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A Highly Specific Assay for the Detection of SARS-CoV-2–Reactive CD4$^+$ and CD8$^+$ T Cells in COVID-19 Patients

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Gaining detailed insights into the role of host immune responses in viral clearance is critical for understanding COVID-19 pathogenesis and future treatment strategies. Although studies analyzing humoral immune responses against SARS-CoV-2 were available rather early during the pandemic, cellular immunity came into focus of investigations just recently. For the present work, we have adapted a protocol designed for the detection of rare neoantigen-specific memory T cells in cancer patients for studying cellular immune responses against SARS-CoV-2. Both CD4$^+$ and CD8$^+$ T cells were detected after 6 d of in vitro expansion using overlapping peptide libraries representing the whole viral protein. The assay readout was an intracellular cytokine staining and flow cytometric analysis detecting four functional markers simultaneously (CD154, TNF, IL-2, and IFN-γ). We were able to detect SARS-CoV-2–specific T cells in 10 of 10 COVID-19 patients with mild symptoms. All patients had reactive T cells against at least 1 of 12 analyzed viral Ags, and all patients had Spike-specific T cells. Although some Ags were detected by CD4$^+$ and CD8$^+$ T cells, VME1 was mainly recognized by CD4$^+$ T cells. Strikingly, we were not able to detect SARS-CoV-2–specific T cells in 18 unexposed healthy individuals. When we stimulated the same samples overnight, we measured significant numbers of cytokine-producing cells even in unexposed individuals. Our comparison showed that the stimulation conditions can profoundly impact the activation readout in unexposed individuals. We are presenting a highly specific diagnostic tool for the detection of SARS-CoV-2–reactive T cells. ***The Journal of Immunology*****, 2021, 206: 580–587.**

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative virus of a respiratory disease termed COVID-19, is a betacoronavirus related to severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) (1–6).

The appearance of SARS-CoV-2 has led to a rapidly spreading pandemic. First cases occurred in December 2019, and by October 1, 2020, more than 1 million deaths and 35 million cases of SARS-CoV-2 infection had been reported worldwide (Johns Hopkins University).

Several attributes of SARS-CoV-2 have contributed to its rapid spread. These characteristics include the capability to transmit already during the asymptomatic phase of infection and its variable incubation time of ∼3–14 d (S. Ma, J. Zhang, M. Zeng, Q. Yun, W. Guo, Y. Zheng, S. Zhao, M. H. Wang, and Z. Yang, manuscript posted on medRxiv, DOI: 10.1101/2020.03.21.20040329) (7). Furthermore, even asymptomatic and presymptomatic SARS-CoV-2–infected individuals can produce high viral loads sufficient for human-to-human transmission (8–10).

Diagnosis of COVID-19 is routinely achieved by detection of SARS-CoV-2 RNA in nasopharyngeal swabs via quantitative RT-PCR (RT-qPCR) (11); however, even symptomatic SARS-CoV-2 infections frequently remain unrecognized. When symptomatic, COVID-19 can range from a mild, common flu-like sickness in ∼85% to a severe respiratory disease in ∼15% of affected patients (12, 13). Mild COVID-19 is characterized by ageusia, fever, sore throat, cough, and mild pneumonia. Severe disease features strong dyspnea, hypoxia, and radiographic evidence of lung involvement. Ultimately, severe COVID-19 can lead to acute respiratory distress syndrome with respiratory failure and multiorgan dysfunction (14).

Similar to other coronaviruses, SARS-CoV-2 infection leads to an activation of the innate and adaptive immune system. Protective immunity is meant to be achieved on the one hand by activated B cells, comprising transient IgM and IgA and persisting IgG responses against the virus. In contrast, activated CD4$^+$ and CD8$^+$ T cells contribute to both the clearance of the acute infection and protective immunity against reinfection by establishing immunological memory (15). Various studies showed that a high percentage of recovered COVID-19 patients (CP) including asymptomatic patients have IgA/IgM and/or IgG Abs against SARS-CoV-2 and that their convalescent plasma has neutralizing capability (16–18) (A. Wajnberg, F. Amanat, A. Firpo, D. Altman, M. Bailey, M. Mansour, M. McMahon, P. Meade, D. Rao Mendu, K. Muellers, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.07.14.20151126). Furthermore, different research groups used convalescent plasma from recovered CP for the treatment of patients with severe illness (19, 20).

Regarding T cell immunity, several reports showed that SARS-CoV-2–specific CD4$^+$ and CD8$^+$ T cells are present in COVID-19

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Abbreviations used in this article: AP3A, protein 3a; CP, COVID-19 patient; HD, healthy unexposed donor; MFI, median fluorescence intensity; NCAP, nucleocapsid; NS6, nonstructural protein 6; RT-qPCR, quantitative RT-PCR; S1, Spike subpool 1; S2, Spike subpool 2; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEB, staphylococcal enterotoxin B; VME1, membrane protein.

patients (21–23) (A. E. Oja, A. Saris, C. A. Ghandour, N. A. M. Kragten, B. M. Hogema, E. J. Nossent, L. M. A. Heunks, S. Cuvalay, E. Slot, F. H. Swaneveld, et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.06.18.159202; and F. Gallais, A. Velay, M. -J. Wendling, C. Nazon, M. Partisani, J. Sibilia, S. Candon, and S. Fafi-Kremer, manuscript posted on medRxiv, DOI: 10.1101/2020.06.21.20132449). Most of these trials were using overlapping peptides for studying T cells responses to work HLA independently. Interestingly, almost all studies were able to detect SARS-CoV-2–specific T cells also in unexposed and/or PCR-negative patients as well as historical controls that were not able to be in contact with the novel coronavirus SARS-CoV-2. This finding is explained by the possibility that pre-existing Spike-reactive T cells in seronegative individuals represent cross-reactive clones against the Spike protein, which might have been acquired as a result of previous exposure to other seasonal human coronaviruses (21–23).

In the current study, we applied a protocol that was initially designed for the detection of rare tumor-associated Ag–specific or neoantigen-specific memory T cells in late-stage cancer patients (24–26). We expanded cryopreserved PBMCs with overlapping peptide libraries and detect virally specific T cells by an intracellular cytokine staining that allowed us to functionally characterize SARS-CoV-2–specific $CD4^+$ and $CD8^+$ memory T cells.

Materials and Methods

Study subjects

Altogether, 18 unexposed donors (nine donors in a discovery cohort and nine in a confirmation cohort) and 10 CP were included in the study. CP included individuals that were tested positive for SARS-CoV-2 RNA in nasopharyngeal swabs by RT-qPCR and symptomatic relatives of those individuals. All but one patient (C10) showed Spike-specific IgG Abs using ELISA. Unexposed donors included individuals tested negative for SARS-CoV-2 by RT-qPCR and asymptomatic individuals with no contact to SARS-CoV-2–infected persons. None of the unexposed individuals showed Spike-specific IgG Abs. Blood was collected between February and September 2020 in Tübingen, Germany. All participants provided written informed consent. The study was approved by the Ethik-Kommission der Landesärztekammer Baden-Württemberg (F-2020-111).

PBMCs isolation

Whole blood was drawn in sodium–heparin collection tubes (Sarstedt). PBMCs were isolated by density gradient centrifugation using BioColl Separation Solution (Biochrom). After isolation, PBMCs were washed and cryopreserved using freezing medium containing 10% DMSO (VWR International) until usage.

Overlapping peptide libraries

Protein-spanning overlapping peptides were obtained for 12 SARS-CoV-2 Ags (PepMix; JPT Peptide Technologies). Ags included Spike (delivered in two subpools of 158 and 157 peptides, subpool [S1] and subpool 2 [S2]), NCAP (NCAP; 102 peptides), protein 3a (AP3A; 66 peptides), envelope small VME1 (VEMP; 16 peptides), VME1 (VME1; 53 peptides), uncharacterized protein 14 (Y14; 16 peptides), ORF10 protein (ORF10; 7 peptides), ORF9b protein (ORF9b; 22 peptides), nonstructural protein 6 (NS6; 13 peptides), nonstructural protein 7A (NS7A; 28 peptides), nonstructural protein 7B (NS7B; eight peptides), and nonstructural protein 8 (NS8; 28 peptides).

Detection of SARS-CoV-2–specific T cells

After thawing, PBMCs were dissolved in TexMACS Medium (Miltenyi Biotec) containing 3 μg/ml DNAse I (Sigma-Aldrich) and were cultivated in a standard incubator (37°C; 5% CO_2; Eppendorf) for 12 h. After this initial preincubation phase, cells were washed and resowed in TexMACS Medium containing 1% penicillin–streptomycin (Sigma-Aldrich) in a 48-well plate. Overlapping peptides (PepMix) were added at a concentration of 1 μg/ml each. Cells were cultivated together with peptides for 6 d. After the first 24 h of cultivation with peptides, 10 U/ml IL-2 (Miltenyi Biotec) and 10 ng/ml IL-7 (Miltenyi Biotec) were added. Medium was changed when necessary. After 5 d of cultivation with peptides, medium without cytokines was added. After 6 d of cultivation with peptides, expanded cells of each well were collected, washed, and resowed in two wells of a 96-well

Table I. Patient characteristics

ID	Gender	RT-qPCR	No. of Mild Symptoms	Time between pos and neg RT-qPCR (d)	Time between pos RT-qPCR/Symptoms and Blood Draw (d)	Spike IgG ELISA
C01	M	pos	2	12	19	pos
C02	M	pos	4	10	79	pos
C03	M	n.a.	4	n.a.	81	pos
C04	F	pos	5	28	5	pos
C05	F	n.a.	3	n.a.	35	pos
C06	M	pos	6	29	10	pos
C07	F	pos	2	21	8	pos
C08	F	pos	6	10	32	pos
C09	F	n.a.	3	n.a.	39	pos
C10	F	pos	Hospitalized	10	107	neg
C11	M	n.a.	0	n.a.	n.a.	neg
C12	F	neg	0	n.a.	n.a.	neg
C13	M	neg	0	n.a.	n.a.	neg
C14	F	n.a.	0	n.a.	n.a.	neg
C15	M	n.a.	0	n.a.	n.a.	neg
C16	M	neg	0	n.a.	n.a.	neg
C17	M	neg	0	n.a.	n.a.	neg
C18	F	neg	0	n.a.	n.a.	neg
C19	F	n.a.	0	n.a.	n.a.	neg
C20	M	neg	0	n.a.	n.a.	neg
C21	M	n.a.	0	n.a.	n.a.	neg
C22	F	n.a.	0	n.a.	n.a.	neg
C23	F	n.a.	0	n.a.	n.a.	neg
C24	M	n.a.	0	n.a.	n.a.	neg
C25	F	n.a.	0	n.a.	n.a.	neg
C26	M	n.a.	0	n.a.	n.a.	neg
C27	F	n.a.	0	n.a.	n.a.	neg
C28	M	n.a.	0	n.a.	n.a.	neg

Mild symptoms included fever, sore throat, coughing, limb aches, ageusia, and dyspnea. Discovery cohort consisted of C11–C19. Confirmation cohort consisted of C20–C28. F, female; ID, identifier; M, male; n.a., not available; neg, negative; pos, positive.

plate. The first well was restimulated with corresponding PepMix, whereas the second well remained unstimulated. Golgi inhibitors (GolgiPlug; BD Biosciences) were added at a concentration of 1 μl/ml, and cells were cultivated for 12 ± 2 h.

Short-term stimulation test

After thawing and preincubation, PBMCs were washed and resowed in TexMACS Medium containing 1% penicillin–streptomycin (Sigma-Aldrich) in a 96-well plate. Overlapping peptides representing the whole Ag (PepMix; JPT Peptide Technologies) were added at a concentration of 1 μg/ml each. Cells were cultivated together with peptides for 12 ± 2 h in presence of GolgiPlug (BD Biosciences) at a concentration of 1 μl/ml.

Flow cytometry

For both approaches, the final readout was an intracellular cytokine staining. After cultivation, cells were washed twice, followed by extracellular staining with fluorochrome-conjugated Abs titrated to their optimal concentrations: CD3-BV785 (clone UCHT1; BioLegend), CD4-FITC (clone RPA-T4; BioLegend), CD8-APC/cyanine (clone SK1; BioLegend), and Zombie Aqua Dye (BioLegend).

After extracellular staining, cells were fixed and permeabilized (BD Biosciences), followed by an intracellular staining with the following Abs: IFN-BV421 (clone 4S.B3; BioLegend), TNF–Alexa Fluor 700 (clone MAb11; BioLegend), IL-2–PE/Cy7 (clone MQ1-17H12; BioLegend), and CD154-BV711 (clone 24-31; BioLegend). Finally, cells were measured on a Novocyte 3005R cytometer (ACEA Biosciences).

For CD25 expression analysis, cells were extracellular stained with CD3-BV785 (clone UCHT1; BioLegend), CD4-FITC (clone RPA-T4; BioLegend), CD8-APC/cyanine (clone SK1; BioLegend), Zombie Aqua Dye (BioLegend), and CD25-BV711 (clone BC96; BioLegend). After extracellular staining, cells were fixed and permeabilized (BD Biosciences), followed by an intracellular staining with IFN-BV421 (clone 4S.B3; BioLegend) and TNF–Alexa Fluor 700 (clone MAb11; BioLegend).

Statistics

Data were analyzed using FlowJo version 10.5.3 (FlowJo). After removal of duplicates using the forward-scatter area versus forward-scatter height plot, dead cells were excluded by gating on the Zombie Aqua–negative cells. Next, $CD4^+$ and $CD8^+$ cells were gated within viable $CD3^+$ lymphocytes and analyzed separately for each functional marker (CD154, IFN-γ, TNF, and IL-2). If applicable, CD25 expression was analyzed on IFN-γ^+ and IFN-γ^- cells. A detailed gating strategy can be found in Supplemental Fig. 1. For each functional marker, we evaluated the percentage of positive cells among all gated $CD4^+$ and $CD8^+$ T cells in sample one (restimulated) and sample two (not restimulated). The donor was defined as having Ag-specific T cells if the stimulation index was ≥2 (sample one divided by sample two) for any functional marker in either $CD8^+$ or $CD4^+$ T cells. The frequency of cytokine-producing cells (or CD154 expressing) cells was determined by subtracting the frequency of cytokine-producing cells in sample two (not restimulated) from sample one (restimulated). All experiments were performed centrally by one investigator (D.W.) and analyzed by another investigator (H.Z.).

Frequencies of total IFN-γ^+ T cells as well as $CD25^+$ IFN-γ^+ T cells within CP and unexposed individuals were compared with unpaired *t* test using Prism 8.4.2 (GraphPad Software).

Results

Demographic and clinical characteristics of the patient cohort

Eighteen unexposed donors (seven female) and ten COVID-19 patients (six female) were included in the study. Detailed characteristics are indicated in Table I. All CP had mild, flu-like symptoms; one patient was hospitalized. The most common symptom was limb aches, followed by sore throat and fever. The mean time between symptoms/PCR^+ and end of symptoms/PCR^- was 17 ± 9 d. The mean time between end of symptoms/PCR^-, and blood draw was 42 ± 36 d.

Presence of SARS-CoV-2–specific T cells

When we analyzed flow cytometry data regardless of phenotype ($CD4^+$ versus $CD8^+$ T cells) and function (CD154, IFN-γ, TNF, and IL-2) according to predefined criteria (21), all CP had specific T cells against at least one viral Ag. Nine of ten patients had

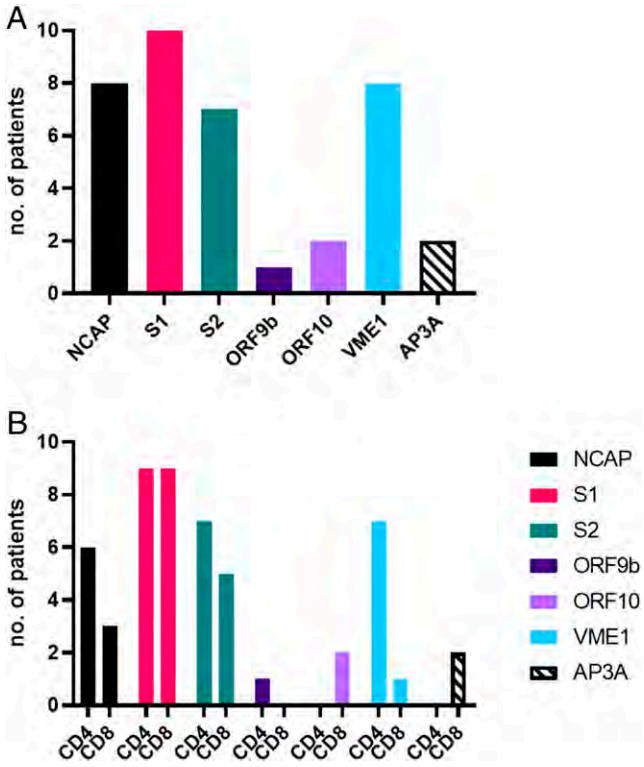

FIGURE 1. SARS-CoV-2–specific T cells in recovered CP (n = 10). Number of patients with SARS-CoV-2–specific T cells according to our predefined response criteria regardless of phenotype (**A**) or separately for $CD4^+$ and $CD8^+$ T cells (**B**) after 6 d of in vitro expansion. SARS-CoV-2–reactive T cells were not detected in unexposed individuals (n = 18).

specific T cells against more than one SARS-CoV-2 Ag. In contrast, none of the nine unexposed donors in the discovery cohort had specific T cells against any of the tested Ags. The most common SARS-CoV-2 Ag recognized by the patients' T cells was Spike, in which Spike1-specific T cells were detected in 10 of 10 patients, and Spike2-specific T cells were detected in 7 of 10 patients. Eight of ten patients had T cells able to recognize the NCAP and VME1. In 2 of 10 patients, we detected ORF10- and AP3A–specific T cells. One patient had ORF9b-specific T cells. None of the CP had T cells against any other viral Ag (Fig. 1A). Based on these results, we compiled a list of minimal SARS-CoV-2 Ags relevant for the detection of recent infections: Spike1, NCAP, membrane, and nonstructural protein 8 (as negative control) and tested PBMCs from a second cohort of unexposed individuals (confirmation cohort). None of the nine unexposed donors in the confirmation cohort had specific T cells against any of these four tested Ags (Table II). For CP C01, C05, and C07, we analyzed a second PBMCs sample, collected ∼5 mo (between 136 and 160 d) after the initial blood draw. Although S1-specific IgG Abs were below detection limit in two of three patients (see Supplemental Fig. 2), we were able to detect SARS-CoV-2–specific T cells in all three patients (Table II).

$CD4^+$ versus $CD8^+$ SARS-CoV-2–specific T cells

The Spike1 Ag was equally recognized by $CD4^+$ and $CD8^+$ T cells (both 9 of 10). This was similar for the Spike2 Ag (7 of 10 patients had $CD4^+$ T cells; 5 of 10 had $CD8^+$ T cells). A majority of NCAP-specific T cells was $CD4^+$ (6 of 10 patients versus 3 of 9 $CD8^+$ T cells). Interestingly, the membrane Ag was mainly detected by $CD4^+$ T cells (7 of 10 as compared with 1 of 10 within $CD8^+$ T cells), whereas AP3A–specific T cells were exclusively $CD8^+$ (2 of 10 patients). The immunogenicity of the VME1 is

Table II. Overview of SARS-CoV-2–specific T cells using our standard method and the commonly used short-term stimulation

ID	Gender	RT-qPCR	Spike IgG ELISA	Presence of Ag-Specific T Cells against SARS-CoV-2 Protein (Short-Term Assay) TP1	Presence of Ag-Specific T cells against SARS-CoV-2 Protein (Long-Term Assay) TP1	Presence of Ag-Specific T Cells against SARS-CoV-2 Protein (Long-Term Assay) TP2
C01	M	pos	pos	S1, S2, Y14	NCAP, S1	NCAP, S1
C02	M	pos	pos	NCAP, S1, S2, ORF9b, ORF10	NCAP, S1, ORF9b, VME1	n.a.
C03	M	n.a.	pos	S1, S2	NCAP, S1, S2, VME1	n.a.
C04	F	pos	pos	NCAP, S1, S2	S1, S2	n.a.
C05	F	n.a.	pos	S1, S2, AP3A	NCAP, S1, S2, ORF10, VME1, AP3A	NCAP, S1, S2, ORF10
C06	M	pos	pos	S1, S2, VME1, VEMP	NCAP, S1, S2, VME1	n.a.
C07	F	pos	pos	S1, S2	NCAP, S1, S2, ORF10, VME1	NCAP, S1, ORF10, VME1
C08	F	pos	pos	S1, S2, NS6, NS7a	NCAP, S1, S2, VME1, AP3A	n.a.
C09	F	n.a.	pos	S1, S2, VME, NS6, AP3A	S1, S2, VME1	n.a.
C10	F	pos	neg	n.a.	S1, NCAP, VME1	n.a.
C11	M	n.a.	neg	S1, S2	—	n.a.
C12	F	neg	neg	S1, S2	—	n.a.
C13	M	neg	neg	S1	—	n.a.
C14	F	n.a.	neg	S1, VME1	—	n.a.
C15	M	n.a.	neg	S2	—	n.a.
C16	M	neg	neg	NCAP, S2	—	n.a.
C17	M	neg	neg	S2	—	n.a.
C18	F	neg	neg	S2, ORF10	—	n.a.
C19	F	n.a.	neg	—	—	n.a.
C20	M	neg	neg	n.a.	—	n.a.
C21	M	n.a.	neg	n.a.	—	n.a.
C22	F	n.a.	neg	n.a.	—	n.a.
C23	F	n.a.	neg	n.a.	—	n.a.
C24	M	n.a.	neg	n.a.	—	n.a.
C25	F	n.a.	neg	n.a.	—	n.a.
C26	M	n.a.	neg	n.a.	—	n.a.
C27	F	n.a.	neg	n.a.	—	n.a.
C28	M	n.a.	neg	n.a.	—	n.a.

Discovery cohort consisted of C11–C19. Confirmation cohort consisted of C20–C28.
—, absence of any Ag-specific T cells; F, female; ID, identifier; M, male; n.a., not available; neg, negative; pos, positive.

comparable to the NCAP protein, which introduces the membrane Ag as interesting target for future Ab studies (Fig. 1B).

Frequency of SARS-CoV-2–specific T cells

Without applying predetermined definitions for the presence of Ag-reactive T cells, we compared the frequency of IFN-γ–producing, SARS-CoV-2–specific $CD4^+$ and $CD8^+$ T cells within CP and unexposed individuals. Looking at $CD4^+$ IFN-γ^+ T cells, highest median frequencies of specific T cells within CP were observed for S2 Ag (0.43%; IQR, 0.19–0.97%), followed by S1 (0.29%; IQR, 0.19–0.97%), NCAP (0.16%; IQR, 0.002–0.93%), and VME1 (0.12%; IQR, −0.06–0.49%). Within unexposed individuals, these frequencies were significantly lower (S2, 0.09%; IQR, −0.02–0.17%. S1, −0.02%; IQR, −0.17–0.07%. NCAP, −0.01; IQR, −0.07–0.04%. VME1, 0.02; IQR, −0.1–0.05%).

Within $CD8^+$ T cells, highest median frequencies within CP were observed for the S1 Ag (0.48%; IQR, 0.10–1.84%), followed by S2 (0.18%; IQR, 0.15–0.84%). Again, frequencies within unexposed individuals were significantly lower (S1, 0.00%; IQR, −0.06–0.08%. S2, 0.06%; IQR, −0.09–0.19%) (Fig. 2).

Differences between long- and short-term stimulation

In contrast to our results, recent publications reported medium to high frequency of unexposed individuals with SARS-CoV-2–specific T cells. To investigate if the observed differences were donor dependent, we performed the same setup within the same donors (both CP and unexposed individuals from the discovery cohort) using a short-term stimulation (i.e., incubation of PBMCs with overlapping peptide libraries for 12 h without previous in vitro expansion for 6 d). When we analyzed flow cytometry data regardless of phenotype and function, we detected SARS-CoV-2–specific T cells in nine of nine CP. However, we have also detected SARS-CoV-2–specific T cells in eight of nine unexposed individuals from the discovery cohort (Table II).

Regarding frequencies of specific T cells, highest median $CD4^+$ IFN-γ^+ T cells within CP were observed for S1 Ag (3.07%; IQR, 0.18–4.83%), followed by S2 Ag (1.92%; IQR, 0.89–8.75%). Frequencies were similar for unexposed individuals in this setting (S1, 2.83%; IQR, 0.35–5.13%. S2, 2.44%; IQR, 1.27–4.37%) (Fig. 3).

Looking at $CD8^+$ T cells, highest median frequencies within CP were observed for the S1 Ag (2.08%; IQR, 0.37–4.33%), followed by S2 (1.43%; IQR, 0.91–5.40%). Again, frequencies were similar for unexposed individuals (S1, 0.09%; IQR, 0.03–3.60%. S2, 1.48%; IQR, 0.46–2.26%). Representative examples of IFN-γ^+ $CD8^+$ T cells of a CP and an unexposed individual after short- and long-term stimulation are shown in Fig. 4.

For selected samples, we repeated the short-term stimulation and compared CD25 expression on IFN-γ–producing T cells between CP and healthy unexposed donors (HD) after Spike2 and staphylococcal enterotoxin B (SEB) stimulation. For S2-stimulated cells, we observed a trend toward higher frequency of $CD25^+$ cells (mean percentage, 60.1 ± 22.3 CP versus 28.2 ± 19.5 HD; $p = 0.136$) and a higher expression level (median fluorescence intensity [MFI], 5923 ± 2670 CP versus 2859 ± 2052 HD; $p = 0.190$) of the IFN-γ–producing cells in CP compared with unexposed individuals. These differences were not observed within SEB stimulated cells (mean percentage: 3.8 ± 1.0 CP versus 5.4 ± 2.0 HD; MFI, 717 ± 490 CP versus 923 ± 228 HD) (Fig. 5, Supplemental Fig. 4).

Discussion

The aim of the current study was the establishment of a highly specific assay enabling the detection of virus-reactive T cells only

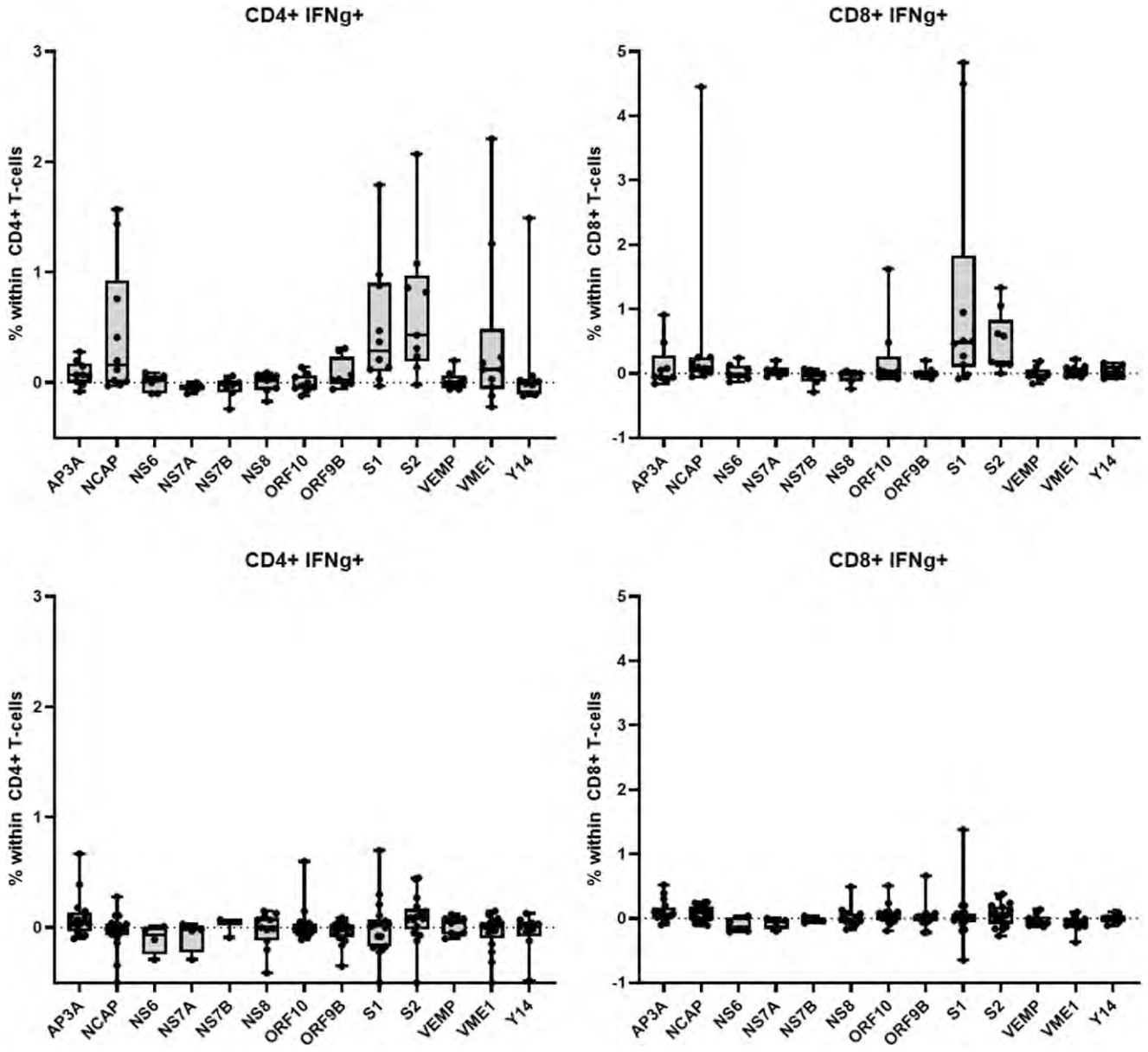

FIGURE 2. Differences between exposed (n = 10) and unexposed donors (n = 18) after long-term stimulation frequency of IFN-γ^+ $CD4^+$ (left) and $CD8^+$ T cells (right) responding upon viral Ag stimulation within recovered CP (upper row) and unexposed individuals (lower row; discovery and confirmation cohort). The frequency was calculated by subtracting the frequency of IFN-γ–producing cells in sample two (not restimulated cells) from sample one (restimulated cells) after 6 d of in vitro expansion. Box–whiskers plot (line, median; box, 25th to 75th percentiles; whiskers minimum [Min] to maximum [Max]; all individuals shown as dots).

in those individuals that underwent an infection with SARS-CoV-2. Our findings confirm results from previous reports showing that both $CD4^+$ and $CD8^+$ SARS-CoV-2–specific T cells can be detected in peripheral blood of recovered CP. We were able to detect Spike-reactive T cells in all CP, confirming its role as major target Ag for vaccination studies. However, we observed that cellular immunity against SARS-CoV-2 is not only limited to the Spike protein but also targets the NCAP and VME1. This highlights the potential importance of non-Spike proteins in future COVID-19 vaccine design.

Although our assay showed an excellent sensitivity, strikingly, we did not detect SARS-CoV-2–specific T cells in unexposed donors. In contrast, Braun et al. detected Spike-reactive $CD4^+$ T cells in 83% of CP, as well as in 34% of SARS-CoV-2–seronegative healthy donors. Cells were stimulated for 16 h, and Ag-specific T cells were detected using CD40L and 4-1BB expression (23). Grifoni et al. detected $CD4^+$ and $CD8^+$ SARS-CoV-2–specific T cells in ∼100 and 70% of COVID-19 convalescent patients, respectively. Targeted Ags included mainly Spike, membrane and NCAP protein. SARS-CoV-2–reactive $CD4^+$ T cells were also detected in 40–60% of unexposed individuals. $CD4^+$ and $CD8^+$ SARS-CoV-2–specific T cells were detected after stimulation for 24 and 9 h, respectively (21). Oja et al. were able to detect $CD4^+$ Spike-specific T cells in 21 of 21 CP with mild symptoms and 14 of 16 unexposed individuals. Cells were stimulated overnight, and SARS-CoV-2–reactive T cells were identified by the upregulation of CD154 and 4-1BB (Oja et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.06.18.159202).

Gallais et al. detected SARS-CoV-2–specific T cells (targeting different Ags) in nine of nine CP and six of eight relatives with neither positive RT-qPCR results nor detectable Abs against

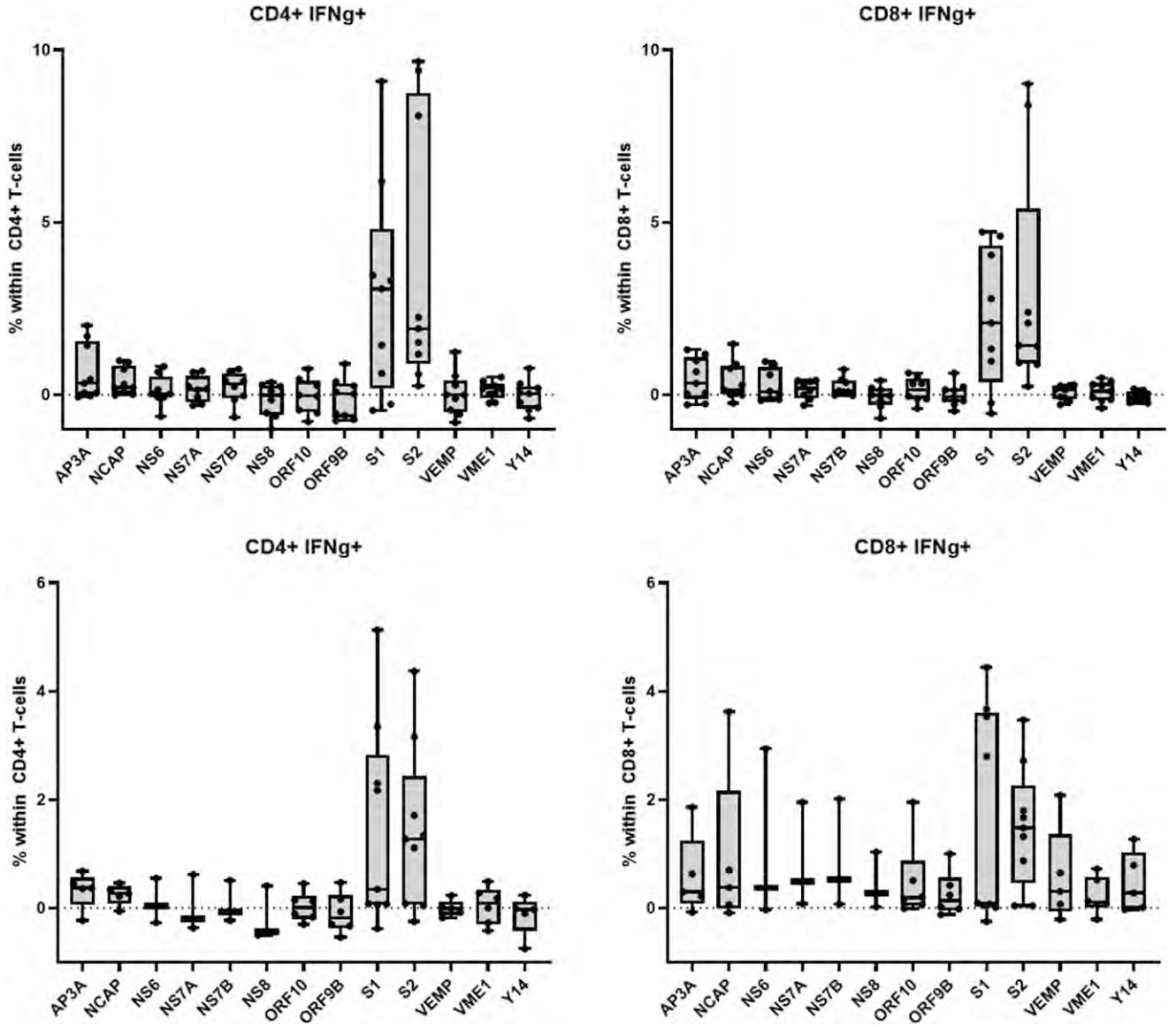

FIGURE 3. Differences between exposed (n = 9) and unexposed donors (n = 9) after short-term stimulation results from the short-term stimulation. Frequency of IFN-γ^+ $CD4^+$ (left) and $CD8^+$ T cells (right) responding upon viral Ag stimulation within recovered CP (upper row) and unexposed individuals (lower row; discovery cohort). The frequency was calculated by subtracting the frequency of IFN-γ–producing cells in sample two (not restimulated cells) from sample one (restimulated cells) after 12 h of stimulation. Box–whiskers plot (line, median; box, 25th to 75th percentiles; whiskers according to Tukey, and outliers shown as individual dots).

SARS-CoV-2. PBMCs were stimulated for ∼20 h, and SARS-CoV-2–specific T cells were detected by an IFN-γ ELISPOT assay (Gallais et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.21.20132449). Weiskopf et al. (22) observed in 10 of 10 and 8 of 10 COVID-10 patients $CD4^+$ and $CD8^+$ T cells responses, respectively. SARS-CoV-2–reactive T cells were also detected in 2 out of 10 healthy controls not previously exposed to SARS-CoV-2. PBMCs were stimulated for ∼20 h and SARS-CoV-2–specific T cells were detected by an IFN-γ ELISPOT assay. PBMCs were stimulated for 20 h, and SARS-CoV-2–specific T cells were detected by the upregulation of CD69 and CD137.

Some of the authors of the previously mentioned studies assumed that those T cells represent cross-reactive common cold coronaviruses–specific T cells. Although early studies supposed that these T cells could even be protective against SARS-CoV-2 and might be responsible for a mild course of disease, recent studies were skeptical about this hypothesis, for example because superspreading events would be very unlikely if cross-reactive T cells are indeed protective (27).

The aforementioned studies have one aspect in common: PBMCs were stimulated for a rather short period of time. Viral epitopes are in principle much more immunogenic as compared with tumor neoantigens for example. Furthermore, especially the Spike protein is a very large protein (1273 aa), bearing a lot of potential class I and class II epitopes. The short incubation time might have led to a weak response of various naive T cells or unspecific T cell responses. Interestingly, using the short-term assay, we observed an increased expression of CD25, the IL-2R α-chain, on S2-specific T cells in CP compared with unexposed individuals. This difference was not observed when T cells were stimulated unspecifically using SEB. Because low-dose IL-2 is present during in vitro expansion, it is possible that the rareness/absence of CD25 is the reason why T cells from unexposed individuals fail to expand. This finding is supported by data from Juno et al. (28), who observed an increased expression of CD25 on

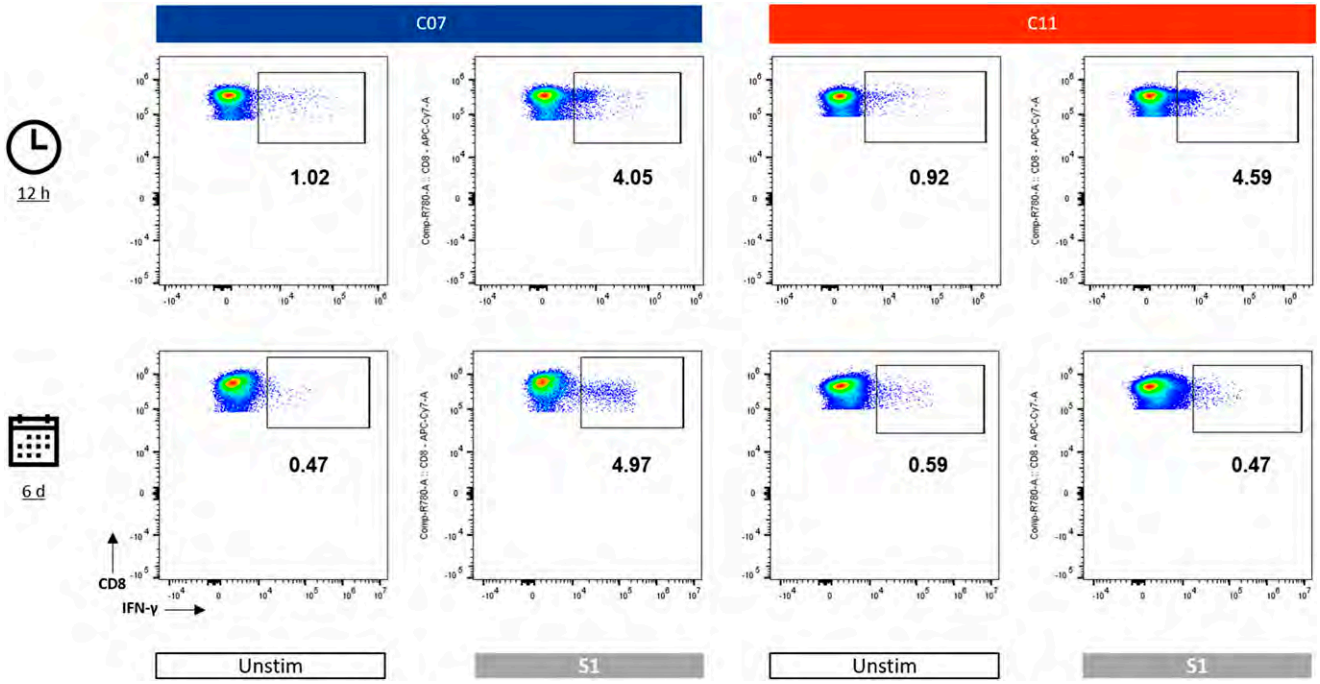

FIGURE 4. Representative examples of IFN-γ^+ $CD8^+$ T cells IFN-γ^+ $CD8^+$ T cells of a COVID-19 patient (C07; see Table I) and an unexposed individual (C11) after short-term (upper row) and long-term (lower row) stimulation. Not restimulated (first and third column) and Spike1-restimulated cells (second and fourth column) are shown. Numbers indicate frequency of IFN-γ^+ cells within all $CD8^+$ T cells.

SARS-CoV-2–specific follicular Th cells from recovered CP. Braun et al. (23) observed that Spike-reactive T cells in CP expressed high levels of CD38 and HLA-DR, two markers that are coexpressed on highly activated T cells. In contrast, Spike-reactive T cells of unexposed individuals did not express CD38 and HLA-DR or only at lower frequencies, probably reflecting their naive origin. Weiskopf et al. (22) showed that SARS-CoV-2–specific $CD4^+$ T cells of CP mainly showed a central memory phenotype, whereas $CD8^+$ T cells mainly showed an effector memory or terminally differentiated effector memory phenotype. The phenotype of SARS-CoV-2–specific T cells of unexposed controls was not discussed. And Rodda et al. observed that only effector and central memory T cells, but not naive T cells, proliferated upon Spike protein stimulation (L. B. Rodda, J. Netland, L. Shehata, K. B. Pruner, P. M. Morawski, C. Thouvenel, K. K. Takehara, J. Eggenberger,

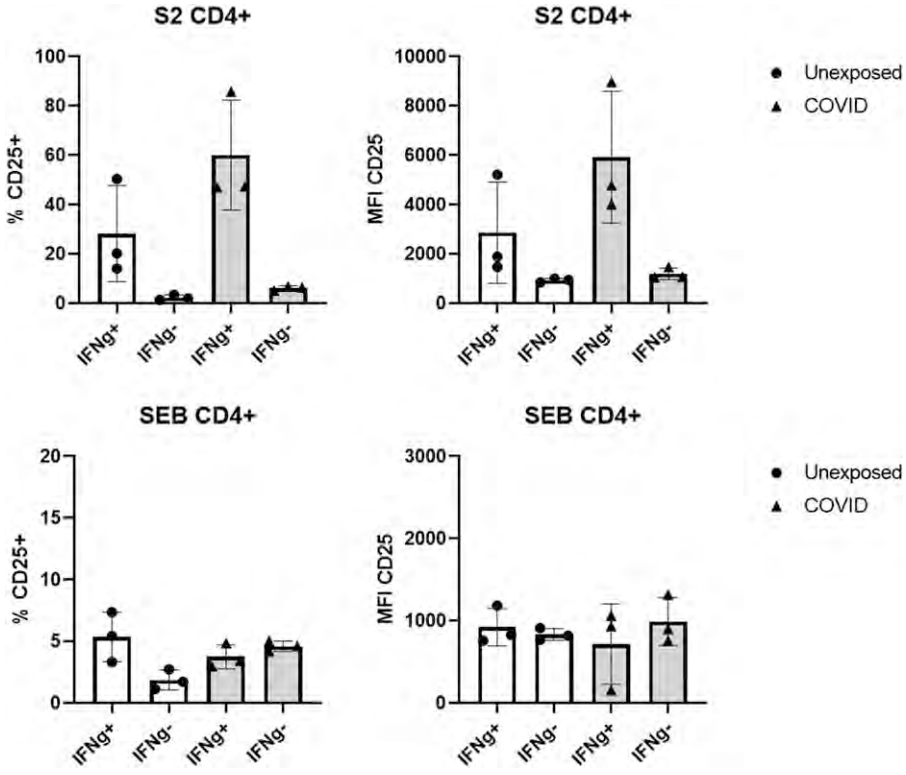

FIGURE 5. CD25 expression on IFN-γ^+ $CD4^+$ T cells percent of $CD25^+$ (left column) and CD25 MFI (right column) within IFN-γ^+ and IFN-γ^- $CD4^+$ T cells of three CP (triangle) and three unexposed individuals (circle) after short-term stimulation using S2 peptide library (upper row) or SEB (lower row). Mean (box) and SD are shown. Results for $CD8^+$ T cells are shown in Supplemental Fig. 3.

E. A. Hemann, H. R. Waterman, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.08.11.20171843).

Regarding the absence of SARS-CoV-2–specific T cells in unexposed individuals, our data are supported by a study by Peng et al. (29), who did also not see significant immune responses against SARS-CoV-2 Ags in 16 HD. Furthermore, a recent human phase I trial (recombinant adenovirus type-5–vectored COVID-19 vaccine) showed that Spike-specific T cell responses before vaccination were not found in 108 participants (30). Taken together, these data are indicating that the observed differences between our results and some previously published data are not donor dependent, but rather assay dependent, and show that stimulation conditions might profoundly impact the activation readout in unexposed individuals.

Our data show that the VME1 might be an interesting target for studying in-depth humoral immune responses against SARS-CoV-2. A high frequency of recovered CP had membrane-specific T cells in our small cohort. Intriguingly, the phenotype of these cells was almost exclusively restricted to the CD4 compartment. Our results are supported by a recently published study in which T cells able to recognize VME1-derived HLA-DR T cell epitopes could be detected in 21 of 22 recovered CP and 0 of 19 unexposed individuals (31). As Th cells play an important role during B cell priming, membrane-specific Abs might be present in these patients.

Deficiencies of our study include the small sample size and the focus on mild COVID-19 cases. With regard to this, we renounce from correlations between immunological and clinical metadata. We have introduced a (to our knowledge) novel approach that can help to identify patients that were previously infected with SARS-CoV-2, so far with very high specificity. The assay can be applied HLA independently and is not relying on epitope prediction algorithms. Especially when monitoring induced immune responses after vaccination trials, assays able to differentiate between cross-reactive, naive, or unspecific and SARS-CoV-2–specific memory responses are most desirable. As compared with neutralization assays, our protocol does not require strict BSL3 safety conditions. Larger studies, correlating true-positive cellular and detailed humoral immune responses with clinical data might help to develop novel treatment strategies.

Disclosures

The authors have no financial conflicts of interest.

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