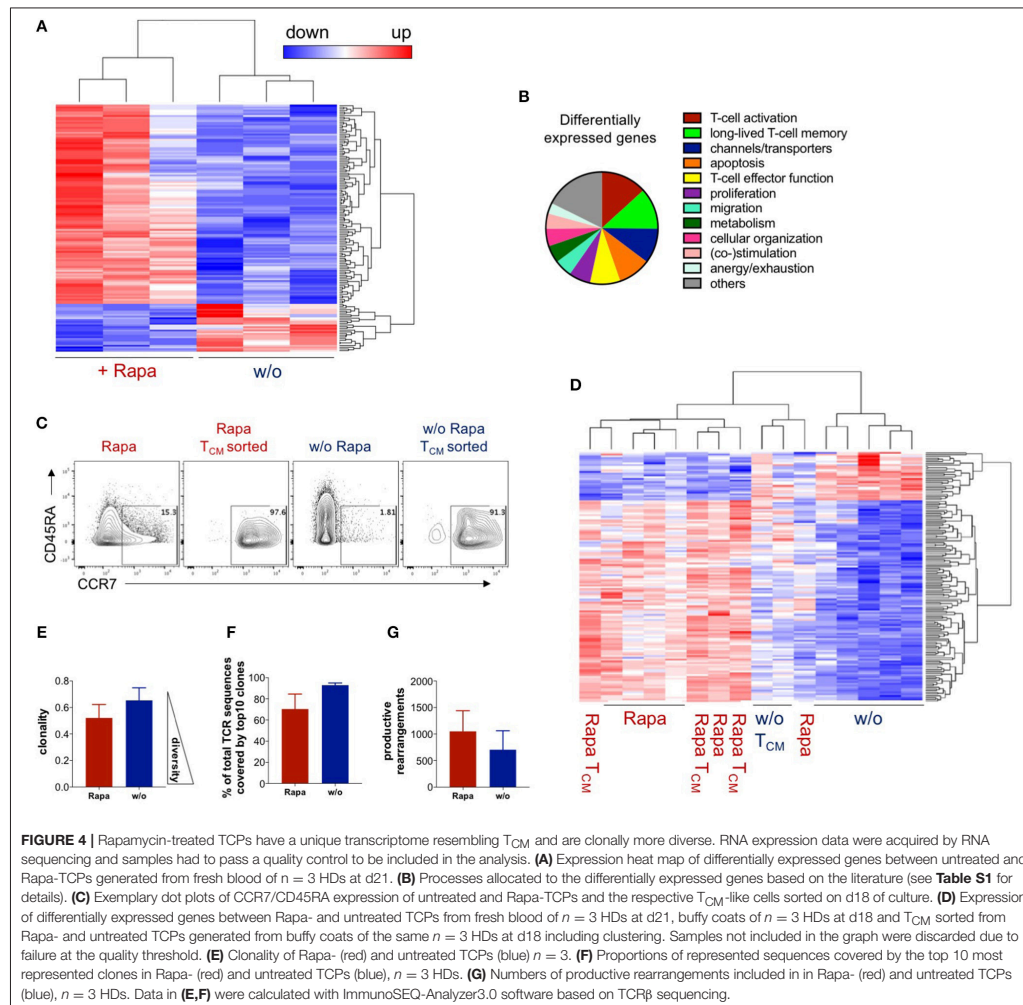
Onset of Immunosuppression in Patients Does Not Influence the Starting Material for TCPs Regarding T-Cell Differentiation and CMV-Specificity

As a prerequisite for clinical translation, we aimed at confirming feasibility of Rapa-TCP generation from patient blood and therefore collected samples from end stage kidney disease (ESRD) patients before and after kidney

FIGURE 3 | Influence of Rapamycin on different T-cell memory subsets. **(A)** Schematic experimental setup: T_{CM}, T_{EM}, and T_{EMRA} were sorted out of lymphocytes singlets CD3^{+} T-cells according to expression of CCR7 and CD45RA, CMV-reactive T-cells were isolated from each subset using an IFNγ secretion assay and CMV-reactive T-cells from each subset were cultured with (Rapa) and without Rapamycin (w/o). Exemplary dot plots of flow cytometry data of sorted subsets of one HD and respective positive fractions of the IFNγ secretion assay are shown. **(B)** Expansion rates of the indicated subsets in the presence of (red, Rapa) and absence of Rapamycin (blue, w/o) calculated from total cell numbers at d21 divided by the seeded cell number. **(C,D)** Proportions of CD4^{+} **(C)** and CD8^{+} CD45RA^{−} CCR7^{+} T_{CM}-like cells **(D)** among Rapa-treated (red) and untreated (blue) cultures of indicated subsets determined from flow cytometric data at d21. **(E,F)** MFIs of Bcl-2 in CD4^{+} **(E)** and CD8^{+} T-cells **(F)** in untreated (blue) and Rapa-treated cultures (red) of isolated T-cell subsets determined in flow cytometry. **(G,H)** To detect CMV-specific cytokine production, cultures were stimulated with $CMV_{IE-1/pp65}$ peptide-loaded autologous LCLs at a ratio of 1:10 for 6 h and BFA was added after 1 h. Proportions of CMV-specific IFNγ-producers among CD4^{+} **(G)** and CD8^{+} T-cells **(H)** in Rapa-treated (red) and untreated (blue) cultures of isolated T-cell subsets determined from flow cytometric data. All graphs contain data from $n = 6$ HDs, normal distribution of data points was tested with Kolmogorov-Smirnov test and paired t-test was used to determine significance in normally distributed samples or Wilcoxon's matched-pairs signed rank test in not normally distributed samples, respectively. P-values below 0.05 are indicated by * and defined to be significant.

transplantation (KTx). To investigate the influence of immunosuppression on the starting material for TCPs, 7 paired samples from ESRD patients before/after KTx were analyzed *ex vivo*. All KTx recipients received standard immunosuppression (characteristics in **Table S2**, *pre/post*-Tx paired samples highlighted in gray). T-cells were divided into five differentiation subsets: CCR7^{+}CD45RA^{+}CD95^{−} T_N (naïve T-cells), CCR7^{+}CD45RA^{+}CD62L^{+}CD45RO^{−}CD95^{+}

T_{SCM}, CCR7^{+}CD45RA^{−} T_{CM}, CCR7^{−}CD45RA^{−} T_{EM}, and CCR7^{−}CD45RA^{+} T_{EMRA} (**Figures S4A,B**) revealing no substantial differences between CD4^{+} and CD8^{+} memory T-cell subset distributions of paired patient samples before/after KTx (**Figures S4C,D**). To assess the phenotypic and functional characteristics of CMV-specific T-cells, PBMCs were stimulated with $CMV_{pp65/IE1}$ peptides showing markedly higher frequencies of CMV-responsive T-cells among CD8^{+}

FIGURE 4 | Rapamycin-treated TCPs have a unique transcriptome resembling T_{CM} and are clonally more diverse. RNA expression data were acquired by RNA sequencing and samples had to pass a quality control to be included in the analysis. **(A)** Expression heat map of differentially expressed genes between untreated and Rapa-TCPs generated from fresh blood of n = 3 HDs at d21. **(B)** Processes allocated to the differentially expressed genes based on the literature (see **Table S1** for details). **(C)** Exemplary dot plots of CCR7/CD45RA expression of untreated and Rapa-TCPs and the respective T_{CM}-like cells sorted on d18 of culture. **(D)** Expression of differentially expressed genes between Rapa- and untreated TCPs from fresh blood of n = 3 HDs at d21, buffy coats of n = 3 HDs at d18 and T_{CM} sorted from Rapa- and untreated TCPs generated from buffy coats of the same n = 3 HDs at d18 including clustering. Samples not included in the graph were discarded due to failure at the quality threshold. **(E)** Clonality of Rapa- (red) and untreated TCPs (blue) n = 3. **(F)** Proportions of represented sequences covered by the top 10 most represented clones in Rapa- (red) and untreated TCPs (blue), n = 3 HDs. **(G)** Numbers of productive rearrangements included in Rapa- (red) and untreated TCPs (blue), n = 3 HDs. Data in **(E,F)** were calculated with ImmunoSEQ-Analyzer3.0 software based on TCRβ sequencing.

compared to CD4$^+$ T-cells (**Figures S4E,F**). However, frequencies of CMV-responsive T-cells were similar before and after KTx (**Figures S4G,H**) and T-cell memory subsets were comparable among CMV-responsive T-cells before and after KTx (**Figures S4I,J**).

Manufacturing Rapa-TCPs Is Feasible Before and After Transplantation

We assessed feasibility of TCP generation from patient material collected before and after KTx. Manufacture of untreated and Rapa-TCPs was successful with respect to yield (**Figure 5A**), although untreated CMV-specific TCPs resulted in higher

yields, which was statistically significant in TCPs generated after KTx (**Figure 5A**). Rapa-TCPs showed beneficial, higher CD4$^+$ T-cell proportions compared to untreated TCPs, which was statistically significant in TCPs generated before KTx (**Figure 5B**). Compared to untreated TCPs, Rapa-TCPs showed higher proportions of CD4$^+$ and CD8$^+$ T_{CM} in KTx recipient-derived TCPs, which was significant in TCPs generated after KTx (**Figures 5C–E**). Upon re-stimulation with CMV-peptide-loaded autologous LCLs, we found enhanced IFNγ-producers (**Figure 5F**) and IFNγ/granzyme B (GZB)-double-producers (**Figure 5I**) among Rapamycin-treated CD8$^+$ T-cells (**Figures 5H,K**), whereas their proportions among

CD4$^+$ T-cells remained stable (**Figures 5G,J**). We recorded comparable frequencies of IFNγ-producers and IFNγ/GZB-double-producers among CD4$^+$ T-cells in TCPs generated before and after KTx (**Figures 5G,J**), whereas both increased among CD8$^+$ T-cells in TCPs generated after KTx (**Figures 5H,K**). Remarkably, Rapamycin increased the frequency of T$_{CM}$ among IFNγ-producers (**Figures 5L,M**), which was statistically significant among CD4$^+$ IFNγ-producers in TCPs generated after KTx (**Figure 5l**). Regarding CMV-specific cytotoxic effects, Rapa-TCPs were as effective as untreated TCPs and TCPs generated *pre-* and *post*-KTx neither showed any differences (**Figure 5N**).

CMV History Affects the Composition of Starting Material From *Post*-KTx Patients

To determine the influence of the CMV infection status on the T-cell subset composition and function of starting material for TCP generation, 19 CMV seropositive KTx patients (**Table S2**) and 13 CMV seropositive healthy donors (HDs) were analyzed in parallel. Patients were categorized according to their CMV reactivation status: No recorded ($n = 9$; 5 m/4f), history of ($n = 6$; 2 m/4f) or very recent CMV-DNAemia within 2 weeks before blood collection ($n = 4$; 4 m/0f) (**Table S2**). The CMV reactivation status had almost no effect on the global CD4$^+$/CD8$^+$ T-cell memory subset distribution (**Figures S5A–C,E,F,H–J**), except for an increase in proportions of CD8$^+$ T$_{SCM}$ and a decrease in CD4$^+$ T$_{EM}$ in the blood of patients with no record of CMV viremia compared to HDs (**Figures S5D,G**).

We found CMV-reactive T-cells in all KTx patients and HDs, with markedly higher frequencies among CD8$^+$ vs. CD4$^+$ T-cells (**Figures S6A,F**). We did neither observe major differences in the magnitude of the CMV-response between KTx patients and HDs nor between the different groups of KTx patients (**Figures S6A,F**). Of note, proportions of below 0.2% of CMV-responsive T-cells among CD8$^+$ T-cells occurred in 38.5% of HDs and only 10.5% of patients without recorded CMV-DNAemia (**Figure S6F**).

The majority of KTx patients showed T$_{SCM}$ frequencies <10% among CMV-reactive CD4$^+$ T-cells. However, patients with a CMV history or recent CMV-DNAemia presented with significantly elevated CMV-reactive CD4$^+$ T$_{SCM}$ compared to KTx patients with no recorded CMV-DNAemia and HDs (**Figure S6B**). T$_{CM}$ proportions among CD4$^+$ CMV-responsive T-cells showed high inter-individual differences among the patients and HDs (**Figure S6C**). T$_{EM}$ proportions among CD4$^+$ CMV-responsive T-cells were significantly lower in the cohort of KTx patients with a record of CMV-DNAemia compared to patients with no recorded CMV-DNAemia and HDs (**Figure S6D**). The proportions of T$_{EMRA}$ among the CD4$^+$ CMV-responsive T-cells were below 5%, except for three patients with recent or previous CMV-DNAemia, who all received virostatic medication (**Figure S6E**, **Table S2**). We found no significant differences in the memory subset distribution among CMV-reactive CD8$^+$ T-cells between the different patient groups

and HDs (**Figures S6G–J**). Notably, we could not detect CMV-responsive CD8$^+$ T$_{CM}$ in the majority of samples (**Figure S6H**).

Impact of CMV Reactivation State on Manufacture of Untreated and Rapa-TCPs

To evaluate the quality of untreated and Rapa-TCPs generated from KTx patients under maintenance immunosuppression with distinct CMV reactivation states, CMV$_{pp65/IE1}$-specific T-cells were expanded with or without Rapamycin (37). We successfully manufactured untreated and Rapa-TCPs from all KTx patients and HDs analyzed, although Rapamycin substantially reduced yields in TCPs from all patients and HDs (**Figure 6A**). Recent CMV reactivation further significantly reduced yields of untreated and Rapa-TCPs compared to HDs and history of CMV reactivation reduced yields of Rapa-TCPs compared to HDs (**Figure 6A**). Interestingly, there was an inverse correlation between expansion rate/yield and age in untreated and Rapa-TCPs (**Figures S7A,B**). Furthermore, the number of records with CMV-DNAemia correlated inversely with the yield of Rapa-TCPs (**Figure S7C**). We found that Rapamycin significantly increased the CD4$^+$/CD8$^+$ T-cell ratio in TCPs of KTx patients without recorded CMV-DNAemia and HDs, which was less pronounced in TCPs of KTx patients with a record of CMV-DNAemia (**Figure 6B**). Rapa-TCPs showed significantly higher proportions of CD8$^+$ T$_{CM}$ in all groups except the KTx patients with recent CMV DNAemia ($n = 4$) (**Figure 6D**), while CD4$^+$ T$_{CM}$ were only significantly enriched in TCPs of KTx patients with no recorded CMV-DNAemia and HDs, being less pronounced in TCPs from the other groups (**Figure 6C**).

Upon CMV-specific re-stimulation, we found increased frequencies of CD8$^+$ IFNγ-producers in Rapa-TCPs, which was statistically significant in the group of KTx patients without record of CMV DNAemia (**Figure 6F**). However, Rapa-TCPs of KTx patients with a history of CMV DNAemia contained significantly lower frequencies of IFNγ-producers compared to Rapa-TCPs from HDs (**Figures 6E,F**). Remarkably, the frequency of IFNγ-producers among CD8$^+$ T-cells, but not CD4$^+$ T-cells (**Figure S8A**), inversely correlated with the time from the last CMV-DNAemia in untreated, but not Rapa-TCPs (**Figure S8B**). Notably, Rapa-TCPs included higher proportions of T$_{CM}$-like among IFNγ-producing CD4$^+$ and CD8$^+$ T-cells compared to the corresponding untreated TCPs (**Figures S8C,D**). This was significant in IFNγ-producing CD4$^+$ T-cells in TCPs of KTx patients without record of CMV DNAemia and IFNγ-producing CD8$^+$ T-cells in TCPs of KTx patients without record and history of CMV DNAemia (**Figures S8C,D**). The T$_{CM}$-like phenotype among IFNγ-producing CD4$^+$ T-cells was significantly more frequent in Rapa-TCPs of KTx patients compared to HDs (**Figure S8C**). IFNγ-producing CD8$^+$ T-cells were significantly more frequent in Rapa-TCPs of KTx patients with history of CMV viremia compared to HDs (**Figure S8D**).

In order to characterize functionality, we co-cultured TCPs with CMV-antigen-loaded LCLs for 14 h and killing was analyzed. Untreated and Rapa-TCPs achieved similar target cell lysis (**Figure 6G**). CMV-specific re-stimulation further characterized up to 65.6% of CD4$^+$ T-cells to be cytotoxic
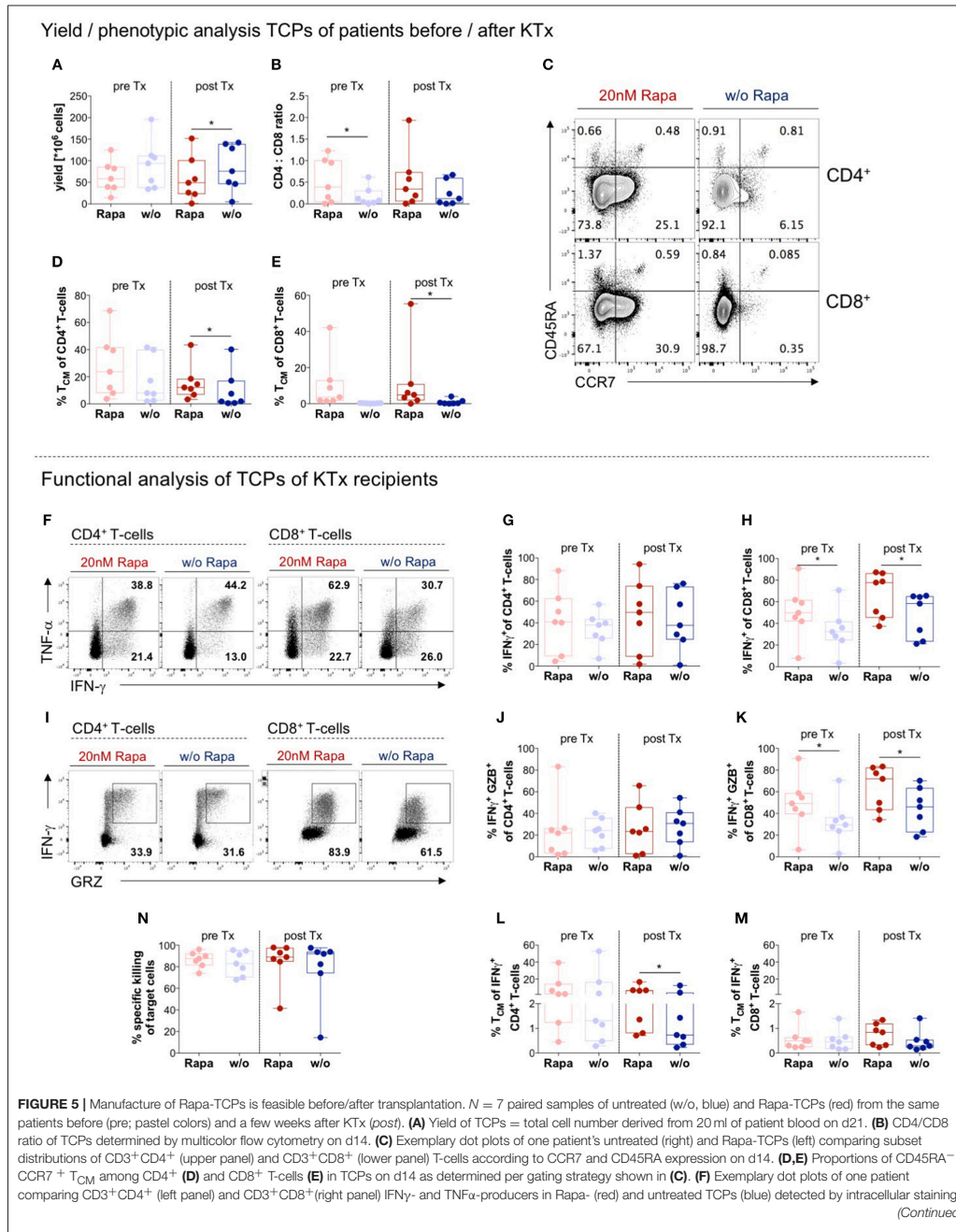
Amini et al.   Advanced CMV-Specific T-Cell Therapy for SOT

Yield / phenotypic analysis TCPs of patients before / after KTx

Functional analysis of TCPs of KTx recipients

FIGURE 5 | Manufacture of Rapa-TCPs is feasible before/after transplantation. $N = 7$ paired samples of untreated (w/o, blue) and Rapa-TCPs (red) from the same patients before (pre; pastel colors) and a few weeks after KTx (*post*). **(A)** Yield of TCPs = total cell number derived from 20 ml of patient blood on d21. **(B)** CD4/CD8 ratio of TCPs determined by multicolor flow cytometry on d14. **(C)** Exemplary dot plots of one patient's untreated (right) and Rapa-TCPs (left) comparing subset distributions of CD3$^+$CD4$^+$ (upper panel) and CD3$^+$CD8$^+$ (lower panel) T-cells according to CCR7 and CD45RA expression on d14. **(D,E)** Proportions of CD45RA$^-$ CCR7 $^+$ T$_{CM}$ among CD4$^+$ **(D)** and CD8$^+$ T-cells **(E)** in TCPs on d14 as determined per gating strategy shown in **(C)**. **(F)** Exemplary dot plots of one patient comparing CD3$^+$CD4$^+$ (left panel) and CD3$^+$CD8$^+$ (right panel) IFNγ- and TNFα-producers in Rapa- (red) and untreated TCPs (blue) detected by intracellular staining
(Continued)

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FIGURE 5 | in multicolor flow cytometry after 6 h stimulation with autologous LCLs loaded with $CMV_{IE-1/pp65}$ peptide pools (gray) or incubation with unloaded autologous LCLs as control (black) and addition of BFA after 1 h on d21. **(G,H)** Summary of background subtracted proportions of $CD4^+$ **(G)** and $CD8^+$ **(H)** CMV-reactive IFNγ-producing T-cells in Rapa- (red) and untreated TCPs (blue) gated as illustrated in **(F)**. **(I)** Exemplary dot plots of one donor comparing $CD4^+$ (left panel) and $CD8^+$ (right panel) CMV-reactive IFNγ- and GZB-producers in Rapa- (red) and untreated TCPs (blue) detected by intracellular staining in multicolor flow cytometry after 6 h stimulation with autologous LCLs loaded CMV_{IE-1} and CMV_{pp65} peptide pools (gray), incubation with unloaded autologous LCLs as control (black) and addition of BFA after 1 h on d21. **(J,K)** Summary of background subtracted proportions of $CD4^+$ **(J)** and $CD8^+$ **(K)** CMV-reactive IFNγ/GZB-double-producers in Rapa- (red) and untreated TCPs (blue) gated as illustrated in **(I)**. **(L,M)** Proportions of $CD45RA^- CCR7^+ T_{CM}$ among CMV-reactive IFNγ-producing $CD4^+$ **(L)** and $CD8^+$ T-cells **(M)**. Gates were applied from gates set for global T-cell subset distribution (see **Figure S4**). **(N)** Specific killing of $CMV_{IE-1/pp65}$ peptide pool loaded autologous LCLs determined by ratio with unloaded allogenic LCLs at a 1:10 ratio with TCPs after incubation overnight. All data tested for normal distribution of data points with Kolmogorov-Smirnov test; significance determined with paired *t*-test if normally distributed or Wilcoxon's matched-pairs signed rank test for not normally distributed samples. *P*-values below 0.05 are indicated by * and defined to be significant.

as defined by GZB/IFNγ-double-production (**Figure S8E**). Interestingly, Rapa-TCPs of KTx patients with no record of CMV viremia contained significantly more GZB/IFNγ double producers among $CD8^+$ T-cells than their untreated counterparts (**Figure S8F**). Rapa-TCPs of KTx recipients with history of CMV viremia contained significantly less GZB/IFNγ double producers among $CD8^+$ T-cells than those of HDs (**Figure S8F**).

Rapa-TCPs From Patients Exhibit Superior Viability After Thawing

Strikingly, every single Rapa-TCP consistently comprised higher proportions of living T-cells compared to its untreated counterpart (**Figure 7A**). By convention, TCPs have to be frozen until GMP-compliant quality controls are accomplished and then are thawed directly before infusion into patients. This procedure is a major stress for the TCPs. We froze and thawed TCPs from ESRD/KTx patients and HDs and observed an increased frequency of living T-cells in the Rapa-TCPs, being detectable immediately and even 1 day after thawing and culture (**Figures 7B,C**). Consistent with the findings from HDs, also Rapa-TCPs of KTx patients showed elevated MFIs of Bcl-2 compared to the untreated TCPs (**Figure S9**).

In summary, we demonstrate that CMV-specific Rapa-TCPs can be generated irrespective of the employed cytokine regiment, show better viability even after thawing, a stable metabolism, beneficial gene expression and increased clonal diversity. We likewise demonstrate the possibility to generate TCPs from patients in ESRD and *post*-KTx despite maintenance immunosuppression containing similar attributes as from HDs. Further, we illustrate that functional CMV-specific T-cells, the prerequisite for manufacture of CMV-specific TCPs, could be identified in all KTx patients investigated and present successful manufacture of untreated and Rapa-TCPs irrespective of the viral replication history.

DISCUSSION

The aim of our study was to demonstrate the benefit of the Rapamycin treatment during manufacture of TCPs with improved properties. We demonstrate stability of Rapa-TCPs irrespective of cytokine combinations administered during expansion, sustained IFNγ production despite withdrawal of Rapamycin and re-stimulation with viral antigen. Furthermore, we found superior viability and partial resistance to death

receptor-induced apoptosis, stable metabolism upon activation, favorable gene expression pattern, and enhanced clonal diversity of Rapa- compared to untreated TCPs. Moreover, we show preferential expansion of T_{CM} in the presence of Rapamycin and partial conversion of other T-cell memory subsets to T_{CM}-like cells. We demonstrate the feasibility of manufacturing autologous anti-CMV Rapa-TCPs from blood of ESRD patients and KTx recipients with distinct CMV reactivation history. We confirm increased CD4/CD8 ratios and T_{CM} proportions in Rapa- compared to untreated TCPs (37), which are associated with long-term clinical efficacy of adoptively transferred TCPs (29, 30, 40, 41). In addition, our results imply that generation of CMV-specific TCPs prior to transplantation is possible, however, not favorable. Moreover, Rapa-TCPs from patients contained more viable cells after freezing/thawing compared to untreated TCPs.

Although the cytokine combination IL-7/IL-15 was reported to preferentially promote T_{CM} (49), we did not reveal substantial differences compared to cultures expanded with IL-2/IL7, suggesting these combinations are equally suited for TCP expansion and do not alter cell intrinsic mTOR-dependent signaling programs. Antigenic re-challenge and concomitant elimination of antigen-loaded APCs decreased T-cell expansion and resulted in T-cell culture contraction. These cultures predominantly contained long-lived memory T-cells. In line with this, among CMV-specific $CD4^+$ T-cells, we recorded significant decreases in T_{EM} and significant increases in T_{SCM} in KTx recipients with a record of CMV DNAemia.

Rapa-TCPs consistently contained more living T-cells than untreated TCPs, even after freezing/thawing. Hence, Rapa-TCPs may entail increased fitness following infusion, as TCPs are frozen until accomplishment of all quality controls in a clinical setting respecting GMP/GCP. As shown previously for B-cell lymphoma lines (51), Rapamycin increased Bcl-2 on protein level and moreover led to partial resistance to Fas-induced apoptosis. We did not identify altered gene expression of *BCL-2* on mRNA level, however, expression of *MYB*, exerting anti-apoptotic effects *via* Bcl-2 (63), was upregulated in Rapa-TCPs. Furthermore, the decreased sensitivity to apoptosis of Rapa-TCPs may also be conferred by additional proteins and pathways as we found many other anti-apoptotic genes, such as *BEX2* (64) and *SIX1* (65), to be overexpressed in Rapa-TCPs compared to untreated TCPs.

We recorded higher OCR/ECAR ratios in untreated TCPs, although inhibition of glycolysis is associated with a long-lived memory phenotype (68) and memory T-cells
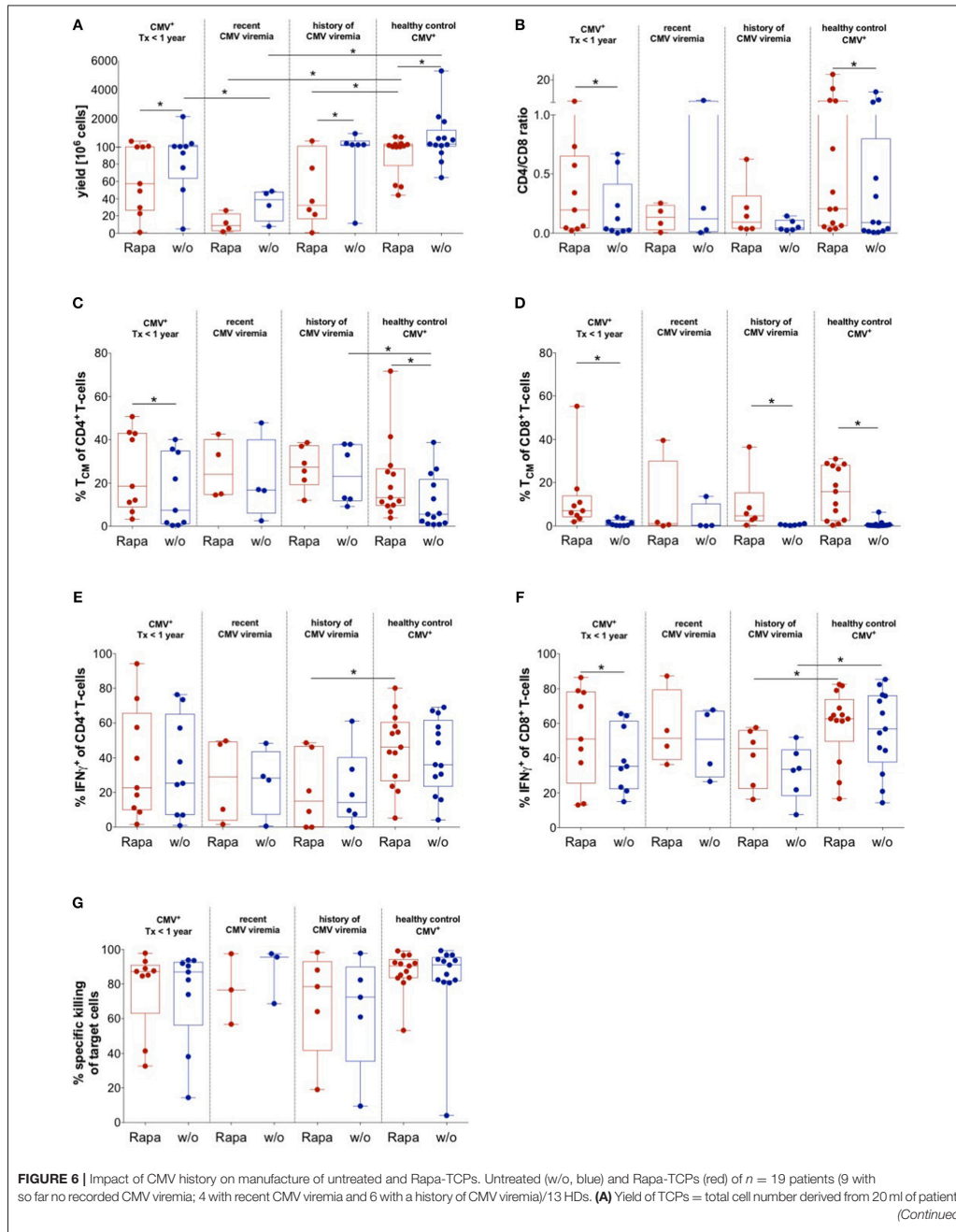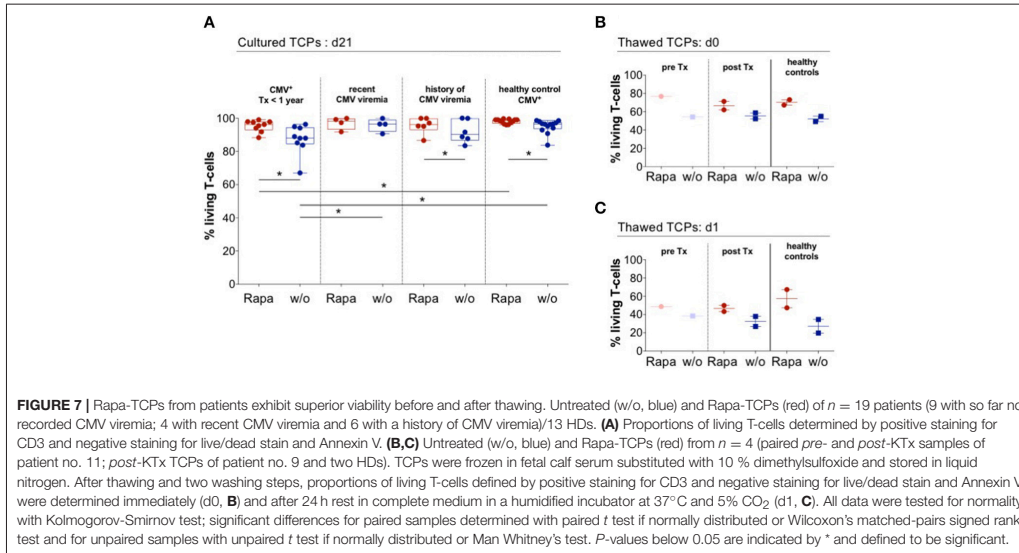
FIGURE 6 | Impact of CMV history on manufacture of untreated and Rapa-TCPs. Untreated (w/o, blue) and Rapa-TCPs (red) of $n = 19$ patients (9 with so far no recorded CMV viremia; 4 with recent CMV viremia and 6 with a history of CMV viremia)/13 HDs. **(A)** Yield of TCPs = total cell number derived from 20 ml of patient
(Continued)

FIGURE 6 | blood on d21. **(B)** CD4/CD8 ratio of TCPs on d14. **(C,D)** Proportions of CD45RA⁻ CCR7 ⁺ T_CM among CD4⁺ **(C)** and CD8⁺ T-cells **(D)** in TCPs as determined per gating strategy shown in **Figure 5C** on d14. **(E,F)** Proportions of CMV-reactive CD4⁺ **(E)** and CD8⁺ **(F)** IFNγ-producers detected by intracellular staining in multicolor flow cytometry after 6 h stimulation with autologous LCLs loaded CMV_IE−1/pp65 peptide pools at a ratio of 1:10 and addition of BFA after 1 h on d21. Gating strategy is shown in **Figure 5F**. **(G)** Specific killing of CMV_IE−1/pp65 peptide pool loaded autologous LCLs determined by ratio with unloaded allogenic LCLs at 1:10 ratio with TCPs after incubation overnight. All data were tested for normality with Kolmogorov-Smirnov test; significant differences for paired samples determined with paired *t*-test if normally distributed or Wilcoxon's matched-pairs signed rank test and for unpaired samples with unpaired *t*-test if normally distributed or Man Whitney's test. *P*-values below 0.05 are indicated by * and defined to be significant.

FIGURE 7 | Rapa-TCPs from patients exhibit superior viability before and after thawing. Untreated (w/o, blue) and Rapa-TCPs (red) of *n* = 19 patients (9 with so far no recorded CMV viremia; 4 with recent CMV viremia and 6 with a history of CMV viremia)/13 HDs. **(A)** Proportions of living T-cells determined by positive staining for CD3 and negative staining for live/dead stain and Annexin V. **(B,C)** Untreated (w/o, blue) and Rapa-TCPs (red) from *n* = 4 (paired *pre*- and *post*-KTx samples of patient no. 11; *post*-KTx TCPs of patient no. 9 and two HDs). TCPs were frozen in fetal calf serum substituted with 10 % dimethylsulfoxide and stored in liquid nitrogen. After thawing and two washing steps, proportions of living T-cells defined by positive staining for CD3 and negative staining for live/dead stain and Annexin V were determined immediately (d0, **B**) and after 24 h rest in complete medium in a humidified incubator at 37°C and 5% CO₂ (d1, **C**). All data were tested for normality with Kolmogorov-Smirnov test; significant differences for paired samples determined with paired *t* test if normally distributed or Wilcoxon's matched-pairs signed rank test and for unpaired samples with unpaired *t* test if normally distributed or Man Whitney's test. *P*-values below 0.05 are indicated by * and defined to be significant.

are reported to preferentially perform fatty acid oxidation (53). Increased glycolysis in Rapa-TCPs was supported by RNA sequencing data showing increased expression of *EPAS1* (66), however, also *CHDH*, a gene involved in fatty acid oxidation (67), showed increased expression in Rapa-TCPs. In fact, glycolysis is reported to allow immediate effector function (69), which is in line with the Rapa-TCPs' enhanced capacity for IFNγ production and increased expression of *MAP3K21* allowing a rapid switch from a rested to an activated state (70). Furthermore, the metabolism of Rapa-TCPs remained more stable upon CMV-specific activation. Memory cell self-renewal might occur at a comparable number to that of effector T-cell generation in Rapa-TCPs, whereas in untreated TCPs, the balance might be extremely skewed toward effector T-cells upon activation, leading to this significant decrease in OCR/ECAR ratio and lack of long-lived memory T-cells.

Intriguingly, 84% of genes differentially expressed between untreated and Rapa-TCPs, whose function we could allocate to impact TCPs, were regulated toward promotion of an effective and long-lived product. Our data imply a T_CM-like transcriptome of long-lived poly-functional memory T-cells for Rapa-TCPs (54–56, 66, 71–79). The fact that Rapa-TCPs show increased clonal diversity may occur due to survival of low frequency

clones. Especially, the preferential expansion of T_CM in Rapa-TCPs may contribute to increased clonal diversity, as this subset was shown to have a higher clonal diversity compared to further differentiated memory T-cells (80). In fact, this may also be the underlying mechanism, why we have more CD4⁺ T-cells in the Rapa-TCP, because, evident from our *ex vivo* data, proportions of CMV-specific T_CM are much higher among CD4⁺ compared to CD8⁺ T-cells.

When CMV-specific T-cells from different memory T-cell subsets were cultured individually, we revealed preferential expansion of T_CM with preserved T_CM phenotype, partially protected from differentiation in the presence of Rapamycin, but partially also conversion of T-cells from other memory T-cell subsets into T_CM-like cells (81). Interestingly, patient 12 lacked CMV-specific early CD8⁺ memory T-cells and his CMV-specific T-cells consisted to 90.6% of T_EMRA. However, the respective Rapa-TCP included a strikingly high proportion of CD8⁺ T_CM suggesting reprogramming of T_EM/T_EMRA to T_CM.

We did not record major differences in the characteristics of the TCPs irrespective of whether the TCPs were generated *post*-KTx or *pre*-KTx. Nonetheless, CD8⁺ T-cells comprised higher proportions of T_CM and cytokine producers in TCPs generated *pre*-KTx. Hence, there is no benefit to generate anti-CMV TCPs prior to KTx.

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Investigating *ex vivo*-T-cell responses to CMV-specific stimuli, we found higher frequencies of CD8$^+$ than of CD4$^+$ CMV-specific T-cells as described previously [82]. Frequencies of below 0.2% of CMV-reactive CD8$^+$ T-cells were found in 2/19 patients compared to 6/13 HDs, which matches findings suggesting an increase of CD8$^+$ CMV-reactive T-cells *post*-Tx [83]. Interestingly, low frequencies of CMV-reactive CD8$^+$ T-cells did not cause low TCP yields, underlining the feasibility of TCP generation. Intriguingly, a high proportion of CD8$^+$ T$_{EMRA}$ among CMV-responsive T-cells did neither cause low expansion/yield nor high CD4/CD8 ratio in the TCP, which stresses the applicability even to patients with a high degree of terminal T-cell differentiation.

The dosage for successful CMV-specific AVTT in the SOT setting is undetermined. Case reports suggest numbers between 30 and 245 million T-cells [19–21, 36]. Importantly, our protocol for TCP generation achieves medians of IFNγ-producers of 20 and 50% in CD4$^+$ and CD8$^+$ T-cells, respectively, and median killing rates of 85%, while these frequencies are much lower in other published approaches reporting a maximum of 8% of CMV-reactive IFNγ-producers in the TCP and specific lysis of ≤50% at higher T-cell/target ratios [19, 20]. Comparable values are only achieved in a recent study [21]. Based on the superiority in function and phenotype of Rapa-TCPs, we assume that also smaller T-cell numbers would be efficient for long-term control of CMV in KTx patients. The patients in this study whose TCPs yielded <5 million T-cells from 20 ml of peripheral blood, included the two patients with the lowest lymphocyte counts (Patients 6/10), suggesting an amendment of the amount of blood collected to the lymphocyte count. Patient 6, with no recorded CMV DNAemia, was diagnosed with acute rejection and treated with ATG before blood collection. ATG administration may be an indication to collect blood for TCP generation preventively, given the patients' risk of developing CMV disease [84, 85]. Indeed, transient CMV DNAemia was recorded in this patient 54 days after blood collection for the study. Patient 10 had a record of recent CMV viremia, was seronegative *pre*-KTx, received a kidney from a seropositive donor and had extensive CMV-associated complications. Notably, he was the oldest patient included and correlation analysis revealed a negative correlation between age and yield of Rapa-TCPs. We also recorded a low yield of the Rapa-TCP of Patient 19, who had a history of CMV-DNAemia and was receiving Acyclovir-treatment at the time of blood collection, which was reported to diminish IFNγ-production in response to CMV$_{pp65}$ peptides [50]. In cases as described above, we suggest to first generate untreated TCPs for patients with acute CMV disease to diminish viral load [36] and then successively generate and infuse Rapa-TCPs for long-term control of the virus. The fact that we recorded significantly lower yields in patients with a recent CMV DNAemia compared to HDs also suggests to follow the proposed approach in these patients and motivates to investigate more than the four patients that we were able to recruit for this study. In fact, we also found a negative correlation of the number of records with viremia and the yield of Rapa-TCPs for the ten patients with detected CMV DNAemia. However, this analysis may be biased by a more thorough screening of problematic patients, as the tests

for CMV DNAemia were not of equal frequency in all patients. Moreover, patient 12, for whom we could also only generate a Rapa-TCP with a yield of around 5 million cells, suffered from chronic hepatitis. These, of course limited data, indicate that problems may occur during manufacture in the case of different chronic infections. It has to be thoroughly overthought whether it is possible to begin with more blood as starting material in these patients with numerous reactivating infections or use first the conventional approach and then generate a Rapa-TCP for long-term protection.

Recently, Smith and colleagues published a study about the application of a comparable autologous CMV-specific TCP in SOT patients [21]. However, their production process varies in many points, as they do not select for CMV-specific T-cells starting their culture with PBMCs, use G-Rex reactors instead of classical well-plates for expansion and have a different cytokine supplementation strategy using IL-21 and IL-2. They infuse multiple doses of TCPs at up to 6 different points in time. Of note, they demonstrate safety and clinical improvement in the majority of patients and could decrease or stop antiviral medication in many patients. Interestingly, compared to the time of infusion, they see an increase in viral load in 9 of 13 patients after infusion of TCPs [21]. In 5 of the 9 monitored patients CD8$^+$ CMV-specific T-cells were reduced by the end of monitoring (max. day 300) and in three of the cases this correlated with an increase in viral load. One of the patients died of CMV disease [21]. These data demonstrate, that there is still room for optimization in the long-term outcome of SOT patients treated with autologous CMV-specific TCPs. Probably, Rapa-TCPs could improve long-term efficacy, however, the actual clinical performance of our TCP has to be demonstrated. Our preclinical data imply a long-lived TCP with beneficial properties maybe even allowing for a single infusion.

A variety of other putative strategies are reported to rejuvenate T-cells with beneficial characteristics for AVTT. These include among others interference with different signaling pathways [86–90], use of different cytokine supplementation strategies [91, 92], employment of certain microRNAs [93], modulation of metabolism [53, 68], inhibition of ion channels [94], and promotion of autophagy [95]. However, most of these are far from being practicable under GMP conditions for contemporary application to a clinical setting. This is also the case for genetic manipulation of T-cells for optimization of AVTT. Proposed strategies for genetic engineering include induction of resistance to immunosuppressive medication [96, 97], introduction of suicide genes as safety switch [49, 98] and knock out of anti-inflammatory signaling components such as PD-1 and LAG-3 [99, 100]. All these suggestions have to be adapted to realistic GMP-feasible conditions and then may be valuable upgrades for even more sophisticated AVTT approaches. In contrast to other approaches, whose translation is less progressed at the moment, our minimally manipulative next-generation anti-CMV AVTT may help many transplanted patients whose endogenous immune system is not capable of defying the virus. Furthermore, the beneficial properties of Rapa-TCPs may also be transferred to other approaches using antigen-specific T-cells, e.g., other viruses or cancer immunotherapy with known antigens [42].

In summary, our study revealed favorable phenotypic and functional properties of Rapa-TCPs as well as their applicability to a variety of ESRD/KTx patient samples. Ultimately, we seek for clinical confirmation of functionality and efficacy of Rapa-TCPs in a clinical proof-of-concept trial.

ETHICS STATEMENT

The Charité Ethics Committee (IRB) approved the study protocol and all blood donors provided written informed consent.

AUTHOR CONTRIBUTIONS

LA, H-DV, PR, and MS-H conceptualized and designed the study. PR and NO provided patient samples and data. LA, TV, DW, and AJ performed experiments supervised by MS-H. LA acquired, analyzed, and interpreted data. LA and MS-H composed figures and manuscript. KJ performed bioinformatics analyses, created the respective graphs and made RNA seq data available at the GEO platform. TV, DW, SL-K, KJ, H-DV, and PR critically revised and all authors approved the final version of the manuscript.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01148/full#supplementary-material

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Conflict of Interest Statement: H-DV, PR, and MS-H own a patent on the manufacture of Rapa-TCPs.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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11. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12.   Complete list of publications

- **Amini L**, Vollmer T, Wendering DJ, Jurisch A, Landwehr-Kenzel S, Otto NM, Jürchott K, Volk HD, Reinke P, Schmueck-Henneresse M; Comprehensive characterization of a next-generation antiviral T-cell product and feasibility for application in immunosuppressed transplant patients; Front Immunol. 2019 May 28. (Impact Factor 2017/2018: 5.511)

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13. Acknowledgements