ORIGINAL ARTICLE

Human CD4^{+} T cells specific for dominant epitopes of SARS-CoV-2 Spike and Nucleocapsid proteins with therapeutic potential

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Abstract

Since December 2019, Coronavirus disease-19 (COVID-19) has spread rapidly throughout the world, leading to a global effort to develop vaccines and treatments. Despite extensive progress, there remains a need for treatments to bolster the immune responses in infected immunocompromised individuals, such as cancer patients who recently underwent a haematopoietic stem cell transplantation. Immunological protection against COVID-19 is mediated by both short-lived neutralizing antibodies and long-lasting virus-reactive T cells. Therefore, we propose that T cell therapy may augment efficacy of current treatments. For the greatest efficacy with minimal adverse effects, it is important that any cellular therapy is designed to be as specific and directed as possible. Here, we identify T cells from COVID-19 patients with a potentially protective response to two major antigens of the SARS-CoV-2 virus, Spike and Nucleocapsid protein. By generating clones of highly virus-reactive CD4^{+} T cells, we were able to confirm a set of nine immunodominant epitopes and characterize T cell responses against these. Accordingly, the sensitivity of T cell clones for their specific epitope, as well as the extent and focus of their cytokine response was examined. Moreover, using an advanced T cell receptor (TCR) sequencing approach, we determined the paired TCR-αβ sequences of clones of interest. While these data on a limited population require further expansion for universal application, the results presented here form a crucial first step towards TCR-transgenic CD4^{+} T cell therapy of COVID-19.

KEYWORDS
COVID-19, epitope, SARS-CoV-2, T cell, T cell receptor

INTRODUCTION

COVID-19 has presented as an unprecedented global health emergency, with 100 million cases worldwide confirmed in little more than 1 year, according to the World Health Organization COVID-19 dashboard at https://covid19.who.int. A huge effort to develop effective vaccines has led to these being introduced at record pace. Despite this success, and assuming that the current vaccines will provide high levels of protection against current and future variants of the virus in

healthy individuals, there will remain a substantial group of individuals who may experience reduced efficacy of a vaccine. From our own perspective, patients with haematological malignancies who have undergone haematopoietic stem cell transplantation remain highly vulnerable to severe complications of infection until 6–9 months post-transplant, when they can be vaccinated successfully. A similar reduction in protection may be true for other immunocompromised individuals. The development of new treatments to boost the immune response to SARS-CoV-2, therefore, remains relevant.

Treatment of established COVID-19 remains challenging. One method of adoptive immunity to the SARS-CoV-2 virus that has been trialled extensively so far is the transfer of convalescent plasma. This approach, which first came to prominence during the outbreak of the Spanish Flu in 1918, relies upon the transfer of neutralizing antibodies. Although various degrees of success have been achieved with this approach in COVID-19, the approach seems mostly successful when plasma with very high titres of antibody is used in mild to moderate disease [1,2]. In more severe disease, where patients require mechanical ventilation, this approach is much less successful. Moreover, a recent report warned that convalescent plasma treatment of severe disease may promote the evolution of the virus to avoid interaction with neutralizing antibodies [3]. This, of course, would be a very worrying development for the wider population, as this may also compromise the efficacy of the current generation of vaccines.

In addition to the production of neutralising antibodies, T cell immunity against a number of SARS-CoV-2 proteins has been found to represent a major component of the healthy immune response [4,5]. This, therefore, opens up another potential avenue of immunotherapy to augment the effect of current approaches. Cooper *et al*. [6] have previously published a method of expanding SARS-CoV-2-specific T cells for allogeneic T cell therapy. However, to limit the risk of adverse effects of cell therapy, it is crucial to direct the T cell response as much as possible. To this end, several groups have previously published immunodominant peptides from the different proteins of the virus [5,7–9], emphasizing the involvement of both spike and non-spike antigens in the T cell response. In addition to the extent to which particular peptide regions are recognized, Bacher *et al*. [10] stressed that the nature of the T cell response is crucial for successful clearance of the virus. In particular, a focused, high-avidity interaction of T helper type 1 (Th1) cells rather than diversity seems crucial to limit disease severity.

With this in mind, we characterized T cells derived from the peripheral blood of COVID-19 patients with strong responses to SARS-CoV-2 by generating hundreds of T cell clones reactive to Spike and Nucleocapsid proteins. Immunodominant epitopes were determined and the responses of 81 antigen-specific clones assessed for sensitivity and diversity of the cytokine response. This, combined with full-length analysis of paired T cell receptor (TCR)-α and -β chains, allows for the selection of a small pool of T cells with highly desirable characteristics that together target multiple epitopes. The information provided here facilitates a significant step towards the generation of a T cell therapy, which will bolster the portfolio of immunotherapies available against COVID-19.

MATERIALS AND METHODS

Donors

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers and patients with confirmed SARS-CoV-2 infection were obtained at the University Hospital Erlangen. The criteria for COVID-19 grades were as follows: severe, intensive care treatment or death; moderate, no criteria for severe disease fulfilled but requiring supplemental oxygen; and mild, no criteria for moderate or severe disease fulfilled. All participants gave their informed, written consent. Human leucocyte antigen (HLA) typing was performed by Illumina sequencing at the European Federation for Immunogenetics (EFI)-accredited Laboratory for Immunogenetics at the University Hospital Erlangen. The study was performed according to the declaration of Helsinki and was approved and monitored by the ethical committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (protocol 118_20B and 174_20B).

Peptides and peptide analysis

Spike and Nucleocapsid 15-mer overlapping peptides, in the form of pooled Pepmix peptides (PM-WCPV-S and PM-WCPV-NCAP) or matrix and individual peptides (EMPS-WCPV-S-1 and EMPS-WCPV-NCAP-1) as well as B.1.1.7 Spike mutant Pepmix (PM-SARS2-SMUT01-1) were purchased from JPT (Berlin, Germany). Predictions of major histocompatibility complex (MHC)-II binding of peptides were generated by analysing the full sequence of Spike and Nucleocapsid proteins with the IEDB-recommended 2.22 prediction method (at: http://tools.immuneepitope.org/mhcii/). Peptides with a percentile ranking of <10 were considered potential strong binders. Alignment of peptides with protein sequences was performed with Geneious Prime software and results collated and visually enhanced in Inkscape version 0.92.4.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) from COVID-19 patients and healthy volunteers were separated by

density gradient centrifugation using Pancoll (PAN Biotech, Aidenbach, Germany) and stored in liquid nitrogen until further processing. Cells were thawed and stimulated with 1 µg/ml of total Spike and Nucleocapsid antigen for 24 h. Subsequently, cells were stained with anti-CD3-fluorescein isothiocyanate (FITC), anti-CD137-phycoerythrin (PE), anti-CD8-Brilliant Violet 421 (BV421), anti-CD4-BV510 and 7-aminoactinomycin D (7AAD) (all from BD Biosciences, San Jose, California, USA). Additionally, for COVID-19 patients only, PBMC were stained *ex vivo* without prior peptide stimulation with anti-HLA-DR-PE, anti-CD3-BV510, anti-CD4-BV421, anti-CD8-allophycocyanin-cyanin 7 (APC-Cy7), anti-CD38-APC and 7AAD (all from BD Biosciences). Fluorescence intensity was measured on a BD FACSCanto II flow cytometer and analysed using FlowJo (version 10) software.

Generation of T cell clones

To generate SARS-CoV-2-specific CD4^{+} T cell clones, single cells were sorted from PBMCs based on CD38 and HLA-DR expression directly *ex vivo* or on CD137 expression after 24-h *in-vitro* stimulation with pooled Spike and Nucleocapsid Pepmix peptides (JPT) in the presence of 10 U/ml recombinant human interleukin-2 (rhIL-2; Proleukin, Vevey, Switzerland). Epstein–Barr virus (EBV)-transformed B cells for antigen-specific stimulation were generated for each donor using standard procedures. Single-sorted CD4^{+} cells were expanded with 0.8 µl/ml phytohaemagglutinin (PHA) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 200 U/ml rhIL-2 and irradiated feeder cells for 10–14 days. Expanded clones were then stimulated with 1 µg/ml of either Spike or Nucleocapsid pooled Pepmix peptides loaded onto autologous EBV-transformed B cells for 24 h to test specificity by interferon (IFN)-γ enzyme-linked immunosorbent assay (ELISA) on the supernatant. After a second round of expansion with PHA and IL-2, epitope mapping was performed by stimulation with peptide matrices from either Spike or Nucleocapsid for 24 h, again followed by IFN-γ ELISA.

Cytokine analysis

IFN-γ ELISAs were performed on culture supernatant using the ELISA Max Deluxe kit (Biolegend, San Diego, California, USA), as per the manufacturer's instructions. Multi-cytokine analysis on 48-h 5× diluted supernatant of T cell clones stimulated with specific Spike or Nucleocapsid peptides was performed in duplicate samples using the 12-analyte Biolegend LegendPlex T helper cytokine panel version 2. Samples were

run on a BD FACSCanto II flow cytometer and analysed using proprietary LegendPlex software.

TCR sequencing

iRepertoire Inc. (Huntsville, Alabama, USA) performed a 'mini-bulk' version of their iPair analysis service to determine paired TCR-α and -β sequences on 81 RNA samples of individual T cell clones. Samples were prepared by isolating RNA from approximately 5×10^5 T cell clones per sample using a Qiagen RNeasy mini kit; 20 µl samples at 10 ng/µl were sent to iRepertoire for analysis. Results were analysed using the proprietary iPair Analyzer software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 software, using the appropriate test as indicated in the figure legends; $p < 0.05$ was considered statistically significant.

RESULTS

SARS-CoV-2-reactive T cells in COVID-19 patients

In order to investigate the T cell response to SARS-CoV-2, we opted to study patients hospitalized due to COVID-19. These patients were reasoned to have a strong and enduring immune response. Eighteen patients (seven ♀ and 11 ♂, average age 69 ± 14.4 years) hospitalized between April and November 2020 were selected at random. The patients varied in disease severity and the overall HLA background of our patient group was characteristic for the German population. Details are listed in Table 1. First, T cells from the peripheral blood of these patients were analysed for the activation markers CD38 and HLA-DR (Figure 1a) *ex vivo*. As described previously [11], a considerable number of CD4^{+} and, in particular, CD8^{+} T cells showed an activated CD38^{+}HLA-DR^{+} phenotype. Subsequently, we looked at up-regulation of the activation marker CD137 on CD4^{+} T cells after *in-vitro* restimulation of PBMCs with 1 µg/ml of SARS-CoV-2 Spike and Nucleocapsid peptides in the presence of 10 U/ml rhIL-2. This was performed not only on the 18 samples from COVID-19 patients, but also on samples from 10 healthy donors collected at the early phase of the outbreak in Germany in March 2020 and on 10 samples taken prior to 2020. As depicted in Figure 1b, only three of 18 COVID-19 patients (16.7%), namely patients 54, 91 and 130, gave a strong response to the antigens with >100 CD137^{+} cells per 10^4

TABLE 1 Details of patients in this study

| Patient ID | Gender | Age (years) | HLA type | | | | | | Disease severity |
			DQA1		DQB1		DRB1		
54	F	92	03:02	05:01	02:01	02:02	03:01	04:05	Moderate
91	M	74	01:01	05:05	03:01	05:01	01:01	11:01	Severe
106	F	88	02:01	05:03	02:02	03:01	07:01	13:19	Severe
130	M	63	01:02	02:01	02:02	06:02	07:01	15:01	Severe
230	F	77	02:01	02:02	02:02	03:03	07:01	07:01	Severe
249	M	73	01:01	05:05	03:01	05:01	01:01	11:01	Severe
383	F	62	01:02	01:03	06:02	06:03	13:01	15:01	Severe
384	M	70	01:01	05:01	02:01	05:01	01:01	03:01	Severe
385	F	62	01:01	01:02	05:01	06:05	13:02	15:02	Severe
389	M	58	01:02	01:02	06:02	06:02	15:01	15:01	Moderate
395	M	72	01:03	05:05	03:01	06:01	11:04	15:02	Severe
406	F	37	01:02	05:01	02:01	06:02	03:01	15:03	Mild
424	M	80	01:01	04:02	04:02	05:01	01:01	08:01	Moderate
425	F	80	01:01	05:05	03:01	05:01	01:01	11:01	Severe
448	M	52	01:02	05:05	03:01	05:02	11:01	16:01	Moderate
465	M	55	01:01	05:05	03:01	05:01	01:03	11:01	Mild
482	M	87	01:01	01:01	03:02	05:03	01:01	01:01	Severe
495	M	56	01:02	03:01	04:02	05:02	04:04	16:01	severe

Abbreviations: F, female; HLA, human leucocyte antigen; M, male.

CD4$^+$ T cells. The former two patients even surpassed 150 CD137$^+$/10^4 CD4$^+$ cells. Healthy volunteers did not show such high responses, although two donors from March 2020 showed an intermediate response (50–100 CD137$^+$/10^4 CD4$^+$ T cells). This level of response may represent cross-reactivity of CD4$^+$ T cells specific for other corona viruses, such as common cold viruses, which would have been circulating at this time of year. This, however, could not be confirmed.

Mapping of immunodominant epitopes from Spike and Nucleocapsid proteins

To study the antigen-specific T cell response of the three highly reactive patients in more detail, we generated clones from CD4$^+$ cells that were either CD38$^+$HLA-DR$^+$ *ex vivo* or expressed CD137 after 24-h *in-vitro* stimulation with a pool of peptides that together span the lengths of both Spike and Nucleocapsid proteins. After expansion, these clones were restimulated with Spike or Nucleocapsid peptide pools for 24 h in the presence of exogenous rhIL-2 to determine specificity for either protein by means of IFN-γ secretion. In this manner, we selected a total of 179 CD4$^+$ T cell clones reactive to Spike (42 activated *ex vivo*, 137 *in vitro*-stimulated), 53 reactive to Nucleocapsid (11 activated *ex vivo*, 42 *in-vitro* stimulated) and one that seemed to recognize both. Very few CD8$^+$ T cell clones were generated, most probably due to the methodology applied, and these were

not analysed further. The CD4$^+$ T cell clones were each restimulated with overlapping matrix peptides from the relevant protein and rhIL-2 to determine specificity down to a 15-mer sequence. Specific T cell clone responses were again detected by IFN-γ ELISA on cell culture supernatant. Although the CD4$^+$ T cell clones recognized peptides throughout the lengths of Spike (Figure 2a) and Nucleocapsid (Figure 2b), a handful of regions were targeted by multiple clones from T cells activated *ex vivo* or *in vitro*, often in two or more of the three donors. We thus classified these regions as immunodominant.

In the next step, we selected nine dominant peptides or peptide regions, two from Nucleocapsid and seven from Spike that were each recognized by multiple CD4$^+$ T cell clones from the two most reactive patients, 54 and 91 (Table 2). T cell clones from patient 130, who died 2 months after contracting a SARS-CoV-2 infection and donating blood for this study, were excluded from further experiments, although it was not clear if death resulted from COVID-19 or was a result of multiple other underlying health issues and declined intensive care treatment. Criteria for the selection of immunodominant peptides were that the peptides were recognized by both remaining donors and/or were recognized by clones generated both from cells that had an activated phenotype *ex vivo* and cells activated after *in-vitro* restimulation. In cases where CD4$^+$ T cell clones recognized two adjacent, overlapping peptides, both peptides were included in further studies and considered as one condition. The nine peptides selected were all similar or identical to
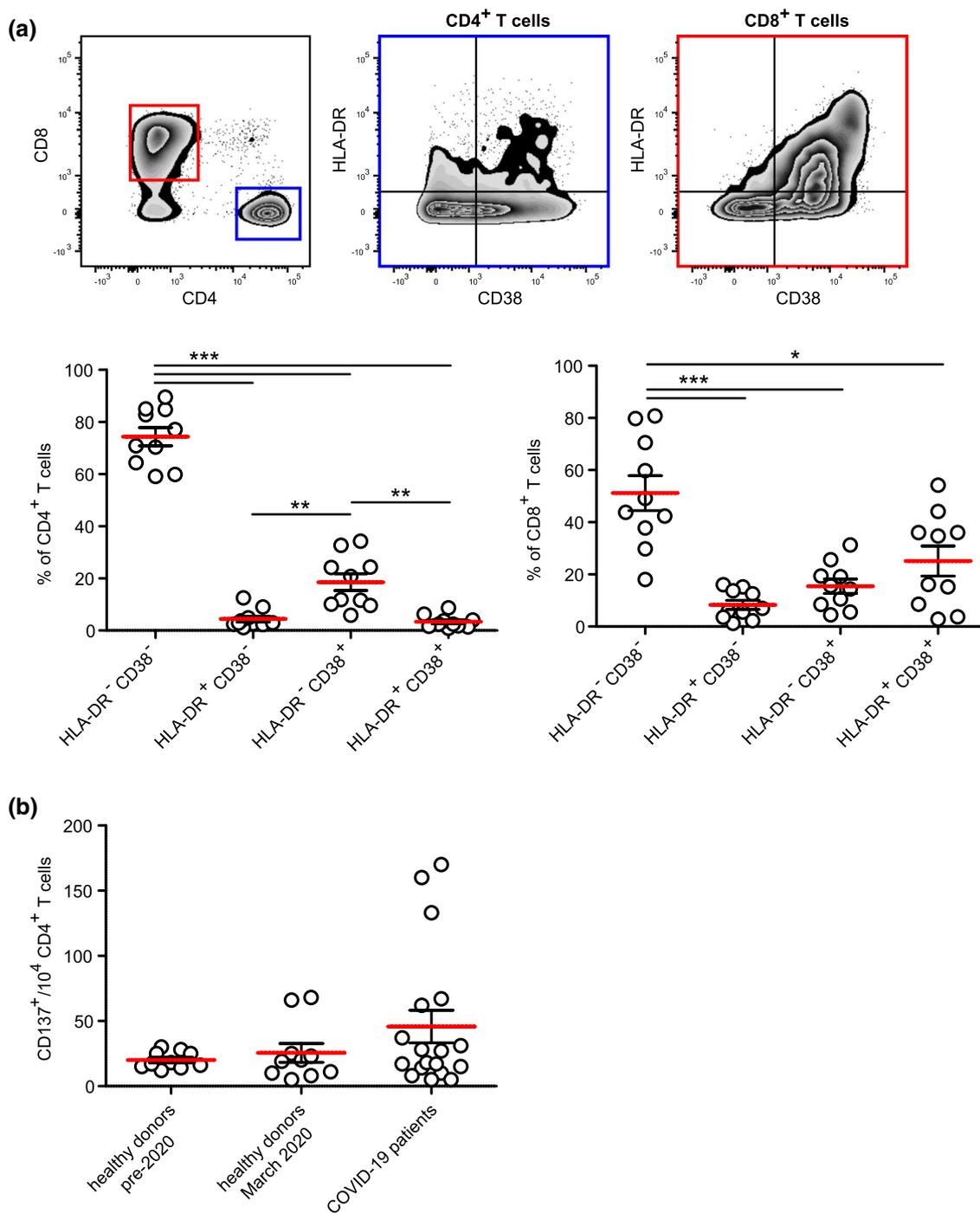
FIGURE 1 T cell activation in COVID-19. (a) Representative flow cytometry plots (top) and cumulative graphs of 10 COVID-19 patients (bottom) showing human leucocyte antigen D-related (HLA-DR) and CD38 expression on CD4⁺ and CD8⁺ T cells *ex vivo*. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Bonferroni multiple comparisons test. (b) CD137 expression on CD4⁺ T cells after 24-h *in-vitro* stimulation with SARS-CoV-2 S + N peptide pools in the presence of exogenous recombinant human interleukin (rhIL)-2. Healthy donors pre-2020, $n = 10$; healthy donors March 2020, $n = 10$; COVID-19 patients, $n = 18$. Red lines and vertical bars represent mean ± standard error of the mean (SEM)

epitopes that have previously been described as immunodominant by at least one other group (Table 2). Additionally, eight of nine peptides (89%) were predicted to have high affinity for the HLA haplotypes in our study population. These nine peptides were recognized by a total of 81 CD4⁺ T cell clones from COVID-19 patients. The sequence of peptide condition

S33-34 is altered near the C terminus by deletion mutation Y144 found in the B.1.1.7 variant of SARS-CoV-2. This, however, did not appear to impair the response of specific T cell clones (Supporting information, Figure S1), so further analysis was performed with the original sequence only. Two clones, namely clone 172 from patient 54 and clone 158 from patient

☐ Activated after *in vitro* restimulation
☐ Activated *ex vivo*
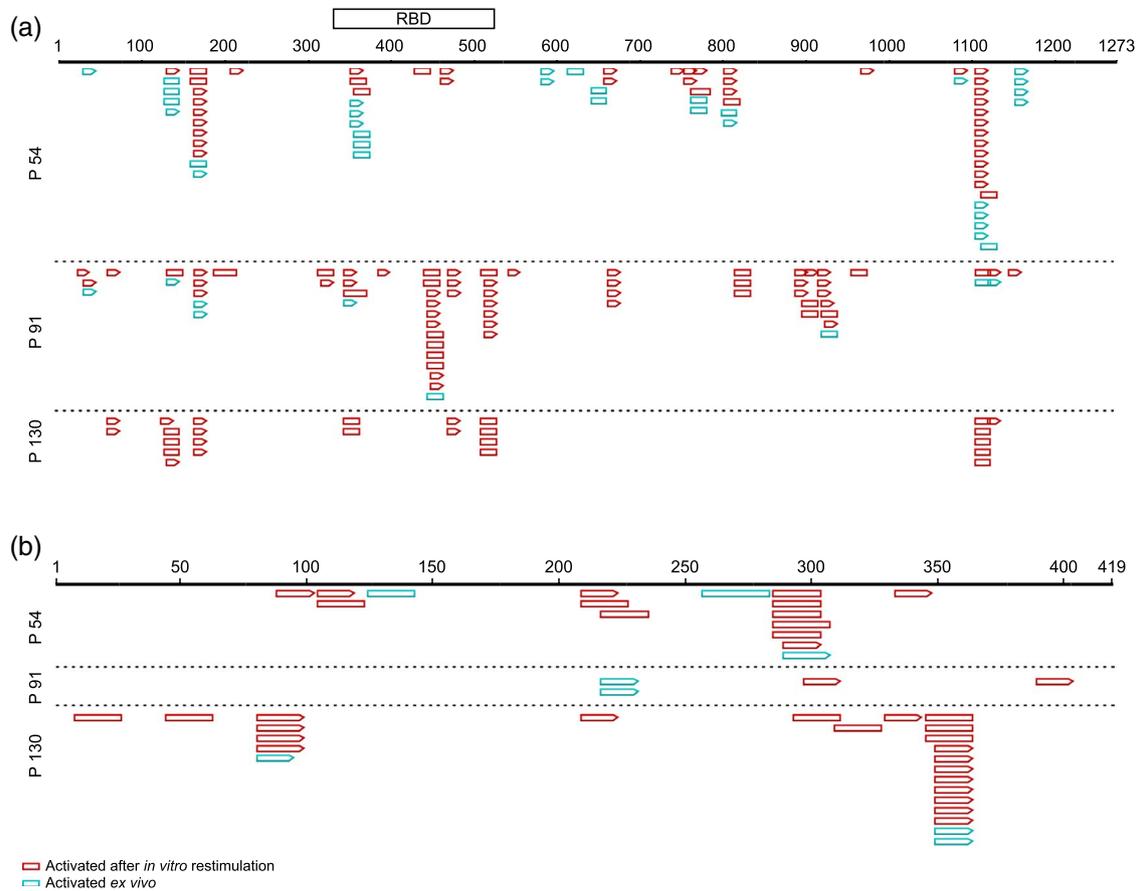
FIGURE 2 Epitope mapping of SARS-CoV-2-reactive CD4$^+$ T cell clones. Identification of 15-mer peptide epitopes in SARS-CoV-2 Spike protein (S) (a) or Nucleocapsid protein (N) (b)-reactive T cell clones from COVID-19 patients 54, 91 and 130. Clones generated from CD4$^+$ T cells activated either *ex vivo* or after *in-vitro* stimulation with S + N peptide mix. Reactive clones identified by interferon (IFN)-γ secretion after 24-h *in-vitro* stimulation with peptide matrices in the presence of rhIL-2. Each icon represents one T cell clone. Icons with pointed right edge indicate a single 15-mer peptide, rectangles represent overlapping 15-mer peptides recognized by the same T cell clone. RBD = receptor binding domain

91, recognized two different peptides within this panel (N72-3 + S42 and S112-3 + S165-6, respectively). TCR analysis, discussed later in this article, however, suggested that clone 172 was not a true clonal expansion. Further analyses were performed to detail the functional characteristics of the CD4$^+$ T cell clones selected.

Sensitivity and focus of the T cell cytokine response

The CD4$^+$ T cells most effective at fighting viral infection are considered those that recognize specific peptides at high affinity and have a highly differentiated Th1 cytokine profile. To test our 81 CD4$^+$ T cell clones each was stimulated with titrated doses of specific peptide, ranging from 1 ng/ml to 1 μg/ml. In these experiments, rhIL-2 was omitted to be able to judge cytokine-producing potential outside a proinflammatory environment. Without the addition of exogenous IL-2, not all clones were able to produce IFN-γ (Figure 3a–i). Moreover, responses to certain peptides, namely N72-73,

S42, S112-113 and S201, were recognized more frequently at lower concentrations than others. We did not detect a consistent difference in pattern between clones generated from T cells activated *ex vivo* and those activated after stimulation *in vitro*. Although the responses of the two donors to most peptides seemed largely distinct, responses to S42 and S276 were broadly similar in both.

All clones were selected initially for IFN-γ production in response to antigen in the presence of exogenous IL-2. However, to be effective in fighting viral infections and to minimize adverse effects, it is important that the T cells demonstrate a highly differentiated Th1 cytokine profile. The avidity of the TCR × pMHC II interaction, which can be affected by the nature of the presented peptide or the characteristics of specific TCRs, might affect T cell polarization [12,13]. Therefore, we examined if some CD4$^+$ T cells specific for immunodominant peptides were more likely than others to demonstrate a skewed cytokine response. Supernatant from cultures stimulated with specific peptide for 48 h without exogenous IL-2 was therefore analysed for a panel of 12 Th cell cytokines by a cytometric

TABLE 2 Selected immunodominant peptides, with predicted HLA affinity and comparison to epitopes described previously

Peptide number(s)	Sequence(s)	Predicted HLA affinity	Described previously
Nucleocapsid			
55	AALALLLLDRLNQLE (N217–231)	HLA-DRB1*03:01	*LLLLDRLNQLE* SKMS [7]; D*AALALLLLDRLNQL* and *LLLLDRLNQLE*SKMS [5]
		HLA-DRB1*11:01	
		HLA-DRB1*13:01	
		HLA-DQA1*01:01/DQB1*05:01	
72 + 73	NFGDQELIRQGTDYK (N285–299)		*FGDQELIRQGTDYKH* and *LIRQGTDYKHWPQ*IA [5]
	QELIRQGTDYKHWPQ (N289–303)		
Spike			
33 + 34	KVCEFQFCNDPFLGV (S129–143)	HLA-DQA1*01:01/DQB1*05:01 (peptide 33)	VVI*KVCEFQFCNDPF* and *CEFQFCNDPFLGVYY* [5]
	FQFCNDPFLGVYYHK (S133–147)		
42	NCTFEYVSQPFLMDL (S165–179)	HLA-DRB1*04:05	*CTFEYVSQPFLMDL* E [8]; SSAN*NCTFEYVSQPF* and *VSQPFLMDL*EGKQGN [5]
		HLA-DQA1*02:01/DQB1*02:02	
		HLA-DQA1*03:02/DQB1*02:02	
		HLA-DQA1*05:01/DQB1*02:01	
89 + 90	WNRKRISNCVADYSV (S353–367)	HLA-DRB1*03:01 (peptide S90)	YA*WNRKRISNCVADY* [8]; *KRISNCVADYSVLYN* and *CVADYSVLYNS*ASFS [5]
	RISNCVADYSVLYNS (S357–371)		
112 + 113	VGGNYNYLYRLFRKS (S445–459)	HLA-DRB1*11:01	*GGNYNYLYRLFRKSN* and *YLYRLFRKSNLKP*FE [8]; *YNYLYRLFRKSNLKP* [9]
	YNYLYRLFRKSNLKP (S449–463)	HLA-DRB1*16:01 (peptide 112)	
		HLA-DRB1*04:05	
		HLA-DRB1*11:01	
		HLA-DRB1*13:01	
		HLA-DRB1*15:01	
		HLA-DRB1*16:01 (peptide 113)	
165 + 166	NNSYECDIPIGAGIC (S657–671)	HLA-DQA1*01:02/DQB1*06:01	V*NNSYECDIPIGAGI* [5]
		HLA-DQA1*05:05/DQB1*03:01 (peptide S166)	
	ECDIPIGAGICASYQ (S661–675)		
201	NFSQILPDPSKPSKR (S801–815)	HLA-DRB1*03:01	*NFSQILPDPSKPSKR* [8]; , *LPDPSKPSKR*SFIED [5]
		HLA-DRB1*04:01	
276	HWFVTQRNFYEPQII (S1101–1115)	HLA-DRB1*04:05	*HWFVTQRNFYEPQII* and *QRNFYEPQII*TTDNT [5]
		HLA-DQA1*01:01/DQB1*05:01	
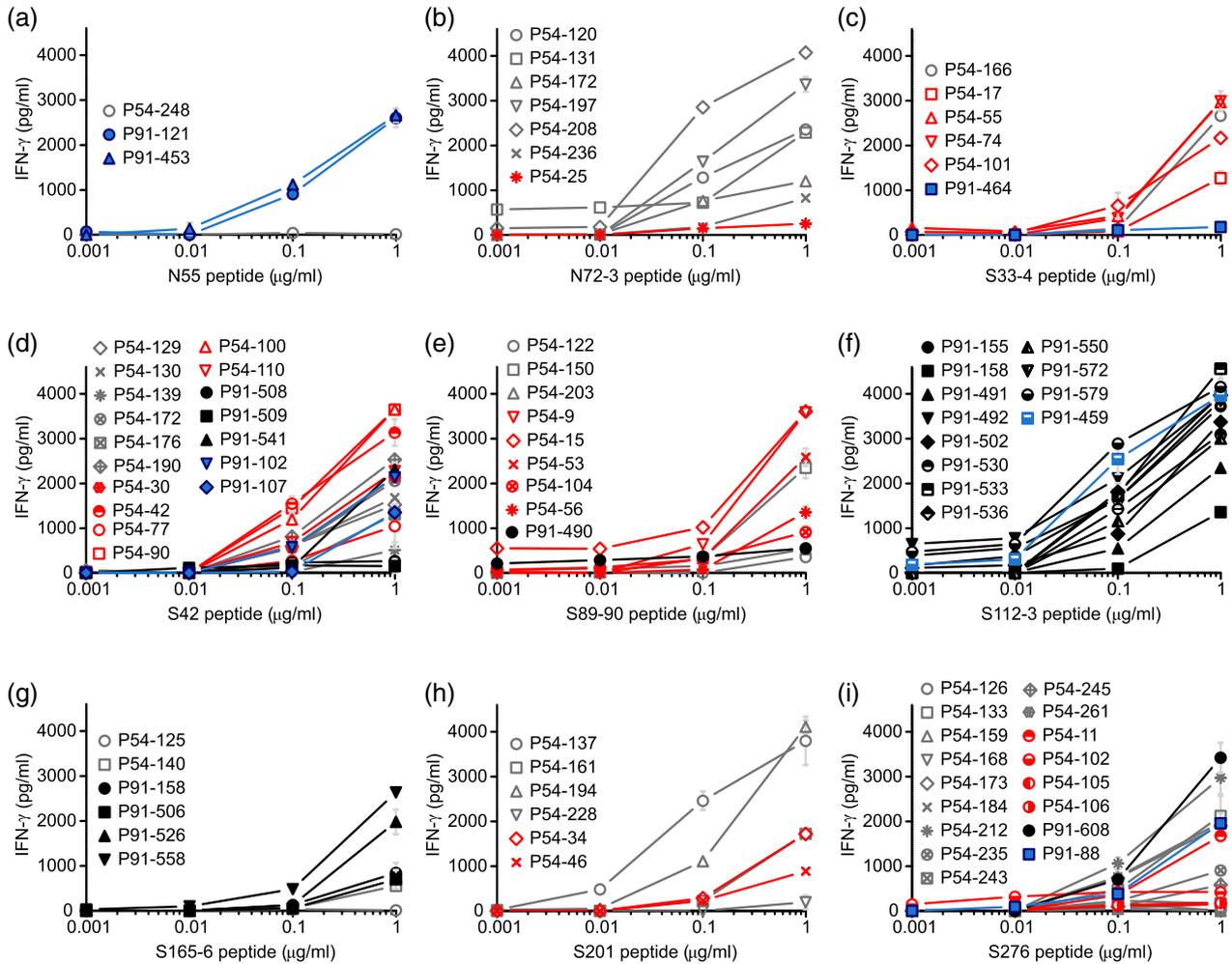
FIGURE 3 T cell clone responses to titrated doses of specific peptide. Interferon (IFN)-γ secretion after 24-h *in-vitro* stimulation of 81 T cell clones, as determined by enzyme-linked immunosorbent assay (ELISA) on cell culture supernatant. Peptides loaded onto autologous Epstein–Barr virus (EBV)-transformed B cells at titrated doses. Data plotted as mean of duplicates ± standard error of the mean (SEM). (a) Nucleocapsid protein (N) peptide 55, (b) peptide 72 and 73 (equivalent), (c) Spike protein (S) peptide 33 and 34 (equivalent), (d) S peptide 42, (e) S peptide 89 and 90 (equivalent), (f) S peptide 112 and 113 (equivalent), (g) S peptide 165 and 166 (equivalent), (h) S peptide 201, (i) S peptide 276. Grey and black lines: T cell clones generated from *in-vitro* activated cells. Red and blue lines: T cell clones generated from *ex-vivo* activated CD4+ T cells

assay. Of the 12 cytokines investigated, IL-17A, IL-17F and IL-22 never exceeded the detection threshold (not shown). As concluded earlier from the ELISA after 24-h activation, most clones were able to produce IFN-γ even in the absence of exogenous IL-2 (Figure 4). However, a substantial number of clones seemed poorly differentiated and additionally produced the Th2 cytokines IL-5 and IL-13, and to a lesser extent IL-4. Although most peptides were detected by T cell clones with various levels of differentiation, T cells specific for some peptides were more likely to demonstrate a mixed Th1/Th2 phenotype than others. T cells specific for peptides S42, S201 and S276, in particular, frequently produced IL-5 and IL-13 (Figure 4 and Supporting information, Figure S2). Additionally, the incidence of IL-10 production varied depending on antigen-specificity, albeit that this cytokine was secreted mainly at

relatively low levels. Nonetheless, this suggests that some CD4+ T cell clones may have an immunosuppressive Tr1-like phenotype that is not desirable for fighting infections. The levels of IL-2 in the supernatants were remarkably low, but it is not clear if this is due to limited production or high consumption. There were no clear differences in polarization between clones from different COVID-19 patients, nor was there a general difference between clones derived from cells activated *ex vivo* or post-*in-vitro* activation. Although we cannot conclude for certain that the cytokine patterns directly reflect either the nature of the antigen or the characteristics of the specific TCR rather than merely the differentiation status of individual T cells, to err on the side of caution we would select TCRs from highly differentiated Th1 cells for potential transgenic T cell therapy.

SARS-COV-2-SPECIFIC HUMAN CD4⁺ CELLS

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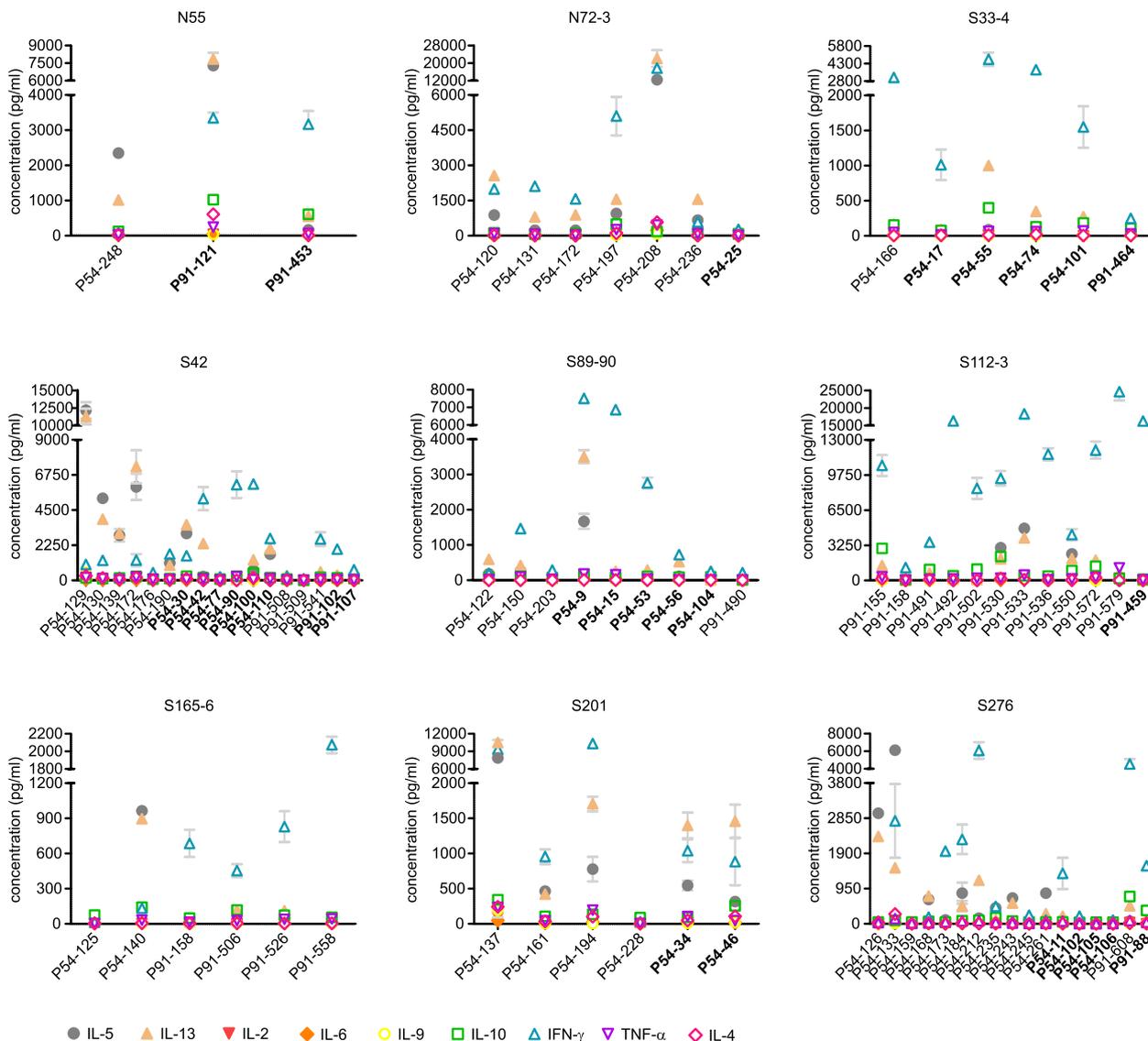
FIGURE 4 Cytokine profile of CD4⁺ T cell clones in response to cognate peptide. Comparison of cytokine profile for each T cell clone selected. CD4⁺ T cell clones were stimulated *in vitro* for 48 h with 1 µg/ml cognate peptide. Cytokines detected with 12-plex cytometric assay. Interleukin (IL)-17A, IL-17F and IL-22 were not detected in any of the samples. Data shown as mean of duplicates ± standard error of the mean (SEM). Names in bold type represent clones derived from *ex-vivo* activated T cells, the remaining from *in-vitro* activated cells

TCR repertoire of SARS-CoV-2-reactive T cell clones

The final step in this study was to determine the TCR-αβ sequence of the 81 T cell clones. This was conducted for two reasons: first, to confirm that the samples are indeed clones and not contaminated with other cells, and secondly to further identify the cells based on their TCR sequence in order to open up the possibility of CD4⁺ T cell therapy with clearly defined populations. By using populations of T cell clones rather than single-sorted cells for TCR sequencing we increased the probability of obtaining paired TCR-α and -β chains and also improved reliability. As a result, we obtained reliable paired sequences for all our samples (Table 3 and Supporting information, Data set S1). The sequences confirmed that four

samples were not clonal T cells. Nevertheless, these were left in the previous sections of the study as they still responded to SARS-CoV-2, and the sequences of very limited diversity found within these samples may still corroborate information from true clones. Sample P54–172 was not clonal, and thus does not represent a dual-specific T cell as earlier data suggested. Non-clonal cells were not used for the next analyses. Interestingly, despite the relatively small number of clones generated, seven paired TCR-α and -β sequences were shared between clones (Table 3). Some T cell clones had two in-frame TCR-α expressed at comparable levels, which seemed to coincide with certain epitopes. The variation in sequences overall seemed highly dependent upon the specific epitope (Figure 5a). For example, whereas 15 of 16 (94%) TRAV sequences from clones generated against peptide S42 were

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TABLE 3 TCR-α and -β chain identification in CD4^{+} T cell clones grouped by specific epitope

Clone no.	TRAV	TRAJ	CDR3	Reads	TRBV	TRBJ	CDR3
N55							
P54–248	38–1	47	ALPREYGNKLV	6344	2	2–3	ASARRTSGEDTQY
	12–2	17	AVVKAAGNKLT	1055			
P91–121	*13–1*	*37*	*AASWGSSNTGKLI*	*5638*	*5–4*	*1–1*	*ASSLGRMNTEAF*
P91–453	*19*	*6*	*ALSEASGGSYIPT*	*3937*	*18*	*2–3*	*ASSPRTLTVPRGTDTQY*
	30	*23*	*GTERVRYNQGGKLI*	*2106*			
N72–3							
P54–C25			Not a clone				
P54–120	8–1	15	AVNGQAGTALI	7176	19	2-3	ASRLTSVSTDTQY
P54–131	35	23	AGHAIYNQGGKLI	1019	2	1–6	ASSEARRNSPLH
P54–172			Not a clone				
P54–197	41	45	AVGTMYSGGGADGLT	2485	9	2–2	ASSVVLAGTPGELF
	12–3	57	TVYI*PQGGSEKLV	534			
P54–208	12–3	10	AMRVGVTGGGNKLT	6353	6–3	1–2	ASSYGGANTGELF
P54–236	4	42	LVVAGGGSQGNLI	4651	19	1–1	ASIRDNQRNTEAF
S33–4							
P54–17	8–4	49	AVSVNTGNQFY	3960	28	1–5	ASRDQDRGHQPQH
P54–74	8–4	49	AVSVNTGNQFY	4708	28	1–5	ASRDQDRGHQPQH
P54–55	8–4	37	AVSDRGSSNTGKLI	4242	7–8	2–4	ASSLAFSGGAGNIQY
P54–166	8–4	37	AVSDRGSSNTGKLI	4975	7–8	2–4	ASSLAFSGGAGNIQY
P54–101	8–4	37	AVSDRGSSNTGKLI	7960	14	1–1	ASSLERGRAEAF
P91–464	*10*	*4*	*VVSPFSGGYNKLI*	*5200*	*20–1*	*2–7*	*SAVDPQNPYEQY*
S42							
P54–30	35	42	AGQNYGGSQGNLI	7026	5–5	1–2	ASSLTGGMGYT
P54–42	35	42	AGQNYGGSQGNLI	4477	6–5	2–2	ASSPRERVNTGELF
P54–90	35	42	AGQNYGGSQGNLI	5613	5–4	2–2	ASSIGTSGGPNTGELF
P54–100	35	42	AGQNYGGSQGNLI	6673	5–1	2–2	ASSKGTSGGPNTGELF
P54–110	35	42	AGQNYGGSQGNLI	9494	5–1	2–2	ASSTRGHNTGELF
P54–176	35	42	AGQNYGGSQGNLI	7000	5–1	2–2	ASTRGSSGGPNTGELF
P54–190	35	42	AGQNYGGSQGNLI	7517	9	2–2	ASSPRDRANTGELF
P54–77	35	42	AGMNYGGSQGNLI	6846	9	2–2	ASSVRDRPNTGELF
P54–130	35	42	AGMNYGGSQGNLI	9242	9	2–2	ASSVRDRVNTGELF
P54–129	35	42	AGMNYGGSQGNLI	7411	7–2	1–2	ASSLRGANGYT
P54–139	35	42	AGMNYGGSQGNLI	10759	4–1	1–2	ASSQGVGYT
P91–508	*35*	*42*	*AGMNYGGSQGNLI*	*1236*	*6–1*	*1–2*	*ASSPGQGAIYGYT*
P91–541	*35*	*42*	*AGLNYGGSQGNLI*	*3822*	*5–1*	*1–4*	*ASSLARQGWGGNEKLF*
P91–102	*35*	*53*	*AGYNSGGSNYKLT*	*3195*	*14*	*1–2*	*ASSPRGDGYT*
P91–107	*35*	*17*	*AGQLYKAAGNKLT*	*2519*	*10–2*	*1–3*	*ASSIQGSGNTIY*
P91–509	*38–2*	*53*	*AYRTLGGGGSNYKLT*	*5770*	*6–3*	*1–5*	*ASSYSQGQPQH*
P54–172			Not a clone				
S89–90							
P54–9	26–2	53	ILRDVSGGGSNYKLT	7851	6–5	1–1	ASSYIHRSNTEAF
P54–15	26–2	53	ILRDVSGGGSNYKLT	7420	6–5	1–1	ASSYIHRSNTEAF
P54–150	26–2	53	ILRSSSGGGSNYKLT	7800	20–1	1–5	SATRQVNQPQH

(Continues)

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TABLE 3 (*Continued*)

Clone no.	TRAV	TRAJ	CDR3	Reads	TRBV	TRBJ	CDR3
P54–56	36	57	AVSQGGSEKLV	2239	6–6	1–5	ASSLQGGGNQPQH
	8–6	36	AVSGRTGANNLF	4412			
P54–104	36	57	AVSQGGSEKLV	2262	6–6	1–5	ASSLQGGGNQPQH
	8–6	36	AVSGRTGANNLF	5828			
P54–122	36	57	AVNQGGSEKLV	6352	6–6	1–5	ASSRQGGGDQPQH
P54–53	12–3	40	AMKEASGTYKYI	5705	6–5	1–4	ASSYVSAGVNEKLF
P54–203	8–2	45	VVSVGGGGADGLT	3522	12–3	1–3	ASSPHPGAAGNTIY
	13–1	45	AATGGGADGLT	2840			
P91–490	*13–1*	*40*	*AATLFSGTYKYI*	*4543*	*19*	*2–5*	*ASSPGAETQY*
S112–3							
P91–155	*2*	*3*	*AVGYSSASKII*	*5861*	*20–1*	*2–1*	*SASGSGSTYNEQF*
P91–579	*2*	*3*	*AVGYSSASKII*	*5266*	*20–1*	*2–1*	*SASGSGSTYNEQF*
P91–492	*2*	*9*	*AVNTGGFKTI*	*4331*	*7–8*	*2–3*	*ASSLQQGAGTDTQY*
P91–550	*2*	*9*	*AVNTGGFKTI*	*4750*	*7–8*	*2–3*	*ASSLQQGAGTDTQY*
P91–158	*8–6*	*27*	*AAALNTNAGKST*	*5811*	*7–2*	*1–6*	*ASSGLVGAGSSYNSPLH*
P91–459	*8–4*	*40*	*AVSEISGTYKYI*	*5441*	*30*	*1–5*	*AWSEGQGVGQPQH*
P91–491	*8–6*	*12*	*AVSLKDSSYKLI*	*4839*	*30*	*1–1*	*AWRRMNTEAF*
P91–502	*17*	*39*	*ATDRGNMLT*	*4318*	*30*	*2–2*	*AWSGPAGSMSGELF*
P91–530	*2*	*8*	*AVMNTGFQKLV*	*5728*	*10–3*	*2–1*	*AISDPGGRSNEQF*
P91–533	*6*	*8*	*ALPNTGFQKLV*	*5847*	*2*	*1–3*	*ASSVDSSSGNTIY*
P91–536	*2*	*26*	*AVRDGQNFV*	*5212*	*5–1*	*2–7*	*ASSLVGGNPSTTTYEQY*
P91–572	*23*	*12*	*AAREDSSYKLI*	*5426*	*5–1*	*2–1*	*ASSLSGGNNEQF*
S165–6							
P91–526	*8–2*	*4*	*VVTPTPFSGGYNKLI*	*2504*	*2*	*2–5*	*ASSPGNVASGRYQETQY*
	1–1	*38*	*AALNAGNNRKLI*	*1341*			
P91–558	*8–2*	*4*	*VVTPTPFSGGYNKLI*	*3219*	*2*	*2–5*	*ASSPGNVASGRYQETQY*
	1–1	*38*	*AALNAGNNRKLI*	*2035*			
P54–125	9–2	29	ALWNSGNTPLV	9481	4–1	2–7	ASSQDSGGQGFYEQY
P54–140	27	40	AGVVSGTYKYI	4980	6–5	1–1	ASSRRQGGVTEAF
P91–158	*8–6*	*27*	*AAALNTNAGKST*	*5811*	*7–2*	*1–6*	*ASSGLVGAGSSYNSPLH*
P91–506	*16*	*26*	*ALSGRNYGQNFV*	*4994*	*9*	*1–1*	*ASSVDPNTEAF*
S201							
P54–34	26–2	28	ILRAYSGAGSYQLT	7185	7–8	2–3	ASRWTSGGADTQY
P54–46	26–1	33	IAHMYDSNYQLI	5862	10–2	1–6	ASKDRANYNSPLH
P54–137	12–2	20	AALGGGYKLS	10539	12–4	2–1	ASSLGERGSYEQF
P54–161	21	6	AVPSSGGSYIPT	4626	7–7	2–7	ASSLESEGEQY
P54–194	2	40	AVEDPGTYKYI	8206	5–1	1–3	ASSLEGLHSGNTIY
P54–228	8–1	37	AVTGSSNTGKLI	4189	7–2	1–1	ASSLDRQAEAF
	2	7	NNRLA	2364			
S276							
P54–126	17	53	ATDAGSNYKLT	8631	10–3	2–5	AISESTSGGRQETQY
P54–212	17	53	ATDRGSNYKLT	5647	10–3	2–5	AISESRTSGQETQY
P54–106	17	53	ATADSGGSNYKLT	8884	15	1–4	ATSSYRGKNEKLF
P54–11	38–2	29	AYRSHNSGNTPLV	4049	19	2–5	ASSSGEGGETQY
	12–1	6	VVTSLSGGSYIPT	1559			
P54–102	36	57	AVSQGGSEKLV	2231	6–6	1–5	ASSLQGGGNQPQH

TABLE 3 (*Continued*)

Clone no.	TRAV	TRAJ	CDR3	Reads	TRBV	TRBJ	CDR3
	8–6	36	AVSGRTGANNLF	6234			
P54–105	26–1	24	IVRAPPDSWGKLQ	6042	20–1	1–1	SARDSEVNTEAF
P54–133	9–2	54	ALTHLQGAQKLV	5419	5–6	1–3	ASSSDLQGGGNTIY
P54–168	9–2	29	ALWNSGNTPLV	3937	20–1	2–1	SPRDPLNNEQF
	14	36	AMREALTGANNLF	2149			
P54–159	12–3	27	ALTDNTNAGKST	4651	11–2	2–5	ASSSVTGPGAQY
P54–173	4	29	LVGEESGGNTPLV	4397	5–1	2–3	ASSRQGRTDTQY
P54–184			Not a clone				
P54–235	26–1	36	IVRVTSDQTGANNLF	6592	20–1	2–7	SARDRVRNEQY
P54–243	35	45	AGPRSGGGADGLT	2254	19	1–2	ASRPLQGSHGYT
P54–245	4	42	LVGYSNYGGSQGNLI	3175	7–2	2–1	ASSSRAEANEQF
P54–261	8–6	29	AVSGVYSGNTPLV	7803	6–3	2–2	ASSYPTGRSTNTGELF
P91–88	*12–2*	*42*	*AVGGSQGNLI*	*2568*	*7–8*	*2–3*	*ASSLVSGWDTDTQY*
	9–2	*29*	*ALSDRGGNTPLV*	*445*			
P91–608	*12–2*	*10*	*AAEGGGGNKLT*	*5647*	*2*	*2–3*	*ASSRAGGASTDTQY*

Identical, matched T cell receptor (TCR)-α and -β pairs are listed first for each epitope. Identical α chains are grouped where possible. Out-of-frame sequences and sequences detected at low frequency due to technical errors are not included in this table, but can be found in the Supporting information. In cases where clones were found to have two highly expressed in-frame α chains, both are listed. TCR-α reads are included to compare expression levels of the two different chains on one cell and to judge robustness of data overall. Sequences from patient 91 are in italic font to distinguish them from patient 54 sequences. Four samples had more than one β chain that could not be explained by technical errors and are listed as 'not a clone'.

identical in TRAV35, clones specific for peptide S276 had 12 different TRAV among a total of 20. Notably, among clones specific for peptide S42, four of five (80%) clones from patient 91 expressed the same TRAV35 also found in all 11 clones from patient 54. Two of the clones of patient 91 shared the TRAV35 × TRAJ42 combination seen in all clones from patient 54, while one clone from patient 91 shared an identical CDR3 sequence with four clones from patient 54. This is remarkable, considering the differences in HLA haplotype between these two individuals, but demonstrates that some TCR sequences are not only dominant but also promiscuous over various HLA backgrounds. It should be noted, however, that despite the similarity in α chain sequences this consistency was not found for the β chain, which may explain the differences in the cytokine responses of individual clones. Finally, we note that, in line with the use of variable and junctional regions, the length of TCR-α CDR3 sequences varied depending on the cognate antigen, with those specific for S112-3 in particular being notably short (Figure 5b). TCR-β CDR3 length was highly variable in all groups (Figure 5c).

DISCUSSION

In this limited study, we define a population of CD4$^+$ T cells with potential for immunotherapy of COVID-19. Although vaccines are currently rolled out at high speed, there remains a notable section of the population who will remain

dependent upon treatment. T cell therapy may bolster the success of current antibody and anti-viral treatments. The adequate and rapid treatment of COVID-19 is essential not only to overcome the initial respiratory disease; concerns are also growing regarding longer-term effects of SARS-CoV-2 infiltration of organs, including the brain and pancreas [14,15].

Here, we focused upon people hospitalized with COVID-19 at a range of severity. As also shown previously by others, these patients have a high level of HLA-DR and CD38 expression on both CD4$^+$ and CD8$^+$ T cells [11,16–18], thus suggesting an ongoing anti-viral T cell response. Because only a limited proportion of these *in-vivo*-activated T cells were expected to be SARS-CoV-2-reactive, we also looked for cells activated by *in-vitro* stimulation. Although Spike is a major immunological target, in particular for antibody responses, others have previously demonstrated that other viral proteins are also targeted by T cells [4]. We therefore examined responses to both Spike and Nucleocapsid proteins. In our study, only three of 18 patients showed a clear response to the viral antigens. Other studies that have looked for immunodominant epitopes have mainly found responders in a much larger fraction of patients [7,8,10]. In part, this may be explained by the fact that most other studies used convalescent patients whose immune responses have developed further. More importantly, studies such as Bacher *et al.*'s [10] have adopted elaborate techniques to detect as many responders as possible. This approach is much more sensitive
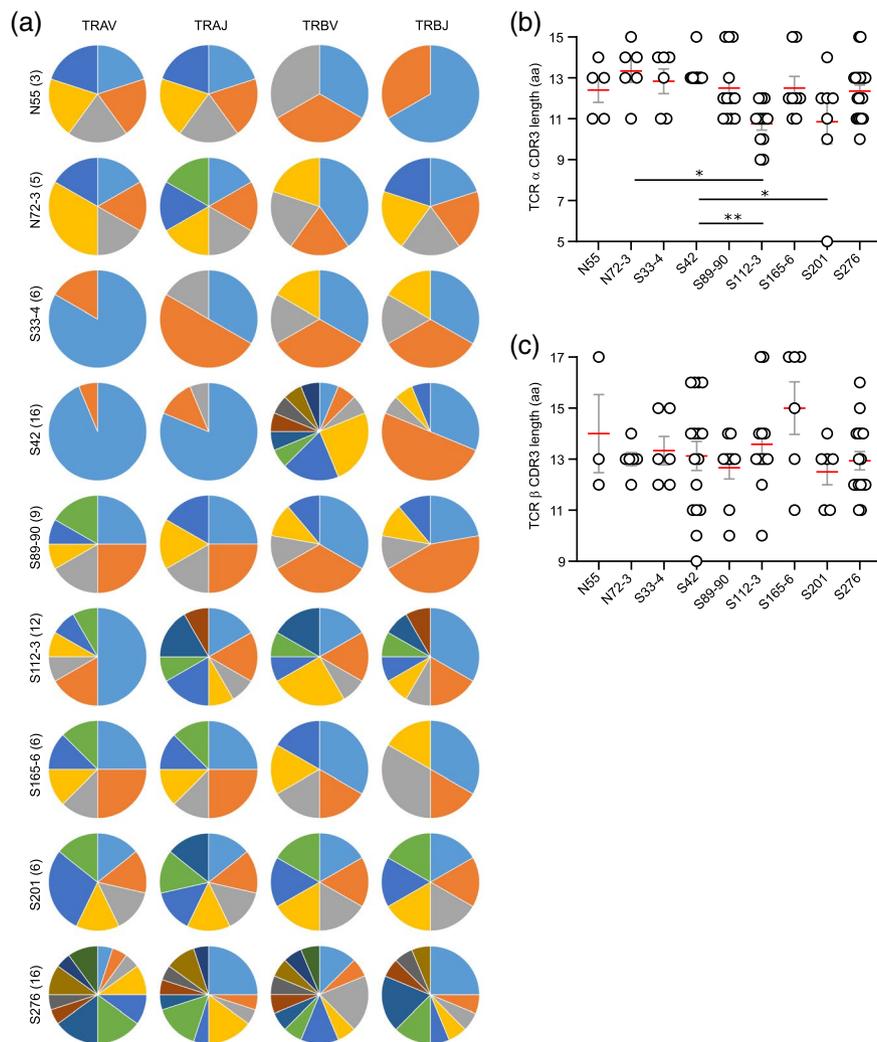
FIGURE 5 Characteristics of T cell receptors in SARS-CoV-2-specific T cell clones. (a) Part-of-whole analysis of distinct TRAV, TRAJ, TRBV and TRBJ in T cell clones grouped by specific epitope. In brackets behind each epitope the number of T cell clones analysed is listed. (b + c) Comparison of CDR3 length in amino acids (aa) for the T cell receptor (TCR)-α (b) and -β chain (c) of each clone, grouped by specific epitope. Only in-frame sequences were used. Where T cell clones had two distinct in-frame TCR-α chains, both were included. Four samples that were not clonal were not included. Red lines and grey bars indicate mean ± standard error of the mean (SEM). $*p < 0.05$, $**p < 0.01$, Tukey's multiple comparisons test

than the analysis of CD137 after 24 h of *in-vitro* stimulation that we adopted. However, the advantage of our approach is that only interactions of relatively high avidity or cells with a low activation threshold are detected. Therefore, although we did not cover the full potential spectrum of the immune responses, we could focus upon the strongest responding T cells that probably are most effective in anti-viral protection. Low-avidity interactions with SARS-CoV-2 antigens, most probably due to cross-reactivity, have been described in both healthy individuals and COVID-19 patients, but these may not provide robust protection [10,17,19]. Indeed, cross-reactive T cells did not demonstrate an overlap with TCR sequences identified in our study [19].

Currently, it is not entirely clear how CD4+ T cells may provide protection or aid recovery from a SARS-CoV-2

infection *in vivo*. Aside from their well-established role in assisting the production and differentiation antibody as well as directing CD8+ T cell responses, cytotoxic CD4+ T cells have been identified in COVID-19 patients [20]. It is not clear, however, whether these cells have a beneficial or detrimental role in disease outcome. Depletion of CD4+ cells in Macaques significantly delayed recovery from SARS-CoV-2 infection but did not abrogate viral clearance [21]. This suggests that, in humans as well, the main boon of CD4+ T cells may be to accelerate recovery rather than provide primary protection. Additionally, a patient with X-linked agammaglobulinaemia (XLA) who demonstrated a seemingly robust T cell response still required anti-viral treatment with Remdesivir for a full recovery [22]. Therefore, CD4+ T cell therapy is a promising novel approach for immunotherapy following SARS-CoV-2

infection but may best be applied in combination with antibody treatment and/or anti-viral drugs rather than as a standalone treatment.

We define and study nine immunodominant epitopes, which all share a sequence of nine or more amino acids with SARS-CoV-2 epitopes described previously [5,7–9]. These studies, including ours, all use peptide pools to identify immunodominant epitopes. A very recent publication by Sallusto and colleagues identifies naturally processed epitopes with a focus on the receptor binding domain (RBD) [23]. The two immunodominant, naturally processed and presented epitopes they identify, S346–365 and S446–485, overlap largely with our two peptides from the same region, S89–90 (353–371) and S112–113 (445–463). This confirms the physiological relevance of at least two of our targets. It is debatable how many epitopes need to be targeted for T cell-mediated immunity to be successful. In the case of our patients a large number of epitopes were targeted, but only a limited number of epitopes were targeted by multiple clones. In the case of a potential T cell therapy, therefore, generating substantial numbers of high-quality clones against a limited number of dominant antigens may suffice.

One current concern is that the level of mutations in the Spike protein could render the neutralizing effect of antibodies induced by the current generation of vaccines less effective. Regarding T cell therapy, this risk can be mitigated by assembling a pool of T cells that targets multiple antigens, including Spike and Nucleocapsid. Spike variants that cause particular concern currently, because they spread more quickly and neutralizing antibodies induced by existing vaccines may be less effective against them, include the UK variant B.1.1.7, the South Africa variant B.1.351 and the Brazil variant P.1. Common mutations within these variants, including K417T, E484K and N501Y, do not affect the immunodominant epitopes defined in this study. The Y144 deletion in the B.1.1.7 variant falls within Spike peptide 33–34 (129–147), but we found that this did not affect the IFN-γ response of specific CD4$^+$ T cell clones. It has been suggested that mutations affect CD4$^+$ T cell immunity less than neutralizing antibodies [5,24,25]. Each patient is thought to harbour T cells recognizing at least 30–40 SARS-Cov-2 epitopes, with significant variability from person to person [5]. Moreover, CD4$^+$ T cell epitopes had minimal overlap with antibody epitopes. It is therefore less likely for any particular mutation that avoids T cell recognition to provide a selective advantage and spread throughout the population. Pre-published studies appear to confirm that while mutations in variants-of-concern of SARS-CoV-2 proteins impair the antibody protection provided by vaccines, the T cell response remains unaffected [24,25]. This corroborates our own findings that the responses to immunodominant epitopes we identified were not affected by common Spike mutations.

In a very recent study of people vaccinated with either Pfizer-BioNTech (BNT162b2) or Moderna (mRNA-1273) mRNA-based COVID-19 vaccine, the authors identify 23 dominant peptides of the Spike protein, five of which overlap significantly with the seven Spike peptides we identified as immunodominant in our patients [26]. Only S201 (801–815) and S276 (1101–1115) were not detected as immunodominant after vaccination. Overall, this implies that the T cell responses we identified are thus also relevant in a healthy, younger population exposed to much-used vaccines rather than the virus. This corroborates the potential of transgenic T cells expressing TCRs based on the sequences we identified for T cell immunotherapy. Because the SARS-CoV-2 Nucleocapsid protein is not currently targeted by any licenced vaccine, a T cell therapy based on our data could offer additional benefits.

We confirm here that when studying immune responses to viral antigen, it is important not to focus solely upon IFN-γ production to identify cells with a desirable phenotype. Many of our clones that produce large amounts of IFN-γ in response to even low concentrations of peptide appeared to be not fully differentiated Th1 cells, but also produced Th2 cytokines such as IL-5 and IL-13. Moreover, some co-produced IL-10, suggesting that they may have immunoregulatory, Tr1-like properties [27]. Certain peptides seem to be more likely to be targeted by pluripotent Th1/Th2 cells than others. In cases where we found two T cell clones with identical TCR-α and -β sequences, these did not necessarily have identical cytokine responses to the same cognate peptide. For example, while clones 55 and 166 from patient 54 have identical TCRs and both produce ample IFN-γ in response to cognate peptide, the former co-produced IL-13. It may thus be disputed to what extent the characteristics of the TCR contribute to Th1/Th2 differentiation [12,13]. Other factors such as, for example, the differentiation status or length of expansion of a clone could also be hypothesized to affect this. This should be kept in mind when translating findings for (transgenic) T cell therapy.

Finally, using a technique developed for single-cell analysis to investigate the TCR repertoire of T cell clones, we managed to reliably obtain paired sequences for all our samples with enough information to clone the exact TCR. This approach of identifying full-length, paired sequences is, to our knowledge, unique when characterizing SARS-CoV-2-specific T cells. Unlike studies where only one TCR chain or only CDR3 regions were sequenced, we can now select the exact TCR sequences from T cell clones with the most desirable properties. By applying techniques established within our facilities we can generate SARS-CoV-2 antigen-specific TCR-transgenic T cells using either a patient's own endogenous T cells or, in the case of haematopoietic stem cell transfer, an HLA-matched donor's. The similarity in TCR sequences that we found between different donors is encouraging for

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the creation of T cell therapy products that may be applicable over a range of HLA backgrounds, thus augmenting the feasibility of this approach. It should be noted, however, that although our donors demonstrated a range of HLA haplotypes characteristic for the German population, wider investigation of antigen-specific T cell responses with donors from more diverse backgrounds will be required for a global approach. Nevertheless, based on the data generated in this study, we will proceed towards the development of a therapeutic approach that can be tested clinically.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Johan Verhagen designed research, conducted experiments, analysed data and wrote the manuscript. Edith D. van der Meijden and Vanessa Lang conducted experiments and analysed data. Simon Völkl, Michael Aigner and Anita N. Kremer designed research, directed the research and contributed to writing the manuscript. Andreas Mackensen contributed to the conception of the project and research. Andreas E. Kremer provided essential materials in the form of patient samples and data.

DATA AVAILABILITY STATEMENT

All relevant data are included with the manuscript. Any reasonable request for further information can be addressed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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