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DISSERTATION

Herstellung von CRISPR-Cas9-editierten Tacrolimus-resistenten SARS-CoV-2-spezifischen T-Zellen für die adoptive Zelltherapie in Transplantationspatienten

Generation of CRISPR-Cas9-edited Tacrolimus-resistant SARS-CoV-2-specific T-cells for adoptive cell therapy in transplant patients

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List of Abbreviations

ACT	adoptive T-cell therapy
AP3a	accessory protein 3a
BeCAT	Berlin Center for Advanced Therapies
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CITE-seq	cellular indexing of transcriptomes and epitopes sequencing
CMV	cytomegalovirus
CNI	calcineurin inhibitor
COVID-19	coronavirus disease 2019
CRISPR	clustered regularly interspaced short palindromic repeats
CsA	cyclosporin A
DMSO	dimethyl sulfoxide
DN cell	double negative cell
DNA	deoxyribonucleic acid
DP cell	double positive cell
EBV	Epstein-Barr virus
FCS	fetal calf serum
FKBP12	FK506 binding protein 12
GFP	green fluorescent protein
GMP	good-manufacturing practice
HCoV	human coronavirus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
IFN-γ	interferon gamma
IL	interleukin
IS	immunosuppressant
KO	knockout
LCLs	lymphoblastoid cell lines
MPA	mycophenolic acid
mRNA	messenger ribonucleic acid
NCAP	nucleocapsid protein

NFAT	nuclear factor of activated T-cells
NK cell	natural killer cell
NS	non-structural protein
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pGFP	plasmid encoding green fluorescent protein
Pred	prednisolone
pSpike	plasmid encoding SARS-CoV-2 wild-type spike protein
rh	recombinant human
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Roswell Park Memorial Institute
RT	room temperature
S.p.	*Streptococcus pyogenes*
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
scRNA-seq	single-cell ribonucleic acid sequencing
SD	standard deviation
SEB	Staphylococcal enterotoxin B
sgRNA	single-guide ribonucleic acid
SOT	solid organ transplantation
Tac	tacrolimus
T_{CM}	central memory T-cell
TCPs	T-cell products
TCR	T-cell receptor
TCR-seq	T-cell receptor sequencing
T_{EM}	effector memory T-cell
T_{EMRA}	terminally differentiated CD45RA-expressing T-cell
T_{NAIVE}	naïve T-cell
TNF-α	tumor-necrosis factor alpha
VEMP	envelope small membrane protein
VME1	membrane protein
Y14	uncharacterized protein 14

Zusammenfassung

Die Empfänger von soliden Organtransplantaten benötigen eine dauerhafte Immunsuppression, um die Abstoßung des Transplantats zu verhindern. Dies beeinträchtigt ihre Immunantwort gegen Infektionen und Impfstoffe, so dass Transplantatempfänger einem erhöhten Risiko ausgesetzt sind, virale Komplikationen zu entwickeln. Transplantatempfänger leiden bei einer Infektion mit dem *severe acute respiratory syndrome coronavirus-2* (SARS-CoV-2) häufiger unter einem schweren Verlauf der Coronavirus Erkrankung 2019 (COVID-19) als die gesunde Bevölkerung. Obwohl die SARS-CoV-2-Impfung den Schweregrad der Erkrankung verringern kann, zeigen Transplantatempfänger teilweise keine, nur eine schwache oder kurzlebige Impfantwort. Daher wird der SARS-CoV-2-spezifische adoptive T-Zell-Transfer als Therapie oder zur Vorbeugung von schweren COVID-19 Verläufen in Transplantatempfängern in Betracht gezogen. Die adoptive antivirale T-Zell-Therapie ist eine attraktive Behandlungsstrategie von Virusinfektionen bei immungeschwächten oder immunsupprimierten Patienten, allerdings beeinträchtigt die Immunsuppression auch deren Wirksamkeit. In der vorliegenden Arbeit wurde die Herstellung von SARS-CoV-2-spezifischen T-Zellprodukten etabliert, die gegen das Immunsuppressivum Tacrolimus (Tac) resistent sind. Diese könnten als neuartiger fortschrittlicher therapeutischer Behandlungsansatz gegen SARS-CoV-2 nach einer Transplantation eingesetzt werden. Darüber hinaus könnte die Kombination aus Tac und Tac-resistenten SARS-CoV-2-spezifischen T-Zellprodukten eine innovative Behandlungsstrategie für schwere COVID-19 Verläufe bei nicht transplantierten Patienten darstellen, da nicht nur eine überschüssige Immunantwort, die als „Bystander“-T-Zell-Aktivierung in der COVID-19-assoziierten Immunpathologie auftreten kann, verhindert, sondern auch eine effiziente Viruseliminierung ermöglicht werden könnte. Tac-resistente SARS-CoV-2-spezifische T-Zellprodukte wurden durch virusfreie CRISPR-Cas9-basierte Gen-Editierung von acht ungeimpften SARS-CoV-2-Rekonvaleszenten hergestellt. Die Funktionalität der Tac-resistenten T-Zellprodukte in Anwesenheit von Tac wurde vergleichend mit nicht-editierten T-Zell-Produkten als Gesamtprodukt auf Proteomebene und auf Einzelzellebene charakterisiert, wobei Methoden wie die Durchflusszytometrie, die zelluläre Indexierung von Transkriptomen und Epitopen (CITE-seq) und die T-Zell-Rezeptor Sequenzierung (TCR-seq) verwendet wurden. Die vielversprechenden Studienergebnisse deuten darauf hin, dass eine Kombination von Tac und dem adoptiven

Transfer von Tac-resistenten SARS-CoV-2-spezifischen T-Zellprodukten schwere COVID-19-Erkrankungen bei immunsupprimierten Transplantationspatienten, sowie Immunpathologien außerhalb des Transplantationsfeldes bei gleichzeitiger Viruseliminierung, verhindern könnte.

Abstract

Solid organ transplant recipients require permanent immunosuppression to prevent graft rejection. This impairs their immune response to infections and vaccines, putting transplant recipients at increased risk of developing viral complications. Transplant recipients are more likely to suffer from severe coronavirus disease 2019 (COVID-19) when infected with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) than the healthy population. Although SARS-CoV-2 vaccination can reduce disease severity, transplant recipients partially show no, weak, or short-lived vaccine response. Therefore, SARS-CoV-2-specific adoptive T-cell transfer is considered as a therapeutic or preventive strategy for severe COVID-19 courses in transplant recipients. Adoptive antiviral T-cell therapy is an appealing treatment strategy for viral infections in immunocompromised or immunosuppressed patients; however, immunosuppression also impairs its efficacy. The present work established the production of SARS-CoV-2-specific T-cell products (TCPs) being resistant to the immunosuppressive drug tacrolimus (Tac). These could be used as a novel advanced therapeutic treatment approach against SARS-CoV-2 after transplantation. In addition, the combination of Tac and Tac-resistant SARS-CoV-2-specific TCPs could provide an innovative treatment approach for severe COVID-19 courses in non-transplant patients by not only preventing excessive immune responses that may occur as "bystander" T cell activation in COVID-19-associated immunopathology, but also by enabling efficient viral elimination. Tac-resistant SARS-CoV-2-specific TCPs were generated by virus-free CRISPR-Cas9-based gene editing of eight unvaccinated SARS-CoV-2 convalescents. The functionality of Tac-resistant TCPs in the presence of Tac was comparatively characterized with non-edited TCPs as a complete product at the proteome and single cell level using methods such as flow cytometry, cellular indexing of transcriptomes and epitopes (CITE-seq), and T cell receptor sequencing (TCR-seq). Promising study results suggest that a combination of Tac and adoptive transfer of Tac-resistant SARS-CoV-2-specific TCPs could prevent severe COVID-19 in immunosuppressed transplant patients, as well as immunopathology outside of the transplant field with concomitant viral elimination.

Schematic overview of PhD Thesis

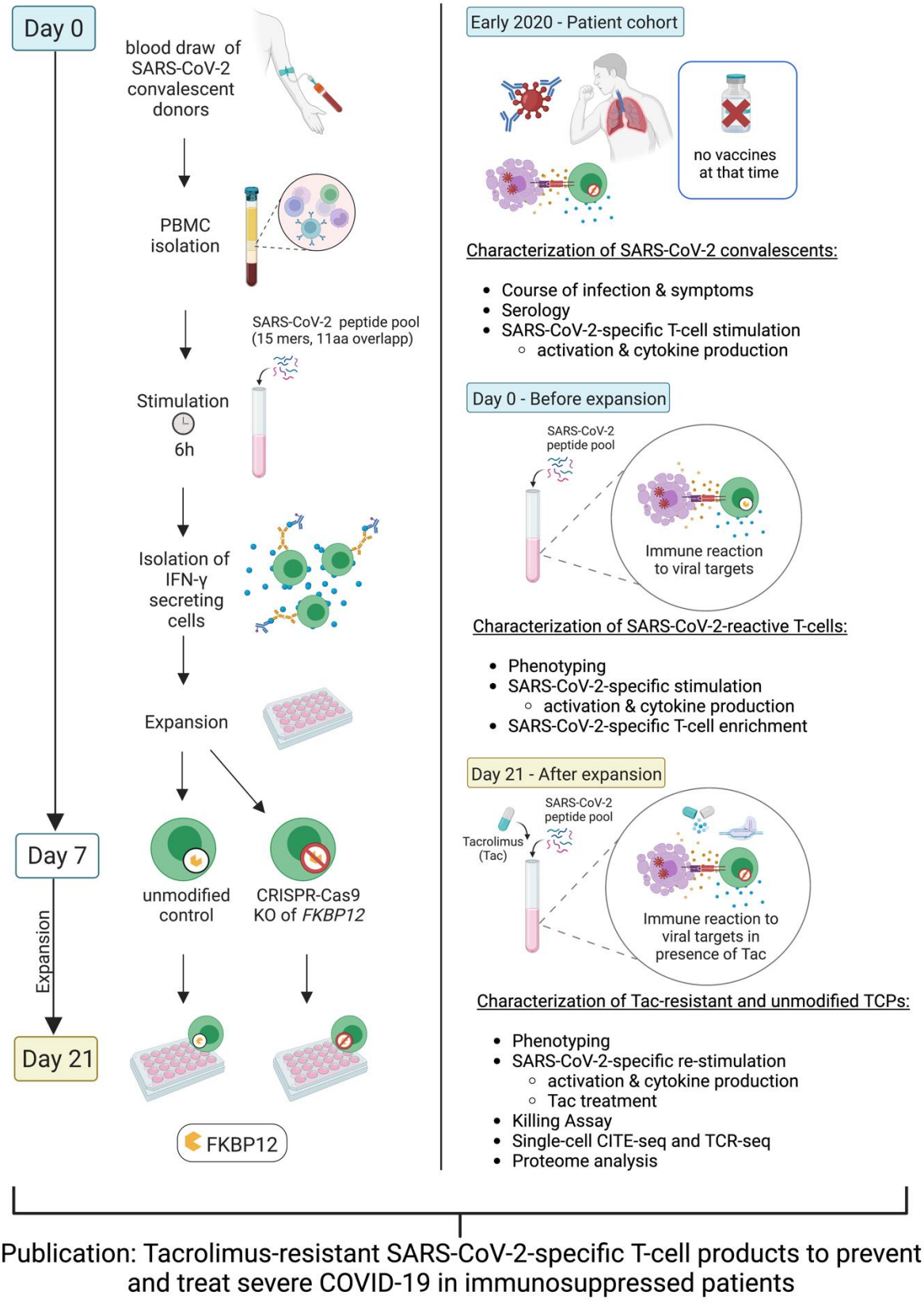

Figure 1: Schematic overview of the experimental procedure and analysis of this PhD project (figure was created by myself, based on the publication [1] using BioRender.com)

1 Introduction

1.1 Transplantation immunology

The recipient's immune system can recognize and attack the transplanted donor tissue resulting into allograft rejection. End-stage organ failure is one if the main risks in solid organ transplantation (SOT) recipients [2]. To minimize the risk of transplant rejection, it is important to consider donor-recipient human leukocyte antigens (HLA)-matching [3] but also pre-screening for infectious diseases to prevent viral re-activation or transmission [4]. Chronic immunosuppression in transplant recipients is a common strategy to improve allograft function by suppressing unwanted immune responses against the transplanted tissue. Classical triple immunosuppression, comprising a combination of calcineurin inhibitors (CNI), corticosteroids, and mycophenolic acid (MPA), are widely used in SOT recipients [5]. CNI, such as Tac or cyclosporine A (CsA), interfere with the calcineurin pathway by binding to the respective intracellular immunophilin preventing the dephosphorylation of nuclear factor of activated T-cells (NFAT) and thus, T-cell receptor mediated T-cell activation [5]. Corticosteroids, such as prednisone, suppress pro-inflammatory cytokines and thereby, reduce activity of both T-cells and B cells, which can result in lymphocyte apoptosis [5]. MPA inhibits the proliferation of T-cells by suppressing purine synthesis [5]. However, systemic immunosuppression does not only target alloreactive immune cells but the entire adaptive immune system, which makes SOT and hematopoietic stem cell transplantation (HSCT) recipients susceptible to chronic (e.g. cytomegalovirus (CMV), Epstein-Barr virus (EBV) or BK virus) but also acute viral infections [6–11]. Therapeutic strategies to cope with viral complications in the transplant setting include the reduction of immunosuppression, administering antiviral medication to target the viral replication machinery or restoring the adaptive immunity by adoptively transferring virus-specific T-cells.

1.2 T-cell immunity towards SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 and is responsible for the ongoing global COVID-19 pandemic [12,13]. The disease severity can range from asymptomatic/mild to severe respiratory failure. Robust T-cell mediated immunity has proven to be crucial in containing SARS-CoV-2 infection and correlates with

disease severity [14–16]. However, severe COVID-19 is characterized by an overshooting cell-mediated immune response (cytokine storm) towards SARS-CoV-2 causing hyperinflammation and severe tissue damage [17–19]. COVID-19-associated hyperinflammation correlates with a dysregulated adaptive immunity resulting in insufficient viral elimination [17,20–22]. Activation of bystander $CD8^+$ T-cells through the pro-inflammatory milieu may considerably contribute to such overshooting immune responses [23]. This phenomenon is linked to an experienced immune system with a high degree of late-differentiated memory T-cells found predominantly in the elderly population [24]. Immunosuppression using dexamethasone has become the standard treatment for patients suffering from severe COVID-19 [25,26]. Although dexamethasone has shown to improve the clinical outcome of patients receiving respiratory support [25,27,28], it prolongs viral shedding by suppressing the functionality of the patient's adaptive immune system [29–31]. Strategies to regenerate SARS-CoV-2-specific immune responses are necessary to contain viral control in the setting of severe COVID-19. The development of highly efficient SARS-CoV-2 vaccines in late 2020, has shown to reduce the risk for a severe COVID-19 course by inducing robust humoral and cellular immunity in healthy individuals [32–34]. However, the continuing emergence of SARS-CoV-2 variants can impair vaccine induced B cell and T-cell responses and may increase the probability of a re-infection in convalescent individuals, which raises concerns about SARS-CoV-2-driven immunological escape [35–37]. Infecting individuals with several SARS-CoV-2 variants has shown that >90% of generated $CD4^+$ and $CD8^+$ T-cells have conserved T-cell epitopes, respectively, that are less dominated by spike protein. Therefore, mutations in the spike protein domain may barely affect T-cell-driven immunity towards SARS-CoV-2 variants, including Delta and Omicron [38].

1.3 SARS-CoV-2 in immunosuppressed patients

Patients receiving constant immunosuppression to prevent allograft rejection are at elevated risk to develop severe COVID-19 with prolonged hospitalization and increased mortality compared to the general population [39–42]. A large US study on SARS-$CoV\text{-}2^+$ SOT recipients has shown that about 43% of SOT recipients were hospitalized with elevated risk to develop acute kidney injury, graft failure as well as organ rejection and cardiologic problems [43]. Moreover, patients that recently underwent SOT have shown to be at higher risk for SARS-CoV-2 infection than those whose transplantation dates back

longer, suggesting that the amount of immunosuppressive drugs influences mortality in SARS-CoV-2 infected SOT recipients [44]. Changes in the immunosuppression regimen of SOT recipients (e.g. reducing the dose or temporarily withholding immunosuppressants) have not shown to correlate with COVID-19 severity or mortality [45]. Some reports suggest that SOT recipients suffering from COVID-19 benefit from reduction or withdrawal of MPA while maintaining Tac treatment [46]. Tac has shown to have beneficial antiviral properties *in vitro* by suppressing pro-inflammatory cytokine production and replication of human coronavirus (HCoV) (e.g. SARS-CoV-1, HCoV-NL63, and HCoV-229E) [47,48]. Further strategies to improve COVID-19 outcome in transplant recipients include antiviral medication as well as treatment with convalescent plasma [49,50] or monoclonal antibodies [51,52], to boost the immune response towards SARS-CoV-2. Both, convalescent plasma, and monoclonal antibodies have shown to improve COVID-19 outcome in the general population [53,54], however, the therapeutic effect can be lower in SOT recipients as it strongly correlates with the immunosuppressive regimen [49–52]. Studies on SARS-CoV-2 mRNA vaccines in SOT recipients have generally proven their safety and effectiveness, although their efficacy depends on the degree of immunosuppression [55–57]. SOT recipients have shown to mount reduced SARS-CoV-2 vaccine responses compared to the general population [56,58–60], in a few cases the effectiveness improved following the third or fourth vaccine dose [55,61,62]. Moreover, there is evidence for predominant impairment of the vaccine induced humoral response in transplant recipients while T-cell responses were largely comparable to those observed in healthy individuals [63]. However, limited responses to vaccination in SOT recipients may not protect from SARS-CoV-2 infection, especially when considering emerging SARS-CoV-2 strains, leaving them at elevated risk to develop severe COVID-19. Indeed, several studies have shown that vaccinated SOT patients are still susceptible to SARS-CoV-2 infection and often require hospitalization [60,64,65]. Another study in a large UK cohort of SOT recipients has reported that COVID-19 mRNA vaccination reduced the risk of death by 20%, implying that SOT recipients have a certain degree of protection, although the effect is still lower than in the general population [57]. These observations underline the necessity for alternative treatments to protect transplant recipients from severe COVID-19.

1.4 Adoptive cell therapy in transplant patients and its progress for the treatment of COVID-19

Transfer for antiviral T-cells to treat viral complications in HSCT and SOT recipients has proven to be safe and feasible [66–68], with very low incidence of adverse events [69]. Antiviral TCPs are generated by isolating and expanding peripheral blood mononuclear cells (PBMCs) in presence of virus-specific antigens *in vitro* following transfusion into the recipient. Allogenic ACT to control viral complications post transplantation is frequently applied in the HSCT setting while autologous antiviral ACT is more common among SOT recipients. Hence, for autologous ACT, the starting material has to be enriched from immunosuppressed SOT patients, which has shown to be feasible [70,71]. In SOT recipients, antiviral ACT resulted in successful treatment of EBV- [72,73] as well as CMV-derived diseases [70,74–76]. Constant immunosuppression is required to prevent allograft rejection after transplantation but may impair long-term survival and engraftment of adoptively transferred T-cells. To overcome this obstacle, several approaches to generate antiviral immunosuppressant-resistant T-cells for ACT in transplant recipients have been described that showed antiviral efficacy in the immunosuppressive environment [77–80].

With emerging evidence that a robust T-cell response is essential for viral elimination and long-term protection against SARS-CoV-2 [14–16], adoptive transfer of SARS-CoV-2-specific T-cells has been proposed to treat or prevent COVID-19 in immunocompromised or immunosuppressed patients as well as for the treatment of acute COVID-19 [1,81–86]. In addition, there is data that ACT can promote antibody responses [87]. Therefore, SARS-CoV-2-specific ACT may not only support viral clearance but also the establishment of cellular and humoral immunity, of which especially those individuals could benefit from that fail to build up a protective immunity post SARS-CoV-2 infection or vaccination. Considering the constant immunosuppression in transplant patients, generation of immunosuppressant-resistant SARS-CoV-2-specific TCPs are a promising approach to provide not only robust viral elimination but also suppressing COVID-19-associated overshooting immune responses in immunosuppressed patients [1,83].

1.5 Study design

The present study hypothesizes that SARS-CoV-2-specific TCPs can be rendered resistant to the IS Tac, to retain their functionality and phenotype in the presence of clinical

doses of Tac. We decided to use the IS Tac as it is frequently administered to transplant recipients to prevent allograft rejection and it may further have beneficial antiviral properties [47,48]. The study aimed at generating Tac-resistant SARS-CoV-2-specific T-cells from SARS-CoV-2$^+$ convalescent donors using a good-manufacturing practice (GMP)-compliant manufacturing process [79]. Therefore, we isolated and expanded SARS-CoV-2-reactive T-cells and performed a vector-free Ribonucleoprotein (RNP)-based CRISPR-Cas9-mediated knockout (KO) of the FK506 binding protein 12 (*FKBP12*) in SARS-CoV-2-specific T-cells.

Due to the lack of a protective SARS-CoV-2-vaccine in early 2020, we planned to examine SARS-CoV-2 convalescent donors for their T-cell reactivity towards SARS-CoV-2-derived structural (NCAP (nucleocapsid), Spike S1, Spike S2, VEMP (envelope small membrane protein), VME1 (membrane protein)) and accessory (AP3a (accessory protein 3a), NS6 (non-structural protein 6), NS7a (non-structural protein 7a), NS7b (non-structural protein 7b), NS8 (non-structural protein 8), ORF9b (open reading frame 9b), ORF10 (open reading frame 10), Y14 (uncharacterized protein 14)) antigens. In the expected instance, that we find SARS-CoV-2-reactive T-cells, we envisioned to expand the cells SARS-CoV-2-specifically and to render them resistant to Tac using CRISPR-Cas9-based gene-editing. At the end of expansion, we strived to comparatively characterize unmodified control and Tac-resistant SARS-CoV-2-specific TCPs with methods such as flow cytometry, CITE-seq and TCR-seq as well as proteome analysis.
We hypothesized to demonstrate that Tac-resistant SARS-CoV-2-specific TCPs possess superior effector cytokine production in presence of clinical doses of Tac compared to unmodified control SARS-CoV-2-specific TCPs. The triple IS (Tac/Prednisolone (Pred)/MPA) as well as CsA may be tested to reduce / abolish effector cytokine production in gene-edited cell products, to provide a potential safety switch *in vivo* post infusion, respectively. The generated Tac-resistant SARS-CoV-2-specific TCP should demonstrate essential antiviral effector functions in the presence of Tac while maintaining the phenotype of unmodified TCPs.

2 Methodology

In the following, the detailed procedure of the methods used for the present study are explained. The methods refer to the attached publication [1] (Peter et al. 2022 – *Tacrolimus resistant SARS-CoV-2-specific T-cell products to prevent and treat severe COVID-19 in immunosuppressed patients*)

2.1 Patient cohort

The study was conducted in early 2020 when no protective SARS-CoV-2 vaccines were available. Peripheral blood was either collected from convalescent SARS-CoV-2 seropositive individuals with a history of asymptomatic/mild COVID-19 (20 donors) or SARS-CoV-2 seronegative healthy donors (19 donors). The procedure to determine the SARS-CoV-2 serology in all blood donors as well as a table comprising COVID-19 symptom severity and potential long-term effects in SARS-CoV-2 convalescent donors can be found within the publication [1]. The study was approved by the Ethics Committee of Charité - Universitätsmedizin Berlin and all subjects had given their written informed consent according to the ‘Declaration of Helsinki'.

2.2 Isolation of Peripheral blood mononuclear cells

PBMCs were isolated from peripheral blood by Biocoll (Biochrom) density gradient centrifugation. Therefore, either heparinized or citrate blood was diluted 1:2 with PBS (Gibco) and transferred onto a layer of Biocoll solution following centrifugation at 800x *g* for 20 min at room temperature (RT) without deceleration. Hereby, cell populations in peripheral blood are separated in layers according to their varying densities. The PBMC layer was carefully collected and washed twice with PBS at 400x *g* for 10 min at 4°C. After determination of cell count using Neubauer chamber and trypan blue (Sigma-Aldrich), cells were diluted with RPMI medium (PAN-Biotech) supplemented with 10% fetal calf serum (FCS, PAA) and 100 U/mL penicillin and 100 µg/mL streptomycin (both Biochrom) and incubated at 37 °C and 5% CO_2 until further analysis.

2.3 Antigen-specific stimulation of PBMCs *ex vivo*

PBMCs were stimulated with overlapping peptide pools of individual antigens of SARS-CoV-2 (JPT Peptide Technologies, *i.e*. NCAP, Spike S1, Spike S2, VEMP, VME1, AP3a, NS6, NS7a, NS7b, NS8, ORF9b, ORF10, Y14; 1 µg/ml each). A purified anti-CD28 antibody (clone CD28.2, Biolegend) was added to ensure proper T-cell activation. An unstimulated sample supplemented with DMSO served as negative control and Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich) and CMV peptide pool (pp65 and IE-1; 0.5 µg/ml each – JPT Peptide Technologies) stimulated samples served as positive controls, respectively. PBMCs were incubated at 37 °C and 5% CO_2 for 2h until 2 µg/ml brefeldin A (Sigma-Aldrich) was added to allow detection of intracellular cytokine production by inhibiting the vesicle transport from the golgi apparatus. PBMCs were incubated for another 14h at 37 °C and 5% CO_2 following antibody staining for flow analysis.

2.4 FACS staining and data acquisition

For extracellular staining, cells were incubated with respective antibodies (human anti-CCR7 (G043H7) and -CD45RA (HI100); both Biolegend) and LIVE/DEAD Fixable Blue Dead Cell Stain (L/D; Invitrogen) to exclude dead cells at 37 °C and 5% CO_2 for 30 minutes and subsequently washed with PBS at 400x *g* for 10 minutes. Afterwards, cells were fixed and premetallized for 30 min at 4 °C using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) and washed with PBS at 400x *g* for 10 minutes. For intracellular staining, human anti-CD3 (OKT3), -CD4 (SK3), -CD8 (RPA-T8), -IFN-γ (4S.B3), -TNF-α (MAb11), -IL-2 (MQ1-17H12), -CD137 (4B4-1) and -CD154 (24-31) antibodies (all Biolegend) were used. Cells were stained intracellularly for 20 min at 4°C and subsequently washed with PBS at 400x *g* for 10 minutes. Flow cytometry data was acquired at the CytoFLEX flow cytometer (Beckman Coulter), and the data analyzed using FlowJo-10 software (Tree Star).

2.5 Generation of SARS-CoV-2 specific T-cell products

To enrich and expand SARS-CoV-2-specific T-cells, PBMCs were isolated from peripheral blood of convalescent donors and stimulated with SARS-CoV-2 peptide pools (JPT Peptide Technologies; 1 mg/mL each) for 6h following IFN-γ Secretion Assay - Cell En-

richment and Detection Kit, which was carried out according to the manufacturer's instructions (Miltenyi Biotec). This method detects and enriches IFN-γ-secreting T-cells with a high sensitivity. The isolated IFN γ-secreting T-cells were the starting material for generating SARS-CoV-2-specific TCPs and were co-cultured with $1*10^7$ irradiated PBMCs (feeder cells) in RPMI medium (PAN-Biotech) supplemented with 10% FCS (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin as well as 10 ng/mL recombinant human IL-7 (rhIL-7) and rhIL-15 (both CellGenix) in 24-well plates and incubated at 37 °C and 5% CO_2. Upon reaching 100% confluency, cells were split 1:1.

2.6 Knockout of *FKBP12* to achieve Tacrolimus-resistance in SARS-CoV-2 specific T-cell products

To render SARS-CoV-2-specific T-cells resistant to the IS Tac, a vector-free RNP-based CRISPR-Cas9-based KO of the adaptor protein *FKBP12* was carried out. At day 7 of culture, SARS-CoV-2-specific T-cells were split 1:1 and one part was left unmodified while the other was used for a CRISPR-Cas9-based KO of *FKBP12*. Therefore, $2\text{-}10*10^6$ T-cells were washed twice with PBS at 400x *g* for 10 min. To generate RNP complexes for electroporation of T-cells, 30 µg of recombinant Alt-R® S.p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies) was combined with 15 µg synthetically modified single-guide RNA (sgRNA) that targets 5'-GGGCGCACCTTCCCCAAGCG-3' and carries 2O'-methyl-3'phosphothioate modifications between the first and last 3 nucleotides (Synthego Corporation). The RNPs were transferred to SARS-CoV-2-specific T-cells by electroporation using Amaxa P3 primary cell 4D-Nucleofector X Kit L (Lonza, according to the manufacturers protocol) and the Amaxa-Nucleofector-4D (Lonza, program CO-115). After electroporation, cells were quickly recovered with pre-warmed antibiotic-free RPMI medium supplemented with 10% FCS, transferred into a 24-well plate and cultured in a humidified incubator at 37 °C and 5% CO_2. After 24h, the cell medium was supplemented with 10 ng/mL rhIL-7 and rhIL-15. Upon reaching 100% confluency, cells were split 1:1.
The KO efficiency was determined at day 21 of culture using peak-shift analysis after Sanger sequencing. The detailed procedure is described within the publication [1].

2.7 Generation of autologous lymphoblastoid cell lines

Immortalized lymphoblastoid cell lines (LCLs) were generated by transfecting $1*10^7$ autologous PBMCs with EBV in presence of 1 µg/ml CsA and 2.5 µg/ml CpG and cultured

in RPMI supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin for 21 days. Upon reaching 100% confluency, cells were either split 1:1 or transferred into a cell culture flask.

2.8 SARS-CoV-2-specific re-stimulation of SARS-CoV-2-specific T-cell products

SARS-CoV-2-specific re-stimulation of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs was carried out using SARS-CoV-2 peptide pool loaded LCLs as antigen-presenting cells in co-culture with SARS-CoV-2-specific T-cells in presence or absence of IS at clinical doses (6 ng/mL Tac (Prograf, Astellas); 120 ng/mL CsA (Sandimmun, Novartis); triple IS = 6 ng/mL Tac + 0.57 mg/mL Pred (Urbason solubile, Sanofi) + 2.7 mg/mL MPA (active substance of mycophenolate mofetil; Sigma-Aldrich)). Unloaded LCLs served as control to exclude unspecific T-cell activation or activation due to presentation of EBV epitopes. Therefore, LCLs and SARS-CoV-2-specific T-cells were harvested separately and washed with PBS at 400x *g* for 10 min. Cell count was determined with a Neubauer chamber and trypan blue and cells were resuspended in respective volume of RPMI medium supplemented with 10% FCS and 100 U/mL penicillin and 100 µg/mL streptomycin. Afterwards LCLs were loaded with SARS-CoV-2 peptide pools (NCAP, spike S1, spike S2, VEMP, VME1, AP3a, NS6, NS7a, NS7b, NS8, ORF9b, ORF10, Y14; JPT Peptide Technologies; 0.5 mg/mL) and co-cultured with SARS-CoV-2-specific T-cells in a 1:10 ratio (LCLs:T-cell). Like for *ex vivo* stimulations, samples were incubated at 37 °C and 5% CO_2 for 2h until 2 µg/ml brefeldin A was added to allow detection of intracellular cytokine production. After a total of 16h stimulation, cells were stained and analyzed via flow cytometry as described earlier.

2.9 VITAL Assay

The procedure of the VITAL assay and the calculation of the SARS-CoV-2-specific killing capacity by SARS-CoV-2-specific TCPs is described in detail within the publication [1].

The capacity of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs to lyse SARS-CoV-2-presenting target cells was determined by co-culturing SARS-CoV-2 peptide pool-loaded autologous LCLs (targets) and allogenic unloaded LCLs (non-targets) in a ratio 1:1 (targets:non-targets) with the respective SARS-CoV-2-specific T-cells in a ratio 1:1 or 1:10 (LCLs:T-cell). To discriminate targets and non-targets, autologous LCLs were

stained with CellTrace CFSE Cell Proliferation Kit, while allogenic ones were labelled with CellTrace Far Red Cell Proliferation Kit (both Life Technologies).

2.10 Cryopreservation of SARS-CoV-2-specific T-cells and LCLs

To cryopreserve SARS-CoV-2-specific TCPs or LCLs, 2-5*10^7 cells were harvested and washed twice with PBS at 400x *g* for 10 min. The cell pellet was resuspended in freezing medium (FCS supplemented with 10% DMSO), up to 2*10^7 cells were transferred into a cryopreservation tube and stored in a freezing container at -80 °C overnight until they were transferred into liquid nitrogen for long-term storage.

2.11 Proteome analysis of SARS-CoV-2 specific TCPs

For proteome analysis, cryopreserved SARS-CoV-2-specific TCPs and autologous LCLs of four donors were quickly thawed in a water bath at 37 °C and washed twice with pre-warmed RPMI medium supplemented with 10% FCS and 100 U/mL penicillin and 100 µg/mL streptomycin at 400x *g* for 10 min. The cell pellet was resuspended in respective amount of medium following cell counting using Neubauer chamber and trypan blue. SARS-CoV-2-specific T-cells were re-stimulated with SARS-CoV-2-loaded autologous LCLs, as described earlier, but in this case the stimulation was performed for a total of 6h in absence of brefeldin A. Subsequently, cells were washed with PBS at 400x *g* for 10 min. To exclude LCLs from further analysis, T-cells were purified by FACSorting at the Sony sorter MA900. Purified T-cells were washed with PBS at 400x *g* for 10 min and cell pellets were frozen in liquid nitrogen and stored at -80 °C until further analysis via nano-liquid chromatography-tandem mass spectrometry at the Imaging Mass Spectrometry Unit at the BIH Center for Regenerative Therapies. A detailed procedure of the analysis is described within the publication [1].

2.12 Single cell CITE-Seq and TCR sequencing of SARS-CoV-2 specific TCPs

For single cell CITE-seq and TCR-seq, expanded unmodified control and Tac-resistant SARS-CoV-2-specific TCPs and autologous LCLs of four donors were harvested separately and washed with PBS at 400x *g* for 10 min. The cell pellet was resuspended in respective amount of RPMI medium supplemented with 10% FCS and 100 U/mL penicillin and 100 µg/mL streptomycin following cell counting using Neubauer chamber and trypan

blue. The unmodified control and Tac-resistant SARS-CoV-2-specific TCPs were re-stimulated with SARS-CoV-2-loaded autologous LCLs, as described earlier, but in this case the stimulation was performed for a total of 6h in absence of brefeldin A. Subsequently, cells were washed with PBS at 400x g for 10 min and prepared for single-cell RNA sequencing (scRNA-seq) which was carried out in collaboration with the German Rheumatism Research Center in Berlin. The preparation procedure and analysis of sequencing data is described in detail within the publication [1].

2.13 Statistical analysis

The detailed procedure for statistical testing is described within the publication [1].

3. Results

The following results of the PhD project are published in the attached publication [1] (Peter et al. 2022 – *Tacrolimus resistant SARS-CoV-2-specific T-cell products to prevent and treat severe COVID-19 in immunosuppressed patients*).

3.1 Detection and Isolation of SARS-CoV-2-reactive T-cells upon antigen-specific stimulation

Prior to the generation of SARS-CoV-2-specific TCPs, the SARS-CoV-2-directed immune response was determined in 20 healthy SARS-CoV-2 seropositive convalescent donors that recovered from mild or asymptomatic COVID-19 as well as 19 seronegative healthy SARS-CoV-2 naïve donors (see Table 1). Upon stimulation with distinct SARS-CoV-2-derived antigens, the T-cell reactivity was determined via flow cytometry and revealed upregulation of activation markers (CD137) and effector cytokine production (IFN-γ and/or TNF-α) in all SARS-CoV-2 convalescent donors. The SARS-CoV-2-derived antigens recognized by T-cells differed among $CD4^+$ and $CD8^+$ T-cell subsets. In seronegative healthy control donors, a $CD4^+$ T-cell response (mostly TNF-α) towards all viral antigens was detected, while $CD8^+$-derived T-cell responses were absent.

Table 1: Main results of *ex vivo* characterization of the immune response towards SARS-CoV-2 in SARS-CoV-2 naïve and convalescent donors. The color code implies whether the corresponding patient cohort shows reactivity towards SARS-CoV-2 – green: positive; red: limited / negative (table was created by myself, based on the publication [1]).

Parameter	**SARS-CoV-2 naïve donors (n=19)**	**SARS-CoV-2 convalescent donors (n=20)**
SARS-CoV-2 Serology (Spike specific IgG &IgA)	negative	positive
T-cell reactivity to SARS-CoV-2 antigens	$CD4^+$: limited $CD8^+$: limited	$CD4^+$: positive $CD8^+$: limited

To generate Tac-resistant SARS-CoV-2-specific TCPs, PBMCs from eight SARS-CoV-2 convalescent donors were stimulated SARS-CoV-2-specifically and IFN-γ secreting cells were isolated with high purity using IFN-γ secretion assay. On day 7 of expansion, a non-

viral, RNP-based CRISPR-Cas9-mediated KO of the adaptor protein *FKBP12*, which renders T-cells resistant to Tac, was performed. Unmodified T-cells were expanded in parallel serving as control for upcoming back-to-back characterizations with the Tac-resistant T-cells. Over 21 days of expansion, no substantial differences in expansion rates or cell yield comparing unmodified and Tac-resistant TCPs were observed (see Table 2). The *FKBP12* KO efficiency in Tac-resistant SARS-CoV-2-specific TCPs on day 21 ranged from 63% – 89% (see Table 2).

Table 2: Main results of expansion frequency and KO efficiency in unmodified and Tac-resistant SARS-CoV-2-specific TCPs. Mean ± SD is presented. The color code implies the potential impact on the potency of the TCPs – green: beneficial /positive; yellow: neutral; red: disadvantageous / negative (table was created by myself, based on the publication [1]).

Parameter	**unmodified TCPs**	**Tac-resistant TCPs**
Expansion rate (n=8)	3010 ± 1458	2981 ± 2317
Cell yield (n=8)	$2.7*10^8 \pm 1.7*10^8$	$3.0*10^8 \pm 2.7*10^8$
FKBP12 KO efficiency (n=8)		63 - 89%

3.2 Activation of SARS-CoV-2-specific TCPs upon stimulation with SARS-CoV-2-derived antigens

To estimate the capacity of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs to recognize SARS-CoV-2 derived antigens, TCPs were re-stimulated at day 21 with distinct SARS-CoV-2-derived antigens, respectively. Both, unmodified control, and Tac-resistant SARS-CoV-2-specific TCPs were comparable in their T-cell activation profile towards the distinct SARS-CoV-2-derived antigens. Similar as for *ex vivo* stimulations, expanded SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T-cells recognized distinct viral antigens. The magnitude by which $CD4^+$ and $CD8^+$ T-cell responded to SARS-CoV-2-derived antigens was comparable between unmodified control and Tac-resistant SARS-CoV-2-specific TCPs.

3.3 Differentiated effector memory phenotype in SARS-CoV-2-specific TCPs

As the state of differentiation impacts T-cell functionality and longevity, the phenotype of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs was estimated using

common markers for T-cell differentiation. Prior to enrichment, the $CD4^+$ T-cell compartment contained predominantly naïve (T_{NAIVE}) and central memory (T_{CM}) T-cells, while right after T-cell enrichment the $CD4^+$ T-cell compartment was dominated by T_{CM} and effector memory (T_{EM}) T-cells. However, at day 21 of culture, the phenotype of $CD4^+$ T-cells in both, unmodified and Tac-resistant SARS-CoV-2-specific TCPs shifted to a more differentiated one with high proportions of T_{EM}. In $CD8^+$ T-cells prior to enrichment, a high frequency of T_{NAIVE}, terminally differentiated RA-expressing (T_{EMRA}) T-cells as well as T_{EM} cells was present, while after enrichment the proportion of T_{EMRA} cells increased in both, unmodified and Tac-resistant SARS-CoV-2-specific TCPs. After expansion, late differentiated T_{EMRA}, followed by T_{EM} cells were predominantly detected in the $CD8^+$ T-cell compartment of both TCPs.

3.4 Superior effector cytokine production of Tac-resistant SARS-CoV-2-specific TCPs under Tac treatment compared to unmodified control TCPs

To validate that Tac-resistance rescues T-cell effector function towards SARS-CoV-2 in presence of Tac, expanded unmodified control and Tac-resistant SARS-CoV-2-specific TCPs were re-stimulated at day 21 with a SARS-CoV-2 peptide pool in presence or absence of either IS. $CD4^+$ and $CD8^+$ T-cells among unmodified control and Tac-resistant SARS-CoV-2-specific TCPs demonstrated comparable frequencies of activated ($CD137^+$) effector cytokine producing (IFN-γ, TNF-α and interleukin-2 (IL2)) in absence of IS. However, Tac treatment suppressed cytokine production in unmodified but not Tac-resistant SARS-CoV-2-specific TCPs. Cytokine production can be abolished in both unmodified control and Tac-resistant SARS-CoV-2-specific TCPs by exposing them to CsA. Upon triple immunosuppression with Tac/Pred/MPA, effector cytokine production in $CD4^+$ and $CD8^+$ T-cells of Tac-resistant SARS-CoV-2-specific TCPs was reduced but other than for unmodified control SARS-CoV-2-specific TCPs not fully abolished. Moreover, SARS-CoV-2-specific polyfunctional T-cells capable of producing multiple effector cytokines at once were detected in unmodified control and Tac-resistant SARS-CoV-2-specific TCPs. Expression of the activation marker CD154 was also upregulated upon SARS-CoV-2-specific stimulation of SARS-CoV-2-specific TCPs and its expression was not affected by Tac treatment in Tac-resistant TCPs.

3.5 Beneficial killing capacity of antigen-expressing target cells by SARS-CoV-2-specific Tac-resistant TCPs in presence of Tacrolimus

Next, the capacity of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs to eliminate SARS-CoV-2-presenting target cells in presence and absence of Tac was evaluated. Using SARS-CoV-2 peptide pools for antigen-presentation by target cells, resulted in comparable cytotoxic killing capacity of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs. Other than for cytokine production, the cytotoxic killing capacity of both unmodified control and Tac-resistant SARS-CoV-2-specific TCPs was not affected by short-term Tac treatment. The nucleocapsid protein (NCAP) and accessory protein 3a (AP3a) were identified as driving antigens of T-cell-mediated cytotoxicity towards antigen-presenting target cells in both unmodified control and Tac-resistant SARS-CoV-2-specific TCPs. Furthermore, $CD8^+$ T-cells of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs were identified to predominantly execute the cytotoxic killing of SARS-CoV-2 peptide-loaded target cells.

To further estimate if unmodified control and Tac-resistant SARS-CoV-2-specific TCPs recognize and kill SARS-CoV-2 infected target cells, the killing assay was performed with target cells co-transfected with a plasmid encoding GFP (pGFP) and a plasmid encoding SARS-CoV-2 wild-type spike protein (pSpike). The unmodified control and Tac-resistant SARS-CoV-2-specific TCPs were able to eliminate GFP^+/pSpike-transfected target cells. Other than for peptide-loaded target cells, exposure to Tac impaired the capacity to eliminate GFP^+/pSpike-transfected target cells in unmodified TCPs which was partially rescued by *FKBP12* KO.

3.6 SARS-CoV-2-specific TCPs are reactive to SARS-CoV-2 variants and indicate limited cross-reactivity to common endemic HCoV

With the continuing emergence of novel circulating SARS-CoV-2 variants it is of vital importance that SARS-CoV-2-specific TCPs can recognize mutated SARS-CoV-2 antigens, including those derived from the Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) strain. Cytokine production by activated $CD4^+$ and $CD8^+$ T-cells towards spike S1 and S2 of SARS-CoV-2 variants was highly comparable to wild-type spike S1 and S2 in both, unmodified control and Tac-resistant SARS-CoV-2-specific TCPs.

To further estimate potential cross-reactivity of SARS-CoV-2-specific TCPs towards spike S1 and S2 of common endemic HCoV, the cytokine profile of SARS-CoV-2-specific TCPs

was analyzed upon re-stimulation with spike S1 and S2 peptide pools derived from HCoV-229E, HCoV-NL63, HCoVOC43, and HKU1. $CD4^+$ and $CD8^+$ T-cells of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs demonstrated only little cross-reactivity towards spike protein of common endemic HCoV. The frequency of activated effector cytokine producers was significantly higher towards SARS-CoV-2-derived spike S1 and S2 compared to spike S1 and S2 of common endemic HCoV among $CD4^+$ and $CD8^+$ T-cells of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs.

3.7 Validation of *FKBP12* KO on transcriptome and protein level using single-cell CITE-sequencing and proteome analysis

To identify potential effects of *FKBP12* KO as well as CNI treatment on transcriptome and surface protein expression of expanded SARS-CoV-2-specific TCPs, unmodified control and Tac-resistant SARS-CoV-2-specific TCPs of four donors were re-stimulated SARS-CoV-2-specifically in presence or absence of CNIs (Tac or CsA) to perform single-cell CITE-seq analysis. In total, 20 different cell clusters were identified, consisting of $CD4^+$ T-cells, $CD8^+$ T-cells, double-negative (DN) T-cells, double-positive (DP) T-cells, natural killer (NK) cells and LCLs. Upon SARS-CoV-2-specific activation, clusters belonging to the $CD4^+$ and $CD8^+$ T-cell compartment showed upregulated expression of mRNA associated with effector function in both unmodified control and Tac-resistant SARS-CoV-2-specific TCPs. Moreover, SARS-CoV-2 activated $CD8^+$ T-cell clusters of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs demonstrated upregulated expression of mRNAs associated with migration, survival and memory formation. Neither CNI treatment nor *FKBP12* KO had an impact on cluster distribution in unstimulated TCPs. However, when re-stimulating SARS-CoV-2-specific TCPs antigen-specifically, the $CD4^+$ and $CD8^+$ T-cell clusters associated with elevated effector function as well as those for $CD8^+$ T-cells that indicate beneficial migration, survival and memory formation were increased. This effect was reversed to the cluster distribution observed in unstimulated state in presence of Tac for unmodified but not Tac-resistant TCPs as well as in presence of CsA for both unmodified and Tac-resistant TCPs. Moreover, downregulation of *FKBP12* in Tac-resistant SARS-CoV-2-specific TCPs was confirmed on the transcriptome level. Further gene expression analysis revealed that $CD4^+$ and $CD8^+$ T-cells of unmodified control and Tac-resistant SARS-CoV-2-specific TCPs shared the same top 25 differentially expressed genes in absence of CNIs and for Tac-resistant SARS-CoV-2-specific TCPs also

when exposed to Tac. Markers for T-cell exhaustion were lower expressed when Tac-resistant SARS-CoV-2-specific TCPs were treated with Tac compared to absence of Tac treatment. Gene expression of common markers for antiviral T-cell function as well as markers indicating support of B cell development and maturation were upregulated in unmodified control and Tac-resistant SARS-CoV-2-specific TCPs in absence of CNI and under Tac exposure for Tac-resistant TCPs.

Proteome analysis of SARS-CoV-2-specific TCPs did not only detect the KO of *FKBP12* in Tac-resistant TCPs but also confirmed the differential expression of some of the mRNA transcripts on the protein level. Upon SARS-CoV-2-specific activation, Tac-resistant SARS-CoV-2-specific TCPs showed increased levels of proteins associated with antiviral effector functions as well as lysosomal secretory capacity. Proteins involved in RNA processing, cell metabolism, and shuttling were higher in unmodified control but not Tac-resistant TCPs. As *FKBP12* KO may influence T-cell clonality, the diversity of the TCR repertoire was determined by performing TCR sequencing on the single-cell level. The KO of *FKBP12* in Tac-resistant TCPs did neither affect the TCR diversity nor induced excessive clonal expansion compared to unmodified control TCPs. Furthermore, the top 5 represented TCR clones of $CD4^+$ and $CD8^+$ T-cells were shared among unmodified control and Tac-resistant SARS-CoV-2-specific TCPs of the same donor.

The main results of the present study, that comparatively analyzed unmodified control and Tac-resistant SARS-CoV-2-specifc TCPs are summarized in the following Table 3.

Table 3: Main results of the comparative characterization of unmodified and Tac-resistant SARS-CoV-2-specific TCPs. The color code implies the potential impact on the potency of the TCPs – green: beneficial /positive; yellow: neutral; red: disadvantageous / negative (table was created by myself, based on the publication [1]).

Parameter	**unmodified TCPs**	**Tac-resistant TCPs**
T cell differentiation phenotypes at **day 0** (CD45-isoforms / CCR7) (n=8)	$CD4^+$: early differentiated (T_{CM} & T_{EM}) $CD8^+$: late differentiated (T_{EM} & T_{EMRA})	
T cell differentiation phenotypes at **day 21** (CD45-Isoforms / CCR7) (n=8)	$CD4^+$: differentiated (T_{EM}) $CD8^+$: late differentiated (T_{EM} & T_{EMRA})	$CD4^+$: differentiated (T_{EM}) $CD8^+$: late differentiated (T_{EM} & T_{EMRA})
Dominant recognition of viral antigens at **day 0** (structural (s)/ accessory (a)) (n=8)	Different for $CD4^+$ and $CD8^+$ T-cells $CD4^+$: NCAP (s), Spike S1 & S2 (s), VME1 (s) $CD8^+$: NCAP (s)	
Dominant recognition of viral antigens at **day 21** (structural (s)/ accessory(a)) (n=8)	Different for $CD4^+$ and $CD8^+$ T-cells $CD4^+$: NCAP (s), Spike S1 & S2 (s), VME1 (s) $CD8^+$: NCAP (s), AP3a (a)	Different for $CD4^+$ and $CD8^+$ T-cells $CD4^+$: NCAP (s), Spike S1 & S2 (s), VME1 (s) $CD8^+$: NCAP (s), AP3a (a)
Cytokine production compared to w/o IS (n=8) + Tac treatment	reduced	no change
Cytokine production compared to w/o IS (n=8) + CsA treatment	abolished	abolished

Table 4: Main results of the comparative characterization of unmodified and Tac-resistant SARS-CoV-2-specific TCPs. The color code implies the potential impact on the potency of the TCPs – green: beneficial /positive; yellow: neutral; red: disadvantageous / negative (table was created by myself, based on the publication [1]).

Parameter	**unmodified TCPs**	**Tac-resistant TCPs**
Cytokine production compared to w/o IS (n=8) + triple IS treatment	abolished	reduced
Cytotoxic killing capacity of SARS-CoV-2-specific TCPs (towards SARS-CoV-2 peptide-loaded target cells) (n=8) + Tac treatment	given cytotoxicity	given cytotoxicity
Cytotoxic killing capacity of separated $CD4^+$/CD8+ SARS-CoV-2-specific TCPs (towards SARS-CoV-2 peptide-loaded target cells) (n=4)	$CD4^+$: limited cytotoxicity $CD8^+$: given cytotoxicity towards NCAP (s), AP3a (a) and SARS-CoV-2 peptide pool (s & a)	$CD4^+$: limited cytotoxicity $CD8^+$: given cytotoxicity towards NCAP (s), AP3a (a) and SARS-CoV-2 peptide pool (s & a)
Cytotoxic killing capacity of SARS-CoV-2-specific TCPs (towards pSpike-transfected target cells) (n=7) + Tac treatment	limited cytotoxicity	given cytotoxicity
TCP reactivity to spike protein of SARS-CoV-2 variants (Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) (n=4)	no difference to wildtype spike (reactivity to Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529)	no difference to wildtype spike (reactivity to Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529)

Table 5: Main results of the comparative characterization of unmodified and Tac-resistant SARS-CoV-2-specific TCPs. The color code implies the potential impact on the potency of the TCPs – green: beneficial /positive; yellow: neutral; red: disadvantageous / negative (table created by myself, based on the publication [1]).

Parameter	**unmodified TCPs**	**Tac-resistant TCPs**
TCP reactivity to spike protein of common endemic HCoV (HCoV-229E, HCoV-NL63, HCoVOC43, and HKU1) (n=8)	no reactivity towards either of the HCoV-derived spike proteins	no reactivity towards either of the HCoV-derived spike proteins
Transcriptome (single-cell CITE-seq) (n=4)	T-cell effector function, survival, migration	T-cell effector function, survival, migration, underrepresented FKBP12 mRNA expression
Proteome analysis (n=4)	T-cell effector function, RNA processing, metabolism, shuttling	T-cell effector function, lysosomal secretory activity *FKBP12* KO
TCR diversity (single-cell TCR-seq) (n=4)	oligoclonality	oligoclonality

4. Discussion

4.1 Summary and interpretation of study results

The data of this study has shown the feasibility of manufacturing SARS-CoV-2-specific TCPs rendered resistant to Tac, thereby, providing superior function towards SARS-CoV-2 under Tac treatment compared to unmodified SARS-CoV-2-specific TCPs. SARS-CoV-2 specific T-cell immune responses were detected in all tested SARS-CoV-2 convalescent individuals while T-cell cross-reactivity towards SARS-CoV-2-derived antigens was limited in SARS-CoV-2 naïve healthy donors. This observation was the driving motivation leading to the generation of a SARS-CoV-2-specific TCP.

Upon SARS-CoV-2-specific stimulation, IFN-γ-secreting cells were successfully isolated to high purity and expanded for one week until performing non-viral CRISPR-Cas9-mediated KO of *FKBP12* to transfer Tac-resistance. Expansion rates and cell yields were highly comparable between Tac-resistant SARS-CoV-2-specific TCPs and unmodified control SARS-CoV-2-specific TCPs, indicating that *FKBP12*-editing did neither impair cell proliferation nor survival. The KO of *FKBP12* was validated on the RNA, DNA and protein level to ensure the specificity of our gene-editing approach. In-depth characterization of SARS-CoV-2-specifc TCPs at day 21 of culture showed that *FKBP12* editing did not affect phenotype or functionality of Tac-resistant SARS-CoV-2-specific TCPs compared to unmodified control SARS-CoV-2-specific TCPs, which is a key requirement for ensuring the safety and efficacy of the Tac-resistant TCP.

$CD4^+$ and $CD8^+$ T-cells of SARS-CoV-2-specific TCPs recognized distinct SARS-CoV-2-dervied antigens on both day 0 and day 21 of culture, which is likely related to the distinct ways of surface and internal antigen processing and presentation to $CD4^+$ and $CD8^+$ T-cells. The phenotype of SARS-CoV-2-enriched $CD4^+$ T-cells shifted to a more differentiated state after expansion, while $CD8^+$ showed already after enrichment a more late-differentiated phenotype that manifested throughout the culture. Highly differentiated T-cells may be less potent for ACT as they are associated with limited persistence and longevity following ACT. However, late differentiated SARS-CoV-2-specific T-cells were also reported by other studies [88–90] and may be a characteristic of the immune re-

sponse towards SARS-CoV-2. Moreover, considering SARS-CoV-2 as an acute viral infection, by which ACT primarily aims at clearing the viral infection to protect the host from severe COVID-19, the T-cell differentiation state may be of minor concern.

Exposing SARS-CoV-2-specific TCPs to clinical doses of Tac inhibited cytokine production in unmodified TCPs, which was partially rescued by *FKBP12* KO in Tac-resistant TCPs. When adding CsA to the culture, cytokine production was abolished in Tac-resistant SARS-CoV-2-specific TCPs, which may serve as an inherent safety switch to counteract potential adverse events *in vivo*. In presence of triple IS, Tac-resistant but not unmodified control SARS-CoV-2-specific TCPs were able to produce effector cytokines, although to lower extend compared to Tac treatment only. Hence, the Tac-resistant SARS-CoV-2-specific TCP may even exert antiviral effects in individuals receiving triple IS, as commonly applied in transplant recipients. The cytotoxic killing capacity of SARS-CoV-2-peptide loaded target cells by SARS-CoV-2-specific TCPs was not affected by Tac treatment, which is in line with previous observations within our laboratory [79]. In contrast, cytotoxic elimination of SARS-CoV-2 spike protein transfected target cells was inhibited in presence of Tac for unmodified control TCPs but remained stable for Tac-resistant TCPs, indicating that Tac-resistant SARS-CoV-2-specific TCPs may be superior in clearing SARS-CoV-2-infected cells within the immunosuppressed host. The contradictory effects of Tac on the killing capacity of T-cells may result from differences in antigen-presentation between the two models. Transfection of target cells with spike protein likely results in the presentation of a reduced set of antigens to T-cells, which is capable to reach the TCR signaling threshold for full T-cell activation [91] much faster compared to the use of antigenic peptide pools.

Moreover, unmodified control and Tac-resistant SARS-CoV-2-specific TCPs were highly cross-reactive to spike protein derived from different SARS-CoV-2 strains supporting reported observations that SARS-CoV-2-specific T-cells of convalescent or vaccinated individuals can also protect from emerging SARS-CoV-2 variants [92–94]. Although several studies reported cross-reactivity of SARS-CoV-2-specific T-cells from convalescent donors towards spike S2 of common endemic HCoV [14,15,90,95], the cross-reactivity of SARS-CoV-2-specific TCPs to spike protein derived from common endemic HCoV was limited. This may likely be a result of SARS-CoV-2-specific T-cell clones having higher

TCR affinity to SARS-CoV-2 antigens and therefore overgrowing cross-reactive HCoV-specific T-cell clones with lower TCR affinity over the period of 21 days [96].

Transcriptome analysis revealed beneficial characteristics of SARS-CoV-2-specific TCPs in terms of antiviral effector function, cell migration, cell survival as well as memory formation and provided some hints for potential support of B cell-derived antibody formation. The transcriptome data once again highlights the auspicious potency of Tac-resistant SARS-CoV-2 specific TCPs to not only provide antiviral T-cell function but also the potential to support formation of humoral immunity [87] in immunosuppressed patients. The diversity of the TCR repertoire of unmodified and Tac-resistant SARS-CoV-2-specific TCPs was highly comparable and gave no hints for excessive proliferation of single T-cell clones, indicating that neither *FKBP12* KO nor potential off-target editing led to abnormal transformation of T-cell clones, which is in line with previous observations within our laboratory [79]. However, to exclude potential off-target editing in Tac-resistant CRISPR-Cas9 gene-edited TCPs, further analysis is currently conducted to ensure safe clinical use.

4.2 Study results and the current state of research

Adoptive antiviral T-cell therapy has already been applied to treat various viral diseases, especially in the setting of transplantation [66–68]. Regarding SARS-CoV-2, several studies, have demonstrated the importance of robust T-cell immunity to fight SARS-CoV-2 infection and prevent severe COVID-19 [14–16]. The feasibility to detect, isolate and expand SARS-CoV-2-specific T-cells upon antigen-specific stimulation of PBMCs of convalescent donors was confirmed by several publications [1,81–86]. A phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT04578210) confirmed the safety of adoptively transferred CD45RA$^-$ memory SARS-CoV-2-specific T-cells derived from a healthy convalescent donor to treat severe COVID-19 [97]. Further clinical trials estimating the safety and efficacy of SARS-CoV-2-specific ACT to treat COVID-19 in non-transplant (ClinicalTrials.gov Identifier: NCT04351659) and cancer patients (ClinicalTrials.gov Identifier: NCT04742595) are currently ongoing. A SOT recipient suffering from acute SARS-CoV-2 infection was successfully treated with an allogenic SARS-CoV-2-specific TCP resulting

in an undetectable nasopharyngeal viral load within 11 days post-infusion [98]. Nevertheless, further studies need to be conducted to validate the safety and effectiveness of SARS-CoV-2-specific ACT in the transplant setting.

Temporary corticosteroid treatment in the setting of severe COVID-19 as well as constant immunosuppression in the transplant setting, may limit the success of SARS-CoV-2-specific ACT, underlining the necessity to develop novel treatment approaches for these patient cohorts. A recent study reported the successful generation of glucocorticoid-resistant SARS-CoV-2-specific TCPs as a novel strategy to improve antiviral T-cell function in corticosteroid-treated COVID-19 patients [83]. However, this approach may not be applicable for transplant patients as Tac treatment is essential to prevent allograft rejection after transplantation and thus impairs T-cell function. In addition, Tac is reported to exert potential beneficial antiviral properties towards coronaviruses [47,48] and observations from more recent studies suggest that SOT recipients benefit from maintained Tac treatment during SARS-CoV-2 infection [46,99–101]. Hence, the Tac-resistant SARS-CoV-2 specific TCPs characterized in the present study may be superior to glucocorticoid-resistant SARS-CoV-2-specific TCPs in treating and preventing COVID-19 in immunosuppressed transplant recipients and potentially even in non-transplant patients.

4.3 Strengths and weaknesses of the study

A major strength of the present study is the focus on the treatment and prevention of COVID-19 using ACT in the transplant setting. Previous studies on SARS-CoV-2-specific ACT have primarily focused on the treatment of severe COVID-19 patients and suggested the treatment of transplant recipients. However, the treatment strategy of a combination of Tac and Tac-resistant SARS-CoV-2-specific TCPs could not only find application in immunosuppressed patients but could also be extended to the general treatment of severe COVID-19 patients. Furthermore, the SARS-CoV-2-specific TCPs were characterized in depth using various methods at different cellular levels to provide an indication of their efficiency and safety. The inherent safety switch by which T-cell activity can be prevented when administering CsA represents a major advantage as it can inhibit potential unwanted effects during *in vivo* application. The GMP-compliant protocol to generate Tac-resistant SARS-CoV-2-specific TCPs can be readily transferred to the GMP facility in our

institute and would thus provide the manufacturing of clinical-grade Tac-resistant SARS-CoV-2-specific TCPs.

Nevertheless, this study has also some limitations, as it primarily showed that it is possible to produce functional Tac-resistant SARS-CoV-2-specific TCPs from healthy SARS-CoV-2 convalescent donors, but this feasibility would still need to be validated in the direct transplantation setting. Unfortunately, at the time the study was conducted, there was no possibility to obtain blood samples from SARS-CoV-2 infected or recovered patients. However, together with the in-house GMP facility, the production of SARS-CoV-2-specifical TCPs from immunosuppressed vaccinated donors could later be validated (unpublished data). Another limitation of this study is the timepoint of blood collection. The blood was collected from SARS-CoV-2 convalescent individuals approximately 5-6 months after infection. In the clinical setting, the timing could vary depending on the approach (allogeneic or autologous) and thus can affect the yield of SARS-CoV-2-specific T-cells. Moreover, due to the lack of a stable *in vivo* animal model mimicking SARS-CoV-2 infection at that time, the study does not include an *in vivo* model to validate the *in vitro* observations.

In general, ACT has proven to be feasible in the setting of transplantation [66–68], with only minor signs of adverse events [69]. Several studies reported the feasibility of generating IS-resistant TCPs reactive towards various viral infection including SARS-CoV-2 and propose their use in transplant recipients [77–80]. However, clinical trials assessing the safety and efficacy of IS-resistant TCPs are still needed. In addition, it is likely that the success of IS-resistant ACT varies among transplant recipients as it may depend on the time post transplantation, the immunosuppressive regimen, and the presence of comorbidities. Nevertheless, Tac treatment was suggested to improve COVID-19 outcome in transplant recipients [46,99–101], which would support the feasibility of our study approach.

4.4 Implication for clinical application and future studies

The general translation of Tac-resistant SARS-CoV-2-specific TCPs into clinical application is feasible when following a GMP-compatible protocol. The present study was carried

out in close collaboration with the in-house GMP facility (Berlin Center for Advanced Therapies (BeCAT)) and once they receive the manufacturing authorization for Tac-resistant antiviral TCPs we aim at clinical translation. Currently, the team around BeCAT is preparing for a first in-human clinical trial using vaccinated donor-derived SARS-CoV-2-specific TCPs in the setting of HSCT to improve COVID-19 vaccination responses early post transplantation. We are confident that the application of SARS-CoV-2 specific TCPs poses little risk, as clinical safety of the application of conventional antiviral TCPs has already been shown within our institute [70]. Moreover, the general approach to manufacture Tac-resistant SARS-CoV-2-specific TCPs can be readily transferred to other viruses (e.g. Influenza) by adapting the antigenic peptide pool used to enrich virus-specific T-cells. In line with this, the spectrum of resistance to IS can also be extended, by replacing the specific sgRNA for the CRISPR-Cas9-mediated KO. Furthermore, the method can be applied to other T-cell subtypes, such as regulatory T-cells. Indeed, these strategies are focus of ongoing collaborative work within our laboratory and the BeCAT.

5. Conclusion

In summary, manufacturing Tac-resistant SARS-CoV-2-specific TCPs with superior effector function under Tac treatment is feasible and demonstrates a promising novel strategy to prevent and treat severe COVID-19 in immunosuppressed transplant but also non-transplant patients. Safety and efficacy of Tac-resistant TCPs *in vivo* must be validated by conducting a clinical trial. The preparation for this is an ongoing process in collaboration with the in-house GMP facility, which will likely pave the way for a widespread acceptance of IS-resistant ACT and may facilitate clinical application of upcoming IS-resistant ACT approaches.

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Affidavit I Eidesstattliche Versicherung

„Ich, Lena Peter, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema *Herstellung von CRISPR-Cas9-editierten Tacrolimus-resistenten SARS-CoV-2-spezifischen T-Zellen für die adoptive Zelltherapie in Transplantationspatienten - Generation of CRISPR-Cas9-edited Tacrolimus-resistant SARS-CoV-2-specific T-cells for adoptive cell therapy in transplant patients* selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/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 ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

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

Datum Unterschrift

Detailed Statement of Contribution

Lena Peter had the following contribution to the following publication:

Publication: Lena Peter, Désirée Jacqueline Wendering, Stephan Schlickeiser, Henrike Hoffmann, Rebecca Noster, Dimitrios Laurin Wagner, Ghazaleh Zarrinrad, Sandra Münch, Samira Picht, Sarah Schulenberg, Hanieh Moradian, Mir-Farzin Mashreghi, Oliver Klein, Manfred Gossen, Toralf Roch, Nina Babel, Petra Reinke, Hans-Dieter Volk, Leila Amini, and Michael Schmueck-Henneresse, *Tacrolimus-resistant SARS-CoV-2-specific T-cell products to prevent and treat severe COVID-19 in immunosuppressed patients*, Molecular Therapy – Methods & Clinical Development, 2022

Contribution: My own contributions to the publication “Tacrolimus-resistant SARS-CoV-2-specific T-cell products to prevent and treat severe COVID-19 in immunosuppressed patients” comprised study design, planning and execution of study experiments, data analysis and statistics, preparation of all figures and tables including the supplementary figures and tables, interpretation of study results and writing of the manuscript.

In the first month of the COVID-19 pandemic little was known on the immune response towards SARS-CoV-2. However, I have done intensive literature research to be always up to date as soon as new findings on SARS-CoV-2 immunogenicity were published. In parallel, I established a SARS-CoV-2-specific T-cell stimulation assay and a multicolor flow cytometry panel to identify SARS-CoV-2-driven T-cell responses. Based on my own findings and the rising number of publications demonstrating that T-cells are essential for controlling SARS-CoV-2 infection, my supervisors, mentors and I decided to setup a study on the feasibility of generating SARS-CoV-2-specific T-cells for ACT. In accordance with, Dr. Stephan Schlickeiser, Dr. Leila Amini, Dr. Michael Schmueck-Henneresse and Prof. Dr. Hans-Dieter Volk, I specified the main study hypotheses and conceptualized the study. I recruited matching donors for the study and coordinated blood sample collection. I created a questionnaire for the study participants to estimate the symptom severity as well as possible long-term consequences of the COVID-19 infection, which can be found in the supplementary table 1 that I created for the publication. I further determined the SARS-CoV-2 serological status of all study participants to discriminate between SARS-CoV-2 convalescent and naïve donors. For the pre-screening of SARS-CoV-2-specific T-cell responses I got experimental supported by Henrike Hoffmann and Sandra Münch. After thorough introduction by Dr. Leila Amini and Dr. Michael Schmueck-Henneresse, I transferred the protocol for producing antiviral Tac-resistant TCPs to the setting of SARS-CoV-2. The CRISPR-Cas9-based KO procedure was carried out under guidance of Dr. Leila Amini and Ghazaleh Zarrinrad, who established the respective standard protocols in our laboratory. Occasionally, Henrike Hoffmann, Sandra Münch, Ghazaleh Zarrinrad, Rebecca Noster, Samira Picht and Sarah Schulenberg helped me with PBMC isolation or cultivation of TCPs. To setup the experiments for functional characterization of TCPs at the end of culture, I took advantage of the knowledge of Dr. Leila Amini and Dr. Michael Schmueck-Henneresse, who are very experienced in this regard. I performed and analyzed all re-stimulation experiments to assess the activation profile of SARS-CoV-2-specific TCPs. The VITAL Assay was carried out

and analyzed with support of Henrike Hoffmann, who routinely performs this assay within the GMP facility. Moreover, Hanieh Moradian provided the plasmid encoding SARS-CoV-2 spike protein and gave advice for plasmid transfection of LCLs. Sample preparation for CITE-seq and TCR-seq was performed together with Dr. Désirée Jacqueline Wendering, who established the respective protocols in our laboratory. The samples were sequenced by Dr. Mir-Farzin Mashreghi of the German Rheumatism Research Center and the data was pre-processed by its bioinformaticians Dr. Pawel Durek and Dr. Frederik Heinrich. In-depth analysis and illustration of the sequencing data was performed by Dr. Stephan Schlickeiser. Moreover, I presented the sequencing data in figures (figure 7 and supplementary figure 7 & 8) and interpretated the results. For proteomics, I performed sample preparation under guidance of Dr. Leila Amini, Dr. Michael Schmueck-Henneresse and Dr. Oliver Klein. The proteome analysis and pre-processing of data was carried out by Oliver Klein, while in-depth analysis and illustration of the proteome data was performed by Dr. Stephan Schlickeiser. Moreover, I presented the proteome data in figure 7 and interpretated the results.
For flow cytometric analysis, I established and compensated panels and discussed gating strategies together with Dr. Leila Amini, Dr. Désirée Jacqueline Wendering and Dr. Michael Schmueck-Henneresse. All data analysis and its graphical design was carried out in close guidance of Dr. Leila Amini and Dr. Michael Schmueck-Henneresse and further discussed with all co-authors of the publication. I generated the graphical abstract, all main (figure 1 – 7) and supplementary figures (supplementary figure 1 – 8) as well as tables (supplementary table 1 & 2) of the publication. Furthermore, I composed and wrote the entire manuscript, which was optimized by all co-authors, but primarily by Dr. Leila Amini and Dr. Michael Schmueck-Henneresse. During the review process, I read and answered reviewer comments and conducted further experimental work as well as adaptions to the manuscript mainly together with Dr. Leila Amini and Dr. Schmueck-Henneresse. The final version of the manuscript was proofread by all co-authors and final language editing was carried out by Dr. Nicola R. Brindle.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

Extract of Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
Selected Categories: **"MEDICINE, RESEARCH and EXPERIMENTAL"**
Selected Category Scheme: WoS
Gesamtanzahl: 140 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	114,401	53.440	0.184050
2	Science Translational Medicine	45,509	17.956	0.103780
3	JOURNAL OF CLINICAL INVESTIGATION	132,296	14.808	0.114560
4	JOURNAL OF EXPERIMENTAL MEDICINE	74,803	14.307	0.062280
5	MOLECULAR ASPECTS OF MEDICINE	8,136	14.235	0.006640
6	Annual Review of Medicine	7,553	13.739	0.009800
7	EMBO Molecular Medicine	11,474	12.137	0.020440
8	TRENDS IN MOLECULAR MEDICINE	13,213	11.951	0.014720
9	Theranostics	23,558	11.556	0.034890
10	Clinical and Translational Medicine	2,201	11.492	0.003110
11	MOLECULAR THERAPY	24,333	11.454	0.030250
12	Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology	3,763	9.182	0.003760
13	Molecular Therapy-Nucleic Acids	8,812	8.886	0.014970
14	EXPERIMENTAL AND MOLECULAR MEDICINE	8,780	8.718	0.013260
15	JOURNAL OF BIOMEDICAL SCIENCE	6,621	8.410	0.007330
16	JCI Insight	15,237	8.315	0.054040
17	EBioMedicine	15,647	8.143	0.040730
18	Inflammation and Regeneration	743	7.354	0.001450
19	npj Vaccines	1,342	7.344	0.003850
20	Molecular Therapy-Oncolytics	1,582	7.200	0.002970

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	AMYLOID-JOURNAL OF PROTEIN FOLDING DISORDERS	2,202	7.141	0.003280
22	Translational Research	5,766	7.012	0.007980
23	Cold Spring Harbor Perspectives in Medicine	10,709	6.915	0.016100
24	Stem Cell Research & Therapy	13,356	6.832	0.018900
25	Molecular Therapy-Methods & Clinical Development	3,268	6.698	0.008200
26	EPMA Journal	1,507	6.543	0.001330
27	BIOMEDICINE & PHARMACOTHERAPY	41,024	6.529	0.056550
28	Nanomedicine-Nanotechnology Biology and Medicine	14,067	6.458	0.013280
29	MOLECULAR MEDICINE	6,239	6.354	0.004460
30	Biomarker Research	1,170	6.148	0.001720
31	CLINICAL SCIENCE	14,200	6.124	0.013580
32	Biomedicines	2,391	6.081	0.003650
33	ALTEX-Alternatives to Animal Experimentation	1,820	6.043	0.001920
34	CANCER GENE THERAPY	3,768	5.987	0.002720
35	mAbs	6,061	5.857	0.010250
36	Stem Cell Reviews and Reports	3,488	5.739	0.003300
37	HUMAN GENE THERAPY	7,074	5.695	0.006860
38	LABORATORY INVESTIGATION	12,539	5.662	0.008240
39	EXPERT REVIEWS IN MOLECULAR MEDICINE	2,042	5.600	0.000790
40	ARCHIVES OF PATHOLOGY & LABORATORY MEDICINE	13,569	5.534	0.013270
41	Journal of Translational Medicine	18,129	5.531	0.022130
42	Nucleic Acid Therapeutics	1,392	5.486	0.003070

Selected JCR Year: 2020; Selected Categories: "MEDICINE, RESEARCH and EXPERIMENTAL"

https://intranet.charite.de/fileadmin/user_upload/microsites/sonstige/medbib/Impact_Faktoren_2020/ISI-WEB-Liste-Kategorie-Medicine__Research_and_Experimental.pdf

Selected Publication: Tacrolimus-resistant SARS-CoV-2-specific T-cell products to prevent and treat severe COVID-19 in immunosuppressed patients

Molecular Therapy
Methods & Clinical Development
Original Article

Tacrolimus-resistant SARS-CoV-2-specific T cell products to prevent and treat severe COVID-19 in immunosuppressed patients

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Solid organ transplant (SOT) recipients receive therapeutic immunosuppression that compromises their immune response to infections and vaccines. For this reason, SOT patients have a high risk of developing severe coronavirus disease 2019 (COVID-19) and an increased risk of death from severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. Moreover, the efficiency of immunotherapies and vaccines is reduced due to the constant immunosuppression in this patient group. Here, we propose adoptive transfer of SARS-CoV-2-specific T cells made resistant to a common immunosuppressant, tacrolimus, for optimized performance in the immunosuppressed patient. Using a ribonucleoprotein approach of CRISPR-Cas9 technology, we have generated tacrolimus-resistant SARS-CoV-2-specific T cell products from convalescent donors and demonstrate their specificity and function through characterizations at the single-cell level, including flow cytometry, single-cell RNA (scRNA) Cellular Indexing of Transcriptomes and Epitopes (CITE), and T cell receptor (TCR) sequencing analyses. Based on the promising results, we aim for clinical validation of this approach in transplant recipients. Additionally, we propose a combinatory approach with tacrolimus, to prevent an overshooting immune response manifested as bystander T cell activation in the setting of severe COVID-19 immunopathology, and tacrolimus-resistant SARS-CoV-2-specific T cell products, allowing for efficient clearance of viral infection. Our strategy has the potential to prevent severe COVID-19 courses in SOT or autoimmunity settings and to prevent immunopathology while providing viral clearance in severe non-transplant COVID-19 cases.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019, causing respiratory tract disorders, referred to as coronavirus disease 2019 (COVID-19), and led to a worldwide pandemic.[1,2] While, in the general population, most SARS-CoV-2 infections show a mild disease course, severe COVID-19 is more common among individuals under chronic immunosuppression, such as transplant recipients, autoimmune patients,[3,4] and the elderly.[5] Chronic immunosuppression increases susceptibility to respiratory viral infections, which are increasingly recognized to be a major cause of morbidity and mortality among transplant recipients.[3,6–8]

Recent studies suggest that SOT recipients are at high risk for complications or death due to SARS-CoV-2 infection.[3,9–13] In a large US cohort (1,925) of SARS-CoV2$^+$ SOT recipients, 42.9% of SOT recipients with SARS-CoV-2 had to be hospitalized and the infection was

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associated with increased risk for acute kidney injury, organ rejection, graft failure, and major cardiologic problems.[14] Furthermore, current findings indicate that immunosuppressed and elderly patients mount weak responses to COVID-19 vaccines. Consequently, vaccination in these populations may not provide protection against severe SARS-CoV-2 infections or to emerging novel SARS-CoV-2 strains, leaving them at risk.[15–20] Indeed, initial studies reported that, despite being vaccinated, SOT patients still became infected with SARS-CoV-2 and required hospitalization, highlighting the necessity of novel strategies to protect this vulnerable population.[21–23]

SARS-CoV-2-specific T cells have been detected in convalescent patients in multiple studies, indicating an important role for T cells in viral clearance and development of protective immunity.[24–26] Thus, SARS-CoV2-specific adoptive T cell therapy (ACT) has been suggested as an early treatment or preventive strategy for COVID-19 in immunocompromised or immunosuppressed individuals[27] as well as more generally for treatment of acute COVID-19.[28,29] Virus-specific ACT has already been administered with a very low incidence of adverse effects to prevent and treat infections in patients after hematopoietic stem cell transplantation and SOT.[30–32] Furthermore, recent studies have shown the feasibility of generating SARS-CoV-2-specific T cell products (TCPs) from the blood of patients who have recovered from SARS-CoV-2 infection.[27,28] Clinical trials are now needed to demonstrate safety, efficiency, and persistence of antiviral TCPs *in vivo* and to determine any therapeutic benefit of ACT for COVID-19 patients. However, current ACT strategies may not benefit COVID-19 patients who are also treated with immunosuppressants, which includes SOT recipients. They might, however, benefit from adoptive transfer of immunosuppression-resistant SARS-CoV-2-specific TCPs, allowing prevention of overshooting immune responses while maintaining some form of viral anti-immunity. Additionally, reported data imply that ACT enhances induction of antibody responses,[33] thus immunosuppression-resistant adoptive SARS-CoV-2-specific ACT may help to establish protective immunity, consisting of both T and B cell responses,[34,35] in immunosuppressed patients, who often fail to mount protective long-term antibody responses after vaccination.[17–20]

Immunosuppression-resistant adoptive SARS-CoV-2-specific ACT could also be beneficial beyond immunosuppressed patient populations. Severe COVID-19 is associated with extrapulmonary systemic hyperinflammation syndrome characterized by an overshooting innate and adaptive immune response (sometimes referred to as cytokine storm) that further results in tissue damage and multi-organ failure.[36,37] Studies indicate that dysregulated T cell function contributes to COVID-19-associated hyperinflammation and impaired viral clearance.[38–42] Consequently, immunosuppressive corticosteroids are currently the first line of treatment for patients with severe COVID-19-associated hyper-inflammation[43,44] and can reduce mortality in patients requiring respiratory support.[45] However, their generalized use in treating coronavirus diseases has been controversial: some reports suggest improved disease outcomes in COVID-19 patients upon corticosteroid treatment,[43,44,46] while others suggest corticosteroids prolong the duration of hospitalization and delay viral elimination.[47–50] Thus, there is an urgent need for effective and safe strategies aiming to support viral clearance while preventing SARS-CoV-2-associated hyperinflammation and tissue damage in patients with severe COVID-19.

The calcineurin inhibitor (CNI) tacrolimus (Tac) may be an attractive alternative to corticosteroids. Tac is reported to inhibit proinflammatory cytokine production and the replication of human coronavirus (HCoV) SARS-CoV-1, HCoV-NL63, and HCoV-229E,[51,52] to reduce T cell-associated hyperinflammation, and to have protective effects in SOT patients infected with Middle East respiratory syndrome coronavirus (MERS-CoV) or SARS-CoV-2.[53–56] Indeed, Tac combined with prednisolone (Pred) pulses (ClinicalTrials.gov Identifier: NCT04341038)[57] and the CNI, cyclosporine A (CsA),[58] are under investigation for treatment of COVID-19 in clinical trials. However, reports imply that immunosuppressed transplant recipients under Tac therapy also show prolonged viral shedding upon SARS-CoV-2 infection, illustrating the need for regeneration of specific immune responses to SARS-CoV-2 in these patients.[59–62] The feasibility of generating glucocorticoid-resistant SARS-CoV-2-specific T cells for ACT has recently been described[29]; however, as tacrolimus is required to prevent organ rejection after SOT transplantation and additionally is reported to support antiviral and anti-inflammatory processes toward coronavirus infections,[51–56] it might be an attractive alternative to corticosteroid-based immunosuppression to reduce or prevent COVID-19-associated hyperinflammation. Thus, we suggest combination therapy for severe COVID-19 using Tac to prevent immunopathology, combined with tacrolimus-resistant adoptive antiviral T cell therapy to improve viral control as a novel treatment concept, both for immunocompromised SOT patients as well as in severe non-transplant COVID-19 cases.[63]

We report the feasibility of generating SARS-CoV-2-specific Tac-resistant antiviral T cells suitable for ACT from convalescent SARS-CoV-2-infected individuals utilizing our vector-free gene-editing approach targeting *FKBP12*, which codes for the adapter protein required for the immunosuppressive function of Tac in antiviral T cells.[63,64] Functional analysis confirmed that *FKBP12* KO SARS-CoV-2-specific T cells are highly resistant to Tac treatment, maintaining their effector function as measured by antigen-specific cytokine production. In contrast, the antiviral cytokine production of these novel *FKBP12* KO SARS-CoV-2-specific T cells was efficiently suppressed by FKBP12-independent immunosuppression via the alternative CNI, CsA. We thus provide an inherent safety switch in case of potential adverse effects of *FKBP12* KO T cells *in vivo*.[43] Our GMP-compatible manufacturing process allows for clinical-grade production of these innovative Tac-resistant TCPs as a pre-requisite for a first-in-human clinical trial investigating the potential of SARS-CoV-2-specific *FKBP12* KO T cells in transplant recipients.

RESULTS

SARS-CoV-2-specific T cells from SARS-CoV-2$^+$ convalescent donors predominantly target the nucleocapsid and spike proteins

To assess the feasibility of isolating SARS-CoV-2-reactive T cells, we analyzed the antiviral T cell responses to SARS-Cov-2 structural and

accessory proteins in individuals with a history of asymptomatic or mild SARS-CoV-2 infection (convalescent donors). Thus, we obtained peripheral blood mononuclear cells (PBMCs) from 20 donors who had cleared an asymptomatic or mild SARS-CoV-2 infection, and 19 SARS-CoV-2-naive control donors (for characteristics of donors, see Table S1). Naive control donors were defined as being seronegative for immunoglobulin G (IgG) and immunoglobulin A (IgA) targeting SARS-CoV-2 spike S1 as detected by ELISA (Figures S1A and S1B). The SARS-CoV-2-specific T cell responses were evaluated by stimulating PBMCs with overlapping peptide pools (15-mers, 11-amino acid [aa] overlap), encompassing the amino acid sequence of structural proteins (NCAP [Nucleocapsid], spike S1 + S2, VEMP [Envelope small membrane protein], VME1) and accessory proteins (AP3a, NS6, NS7a, NS7b, NS8, ORF9b, ORF10, Y14) of SARS-CoV-2. Cells were stimulated for 16 h to analyze the reactivity of T cells by flow cytometry using a set of markers for T cell activation and effector cytokine production (Figures 1A–1C). In all SARS-CoV-2 convalescent donors, we observed upregulation of cluster of differentiation (CD) 137 (4-1BB) and production of either interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), or both cytokines (Figures 1D–1G), which is consistent with effector T cell activation following SARS-CoV-2-specific stimulation. Furthermore, we found SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells responded to different viral antigens. $CD4^+$ T cells predominantly reacted to NCAP, spike S1 and S2, and to a lesser extent to VME1 (Figures 1D and 1E). In contrast, $CD8^+$ T cells predominantly reacted to NCAP, as illustrated in Figures 1F and 1G. Healthy SARS-CoV-2-seronegative control donors presented few IFN-γ-positive $CD4^+$ T cells, whereas TNF-α producers responded to all tested antigens (Figures S1C and S1D). SARS-CoV-2-reactive T cells were undetectable in the $CD8^+$ T cell fraction of the seronegative control donors (Figures S1E and S1F).

Vector-free CRISPR/Cas9-based *FKBP12* KO in SARS-CoV-2-specific TCPs generated from convalescent donors

We have previously described a vector-free protocol for electroporation and ribonucleoprotein (RNP)-based knockout (KO) of *FKBP12* to generate Tac-resistant antiviral TCPs, which was now applied to generate SARS-CoV-2-specific Tac-resistant TCPs from eight convalescent donors (CD 1–3, 15, 17–20; Table S1).[63] We isolated SARS-CoV-2-specific T cells with a high purity from PBMCs, based on their IFN-γ secretion after stimulation with SARS-CoV-2 peptide pools (NCAP, spike S1 + S2, VEMP, VME1, AP3a, NS6, NS7a, NS7b, NS8, ORF9b; ORF10, Y14) (Figures 2A–2C). These SARS-CoV-2-specific T cells were expanded and split on day 7. One-half of the culture was subsequently electroporated with RNP complexes of Cas9 and a single guide RNA (sgRNA), whereas the other half served as the unmodified control (Figure 2A). The unmodified and *FKBP12* KO SARS-CoV-2-specific T cells were expanded for a further 2 weeks (Figure 2A). Expansion rates and cell yields were similar for both fractions at days 14 and 21, illustrated in Figures 3A and 3D. Similarly, the expansion rates and total counts of $CD4^+$ and $CD8^+$ T cells of the TCPs at day 21 were comparable between unmodified and $FKPB12^{KO}$ fractions (Figures 3B and 3E). Although on day 0 we found the $CD4^+/CD8^+$ ratios were high among the SARS-CoV-2-reactive T cells, these gradually became more balanced during expansion in both unmodified and *FKBP12* KO TCPs (Figure 3F). On day 21, the KO efficiency of *FKBP12* ranged from 63% to 89% in the SARS-CoV-2-specific TCPs, as assessed by peak-shift analysis after Sanger sequencing (Figure 3C).

Expanded SARS-CoV-2-specific $CD4^+$ and $CD8^+$ TCPs recognize multiple SARS-CoV-2-derived antigens

Subsequently, we sought to identify the antigenic targets driving the expansion of SARS-CoV-2-specific T cells and compared their antigen specificities before and after expansion. We addressed this by re-stimulating the TCPs from both cultured unmodified controls and *FKBP12* KO with individual peptide pools of the different structural and accessory proteins of SARS-CoV-2 for 16 h.

Ex vivo (day 0) and post-expansion (day 21) $CD4^+$ T cells were predominantly activated by NCAP, spike S1 and S2, and VME1 (Figures 4A and 4B, S2A, S2E, and S2F). The frequency of $CD4^+$ T cell reactivity toward the different SARS-CoV-2 antigens tested was similar between *FKBP12* KO and unmodified TCPs (Figures 4B and S2C–S2F). $CD8^+$ T cells exhibited a discernably different response to the antigens tested; however, the response of the *FKBP12* KO and unmodified TCPs was again similar (Figure 4C and S2B). $CD8^+$ T cells showed greater proportions of cells reactive to NCAP and spike S1 *ex vivo* (day 0); however, after expansion (day 21), $CD8^+$ T cells responded principally to NCAP and AP3a peptide pools, whereas spike S1- and S2-specific T cells were detected at much lower frequencies than NCAP- and AP3a-specific T cells (Figures 4D, S2G, and S2H). After expansion, AP3a-specific $CD8^+$ T cells were more abundant in both unmodified and *FKBP12* KO TCPs (Figures 4D, S2C, S2D, S2G, and S2H). Interestingly, no specific activation of $CD4^+$ or $CD8^+$ T cells was observed in response to any of the other accessory proteins NS6, NS7a, NS7b, NS8, ORF9b, ORF10, and Y14 or the structural VEMP protein in both unmodified and *FKBP12* KO TCPs (Figures 4B, 4D, and S2E–S2H).

SARS-CoV-2-specific T cells expanded from convalescent donors display a differentiated memory phenotype

We next evaluated the cell surface expression of T cell differentiation markers of expanded unmodified control and *FKBP12* KO TCPs *ex vivo* (day 0) and after culture (day 21) (Figures 4E and 4F). In brief, the order of frequency in descending order, illustrated in Figures 4E and 4G, was observed to be: high $CCR7^+/CD45RA^+$ naive T cells (T_{NAIVE}), $CCR7^+/CD45RA^-$ central memory T cells (T_{CM}), $CCR7^-CD45RA^-$ effector memory T cells (T_{EM}), $CCR7^+/CD45RA^+/CD95^{dim}$ stem cell-like memory T cells (T_{SCM}), and $CCR7^-/CD45RA^+$ terminally differentiated effector memory T cells (T_{EMRA}) (Figures 4E and 4G). Post enrichment, the SARS-CoV-2-specific $CD4^+$ T cells contained a high percentage of T_{CM} and T_{EM} (Figures 4E and 4G). On day 21 SARS-CoV-2-specific $CD4^+$ T cells exhibited a more differentiated phenotype, with the majority being T_{EM} in both bulk and $CD137^+$ and IFN-γ^+ T cells and a lack of T_{CM} (Figures 4F and 4H). Before enrichment, $CD8^+$ T cells presented

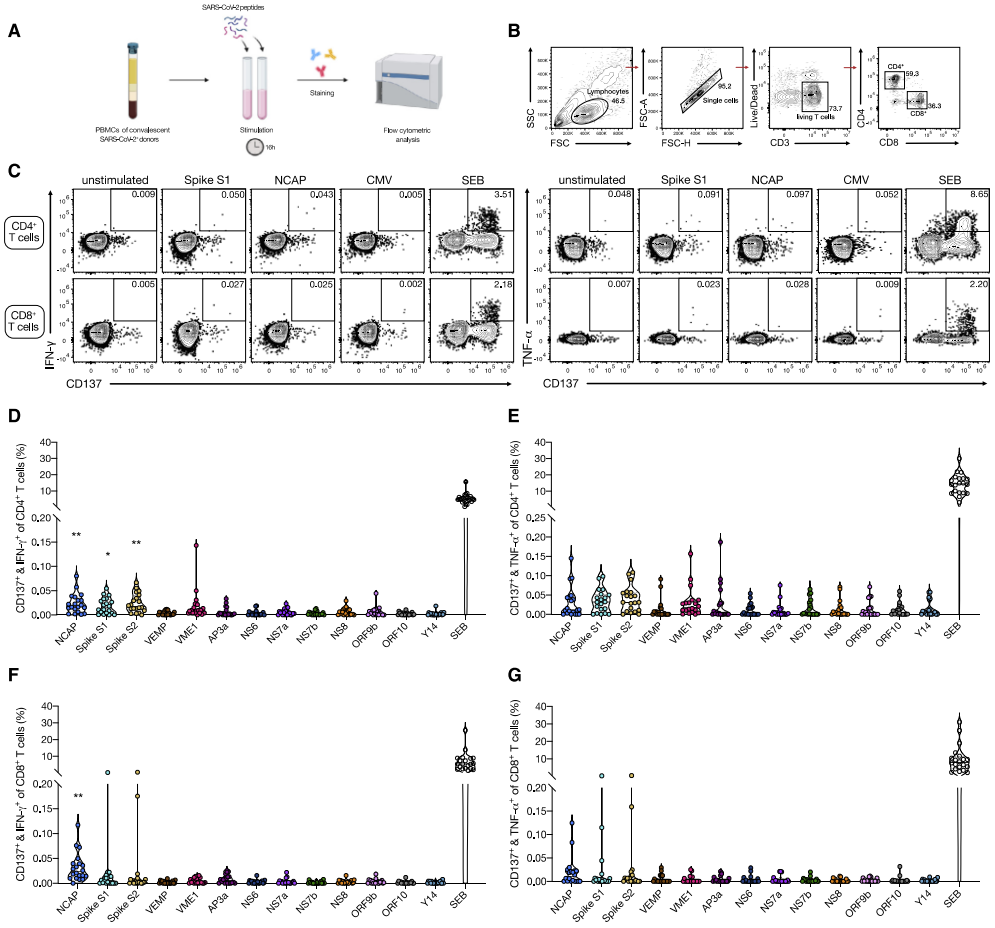


Figure 1. Frequencies of SARS-CoV-2-reactive T cells in blood of convalescent SARS-CoV-2$^+$ donors

SARS-CoV-2-specific stimulation of PBMCs of convalescent SARS-CoV-2$^+$ donors with individual structural and accessory proteins of SARS-CoV-2. n = 20; *p < 0.05, **p < 0.01 (statistics refer to data of seronegative healthy donors in Figures S1C–S1F). Staphylococcal enterotoxin B (SEB) serves as positive control. (A) Schematic outline of the experimental setup. Created with BioRender.com. (B) Representative gating strategy to select lymphocytes, single cells, and living CD4$^+$ and CD8$^+$ T cells. (C) Representative gating strategy to select antigen-reactive (CD137$^+$) cytokine producers (IFN-γ$^+$ or TNF-α$^+$) among CD4$^+$ and CD8$^+$ T cells. (D) IFN-γ production of SARS-CoV-2-activated (CD137$^+$) CD4$^+$ T cells after 16 h of stimulation with individual peptides of SARS-CoV-2 in convalescent SARS-CoV-2$^+$ donors. (E) TNF-α production of SARS-CoV-2-activated (CD137$^+$) CD4$^+$ T cells after 16 h of stimulation with individual peptides of SARS-CoV-2 in convalescent SARS-CoV-2$^+$ donors. (F) IFN-γ production of SARS-CoV-2-activated (CD137$^+$) CD8$^+$ T cells after 16 h of stimulation with individual peptides of SARS-CoV-2 in convalescent SARS-CoV-2$^+$ donors. (G) TNF-α production of SARS-CoV-2-activated (CD137$^+$) CD8$^+$ T cells after 16 h of stimulation with individual peptides of SARS-CoV-2 in convalescent SARS-CoV-2$^+$ donors.

with an overall high frequency of T_{NAIVE}, followed by T_{EMRA} and T_{EM}, whereas T_{CM} as well as T_{SCM} were present at lower frequencies (Figures 4E and 4G). After SARS-CoV-2-specific enrichment, the CD8$^+$ T cells contained a high proportion of T_{EMRA} and T_{EM}. On day 21 of expansion, SARS-CoV-2-specific CD8$^+$ T cells expressed a similar phenotype, with the majority being T_{EMRA} and T_{EM} in both bulk and CD137$^+$ and IFN-γ$^+$ T cells (Figures 4F and 4H). Overall, the *FKBP12* KO did not have a major effect on T cell differentiation and the subset composition of the TCPs, nor did it confer a discernible advantage to any particular subset.

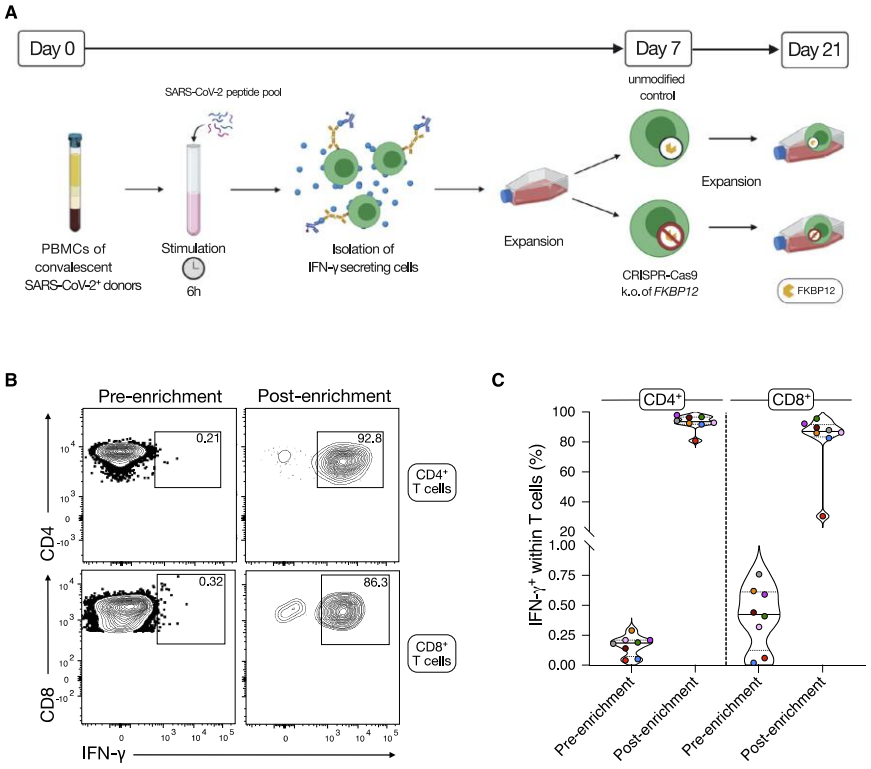


Figure 2. Schematic outline of the experimental setup to isolate and expand SARS-CoV-2-specific T cells following CRISPR-Cas9-mediated KO of *FKBP12* to induce tacrolimus-resistance

(A) Timeline and individual steps of the procedure to isolate and expand SARS-CoV-2-specific T cells following CRISPR-Cas9-mediated KO of *FKBP12* to induce tacrolimus-resistance. Created with BioRender.com. (B) Representative dot plot of purities of SARS-CoV-2-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells pre and post enrichment. (C) Purities of SARS-CoV-2-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells pre and post enrichment, where each dot color represents one individual donor (n = 8).

Effector cytokine production in the presence of tacrolimus is rescued by *FKBP12* KO in SARS-CoV-2-specific TCPs

To demonstrate both efficacy against SARS-CoV-2 and to confirm Tac resistance of our *FKBP12* KO TCPs, we re-stimulated the distinct TCPs with SARS-CoV-2 peptide pools and analyzed production of antiviral cytokines (IFN-γ, TNF-α, and interleukin [IL]-2) in the presence or absence of clinical doses of immunosuppressive drugs. To confirm the specificity of the *FKBP12* KO approach, we re-stimulated unmodified control and *FKBP12* KO TCPs in presence of Tac as well as an alternative CNI, CsA, which depends on the adaptor protein, peptidylprolyl isomerase A (PPIA). Thus, *FKBP12* KO should not affect the immunosuppressive function of CsA in edited TCPs. We also tested the functionality of SARS-CoV-2-specific T cells in the unmodified and *FKBP12* KO TCPs by exposure to triple immunosuppression (IS) therapy commonly administered post solid organ transplantation, namely, Tac, prednisolone (corticosteroid), and mycophenolic acid (MPA). Upon SARS-CoV-2-specific re-stimulation on day 21, unmodified control and *FKBP12* KO TCPs showed comparable frequencies of activated ($CD137^+$) cytokine producers among $CD4^+$ and $CD8^+$ T cells (Figures 5A–5G and S3A–S3F). Exposing TCPs to immunosuppressive drugs during stimulation resulted in a significant decrease in activated cytokine producers among $CD4^+$ (Figures 5A–5D and S3A–S3C) and $CD8^+$ T cells (Figures 5A, 5E–5G, and S3D–S3F). These were partially rescued by the *FKBP12* KO (Figures 5A–5G and S3A–S3F). Both $CD4^+$ and $CD8^+$ *FKBP12* KO T cells produced effector cytokines in the presence of Tac but not in the presence of CsA (Figures 5A–5G and S3A–S3F). The proportions of cytokine producers among $CD4^+$ and $CD8^+$ T cells in *FKBP12* KO TCPs were similar in Tac-treated

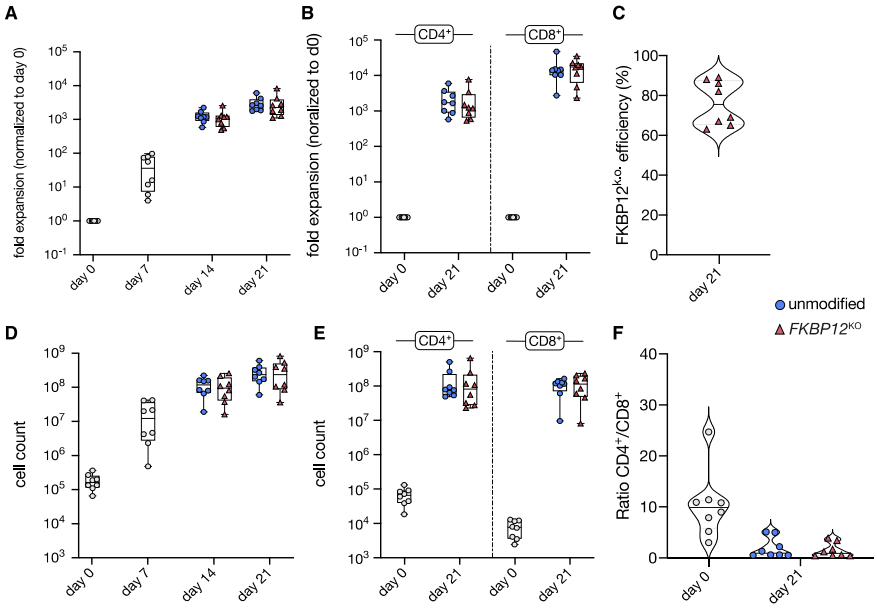


Figure 3. Expansion rates and CD4$^+$/CD8$^+$ T cell ratio of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs over 21 days of culture

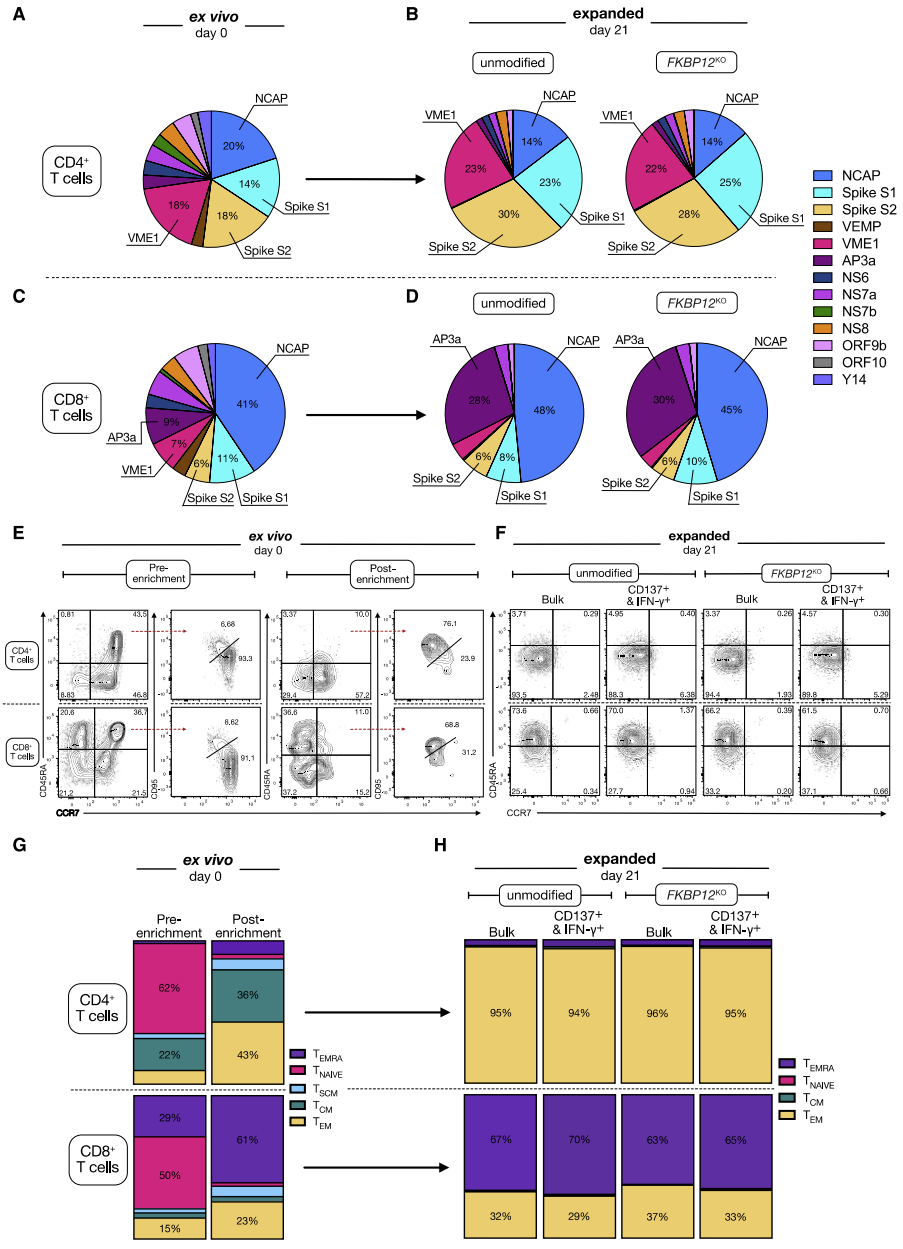
Experimental setup same as in Figure 2. n = 8. (A) Expansion rates (fold expansion) from day 0 to day 21 of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs normalized to day 0. (B) Expansion rates (fold expansion) from day 0 to day 21 of CD4$^+$ and CD8$^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs normalized to day 0. (C) KO efficiency of *FKBP12* KO SARS-CoV-2-specific TCPs at day 21. (D) Expansion (cell count) from day 0 to day 21 of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs. (E) Expansion (cell count) from day 0 to day 21 of CD4$^+$ and CD8$^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs. (F) Ratio of CD4$^+$/CD8$^+$ T cells at day 0 and day 21 of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs.

and untreated TCPs. Exposure to triple IS during stimulation decreased the capacity to produce effector cytokines among both the SARS-CoV-2-stimulated CD4$^+$ and CD8$^+$ *FKBP12* KO T cells (Figures 5A–5G and S3A–S3F). Among the CD4$^+$ and CD8$^+$ T cells of unmodified control and *FKBP12* KO TCPs, we identified polyfunctional T cells based on their ability to secrete multiple cytokines, including IFN-γ, TNF-α, and IL-2 (Figures 5D, 5G, S3B, S3C, S3E, and S3F). Unlike CD4$^+$ T cells, the frequency of activated SARS-CoV-2-specific CD8$^+$ T cells, identified by CD137 (4-1BB) expression, did not significantly decrease when TCPs were exposed to CsA during stimulation (Figures S3G and S3H). However, both CD4$^+$ and CD8$^+$ CD137-expressing T cells were unable to produce effector cytokines in the presence of CsA, both in unmodified and *FKBP12* KO TCPs (Figures 5A–5G and S3A–S3F). Expression of the activation marker CD154 (CD40L) among both the SARS-CoV-2-stimulated CD4$^+$ and CD8$^+$ unmodified and *FKBP12* KO T cells was comparable. Exposing TCPs to IS during stimulation resulted in a significant decrease in CD154 expression among CD4$^+$ (Figure S3I) and CD8$^+$ T cells (Figure S3J), which was partially rescued by *FKBP12* KO.

FKBP12 KO SARS-CoV-2-specific TCPs demonstrate killing capacity comparable with unmodified control SARS-CoV-2-specific TCPs

Since targeted elimination of virus-infected cells is an essential characteristic of antiviral T cells, we tested the cytotoxic killing capacity of SARS-CoV-2-specific TCPs. Although short-term incubation with the IS Tac showed a strong effect on antiviral cytokine production in TCPs (Figure 5), we found that the T cell-mediated cytotoxic killing of target cells loaded with SARS-CoV-2 peptides was not affected by short-term treatment with Tac, neither in unmodified nor in *FKBP12* KO TCPs (Figures 6A–6C).

To identify the dominant antigens driving T cell-mediated killing of SARS-CoV-2 peptide-loaded target cells, we analyzed the killing capacity of TCPs with regard to individual antigens of SARS-CoV-2. Both unmodified control and *FKBP12* KO TCPs showed efficient killing of NCAP as well as AP3a peptide-loaded target cells, followed by target cells loaded with VME1 and spike S1 and S2 peptides (Figure 6D). We also found T cell-mediated killing toward target cells loaded with peptides from the accessory proteins NS7a and ORF9b



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for some of the TCPs, but not toward target cells loaded with peptides from the remaining accessory proteins NS6, NS7b, NS8, ORF10, and Y14 or the structural protein VEMP (Figure 6D). This was in contrast to antiviral cytokine production, which we did not observe in response to accessory-protein-derived peptides in the expanded unmodified control and *FKBP12* KO TCPs, with the exception of the AP3a peptide pool (Figures 4B, 4D, and S2D–S2H). We then examined the killing capacity of $CD4^+$ and $CD8^+$ T cells separately to determine whether T cell-mediated cytotoxic killing of the dominant target antigens is executed by $CD4^+$ or $CD8^+$ T cells. Our observations suggested $CD8^+$ T cells were the main drivers of cytotoxic elimination of SARS-CoV-2 peptide-loaded target cells (Figures 6E and 6F). Among $CD8^+$ T cells of unmodified control and *FKBP12* KO TCPs, we detected the most efficient killing of NCAP, AP3a, and SARS-CoV-2 peptide-pool-loaded target cells, followed by target cells loaded with VME1, spike S1, and spike S2 peptide pools (Figures 6E and 6F).

To add another model of SARS-CoV-2 infection and to determine if the SARS-CoV-2-specific TCPs can recognize and kill these cells, we co-transfected target cells with a plasmid encoding the full sequence of the SARS-CoV-2 wild-type (WT) spike protein (pSpike) (Figure S4A) and a plasmid encoding GFP (pmaxGFP by Lonza). We sorted for GFP^+ target cells and co-cultured them with the distinct SARS-CoV-2-specific TCPs to determine the T cell-mediated cytotoxicity. Expression of spike was confirmed by flow cytometry (Figure S4B). We observed T cell-mediated killing of pSpike-transfected target cells by unmodified and *FKBP12* KO TCPs. Although the cytotoxic killing of SARS-CoV-2 peptide-loaded target cells by our TCPs was not affected by short-term incubation with the IS Tac (Figure 6C), we found that cytotoxic elimination of target cells transfected with pSpike was reduced in presence of Tac for unmodified but not *FKBP12* KO TCPs (Figure 6G).

SARS-CoV-2-specific unmodified and *FKBP12* KO TCPs recognize spike S1 and S2 of SARS-CoV-2 variants but show little cross-reactivity to spike S1 and S2 proteins of common endemic HCoVs

Considering the ongoing occurrence of SARS-CoV-2 variants, it becomes increasingly important that TCPs also recognize antigens of the mutant SARS-CoV-2 strains without the need to determine the exact variant. Therefore, we re-stimulated the TCPs with peptide pools of the distinct spike proteins S1 and S2 of SARS-CoV-2 variants Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) and analyzed production of antiviral cytokines (IFN-γ and TNF-α). Upon re-stimulation with peptide pools of the spike protein S1 and S2 of SARS-CoV-2 variants, frequencies of activated ($CD137^+$) cytokine producers among $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO TCPs were comparable with those elicited by the WT spike S1 and S2 (Figures S5A–S5H).

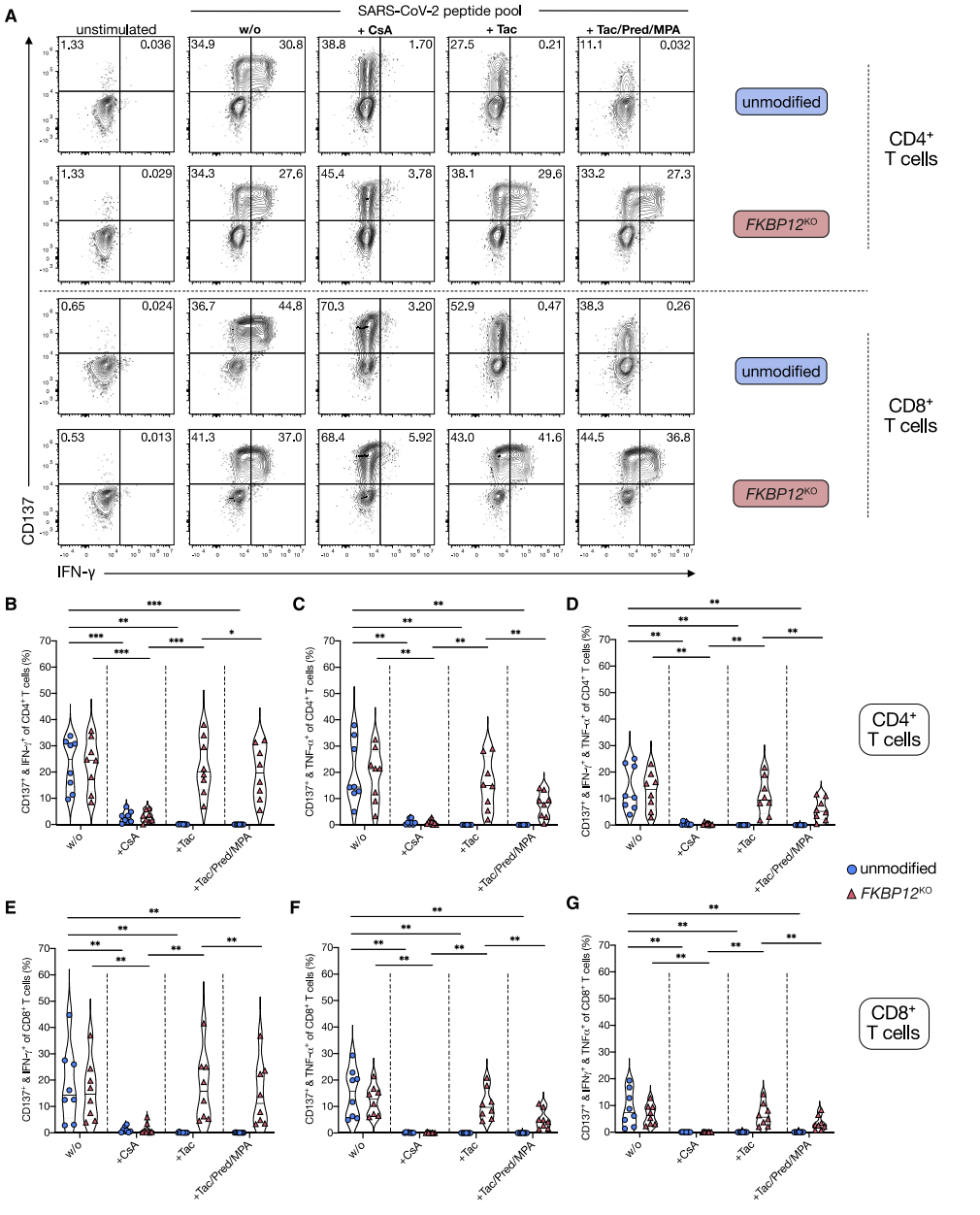
Numerous studies have suggested SARS-CoV-2 cross-reactive T cells in non-exposed individuals directed against the S2 subunit of the spike protein occur due to its partial sequence homology with common endemic HCoVs.[24,25,65,66] We investigated this notion by re-stimulating unmodified control and *FKBP12* KO TCPs with a peptide pool derived from spike S1 and S2 of HCoV-229E, HCoV-NL63, HCoV-OC43, and HKU1. We found that unmodified control and *FKBP12* KO TCPs showed little cross-reactivity toward spike S1 and S2 peptide pools of the common endemic HCoV (Figures S6A–S6D). For $CD4^+$ T cells, both spike S1 and S2 peptide pools of SARS-CoV-2 induced significantly higher frequencies of activated IFN-γ as well as TNF-α producers in unmodified control and *FKBP12* KO TCPs compared with spike S1 and S2 peptide pools of common endemic HCoV (Figures S6A and S6B). Among the $CD8^+$ T cells, the spike S2 peptide pool of SARS-CoV-2 induced significantly higher frequencies of activated IFN-γ as well as TNF-α producers in unmodified control and *FKBP12* KO TCPs compared with spike S1 and S2 peptide pools of common endemic HCoV (Figures S6C and S6D).

Cellular indexing of transcriptomes and epitopes sequencing and proteome analyses confirm resistance of *FKBP12* KO TCPs to tacrolimus

To determine whether *FKBP12* editing affects the transcriptome and specific surface protein levels and how these are influenced by different CNIs, we performed single-cell Cellular Indexing of Transcriptomes and Epitopes sequencing (CITE-seq) on unmodified control and *FKBP12* KO TCPs of CD17 to CD20 (Table S1). SARS-CoV-2-specific unmodified control and *FKBP12* KO TCPs

Figure 4. Antigen-specific T cell distribution before and after expansion of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs

Experimental setup to define antigen specificity the same as in Figure 1, selection and expansion the same as in Figure 2. n = 8. (A) Proportional distribution of IFN-γ-producing SARS-CoV-2-reactive ($CD137^+$) $CD4^+$ T cells from convalescent SARS-CoV-2^+ donors *ex vivo* on day 0 after 16 h of stimulation with the individual structural and accessory proteins of SARS-CoV-2. (B) Proportional distribution of IFN-γ-producing SARS-CoV-2-reactive ($CD137^+$) $CD4^+$ T cells of unmodified control (left pie chart) and *FKBP12* KO TCPs (right pie chart) on day 21 of expansion after 16 h of stimulation with the individual structural and accessory proteins of SARS-CoV-2. (C) Proportional distribution of IFN-γ-producing SARS-CoV-2-reactive ($CD137^+$) $CD8^+$ T cells from convalescent SARS-CoV-2^+ donors *ex vivo* on day 0 after 16 h of stimulation with the individual structural and accessory proteins of SARS-CoV-2. (D) Proportional distribution of IFN-γ-producing SARS-CoV-2-reactive ($CD137^+$) $CD8^+$ T cells of unmodified control (left pie chart) and *FKBP12* KO TCPs (right pie chart) on day 21 of expansion after 16 h of stimulation with the individual structural and accessory proteins of SARS-CoV-2. (E) Representative flow cytometric dot plots illustrating T cell memory subsets of $CD4^+$ and $CD8^+$ T cells pre and post enrichment. (F) Representative flow cytometric dot plots illustrating T cell memory subsets of either bulk or $CD137^+$ and IFN-γ^+ $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs after 21 days of expansion. (G) Proportional distribution of T cell memory subsets of $CD4^+$ and $CD8^+$ T cells pre and post enrichment. T_{Naive}, naive T cells ($CCR7^+$/$CD45RA^+$/$CD95^-$); T_{SCM}, stem-cell-like memory T cells ($CCR7^+$/$CD45RA^+$/$CD95^+$); T_{CM}, central memory T cells ($CCR7^+$/$CD45RA^-$); T_{EM}, effector memory T cells ($CCR7^-$/$CD45RA^-$); T_{EMRA}, terminally differentiated effector memory T cells ($CCR7^-$/$CD45RA^+$). (H) Proportional distribution of T cell memory subsets of either bulk or $CD137^+$ and IFN-γ^+ $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs after 21 days of expansion. T_{Naive}, naive T cells ($CCR7^+$/$CD45RA^+$); T_{CM}, central memory T cells ($CCR7^+$/$CD45RA^-$); T_{EM}, effector memory T cells ($CCR7^-$/$CD45RA^-$); T_{EMRA}, terminally differentiated effector memory T cells ($CCR7^-$/$CD45RA^+$).



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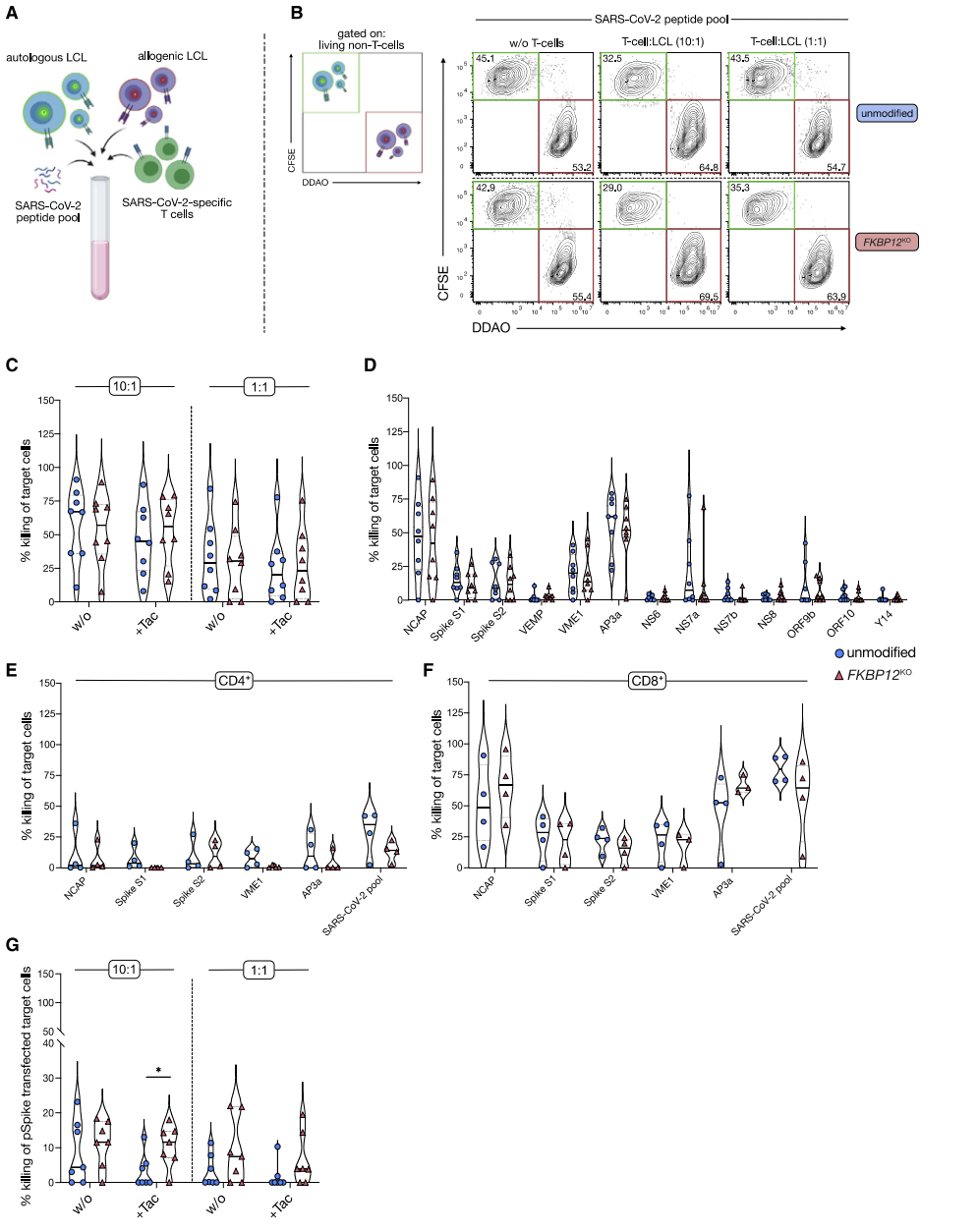
were re-stimulated with SARS-CoV-2 peptide-pool-loaded target cells for 6 h on day 21, either in the presence or absence of CNIs. According to their transcriptomes and specific protein expression identified by CITE-seq, cells were clustered by Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP [Uniform manifold approximation and projection]). Twenty distinct cell clusters were identified, comprising $CD4^+$ T cells (clusters 15, 12, 2, 9, and 6), $CD8^+$ T cells (clusters 13, 17, 11, 19, 1, 5, 18, 14, and 7), double-negative (DN) T cells (cluster 16), double-positive (DP) T cells (clusters 4b, 4a, and 3), natural killer (NK) cells (clusters 20 and 10), and lymphoblastoid cell lines (LCLs) (cluster 8) (Figure 7A). RNA transcripts of genes associated with effector function (e.g., *IFNG*, *IL2*, *TNFA*, and *GZMB*) were upregulated in clusters 13 and 15 upon SARS-CoV-2-specific activation of unmodified and *FKBP12* KO TCPs (Figure 7B). However, cluster 15 but not 13 showed upregulation of *PD-1* (*PDCD1*) as well as *IL-4* mRNA. Cluster 13 additionally showed strong expression of *XCL1*, *XCL2*, *CD226*, and *IRF8*, transcripts associated with migration, survival, and memory formation,[67–69] which was less pronounced for cluster 15. Among both $CD4^+$ and $CD8^+$ T cells, CNI treatment as well as *FKBP12* KO had no impact on the cluster distribution in unstimulated TCPs. In contrast, upon SARS-CoV-2-specific re-stimulation, the frequency of $CD4^+$ T cell cluster 15 and $CD8^+$ T cell clusters 13 and 17 of both unmodified control and *FKBP12* KO TCPs increased. The increased frequencies of $CD4^+$ T cell cluster 15 and $CD8^+$ T cell clusters 13 and 17 were inhibited upon treatment with Tac in the unmodified control but not in *FKBP12* KO TCPs (Figure 7C). However, in the presence of CsA, $CD4^+$ T cell cluster 15 and $CD8^+$ T cell cluster 13 were under-represented in both unmodified control and *FKBP12* KO TCPs, whereas the proportion of cells falling into $CD4^+$ T cell cluster 2 and $CD8^+$ T cell clusters 17 as well as 18 increased, respectively (Figure 7C). Interestingly, cluster 2 was characterized by upregulation of *CXC3CR1* and *IL7R* mRNA, whereas cluster 17 overexpressed *ID2*, *ID3*, and *HAVCR2* (coding for TIM-3) mRNA and cluster 18 showed slightly elevated levels of *CD247* mRNA, respectively (Figure 7B). Gene expression analysis confirmed downregulation of *FKBP12* mRNA in $CD4^+$ and $CD8^+$ T cells of *FKBP12* KO TCPs (Figures S7A and S7B). Gene expression analysis also showed that, upon SARS-CoV-2-specific stimulation, the top 25 differentially expressed genes were similar between unmodified and *FKBP12* KO TCPs in the absence of CNIs as well as under Tac treatment in the *FKBP12* KO TCPs. This was the case for both $CD4^+$ and $CD8^+$ T cell populations (Figures S7A and S7B). Remarkably, the presence of Tac further upregulated *SLA* mRNA in $CD4^+$ and *IL2* mRNA in $CD8^+$ $FKBP12^{KO}$ T cells respectively, compared with untreated *FKBP12* KO and unmodified controls. To characterize the functional capacity of *FKBP12* KO TCPs in more detail, we performed gene expression analysis of markers associated with antiviral T cell function as well as T cell exhaustion. Upon SARS-CoV-2-specific re-stimulation, $CD4^+$ T cells of unmodified and *FKBP12* KO TCPs show increased expression of *TOX2*, *EOMES*, *PDCD1*, *CTLA4*, *IL-10*, and *LAG3*, which are common markers to define T cell exhaustion (Figure S7C). When *FKBP12* KO TCPs were exposed to Tac, expression of *TOX2*, *CTLA4*, and *IL-10* was lower compared with *FKBP12* KO TCPs without Tac. $CD8^+$ T cells of unmodified and *FKBP12* KO TCPs showed increased expression of *PDCD1*, *CTLA4*, *LAG3*, and *IL10*, while the expression of *TOX2* and *EOMES* was only upregulated in unmodified but not *FKBP12* KO TCPs (Figure S7D). Moreover, $CD4^+$ and $CD8^+$ T cells of unmodified and *FKBP12* KO TCPs showed increased expression of common markers defining antiviral T cell function, including *CD40LG*, *IL21*, *IFNG*, *TNF*, *IL2*, *PRF1*, and *GZMB* in the absence of CNIs as well as under Tac treatment (Figures S7C and S7D).

To further characterize the proteomes of SARS-CoV-2-specific TCPs, we performed proteome analysis based on mass spectrometry. We confirmed the gene KO of *FKBP12* on protein level (Figure 7D). Furthermore, we detected 11 of the differentially expressed mRNA transcripts (Figures S7A and S7B) in the proteome (Figure 7D). Among those, we observed increased expression of DDX21, NAMPT, NCL, PGAM1, and PPA1 in SARS-CoV-2-activated unmodified but not *FKBP12* KO TCPs (Figure 7D). In SARS-CoV-2-activated *FKBP12* KO TCPs, we found upregulated protein expression of RAB27A under Tac treatment (Figure 7D). High levels of GZMB were detected in *FKBP12* KO TCPs in the presence and absence of Tac, which was to a lesser extent also observed in SARS-CoV-2-activated unmodified TCPs in the absence of IS (Figure 7D).

Figure 5. Functional analysis of SARS-CoV-2-specific unmodified control and *FKBP12* KO TCPs

SARS-CoV-2-specific stimulation of unmodified control and *FKBP12* KO TCPs on day 21 of culture. Immunosuppressants were added where indicated: CsA, cyclosporine A; Tac, tacrolimus; Tac/Pred/MPA, tacrolimus + prednisolone + mycophenolic acid. n = 8; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (A) Representative flow cytometry plots of antigen-reactive ($CD137^+$) IFN-γ producers among $CD4^+$ and $CD8^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs. IFN-γ production upon SARS-CoV-2-specific re-stimulation of TCPs is shown in the presence or absence of the indicated immunosuppressants. (B) Quantified data for the IFN-γ production of SARS-CoV-2-activated ($CD137^+$) $CD4^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants. (C) Quantified data for the TNF-α production of SARS-CoV-2-activated ($CD137^+$) $CD4^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants. (D) Quantified data for the IFN-γ and TNF-α production of SARS-CoV-2-activated ($CD137^+$) $CD4^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants. (E) Quantified data for the IFN-γ production of SARS-CoV-2-activated ($CD137^+$) $CD8^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants. (F) Quantified data for the TNF-α production of SARS-CoV-2-activated ($CD137^+$) $CD8^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants. (G) Quantified data for the IFN-γ and TNF-α production of SARS-CoV-2-activated ($CD137^+$) $CD8^+$ T cells in unmodified and *FKBP12* KO SARS-CoV-2-specific TCPs after 16 h of stimulation with SARS-CoV-2 peptide pool in the presence or absence of respective immunosuppressants.



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We additionally performed T cell receptor (TCR) repertoire analysis on the single-cell level to determine the effect of *FKBP12* editing on TCR diversity. Comparing the TCR clonality and total number of clones included in SARS-CoV-2-specific unmodified control and *FKBP12* KO TCPs revealed higher variations within largely expanded but low variations within single small and medium expanded clone types (Figure 7E). Importantly, there was no overrepresentation of largely expanded TCR clones within the *FKBP12* KO TCPs (Figure 7E). The TCR diversity represented by Shannon entropy was largely comparable between unmodified control and *FKBP12* KO TCPs (Figure 7F). The five most represented TCR clones within both $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO TCPs, respectively, revealed a shared TCR repertoire with overall comparable representation of TCR clones between unmodified and *FKBP12* KO TCPs of one donor (Figure S8A). The proportion of all TCR sequences represented by the top five clones ranged from around 5% to a maximum of 32% but was comparable between *FKBP12* KO and the corresponding unmodified TCPs (Figure S8B).

DISCUSSION

ACT is an attractive treatment strategy to prevent and treat viral infections in immunocompromised or immunosuppressed patients[30–32] and has been suggested as an early treatment strategy for SARS-CoV-2 infection[27] or even to treat acute COVID-19.[28,29] Immunosuppressants likely suppress endogenous antiviral immunity and could undermine the benefits of antiviral ACT, especially in patients under constant immunosuppressive treatment, such as SOT recipients.[70] Here, we demonstrate the feasibility of generating Tac-resistant SARS-CoV-2-specific TCPs from the blood of donors who have cleared SARS-CoV-2 infection. Tac-resistant SARS-CoV-2-specific TCPs show superior cytokine production when exposed to therapeutic doses of Tac or triple IS compared with unmodified TCPs. Tac-resistant SARS-CoV-2-specific TCPs could be used for the prevention or acute treatment of COVID-19 and enhancement of active vaccination in immunosuppressed patients on Tac therapy (transplant recipients and autoimmune patients) as well as for patients with severe COVID-19, in combination with Tac, to prevent immunopathology while achieving viral control.[63]

Upon SARS-CoV-2-specific stimulation of *ex vivo* PBMCs from SARS-CoV-2 convalescent donors, we detected antigen-specific $CD4^+$ and $CD8^+$ T cells, which are a pre-requisite for manufacturing of Tac-resistant TCPs. In line with recent studies,[24–26,65,66,71] we found that SARS-CoV-2-specific $CD4^+$ T cells predominantly target the structural proteins NCAP, spike S1 and S2, and to a lower extent VME1, whereas $CD8^+$ T cells predominantly show specificity toward NCAP during characterization of the starting material. Similar to our observations of T cells stimulated *ex vivo* on day 0, expanded $CD4^+$ and $CD8^+$ T cells preferentially targeted different antigens of SARS-CoV-2 yet with a clear overall preference of both $CD4^+$ and $CD8^+$ T cells for the structural proteins, in line with previous reports.[27,29] The distribution of specificities among T cells before and after SARS-CoV-2-specific T cell expansion was largely comparable for $CD4^+$ and $CD8^+$ T cells, except for AP3a, which was found to be a relevant driver of $CD8^+$ T cell expansion and was not affected by KO of *FKBP12*. Supporting previous reports, expanded SARS-CoV-2-specific $CD4^+$ T cells recognized a broader set of viral surface antigens,[27,29] whereas antigen specificity for $CD8^+$ T cells was limited to internal NCAP[29] and AP3a.[72] The differences in antigen specificity between SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells may relate to distinct processing and presentation of viral surface and internal antigens. However, it is also reported that apoptosis and autophagy are upregulated in PBMCs of SARS-CoV-2-infected individuals,[73] which may promote presentation of phagocytosed antigens via major histocompatibility complex (MHC) class II molecules on professional antigen-presenting cells. In line with our observations, the VEMP protein is reported to be barely recognized by the host's adaptive immune defense.[74] A recent report suggests that ORF8 protein downregulates the expression of MHC class I molecules on several cell types, resulting in impaired antigen presentation to $CD8^+$ T cells.[75] While $CD8^+$ T cells are indispensable for elimination of infected cells, $CD4^+$ T cells contribute to affinity-maturated and protective antibody responses, and thus Tac-resistant TCPs may help to establish improved antibody responses in immunosuppressed individuals. Indeed, it was shown that spike-specific $CD4^+$ T cell responses correlate with serum levels of anti-spike IgG titers in recovered SARS-CoV-2-infected donors.[76] Moreover, VME1-specific antibodies have been suggested as an additional target for immune monitoring due to the relatively high frequency of VME1-specific $CD4^+$ T cells in the blood of convalescent COVID-19 patients.[26]

With the continued emergence of SARS-CoV-2 variants, we were interested in whether the SARS-CoV-2-specific TCPs we generated are cross-reactive to mutant SARS-CoV-2 strains abolishing the need for

Figure 6. Killing capacity of SARS-CoV-2 peptide-loaded autologous target cells by unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs

SARS-CoV-2-specific T cell-mediated cytotoxicity of unmodified control and *FKBP12* KO TCPs at day 21 of culture. Immunosuppressant Tac was added where indicated. n = 8 for (C) and (D); n = 4 for (E) and (F) (except for VME1 and AP3a of *FKBP12* KO TCPs: n = 3); n = 7 for (G) *$p < 0.05$. (A) Schematic illustration of the experimental setup. Created with BioRender.com. (B) Representative flow cytometry plots illustrating the selection of autologous (CFSE-labeled) and allogenic (DDAO [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)]-labeled) LCLs in the different conditions indicated above the plots. (C) Percentage killing of SARS-CoV-2 peptide-pool-loaded autologous target T cells by unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs at 10:1 and 1:1 ratios (T cells:LCLs) in the presence or absence of Tac. (D) Percentage killing of autologous target cells loaded with indicated structural and accessory proteins of SARS-CoV-2 by unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs at 10:1 ratio (T cells:LCLs). (E) Percentage killing of autologous target cells loaded with indicated structural (NCAP, spike S1, spike S2, VME1, SARS-CoV-2-pool) and accessory proteins (AP3a) of SARS-CoV-2 by purified $CD4^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs at 10:1 ratio (T cells:LCLs). (F) Percentage killing of autologous target cells loaded with indicated structural (NCAP, spike S1, spike S2, VME1, SARS-CoV-2-pool) and accessory proteins (AP3a) of SARS-CoV-2 by purified $CD8^+$ T cells of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs at 10:1 ratio (T cells:LCLs). (G) Percentage killing of autologous target cells transfected with a plasmid encoding the full spike protein of SARS-CoV-2 by unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs at 10:1 and 1:1 ratio (T cells:LCLs) in the presence or absence of Tac.

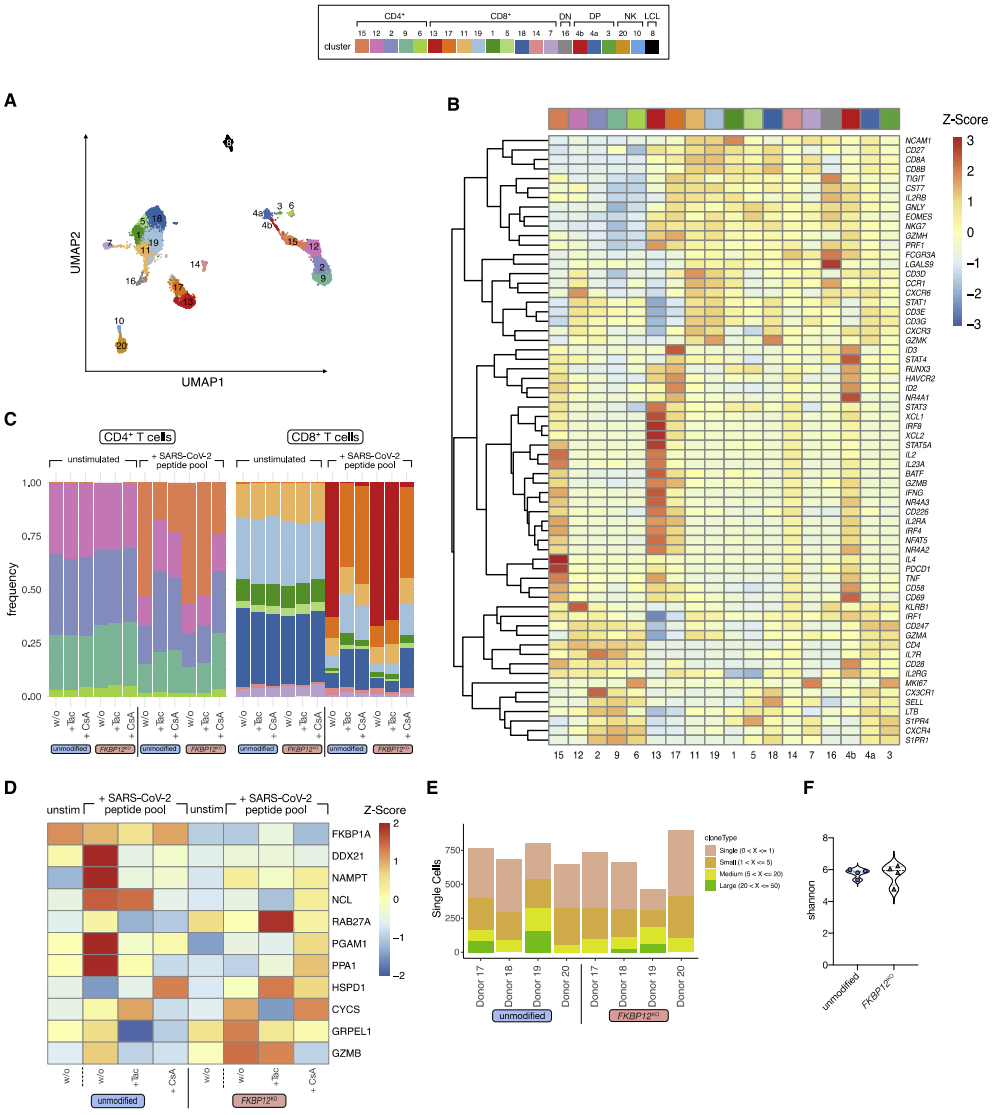

Figure 7. Single-cell transcriptomes, proteome data, and TCR repertoire of unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs

CITE-seq, proteome, and TCR analysis of SARS-CoV-2-specific TCPs after SARS-CoV-2-specific re-stimulation at day 21 of culture. Immunosuppressant Tac or CsA were added where indicated. n = 4, n = 3 for (D) (A) UMAP representation of unmodified and *FKBP12* KO TCPs. Transcriptionally similar clusters were identified using shared nearest neighbor (SNN) modularity optimization. (B) Heatmap of RNA expression of T cell-associated genes within different clusters. (C) Cluster distribution within unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs in the presence or absence of IS as well as in unstimulated and stimulated conditions. (D) Heatmap of proteins differentially expressed upon SARS-CoV-2-specific stimulation in T cells of unmodified control and *FKBP12* KO TCPs in presence or absence of IS. (E) Distribution of different clone types within unmodified control and *FKBP12* KO TCPs. (F) TCR diversity represented by Shannon entropy of unmodified control and *FKBP12* KO TCPs.

variant identification prior to TCP manufacturing. Both unmodified control and *FKBP12* KO TCPs recognized spike protein S1 and S2 of SARS-CoV-2 variants Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529), and elicited effector cytokine levels similar to WT spike S1 and S2 peptide pools, which is in line with recent reports.[77,78] This observation confirms that the T cell response in SARS-CoV-2 convalescent or vaccinated individuals remains largely stable even against mutants of SARS-CoV-2, whereas neutralizing antibodies are reported to be weakened in their effect.[77,79]

Cross-reactive SARS-CoV-2 T cell epitopes with predominant spike specificity have been described in unexposed individuals, which might be due to previous infections with common endemic HCoV.[80] In agreement with recent studies, we observed higher frequencies of SARS-CoV-2 cross-reactive T cells among $CD4^+$ compared with $CD8^+$ T cells[65,80–82] in the seronegative healthy individuals from our study. There is evidence that pre-formed SARS-CoV-2-directed immunity to structural proteins is not driven by cross-reactivity to common endemic HCoV but rather by other frequently encountered pathogens.[83] Hence, the exact source of pre-formed T cell immunity in SARS-CoV-2-naive individuals remains to be elucidated. To date, it remains uncertain whether cross-reactive memory T cells possess protective features to fight SARS-CoV-2 infection.[84] Our data from convalescent donors indicate little cross-reactivity of SARS-CoV-2-specific TCPs toward spike S1 and S2 of common endemic HCoV after expansion. In line with this, when stimulating PBMCs of convalescent SARS-CoV-2-infected donors with spike peptide pools from either SARS-CoV-2, common endemic HCoV-229E, or HCoV-OC43, it was reported that frequencies of SARS-CoV-2 spike-specific $CD4^+$ T cells were significantly higher than spike-299E- or spike-OC43-specific $CD4^+$ T cells.[74] This indicates that the T cell response toward spike protein is predominantly directed against SARS-CoV-2 in these donors. Although it has been reported that, after coronavirus infection, spike-specific T cells can persist for as long as 4 years,[85] the frequency of cross-reactive T cells in the blood might be limited since memory T cells are known to reside in the bone marrow and, without substantial viral re-stimulation, it is unlikely that they egress into the blood stream.[86]

Patients who experience mild symptoms following SARS-CoV-2 infection show higher proportions of $CD8^+$ T cell responses compared with those suffering from severe infection,[42,76] suggesting a potential protective role of $CD8^+$ T cell immunity against SARS-CoV-2. $CD8^+$ T cells are known to contribute to effective viral clearance to terminate acute viral infections, whereas cytotoxic $CD4^+$ T cells are required to control chronic infections by, e.g., human immunodeficiency virus or herpes viruses.[87] We found that $CD8^+$ T cells were the main drivers of SARS-CoV-2-directed cytotoxicity to SARS-CoV-2-loaded target cells. Although starting with a small proportion of SARS-CoV-2-reactive $CD8^+$ T cells on day 0, we obtained a more balanced $CD8^+$ to $CD4^+$ T cell ratio on day 21 of *in vitro* expansion. Contrary to our observations, Keller et al. (2020) did not obtain strong SARS-CoV-2-specific $CD8^+$ T cell responses using peptide libraries consisting of only SARS-CoV-2 structural proteins to isolate SARS-CoV-2-specific T cells, neither immediately after isolation nor after expansion.[27] It may be that a combination of structural and accessory antigens of SARS-CoV-2 is superior in driving expansion of both $CD4^+$ and $CD8^+$ SARS-CoV-2-directed T cells.[29] Furthermore, the cytokine cocktail within the culture medium may affect the expansion of either $CD4^+$ or $CD8^+$ T cells.[88] Therefore, the use of different cytokine cocktails may explain the observed variations in the expansion of SARS-CoV-2-specific $CD8^+$ T cells.

Upon SARS-CoV-2-specific *ex vivo* stimulation, multiple studies have reported high proportions of T_{EMRA} in the SARS-CoV-2-specific $CD8^+$ T cell compartment of patients who have recovered from SARS-CoV-2 infection.[65,76,89] Prior to expansion, SARS-CoV-2-specific $CD8^+$ T cells comprised a substantial proportion of T_{EMRA}.[28] This highly differentiated memory phenotype might therefore be characteristic of SARS-CoV-2 infection, suggesting short-term $CD8^+$ T cell immunity. T cells with effector memory and central memory phenotypes have previously been shown to exhibit increased *in vivo* persistence after adoptive transfer and to induce long-term immunity,[90] and would therefore be desirable for ACT, whereas a terminally differentiated phenotype would not.[91] Analysis of the T cell phenotype of the *FKBP12* KO TCPs revealed a predominant T_{EM} and T_{CM} phenotype among $CD4^+$ T cells before and after SARS-CoV-2-induced T cell expansion, whereas $CD8^+$ T cells displayed mainly a T_{EMRA} phenotype followed by T_{EM} as the second most represented memory T cell subset, which was also the case for unmodified control TCPs. This may raise concerns about the efficacy, longevity, and induction of functional memory by TCPs. However, in patients suffering from severe COVID-19, parameters including longevity and memory formation of the TCPs may not necessarily be required to clear an acute SARS-CoV-2 infection. Moreover, T_{EMRA} can be divided into several subsets that have been shown to differ in their ability to differentiate, proliferate, and produce effector cytokines[92–94] and might even comprise cells evolved from a naive differentiation stage.[93] It is conceivable that SARS-CoV-2-specific $CD8^+$ T cells with a late-differentiated effector phenotype could de-differentiate into long-lived memory cells.[94] Low-dose rapamycin supplementation during cell expansion may be a potential strategy to arrest differentiation of SARS-CoV-2-reactive T cells or to generate early-differentiated T cells.[88,95] Ultimately, clinical trials are needed to answer the question of efficacy of TCPs with high T_{EMRA} content to fight or prevent SARS-CoV-2 infection.

Our functional analysis of the SARS-CoV-2-specific TCPs has shown that the innovative *FKBP12* KO TCPs possess superior cytokine production upon SARS-CoV-2-specific re-stimulation in the presence of Tac and triple IS compared with unmodified SARS-CoV-2-specific TCPs. The sensitivity of *FKBP12* KO SARS-CoV-2-specific TCPs toward CsA shows specificity of the approach and represents an important safety switch, which could limit undesired toxicity associated with Tac-resistant *FKBP12* KO SARS-CoV-2-specific TCPs *in vivo*.[43] In line with previous observations, Tac did not influence the cytotoxic killing capacity of either unmodified or *FKBP12* KO SARS-CoV-2-specific TCPs when confronted with peptide-loaded target cells.[63,96]

However, Tac treatment did influence T cell-mediated cytotoxic killing of pSpike-transfected target cells. The transfection of spike protein likely leads to a reduced or selective antigen presentation by target cells compared with the usage of a peptide pool. Indeed, Tac has been reported to interfere with antigen presentation.[97,98] Thus, the system, which requires active antigen presentation by the LCLs as opposed to the passive presentation of peptides, may be more susceptible to Tac, since a certain TCR signaling threshold is necessary to achieve full T cell activation.[99] Re-stimulation using a SARS-CoV-2 peptide pool may be able to continuously exceed this threshold irrespective of the presence of Tac, whereas it is not reached in the case of transfection with pSpike due to the diminished antigen presentation in the presence of Tac.

FKBP12 KO TCPs may also support antibody production in Tac-treated patients as they show CD154 expression upon SARS-CoV-2-specific re-stimulation in the presence of Tac and triple IS, which was not the case for unmodified SARS-CoV-2-specific TCPs. We also confirmed upregulation of CD154 (CD40LG) mRNA transcripts upon SARS-CoV-2-specific re-stimulation of both unmodified and *FKBP12* KO TCPs as well as expression of IL-21 mRNA, which are both important for antigen-specific B cell development and maturation as well as the formation of long-lived plasma cells and memory B cells.[100,101] Virus-specific T cell polyfunctionality is a correlate of T cell efficacy and immune protection.[102,103] Since *FKBP12* KO SARS-CoV-2-specific TCPs contain multiple effector cytokine producers (even in the presence of Tac), our approach holds promise for adoptively inducing protective immunity against SARS-CoV-2.

Single-cell CITE-seq identified distinct cell clusters based on specific cell surface protein expression and their transcriptomes. Cluster distributions were comparable among $CD4^+$ and $CD8^+$ T cells between non-activated unmodified control and *FKBP12* KO SARS-CoV-2-specific TCPs even in the presence of Tac or CsA. Upon SARS-CoV-2-specific activation, a distinct cluster distribution was observed. For $CD4^+$ T cells, the frequency of cluster 15, and for $CD8^+$ T cells the frequency of clusters 13 and 17, increased in the absence of CNIs for unmodified and *FKBP12* KO TCPs as well as in the presence of Tac for *FKBP12* KO TCPs, which demonstrates that neither *FKBP12* editing nor Tac treatment affected the transcriptome or expression of specific proteins of the inventive *FKBP12* KO TCPs. Gene expression signatures revealed $CD4^+$ T cell cluster 15 and $CD8^+$ T cell cluster 13 represented T cells with effector functions. Upregulated expression of *PD-1* was detected for cluster 15 but not cluster 13, indicating that expanded SARS-CoV-2-specific $CD4^+$ effector T cells might be more prone to T cell exhaustion than their $CD8^+$ counterparts. Furthermore, high IL-4 expression in T cells allocated to cluster 15 points out the presence of $CD4^+$ T_{H2} cells known to suppress IFN-γ-producing $CD4^+$ T_{H1} cells.[104] However, IL-4 is also involved in immunoglobulin class switching and therefore the development and maturation of antigen-specific B cells and the formation of long-lived plasma cells and memory B cells.[101] Within $CD8^+$ T cell cluster 13, we observed higher expression of *XCL1*, *XCL2*, *CD226*, and *IRF8*, which was less pronounced for $CD4^+$ T cell cluster 15. These genes are known to be expressed by $CD8^+$ effector T cells and support cell migration as well as memory formation and cell survival.[67–69] Gene expression patterns confirmed downregulation of *FKBP12* mRNA in *FKBP12* KO TCPs. The top 25 differentially expressed genes among SARS-CoV-2-specific activated $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO TCPs in the absence of CNI as well as under Tac treatment in the *FKBP12* KO TCPs comprised genes mainly associated with metabolism, zinc-finger proteins, as well as proteins of the transcriptional machinery. Interestingly, *IL-2* was higher in SARS-CoV-2-stimulated *FKBP12* KO $CD8^+$ T cells exposed to Tac compared with non-exposed *FKBP12* KO $CD8^+$ T cells and unmodified controls. IL-2-producing $CD8^+$ antiviral T cells are associated with high proliferative potential and are promising candidates to induce sustained immunity after adoptive transfer.[105] Moreover, upregulation of *CXC3CR1* and *IL7R* mRNA in $CD4^+$ T cell cluster 2 implies T cells with high effector function and migratory capacity.[106–108] For $CD8^+$ T cell cluster 17, we found overexpression of *ID2* and *ID3*, which are reported to promote survival and differentiation of mature effector $CD8^+$ T cells.[109] Furthermore, upregulation of *HAVCR2* mRNA in $CD8^+$ T cell cluster 17 could have beneficial effects on TCR-dependent activation and, therefore, could enhance effector functions of our TCPs.[110] A slight increase in *CD247* mRNA levels within $CD8^+$ T cell cluster 18 could result in reduced susceptibility to apoptosis.[111] Gene expression analysis of markers associated with antiviral T cell function or T cell exhaustion revealed upregulation of TOX2, a key player in T cell development, which regulates T cell persistence and exhaustion and is induced upon strong antigen stimulation in T cells,[112] and EOMES, an important transcription factor for memory T cells, although high levels of EOMES can promote T cell exhaustion.[113] The unmodified TCPs show higher TOX2 and EOMES expression compared with *FKBP12* KO TCPs, indicating that the *FKBP12* KO TCPs tend to be less exhausted, presumably due to reduced Ca^{2+} influx into the cytoplasm and thus reduced Ca^{2+}-dependent activation.[114] We also observed upregulation of some co-inhibitory receptors among $CD4^+$ and $CD8^+$ T cells in both unmodified and *FKBP12* KO TCPs, including PD-1 (*PDCD1*), CTLA-4, LAG-3, and IL-10. However, we also detected upregulated expression of genes associated with antiviral T cell function, including IFN-γ, TNF-α, IL-2, as well as perforin and granzyme B (*GZMB*). Exhausted T cells are characterized by the loss of effector cytokine production combined with high co-expression of inhibitory receptors.[115] Although we observed upregulation of some exhaustion markers in both of our SARS-CoV-2-specific TCPs, we also found that they retain their antiviral effector function, furthermore, many of the exhaustion markers are not exclusive to exhausted cells but are also upregulated upon activation. Thus, we conclude that our TCPs show strong antiviral function and only minor signs of T cell exhaustion. Moreover, proteome data confirmed downregulation of *FKBP12* on the protein level in *FKBP12* KO TCPs. SARS-CoV-2-activated unmodified but not *FKBP12* KO TCPs showed upregulated expression of proteins involved in RNA processing, cell metabolism, and shuttling, such

as DDX21,[116] NAMPT,[117] NCL,[118] PGAM1,[119] and PPA1.[120] We observed upregulated expression of RAB27A in SARS-CoV-2-specific *FKBP12* KO TCPs under Tac treatment, indicating increased lysosomal secretory capacity compared with unmodified TCPs.[121] We also confirmed upregulation of the antiviral T cell marker GZMB on the protein level in *FKBP12* KO TCPs in the presence and absence of Tac. On the mRNA level, we observed upregulation of all these genes in SARS-CoV-2-activated unmodified and *FKBP12* KO TCPs. The differences between mRNA and protein expression patterns might be due to post-translational stabilization or preferential degradation of certain proteins. In summary, the proteome data confirmed the observed Tac resistance of SARS-CoV-2-specific *FKBP12* KO TCPs as well as upregulation of GZMB, and further indicate superior lysosomal function compared with unmodified TCPs.

TCR diversity was largely comparable between unmodified control and *FKBP12* KO TCPs. The distribution of the top five most represented TCR clones within $CD4^+$ and $CD8^+$ T cells of unmodified control and *FKBP12* KO TCPs revealed a shared TCR repertoire with no signs of abnormal proliferation of individual clones, which would be the case if *FKBP12* KO or potential off-target editing transformed certain clones. Therefore, we conclude that our novel strategy of *FKBP12* editing neither skewed the TCR repertoire of TCPs nor induced excessive clonal expansion.

Taken together, we have demonstrated the feasibility of manufacturing GMP-compatible Tac-resistant SARS-CoV-2-specific TCPs with superior function in the presence of Tac and triple IS compared with unmodified control SARS-CoV-2-specific TCPs. The retained sensitivity to CsA represents an important safety switch to inhibit potential adverse effects elicited by *FKBP12*-edited TCPs *in vivo*. Our innovative GMP-compliant protocol to generate *FKBP12* KO SARS-CoV-2 specific TCPs qualifies for transfer into clinical application,[63] since we have already shown this is feasible in our previous study.[88,122] We are preparing for clinical translation of Tac-resistant SARS-CoV-2-specific TCPs for first-in-human use in SOT recipients or autoimmune patients on Tac therapy as a proof of concept. Transplant patients may specifically benefit from Tac-resistant ACT as their responses toward active SARS-CoV-2 vaccination are reported to be poor[18,123] and therefore could be improved if patients have previously undergone Tac-resistant ACT as a passive vaccination strategy. Importantly, Tac therapy combined with adoptive transfer of Tac-resistant SARS-CoV-2 TCPs may be an attractive novel treatment concept to prevent undesired immune reactions such as alloreactivity, autoimmunity, or hyperinflammation while efficiently treating viral infections such as severe COVID-19.

MATERIALS AND METHODS

Blood sampling and PBMC isolation

The study was approved by the Charité – Universitätsmedizin Berlin Ethics Committee and peripheral blood was obtained from convalescent individuals with a history of asymptomatic or mild COVID-19 or SARS-CoV-2 seronegative healthy donors, who had given their written informed consent. PBMCs were isolated using Biocoll (Biochrom) gradient centrifugation.

Determining SARS-CoV-2-specific T cell responses

PBMCs (2×10^6) were stimulated with peptide pools of individual proteins of SARS-CoV-2 (JPT Peptide Technologies; i.e., NCAP, spike S1, spike S2, VEMP, VME1, AP3a, NS6, NS7a, NS7b, NS8, ORF9b, ORF10, Y14) at 1 μg/mL each in the presence of 1 μg/mL purified anti-CD28 antibody (clone CD28.2, BioLegend). Unstimulated controls were supplemented with equal concentrations of DMSO and 4 mg/mL staphylococcal enterotoxin B (SEB) (Sigma-Aldrich), and CMV peptide pool (pp65 and IE-1; 0.5 μg/mL each; JPT Peptide Technologies) served as positive controls. Stimulated PBMCs were incubated in a humidified incubator at 37°C, 5% CO_2 for 16 h. Intracellular cytokine production was captured by addition of 2 μg/mL Brefeldin A (Sigma-Aldrich) after 2 h of stimulation, and cells were stained using antibodies (all from BioLegend) and the FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Staining was performed using fluorophore-conjugated human anti-CD3 (OKT3), -CD4 (SK3), -CD8 (RPA-T8), -IFN-γ (4S.B3), -TNF-α (MAb11), -IL-2 (MQ1-17H12), -CD137 (4B4-1), -CD154 (24–31), -CCR7 (G043H7), and -CD45RA (HI100) antibodies. LIVE/DEAD Fixable Blue Dead Cell Stain (L/D; Invitrogen) was used to exclude dead cells. Samples were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and FlowJo-10 software (Tree Star).

Serology

Serum IgG and IgA levels of antibodies targeting the S1 domain of the spike glycoprotein were determined by using anti-SARS-CoV-2 spike 1 IgG and IgA ELISA and carried out according to the manufacturers protocol (EUROIMMUN).

Isolation and culture of SARS-CoV-2-specific T cells

Virus-specific T cells were isolated from PBMCs derived from 100 mL of peripheral blood from convalescent donors following a 6-h stimulation with overlapping SARS-CoV-2-specific peptide pools (JPT Peptide Technologies; 1 μg/mL each) using an IFN-γ Secretion Assay—Cell Enrichment and Detection Kit according to the manufacturer's instructions (Miltenyi Biotec). Isolated virus-specific T cells were cultured in complete medium (VLE RPMI 1640 [PAN-Biotech] supplemented with penicillin [100 IU/mL], streptomycin [Biochrom], 10% fetal calf serum [FCS, PAA], 10 ng/mL recombinant human IL-7 [rhIL-7] and rhIL-15 [CellGenix]) in 24-well plates, in humidified incubators at 37°C and 5% CO_2 as described previously.[88,122] Cells were split 1:1 upon reaching 100% confluency.

KO procedure

Two million to 10 million antiviral T cells (half of the culture derived from 100 mL of peripheral blood) were electroporated with RNPs on day 7 of expansion using Amaxa P3 primary cell 4D-Nucleofector X Kit L and the Amaxa-Nucleofector-4D (Lonza, program CO-115) to transfer ribonucleoprotein complexes of 30 μg of recombinant Alt-R S.p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies)[124]

precomplexed with 15 μg of synthetically modified sgRNA targeting 5'-GGGCGCACCTTCCCCAAGCG-3′ with 2O'-methyl-3'phosphothioate modifications between the first and last three nucleotides (Synthego Corporation). The same number of unmodified antiviral T cells were expanded as controls.

Phenotypic and functional assays assessed by flow cytometry

For assessing SARS-CoV-2-specific cytokine production/activation, LCLs were generated as described previously[125] and used as antigen-presenting cells at a 1:10 ratio for a 16-h SARS-CoV-2-specific stimulation with 0.65 μg/mL of pooled SARS-CoV-2-specific peptides (JPT Peptide Technologies) in the presence or absence of immunosuppressants at clinical doses (6 ng/mL tacrolimus [Prograf, Astellas]; 120 ng/mL CsA [Sandimmun, Novartis]; triple IS = 6 ng/mL tacrolimus + 0.57 μg/mL prednisolone [Urbason solubile, Sanofi] + 2.7 μg/mL MPA [active substance of mycophenolate mofetil; Sigma-Aldrich]). Additionally, T cells were re-stimulated using peptide pools of individual SARS-CoV-2 proteins (NCAP, spike S1, spike S2, VEMP, VME1, AP3a, NS6, NS7a, NS7b, NS8, ORF9b, ORF10, Y14) (JPT Peptide Technologies; 0.5 μg/mL). To determine cross-reactivity to SARS-CoV-2 variants, cryopreserved TCPs were thawed and re-stimulated with a pool of peptides spanning the sequences of spike proteins of Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) (JPT Peptide Technologies; 0.5 μg/mL). To assess potential cross-reactivity to other common HCoVs, TCPs were re-stimulated with a pool of peptides spanning the sequences of spike proteins of common endemic HCoVs (HCoV-229E, HCoV-NL63, HCoV-OC43, HKU1) (JPT Peptide Technologies; 0.5 μg/mL). Re-stimulation with CEFX Ultra Superstim pool (JPT Peptide Technologies; 0.5 μg/mL) served as control to exclude nonspecific T cell expansion. Unstimulated controls included LCLs without SARS-CoV-2-specific peptides. Intracellular cytokine production was captured by addition of 2 μg/mL of Brefeldin A (Sigma-Aldrich) after 2 h of stimulation and cells were stained using antibodies (all from BioLegend, unless stated otherwise) and the FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Staining was performed using fluorophore-conjugated human anti-CD3 (OKT3), -CD4 (SK3), -CD8 (RPA-T8), -IFN-γ (4S.B3), -TNF-α (MAb11), -IL-2 (MQ1-17H12), -CD137 (4B4-1), -CD154 (24–31), -CCR7 (G043H7), and -CD45RA (HI100) antibodies. LIVE/DEAD Fixable Blue Dead Cell Stain (L/D; Invitrogen) was used to exclude dead cells.

A VITAL assay was performed to assess the killing capacity of TCPs.[88,126,127] Briefly, cells from TCPs were incubated at distinct ratios with autologous LCLs loaded with SARS-CoV-2-peptide pool or with peptide pools of individual SARS-CoV-2 proteins and stained with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Sigma-Aldrich) for 4 min, whereas unloaded allogenic LCLs serving as non-target controls were stained with 5 μM CellTrace Far Red (Invitrogen) for 10 min. T cell-free LCL mixtures served as internal controls to calculate the SARS-CoV-2-specific killing capacity. After 14 h of incubation, co-cultures were stained with L/D.

To assess the killing capacity of SARS-CoV-2 spike protein-transfected target cells by TCPs, we performed another VITAL assay. From 8×10^6 to 10×10^6 autologous LCLs were co-transfected with 4 μg of plasmid encoding SARS-CoV-2 spike protein (pSpike) and 4 μg of pmaxGFP (Lonza) via electroporation using Amaxa P3 primary cell 4D-Nucleofector X Kit L and the Amaxa-Nucleofector-4D (Lonza, program DS-104). After 24 h, GFP^+ LCLs were sorted at the Sony Sorter MA900. Spike expression of sorted GFP^+ target cells was confirmed by flow cytometry using an anti-spike-RBD AF647 antibody (Invitrogen). Cryopreserved TCPs were thawed and incubated at distinct ratios with GFP^+/pSpike-transfected LCLs, whereas unloaded allogenic LCLs serving as non-target control were stained with 5 μM CellTrace Far Red (Invitrogen) for 10 min. T cell-free LCL mixtures served as internal controls to calculate the SARS-CoV-2-specific killing capacity. After 14 h of incubation, co-cultures were stained with L/D.

The SARS-CoV-2-specific killing capacity was calculated according to the following formulas:

$$\text{Ratio T} - \text{cell} - \text{free LCL mixtures: } \frac{\%\ target\ cells}{\%\ non - target\ cells}$$

$$\%\ \text{killing of target cells} = 100 - \frac{\dfrac{\%\ target\ cells}{\%\ non - target\ cells}}{Ratio\ \text{T} - \text{cell} - \text{free LCL mixtures}} * 100$$

All flow cytometry samples were analyzed using either a CytoFLEX or Navios flow cytometer (both Beckman Coulter) and FlowJo-10 software (Tree Star).

KO efficiency analysis

Analysis of on-target gene editing was performed on isolated DNA (Zymo Research) from day 21 cell samples. The *FKBP12* locus was amplified using KAPA HiFi HotStart ReadyMix (Roche) and the following primer pairs: TCTGACGGGTCAGATAACACCTAG (F) and TCTTCCGGAGGCCTGGGTTT (R) with the following touchdown-PCR program in an automated thermocycler: (1) 95°C, 3 min; (2) 98°C, 30 s; (3) 72°C to 64°C, 15 s (−0.5°C for each cycle starting at the highest until the lowest temperature was reached; 20 cycles, 64°C); (4) 72°C, 15 s; (5) repeat from step (2) with decreasing annealing temperature (as specified); (6) 72°C, 1 min; (7) 4°C. PCR products were purified using DNA purification and enrichment kit (Zymo Research) prior to Sanger sequencing with primer F by LGC Genomics. Editing frequencies were calculated using the Inference of CRISPR Edits (ICE) algorithm (Synthego Corporation).

Proteomics

Cryopreserved *in vitro* expanded SARS-CoV-2-specific unmodified and *FKBP12* KO T cells were thawed and re-stimulated with SARS-CoV-2 peptide pool-loaded LCLs for 6 h and subsequently T cells were purified with the Sony sorter MA900 and cell pellets were frozen in liquid nitrogen and stored at −80°C. Further sample preparation

and analysis via nano-liquid chromatography-tandem mass spectrometry was performed as already described elsewhere.[128] The acquired raw files were analyzed by data analysis (Version 3.0, Bruker Daltonic, Bremen, Germany). The derived peak lists were searched against the human Swiss-Prot database using PEAKS studio proteomics software version 7.5 (Bioinformatics Solutions, Waterloo, Canada). Default settings were used with PEAKS studio proteomics software version 7.5 without merging the scans. Correct precursor was detected using mass only. Peptide identifications were performed within PEAKS using its own search engine PEAKS DB combined with PEAKS *de novo* sequencing. PEAKS PTM search tool was used to search for unspecified peptides that are homologous to peptides in the protein database. The default maximum number of variable post-translational modifications per peptide was three. Retention time shift tolerance was 1 min. All the search tools are included in the PEAKS studio software. False discovery rate (FDR) was estimated with target decoy fusion and set to 0.01. Label-free quantification with PEAKS Q was used. PEAKS was allowed to autodetect the reference sample and automatically align the sample runs. To allow the exporting of complete results, protein significance filter was set to 0, protein fold change filter to 1, and unique peptide filter to 1 in the export settings. They were considered just for high-confidence interaction of active interaction sources by experiments, databases, co-expression, and co-occurrences. Differentially abundant proteins were identified using ANOVA. To validate genes at the protein level, top differentially regulated genes in $CD4^+$ and $CD8^+$ T cells and a manual selection of exhaustion/activation genes were intersected with the complete list of proteins. The shortlisted proteins were then tested for interaction between *FKBP12* KO and tacrolimus-treatment in LCL-stimulated T cells.

Single-cell CITE-seq and TCR sequencing

In vitro expanded SARS-CoV-2-specific unmodified and *FKBP12* KO T cells were labelled with anti-human TotalSeq-C hashtag antibodies (BioLegend) allowing the pooling of samples followed by labelling with anti-human TotalSeq-C antibodies (BioLegend) targeting a selection of extracellular proteins (Table S2).[129] Single-cell suspensions were loaded onto Next GEM Chip G (10X Genomics), which were placed into a 10X Genomics controller for initiation of the 10X workflow. Transcriptome, antibody-derived, and TCR libraries were prepared using the Chromium Single Cell 5′ Library and Gel Bead Kit as well as the Single Cell 5′ Feature Barcode Library Kit (10X Genomics). TCR targeting was performed using the Chromium Single Cell V(D)J Enrichment Kit for Human T cells (10X Genomics). Gene expression and TCR libraries were prepared using the Single Index Kit T Set A, whereas the Single Index Kit N Set A (10X Genomics) was used for antibody-derived libraries.

Qubit HS DNA assay kit (Life Technologies) was used for library quantification, and fragment sizes were obtained using the 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent). Sequencing was performed on a NextSeq500 device (Illumina) using High Output v2 Kits (150 cycles) with the recommended sequencing conditions for 5′ GEX libraries (read1, 26 nt; read2, 98 nt; index1, 8 nt; index2, not available [n.a.]) and Mid Output v2 Kits (300 cycles) for TCR/BCR libraries (read1, 150 nt, read2, 150 nt, index1, 8 nt, index2, n.a., 20% PhiX spike-in).

Single-cell CITE-seq and TCR sequencing analysis

Raw sequence reads were processed using Cell Ranger 5.0.0, including the default detection of intact cells. Mkfastq and count were used in default parameter settings for demultiplexing and quantifying the gene expression. Refdata-cellranger-hg19–1.2.0 was used as reference. Raw UMI (Unique Molecular Identifier) counts were further processed and analyzed using R 4.0.2 according to the osca workflow by Lun et al.,[130] including normalization, filtering of low-quality cells, clustering, and UMAP dimensionality reduction. Differentially abundant genes and clusters were identified using edgeR's quasi-likelihood methods and test for interaction between *FKBP12* KO and tacrolimus treatment in LCL-stimulated cells. TCR repertoire analysis was conducted on the filtered_contig_annotations.csv outputs from the 10X Genomics Cell Ranger pipeline using the scRepertoire package.[131] Clonotypes were called using the combination of CDR3 nucleotide sequences and genes.

Statistics

p values were determined by tests for normal distribution (Shapiro-Wilk and Kolmogorov-Smirnov tests), followed by one-way ANOVA (normally distributed data sets) or Friedman test (not normally distributed data sets) and paired t tests (normally distributed data sets) or Wilcoxon matched pairs signed rank tests (not normally distributed data sets) or Mann-Whitney-U test (not normally distributed data sets) as posttests. GraphPad Prism 9 (GraphPad Software) and R (version 3.5.2) were used to generate graphs and carry out the statistical analysis of data. Graphical schemes were designed using BioRender 2022 (www.biorender.com).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.02.012.

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AUTHOR CONTRIBUTIONS

L.P. planned and performed experiments, composed the figures, analyzed results, interpreted the data, and wrote the manuscript. D.J.W., H.H., R.N., D.L.W., G.Z., S.M., S.P., and S.Schlickeiser. performed experiments and revised the manuscript. S. Schlickeiser performed statistical analyses, and analyzed and interpreted the data. M.-F.M. supervised single-cell data acquisition and provided the necessary infrastructure. O.K. performed proteomics data acquisition and analysis. H.M., M.G., T.R., and N.B. developed and provided the plasmid encoding SARS-CoV-2 spike protein and supported the experimental procedure. H.-D.V. and P.R. provided infrastructure, interpreted the data, and edited the manuscript. L.A. and M.S.-H. led the project, designed the research, interpreted the data, and wrote the manuscript. All authors discussed, commented on, and approved the manuscript in its final form.

DECLARATION OF INTERESTS

D.L.W., H.-D.V., P.R., L.A., and M.S.-H. have a patent pending on immunosuppressant-resistant T cells for adoptive immunotherapy. All other authors declare no competing interests.

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Curriculum Vitae

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

Publication list

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