

Profound CD8 T cell responses towards the SARS-CoV-2 ORF1ab in COVID-19 patients

Anastasia Gangaev

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Steven L. C. Ketelaars

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Sanne Patiwael

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Anna Dopler

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Olga I. Isaeva

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Kelly Hoefakker

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Sara De Biasi

Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia School of Medicine, Italy

Cristina Mussini

Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia School of Medicine, Italy

Giovanni Guaraldi

Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia School of Medicine, Italy

Massimo Girardis

Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia School of Medicine, Italy

Cami M. P. Talavera Ormeno

Department of Cell and Chemical Biology, Leiden University Medical Center, The Netherlands

Paul J. M. Hekking

Department of Cell and Chemical Biology, Leiden University Medical Center, The Netherlands

Neubury M. Lardy

Department of Immunogenetics, Sanquin Diagnostics B.V., The Netherlands

Mireille Toebes

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Robert Balderas

Department of Biological Sciences, BD Bioscience, USA

Ton N. Schumacher

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Huib Ovaa

Department of Cell and Chemical Biology, Leiden University Medical Center, The Netherlands

Andrea Cossarizza

Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia School of Medicine, Italy

Pia Kvistborg (✉ p.kvistborg@nki.nl)

Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute, The Netherlands

Research Article

Keywords: SARS-CoV-2, COVID-19, CD8 T cell reactivity, ORF1ab polyprotein 1ab

Posted Date: June 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-33197/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

A large global effort is currently ongoing to develop vaccines against SARS-CoV-2, the causative agent of COVID-19. While there is accumulating evidence on the antibody response against SARS-CoV-2, little is known about the SARS-CoV-2 antigens that are targeted by CD8 T cells. To address this issue, we have analyzed samples from 20 COVID-19 patients for T cell recognition of 500 predicted MHC class I epitopes. CD8 T cell reactivity against SARS-CoV-2 was common. Remarkably, a substantial fraction of the observed CD8 T cell responses were directed towards the ORF1ab polyprotein 1ab, and these CD8 T cell responses were frequently of a very high magnitude. The fact that a major part of the SARS-CoV-2 specific CD8 T cell response is directed against a part of the viral genome that is not included in the majority of vaccine candidates currently in development may potentially influence their clinical activity and toxicity profile.

Introduction

COVID-19, the disease caused by the novel corona virus SARS-CoV-2, is a global emergency. The first cases of COVID-19 were reported in December 2019, and as of May 22nd 2020 there are more than 5.200.000 confirmed cases and 335.000 deaths. Due to the measures undertaken in attempt to contain the rapid spread of the infection, this global pandemic is having tremendous health and socioeconomic consequences, and there is an urgent need for vaccines. As many as 78 candidate vaccines are currently under development^{2,3}. While there is accumulating evidence on the antibody response against SARS-CoV-2, we are only beginning to acquire knowledge regarding the SARS-CoV-2 specific T cell response. Therefore, it is an urgent matter to gain a deeper insight into the virus specific T cell response to both, assist vaccine design and provide tools to evaluate the vaccine-induced T cell responses.

It has been demonstrated in multiple studies that T cells isolated from COVID-19 patients have impaired effector functions and express higher levels of inhibitory receptors in comparison to T cells from healthy individuals, which was found to increase with disease severity^{5,6}. Seminal work from Sette and colleagues has provided first evidence that T cells specifically recognize SARS-CoV-2. They demonstrated that both CD4 and CD8 T cells from recovered COVID-19 patients can recognize large pools of SARS-CoV-2 derived peptides⁷ which is supported by multiple studies currently in preprint archives⁸. Due to the fact that these first studies have been focused on large peptide pools, it is currently unknown which exact epitopes are driving the T cell response towards SARS-CoV-2. Identification of the exact viral epitopes recognized by T cells, and among the T cell recognized epitopes which are most immunogenic (i.e. the immunodominant epitopes), is of immediate relevance as such knowledge will help guide vaccine design, and allow the development of tools for monitoring SARS-CoV-2 specific T cell responses. There has been (and still is) a substantial effort ongoing to predict potentially immunogenic SARS-CoV-2 epitopes restricted to both, major histocompatibility complex (MHC) class I and II⁹⁻¹³. However, there is very limited data available confirming which of these epitopes can induce T cell responses. To date, one study tested for CD8 T cell recognition of 30 epitopes derived from the spike protein restricted to HLA-A*02:01. Analyzing material from two COVID-19 patients, a few immunogenic

epitopes were identified¹⁴. However, more systemic efforts are necessary to dissect which of the many potential immunogenic SARS-CoV-2 derived epitopes are in fact recognized by CD8 T cells. In the current study we probed for CD8 T cell recognition towards 500 SARS-CoV-2 derived epitopes restricted to 10 of the most common HLA class I alleles. CD8 T cell reactivity against SARS-CoV-2 was common. Remarkably, a substantial fraction of the observed CD8 T cell responses were directed towards the ORF1ab polyprotein, and ORF1ab specific CD8 T cell responses were frequently of a very high magnitude.

Results

Epitope selection

To cover as many HLA alleles as possible in a patient-specific, and in a high throughput manner, we focused our analysis on 10 common HLA alleles of the Italian population including HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*11:01 and HLA-A*24:02 together with HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*18:01 and HLA-B*51:01. This collection of HLA alleles gives a coverage of 95% of the Italian population¹⁵. For each HLA allele, the top 50 epitopes were selected based on predicted binding affinity (NetMHCpan- 4.0)¹⁶ and likelihood of successful proteasomal processing (NetChop-3.1)¹⁷. In addition, SARS-CoV-2 epitope predictions shared by the science community were considered, and epitopes shared between SARS-CoV and SARS-CoV-2 for which T cell reactivity had previously been reported were included (Table S. 1). So far, most efforts have been focused on identifying epitopes and T cell responses towards in particular the spike protein but also the nucleo-and membrane proteins of SARS-CoV-29-13, however, we decided to broaden our analysis spanning the entire proteome of the virus, which resulted in a high representation of epitopes from the polyprotein encoded by ORF1ab (Fig 1A). Of note, the contribution of epitopes derived from the spike protein and from ORF1ab roughly reflects the difference in size of the proteins.

The SARS-CoV-2 specific CD8 T cell response

To investigate which epitopes from SARS-CoV-2 are recognized by CD8 T cells, PBMC samples collected from 22 patients hospitalized were analyzed (Table 1). All samples were collected during hospitalization and included 10 patients with severe disease that required non-invasive ventilation (NIV, in Infectious Disease Clinics, ID), 9 patients with critical disease (requiring intubation and mechanical ventilation, hospitalized in intensive care unit, ICU), and 3 patients who had recovered from the disease and were off therapies for a week. To probe for T cell recognition of the selected epitopes, we made use of our in-house technology based on multiplexing of peptide HLA (pHLA) multimers conjugated to fluorescent dyes. We have previously successfully used this technology to probe for T cell recognition towards shared self-antigens and neoantigens in cancer patients^{18,19}. Utilizing 14 different fluorescent dyes to make unique dual fluorescent codes made it feasible to probe for T cell recognition of 75 epitopes in parallel. Patients were typed for HLA-A and HLA-B loci, and with the 10 selected HLA alleles we could cover 1 HLA allele for 13 patients, 2 HLA alleles for 5 patients, and 3 alleles for 2 patients (Table 1).

In total we performed 1500 individual antigen-specific CD8 T cell analyses. We detected a total of 16 SARS-CoV-2 specific CD8 T cell responses in 9 of 18 patients towards 9 different epitopes (two patients were excluded from the data analysis due to insufficient CD8 T cell count: <1000). Examples of SARS-CoV-2 specific T cell responses are shown in Fig 1B. Strikingly, a subset of the identified CD8 T cell responses were of profound magnitude (above 10% of total CD8+ cells). The average magnitude of the detected responses was 2.4% of total CD8+ cells (range: 0.006 to 18.4% of total CD8+ cells) (Fig 1C). Eleven of the detected responses were restricted to HLA-A*01:01, 3 responses were restricted to HLA-A*02:01, 1 response was restricted to HLA-A*03:01, and 1 to HLA-A*24:02. Interestingly, among the COVID-19 patients, there was a complete lack of T cell responses restricted to the included HLA-B alleles.

To validate the identified CD8 T cell responses, PBMC samples from 2 COVID-19 patients with none of the HLA alleles included in the study were analyzed. All the epitope and HLA combinations for which we had identified CD8 T cell responses were included, and no non-specific signals were observed (data not shown). Furthermore, for 3 patients we had sufficient PBMC material to validate the high magnitude responses detected. This validation was carried out using altered dual fluorescent codes compared to the initial detection and all responses were confirmed (representative examples are shown Fig S3). Taken together these data strongly indicate that the identified responses are indeed true antigen specific CD8 T cell responses.

It has recently been demonstrated that T cell responses towards SARS-CoV-2 derived epitopes can be detected in PBMCs of unexposed healthy individuals⁷. To test if there was cross-reactivity towards the SARS-CoV-2 epitopes included in our analysis, PBMC samples from 4 healthy individuals collected prior to October of last year were analyzed. In total, we could cover 7 of the 10 included HLA alleles with these analyses (i.e. HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, HLA-B*08:01 and HLA-B*15:01).

We identified a single response in healthy donor 1 (HD1) restricted to HLA-B*15:01 which was of low magnitude (0.008% of total CD8+ cells), and thus observed a considerably lower level of antigen-specific CD8 T cell responses in healthy donors compared to COVID-19 patients (Fig 1D). This lack of responses in healthy donors compared to work from Grifoni et al.⁷ may be due to the low number of epitopes (11 of 500) included with known overlap with coronaviruses causes 'common colds' (Table S1).

The analyzed patient cohort included 6 patients hospitalized with severe disease, 9 patients with critical disease (admitted to ICU) and 3 patients who were recovering in the hospital. This allowed us to compare differences in magnitude and number of detected CD8 T cell responses between these groups. We found that the recovering patients had a trend towards higher magnitude T cell responses compared to the two other groups of patients, and that the magnitude of T cell responses decreased with increasing disease stage, although not significantly (Fig 1E). Intriguingly, 13 of the 16 identified T cell responses were detected in patients with severe disease or recovering from severe disease while only 3 of 13 responses were detected in patients with critical disease (Fig 1C).

Immunogenicity hierarchy within T cell recognized SARS-CoV-2 epitopes

A striking observation was that the vast majority of identified responses were restricted to the HLA-A*01:01 allele (11 of 16) (Fig 1C). Furthermore, a CD8 T cell response towards the ORF1ab epitope TTDP SFLGRY was detected in all 5 HLA-A*01:01 positive patients, and these responses were of significantly higher magnitude compared to the magnitude of all other detected CD8 T cell responses (Fig 1F, $p = 0.0002$). Furthermore, for 3 out of the 5 HLA-A*01:01+ patients, additional responses were identified (likewise restricted to HLA-A*01:01), and the magnitude of these additional responses was at least 20-fold lower compared to the response towards the TTDP SFLGRY epitope within each patient. This observation strongly indicates that this epitope is likely to drive a large part of the CD8 T cell response towards SARS-CoV-2 in HLA-A*01:01 positive patients, and is likely the immunodominant epitope in this subgroup of patients. Of note the bias of detected CD8 T cell responses towards HLA-A*01:01 was not a reflection of differences in the quality of epitope prediction (measured by predicted binding affinity to HLA) (Fig 1SA).

The origin of the CD8 T cell recognized SARS-CoV-2 epitopes

Of the 9 CD8 T cell recognized epitopes identified in COVID-19 patients, 4 are unique for SARS-CoV-2 and 5 are shared between SARS-CoV-2 and SARS-CoV (Table S. 1). Of the 5 epitopes shared between SARS-CoV-2 and SARS-CoV, 1 was previously demonstrated to be immunogenic²⁰. Noteworthy, a substantial part of identified CD8 T cell recognized epitopes (4 of the 9) were derived from ORF1ab. However, CD8 T cell recognized epitopes derived from the spike protein (S) were also well-represented (3 of 9). In contrast, only 1 epitope was derived from the nucleoprotein (N) and 1 from the membrane protein (M). Importantly, the CD8 T cell responses specific for the epitopes derived from ORF1ab were of significantly higher magnitude compared to the CD8 T cell responses towards S, N and M combined (Fig 1G, $p = 0.0027$). This is potentially a very important observation for design of vaccine candidates, as the majority of current vaccine candidates are focused on the spike protein which may result in an induction of a more limited CD8 T cell response compared to the naturally induced CD8 T cell response.

CD8 T cell recognized SARS-CoV-2 epitopes are not located in hotspot regions of the SARS-CoV-2 genome

Next, we examined whether the SARS-CoV-2 epitopes recognized by CD8 T cells were spanning the positions of the SARS-CoV-2 genome demonstrated to contain a high level of single nucleotide polymorphisms (SNPs), the so-called 'hotspots'. Such information is of key importance for the utilization of the obtained information for both vaccine development and monitoring efforts. For this purpose, we used the SARS-CoV-2 Alignment Screen tool (version as per 14-05-2020) containing sequencing information of 7667 SARS-CoV-2 isolates²¹. Importantly, the 9 T cell recognized epitopes identified in the COVID-19 patients did not overlap with these 'hotspots' (Fig S4). Furthermore, between 7667 and

6996 of the virus isolates contained information regarding the potential consequence of the SNPs on the amino acid level in the regions encoding for the 9 CD8 T cell recognized epitopes. The median fraction of these isolates encoding the T cell epitopes were 99.98% with a range of 97.13% to 100%. These findings strongly indicate that the identified immunogenic epitopes are highly preserved, making them excellent candidates to be included in the development of tools for monitoring the SARS-CoV-2 specific T cell responses in COVID-19 patients and evaluation of vaccine-induced immunity.

SARS-CoV-2 specific T cells display high expression levels of inhibitory receptors

Finally, we evaluated the expression level of 4 inhibitory receptors associated with T cell activation and exhaustion (NKG2A, PD-1, TIM-3, and 2B4). The fraction of CD8 T cells expressing NKG2A has been shown to be increased on bulk CD8 T cells in COVID-19 patients compared to healthy donors⁶. We found no significant differences in the frequency or expression levels of these inhibitory receptors in bulk CD8 T cells of COVID-19 patients in comparison to healthy donors which may reflect low sample numbers (Figure S. 5). However, when focusing the analysis on the SARS-CoV-2 specific CD8 T cells in comparison with bulk CD8 T cells, we found that the fraction of NKG2A positive cells was significantly higher among SARS-CoV-2-specific CD8 T cells (Fig 2A and B). NKG2A expression can be induced by cytokines such as IL-6 and IL-10, that both have been detected at high levels in COVID-19 patients compared to healthy donors^{22,23}. Interestingly, NKG2A expression on CD8 T cells has been found to play a role in the control of viral infections, as the expression can preserve the virus-specific T cells by limiting excessive activation, potentially leading to apoptosis of T cells²⁴. However, the expression of NKG2A has also been shown to have opposing effects in different viral infections²². It remains to be determined whether NKG2A expression on SARS-CoV-2 specific CD8 T cells help sustain the virus-specific T cells resulting in an ongoing protective T cell response against the infection or whether the NKG2A expression mainly hampers the functionality of a protective CD8 T cell response. In case of the latter, therapy with NKG2A blocking antibodies may prove valuable.

While we did not observe a significant difference in either the expression level of PD-1 or the fraction of PD-1+ SARS-CoV-2-specific T cells compared to bulk CD8 T cells (Figure 2B), we did observe a significant correlation between PD-1 expression level (MFI) on the SARS-CoV-2 specific CD8 T cells and the magnitude of the SARS-CoV-2-specific T cell response (*Error! Reference source not found.*B and C). PD-1 expression has been described to increase with persistent antigen driven stimulation via the T cell receptor²⁵, and these data may suggest that the high magnitude SARS-CoV-2 specific CD8 T cells more frequently encountered their cognate antigen during the course of the infection. Together, these observations underline the importance to focus the analysis on the virus specific T cells in COVID-19 patients.

Discussion

In this study, we report on a systemic effort to identify CD8 T cell recognized epitopes from SARS-CoV-2. This is to our knowledge a first effort dissecting which of the many potential HLA class I restricted SARS-CoV-2 epitopes are recognized by CD8 T cells.

A first observation based on our data is that CD8 T cell reactivity towards SARS-CoV-2 was common in our patient cohort. These findings are in line with the data from Sette and colleagues demonstrating SARS-CoV-2 reactive CD8 T cell responses in approximately 70% of recovered COVID-19 patients²⁶. Strikingly, 3 of the 9 identified T cell recognized epitopes in our analysis yielded responses in up to 5 patients. Specifically, we have identified 2 epitopes restricted to HLA-A*01:01, TTDPNFLGRY and PTDNYITTY, which induced detectable T cell responses in 5 and 3 patients, respectively. In addition, we have also identified an HLA-A*02:01-restricted epitope, YLQPRTFLL, that induced CD8 T cell responses in 2 patients. Together with the data from Heath and colleagues¹⁴, we clearly demonstrate the feasibility to develop tools for monitoring of SARS-CoV-2 specific T cell responses across patients in a high throughput manner.

It has been demonstrated that CD4 T cells can play a significant role in the immunopathology observed in COVID-19 patients^{27,28}. However, at this point in time it is not clear whether CD8 T cell responses towards SARS-CoV-2 primarily have a protective role or whether these responses can also contribute to the immunopathology. In our analysis, we detected a higher number of SARS-CoV-2 specific CD8 T cell responses (13 of 16) in patients who had a relatively milder clinical course of disease (patients with severe disease or recovering from severe disease) compared to patients with critical disease (admitted to ICU) where we only detected 3 of 16 responses. Together with the observation that the patients in the first group also had CD8 T cell responses of higher magnitude compared to the latter group, it is tempting to speculate that CD8 T cell responses have a protective role. However, our data set is limited in size (and only one timepoint was available per patient), and it is not possible to differentiate between cause and consequence.

Based on our data it seems that certain HLA alleles can induce strong CD8 T cell responses. The magnitude of 6 of 11 CD8 T cell responses restricted to HLA-A*01:01 was above 0.5% of total CD8+ cells, whereas the magnitude of T cell responses restricted towards all other HLA alleles (5 responses in total) was between 0.006 and 0.085% of total CD8 T cells. This observation suggests that immunodominant epitopes may not exist for all HLA alleles (or were simply not included in our epitope selection), and that patients with a specific (set of) HLA alleles may have a strong CD8 T cell response towards SARS-CoV-2. Such a scenario is well described for other viral infections including HIV where patients positive for HLA-B*57:01 are more likely to be long term nonprogressors²⁹. If this is also the case for COVID-19 patients, one would expect that certain subgroups of patients with specific HLA-alleles are likely to experience milder disease courses (given that a strong CD8 T cell response is indeed protective), and that HLA-A*01:01 is one such allele. However, the current data is limited in size and therefore merely hypothesis-generating, and this needs to be addressed in larger patient cohorts in the future.

Remarkably, a substantial fraction of the identified CD8 T cell responses were directed towards ORF1ab, and these responses were of profound magnitude, and significantly higher compared to the CD8 T cell responses directed towards spike, nucleo- and membrane proteins combined. These findings suggest that the ORF1ab epitopes can be highly immunogenic. In particular, the TTDPSFLGRY epitope from the ORF1ab, which is unique to SARS-CoV-2, induced CD8 T cell responses of profound magnitude. These observations are in line with data from Grifoni et al. demonstrating that CD8 T cells from patients who recovered from COVID-19 respond to peptide pools spanning the ORFs 3a, 8 and part of 1ab, and that T cell responses towards ORF3a accounted for 7% of the total SARS-CoV-2 specific CD8 T cell response²⁶. Together, these data clearly demonstrate the potent immunogenicity of SARS-CoV-2 epitopes derived from viral proteins other than the spike protein, which is currently the focus for vaccine development^{2,3}.

In conclusion, we have provided compelling evidence supporting that SARS-CoV-2 specific CD8 T cell responses can be of profound magnitude, in particular in patients experiencing milder disease course. The fact that a major part of the SARS-CoV-2 specific CD8 T cell response is directed against a part of the viral genome not included in the majority of the currently developed vaccine candidates may potentially influence their clinical activity and toxicity profile.

Materials

Blood collection and PBMC isolation

The samples from both COVID-19 patients and healthy donors were collected in accordance with the Declaration of Helsinki after approval by the institutional review boards. Each participant provided informed consent. Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes, and isolation of peripheral blood mononuclear cells (PBMCs) was immediately performed using Ficoll-Paque density centrifugation according to standard protocol. PBMCs were suspended in fetal bovine serum (FCS) with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until analysis.

HLA typing of study participants

PBMCs isolated from COVID-19 patients were thawed and washed with RPMI 1640 supplemented with 10% FCS, 1% Penicillin-Streptomycin solution and Benzoyl-DL-homoserine nucleoside (Merck-Millipore, 2500 U/mL), resuspended and incubated at 37°C for 30 minutes. PBMCs were counted and up to 1,000,000 cells were aliquoted for subsequent DNA isolation. DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, cat. #69506) according to manufacturer's protocol. HLA typing was done using next-generation sequencing according to the manufacturer's protocol (GenDx). For the healthy donor samples, DNA was isolated directly from whole blood and HLA typing was performed in the same way as for the COVID-19 patients.

SARS-CoV-2 peptide selection and synthesis

Fifty SARS-CoV-2 peptides were selected for each of the top ten most prevalent HLA alleles in Italy. The selection was primarily based on SARS-CoV-2 epitopes that had the highest predicted binding affinity to the MHC according to NetMHCpan-4.016, as well as receiving a prediction score higher than 0.5 using NetChop-3.117. The SARS-CoV-2 proteome was obtained from UniProt (Proteome ID: UP000464024). Thirteen proteins (pp1ab (ORF1ab), protein 3a, non-structural protein 6, protein 7a, non-structural protein 7b, non-structural protein 8, protein 9b, ORF10 protein, uncharacterized protein 14, envelope small membrane protein (E), membrane protein (M), nucleoprotein (N) and spike glycoprotein (S)) were considered as sources of potential epitopes. All possible 9–11mer peptide sequences were derived from the thirteen SARS-CoV-2 proteins, and epitopes were ranked and selected as described above. In addition, SARS-CoV-2 epitopes that were predicted to be most immunogenic by the science community^{9–11,37} were included for analysis (Table S. 1). Selected peptides were synthesized by the Chemical Biology group, Leiden University Medical Centre.

Generation of UV-cleavable pHLA monomers

The UV-cleavable peptides were synthesized in-house as described previously (REF). Recombinant HLA-A*01:01, A*02:01, A*03:01, A*11:01, A*24:02, B*07:02, B*08:01, B*15:01, B*18:01 and B*51:01 heavy chains and human beta-2 microglobulin (B2M) were produced in *Escherichia coli* and isolated from resulting inclusion bodies³⁰. MHC class I refolding reactions and purification by gel filtration HPLC were performed, and HLA-A and B heavy chains and B2M were refolded in the presence of UV-cleavable peptides (Table S. 3) following subsequent biotinylation as described previously³¹.

Generation of fluorescent pHLA multimers

MHC complexes were loaded with the selected SARS-CoV-2 peptides via UV-induced ligand exchange^{32,33}. In brief, pHLA complexes with UV-sensitive peptide were subjected to 254/366 nm UV light for 1 h at 4°C in the presence of a rescue peptide. The following amounts of 14 different fluorescent streptavidin conjugates were added to 10 µl of pHLA monomer (100 µg/ml): 1 µl of SA-BB790 (BD, custom), 1 µl of SA-BB630 (BD, custom), 1 µl of SA-APC-R700 (BD, 565144), 0.6 µl of SA-APC (Invitrogen, S868), 1 µl of SA-BV750 (BD, custom), 2 µl of SA-BV650 (BD, 563855), 2 µl of SA-BV605 (BD, 563260), 2 µl of SA-BV480 (BD, 564876), 2 µl of SA-BV421 (BD, 563259), 1 µl of SA-BUV615 (BD, 613013), 1.5 µl of SA-BUV563 (BD, 565765), 2 µl of SA-BUV395 (BD, 564176), 1.25 µl of SA-BV711 (BD, 563262) and 0.9 µl of SA-PE (Invitrogen, S866). For each pHLA monomer, conjugation was performed with two of these fluorochromes resulting in up to 75 dual fluorescent colour codes. Subsequently, milk (1% w/v, Sigma) was added to block and capture unspecific peptide binding residues, and fluorescently labelled pHLA multimers were incubated for 30 min on ice. Finally, D-biotin (26.3 mM, Sigma) in PBS and NaN₃ (0.02% w/v) was added to block residual binding sites.

Combinatorial encoding of pHLA multimers and surface marker staining

PBMCs isolated from COVID-19 patients were thawed and washed with RPMI 1640 supplemented with 10% FCS, 1% Penicillin-Streptomycin solution and Benzonase nuclease (Merck-Millipore, 2500 U/mL), resuspended and incubated at 37°C for 30 minutes. The following amounts of fluorescently labelled pHLA multimers were used to stain T cells: 1 µl of SA-BB790-pHLA, SA-BB630-pHLA, SA-APC-R700-pHLA, SA-BV750-pHLA, SA-BV650-pHLA, SA-BV605-pHLA, SA-BV480-pHLA, SA-BV421-pHLA, SA-BUV615-pHLA, SA-BUV563-pHLA, SA-BUV395-pHLA, SA-BV711-pHLA, SA-PE-pHLA and 2 µl of SA-APC-pHLA. The cells were stained in Brilliant Staining Buffer Plus (BD, 563794) according to manufacturer's protocol. Final staining volume was 100 up to 194 µl depending on the amount of fluorescent pHLA multimers for each individual sample. Cells were incubated for 15 min at 37 °C. Subsequently cells were stained with 2 µl of anti-CD8-BUV805 (BD, 564912), 1 µl of anti-CD4-APC-H7 (BD, 641398), 1 µl of anti-CD14-APC-H7 (BD, 560180) 1 µl of CD16-APC-H7 (BD, 560195), 1 µl of anti-CD19-APC-H7 (BD, 560252), 1 µl of 2B4-FITC (BD, 550815), Tim-3-BV786 (BD, 742857), PD1-BUV737 (BD, 612791), NKG2A-PE-Cy7 (Beckman, B10246) and 0.5 µl of LIVE/DEAD Fixable IR Dead Cell Stain Kit (Invitrogen, L10119) and incubated on ice for 20 min. Samples were analysed on the BD FACSymphony A5.

Identification of antigen-specific CD8 T cell responses

Analysis of antigen-specific CD8 T cell responses was carried out without prior knowledge about clinical patient characteristics to avoid experimental bias. The following gating strategy was applied to identify CD8⁺ T cells: (i) selection of live (IRDye low-dim) single-cell lymphocytes [forward scatter (FSC)-W/H low, side scatter (SSC)-W/H low, FSC/SSC-A], (ii) selection of anti-CD8⁺ and 'dump' (anti-CD4, anti-CD14, anti-CD16, anti-CD19) negative cells. Antigen-specific CD8 T cell responses that were positive for two none of the other pHLA multimer channels were identified using Boolean gating. The full gating strategy used on the BD FACSymphony A5 is shown in *Error! Reference source not found.* Cut-off values for the definition of positive responses were $\geq 0.005\%$ of total CD8 T cells and ≥ 5 events. A minimum of 1,000 CD8 T cells were acquired per sample. To reduce researcher-bias caused by manual gating, only positive responses that were confirmed by three independent people were defined as real. Data was analysed using either the BD FACSDiva v.8.0.1 or the FlowJo 10.6.2 software. To monitor the reproducibility of the assay system, reference samples with up to 10 CD8 T cell responses present at varying frequencies were included in each analysis.

Flow cytometer settings

The following 21-color instrument settings were used on the BD FACSymphony A5: blue laser (488 nm at 200 mW): FITC, 530/30BP, 505LP; BB630, 600LP, 610/20BP; BB790, 750LP, 780/60BP. Red laser (637 nm at 140 mW): APC, 670/30BP, APC-R700, 690LP, 630/45BP, IRDye and APC-H7, 750LP, 780/60BP. Violet laser (405 nm at 100 mW): BV421, 420LP, 431/28BP; BV480, 455LP, 470/20BP; BV605, 565LP, 605/40BP;

BV650, 635LP, 661/11BP; BV711, 711/85, 685; BV750, 735LP, 750/30BP, BV786, 780/60BP, 750LP. UV laser (355 nm at 75 mW): BUV395, 379/28BP, BUV563, 550LP, 580/20BP; BUV615, 600LP, 615/20BP; BUV737, 735/44BP, 770LP; BUV805, 770LP, 819/44BP. Yellow-green laser (561 nm at 150mW): PE, 586/15BP; PE-Cy7, 750LP, 780/60BP. Appropriate compensation controls were included in each analysis.

Statistical analysis

Differences between two or multiple patient groups were assessed using the non-parametric Mann-Whitney *U*-test or the ordinary one-way ANOVA test, respectively. The data cut-off for all analyses was 21 May 2020. Statistical analysis was performed using Excel 16.36 and PRISM 8 (Version 8.4.0).

Declarations

Acknowledgments

We would like to thank Kees Korbee for helping to set up a work environment according to the safety regulations, Frank van Diepen and Martijn van Baalen for flow cytometry support with the BD FACSymphony which was partly funded by the Louise Vehmeijer Stichting. We are also grateful for the help from Stephanie Timmer and Patty Lagerweij for getting the COVID-19 patient samples shipped to Amsterdam.

Author contributions

Conceptualization: PK; Investigation: AG, SK, SP, AD, KH, PK; Data analysis: AG, SK, OI, PK; Reagents and samples: SDB, HO, RB, MT, TNS, AC; Clinical activities: AC, CM, GG, MG; Interpretation of data: AG, SK, TNS, AC, PK

This manuscript is dedicated to Huib Ovaa

Competing interests: The authors declare no conflict of interest

Data accessibility: All data will be shared upon request

References

1. <https://worldometers.info/coronavirus/>.
2. Thanh Le, T. *et al.* The COVID-19 vaccine development landscape. *Nature Reviews Drug Discovery* **19**, 305–306 (2020).
3. Amanat, F. & Krammer, F. SARS-CoV-2 Vaccines: Status Report. *Immunity* **52**, 583– 589 (2020).
4. Long, -X. *et al.* Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med* **62**, 1–15 (2020).

5. Diao, B. *et al.* Reduction and Functional Exhaustion of T Cells in Patients with Coronavirus Disease 2019 (COVID-19). *medRxiv* 1–14 (2020). doi:10.1101/2020.02.18.20024364
6. Zheng, M. *et al.* Functional exhaustion of antiviral lymphocytes in COVID-19 *Cellular & Molecular Immunology* **382**, 1–3 (2020).
7. Grifoni, A. *et al.* Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell* 1–33 (2020). doi:10.1016/j.cell.2020.05.015
8. Weiskopf, D. *et al.* Phenotype of SARS-CoV-2-specific T-cells in COVID-19 patients with acute respiratory distress syndrome. *medRxiv* 1–20 (2020). doi:10.1101/2020.04.11.20062349
9. Campbell, M., Steiner, G., Wells, D. K., Ribas, A. & Kalbasi, A. Prediction of SARS- CoV-2 epitopes across 9360 HLA class I alleles. *bioRxiv* 1–12 (2020). doi:10.1101/2020.03.30.016931
10. Poran, A. *et al.* Sequence-based prediction of vaccine targets for inducing T cell responses to SARS-CoV-2 utilizing the bioinformatics predictor RECON. *bioRxiv* **186**, 7264–30 (2020).
11. Grifoni, A. *et al.* A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2. *Cell Host and Microbe* **27**, 671–680.e2 (2020).
12. Nerli, S. & Sgourakis, N. G. Structure-based modeling of SARS-CoV-2 peptide/HLA- A02 antigens. *bioRxiv* **8**, S2–14 (2020).
13. Baruah, V. & Bose, S. Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-nCoV. *J Med Virol* **92**, 495–500 (2020).
14. Chour, W. *et al.* Shared Antigen-specific CD8+ T cell Responses Against the SARS- COV-2 Spike Protein in HLA A*02:01 COVID-19 Participants. *medRxiv* 1–17 (2020). doi:10.1101/2020.05.04.20085779
15. <http://www.allelefreqencies.net>.
16. Jurtz, *et al.* NetMHCpan-4.0: Improved Peptide-MHC Class I Interaction Predictions Integrating Eluted Ligand and Peptide Binding Affinity Data. *J. Immunol.* **199**, 3360– 3368 (2017).
17. Nielsen, M., Lundegaard, C., Lund, O. & Kesmir, C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics* **57**, 33–41 (2005).
18. Kvistborg, P. *et al.* Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Science Translational Medicine* **6**, 254ra128–254ra128 (2014).
19. Kvistborg, *et al.* TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *Oncoimmunology* **1**, 409–418 (2012).
20. Wang, B. *et al.* Identification of an HLA-A*0201-restricted CD8+ T-cell epitope SSp- 1 of SARS-CoV spike protein. *Blood* **104**, 200–206 (2004)
21. van Dorp, L. *et al.* Emergence of genomic diversity and recurrent mutations in SARS- CoV-2. *Genet. Evol.* 104351 (2020). doi:10.1016/j.meegid.2020.104351
22. Antonioli, L., Fornai, M., Pellegrini, C. & Blandizzi, C. NKG2A and COVID-19: another brick in the wall. *Cellular & Molecular Immunology* **55**, 1–3 (2020).

23. Cao, & Li, T. COVID-19: towards understanding of pathogenesis. *Cell Research* **30**, 367–369 (2020).
24. Rapaport, S. *et al.* The Inhibitory Receptor NKG2A Sustains Virus-Specific CD8⁺ T Cells in Response to a Lethal Poxvirus Infection. *Immunity* **43**, 1112–1124 (2015).
25. Kahan, S. M., Wherry, J. E. & Zajac, A. J. T cell exhaustion during persistent viral infections. *Virology* **479-480**, 180–193 (2015).
26. Grifoni, A. *et al.* Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell* 1–33 (2020). doi:10.1016/j.cell.2020.05.015
27. De Biasi, *et al.* Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with Covid-19 pneumonia. *Nature* 1–32 (2020). doi:10.21203/rs.3.rs-23957/v1
28. Peeples, News Feature: Avoiding pitfalls in the pursuit of a COVID-19 vaccine. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 8218–8221 (2020).
29. Flores-Villanueva, P. O. *et al.* Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci USA* **98**, 5140–5145 (2001).
30. Garboczi, D. N., Hung, D. T. & Wiley, D. C. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc Natl Acad Sci USA* **89**, 3429–3433 (1992).
31. Toebes, *et al.* Design and use of conditional MHC class I ligands. *Nat Med* **12**, 246–251 (2006).
32. Rodenko, *et al.* Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* **1**, 1120–1132 (2006).
33. Hadrup, S. R. *et al.* Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Meth* **6**, 520–526 (2009).
34. Frøsig, T. M. *et al.* Design and validation of conditional ligands for HLA-B*08:01, HLA-B*15:01, HLA-B*35:01, and HLA-B*44:05. *Cytometry A* **87**, 967–975 (2015).
35. Chang, X. L. *et al.* Conditional ligands for Asian HLA variants facilitate the definition of CD8⁺ T-cell responses in acute and chronic viral diseases. *Eur. J. Immunol.* **43**, 1109–1120 (2013).
36. Bakker, A. H. *et al.* Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Natl. Acad. Sci. U.S.A.* **105**, 3825–3830 (2008).
37. Prachar *et al.*, COVID-19 Vaccine Candidates: Prediction and Validation of 174 SARS-CoV-2 Epitopes. *bioRxiv.* **7**, 13404–14 (2020).

Table

Table 1. Characteristics of patients and healthy donors.

Covered HLA alleles are indicated in bold. The outcome of COVID-19 patient 42 is unknown as this patient was transferred to a different hospital. HLA: human leukocyte antigen, RH: Recovering in hospital, ID ward: Infectious disease ward, ICU: Critical: Intensive care unit. N/A: not available/applicable

Donor ID	Age (years)	Therapy	Disease severity	Days on therapy prior to sampling	Patient outcome	HLA-A1	HLA-A2	HLA-B1	HLA-B2
COVID-143	73	Tocilizumab	RH	10	Discharged	A*01:01	A*02:01	B*44:03	B*51:01
COVID-096	43	Tocilizumab	RH	7	Discharged	A*01:01	A*23:01	B*49:01	B*57:01
COVID-153	52	Tocilizumab	RH	7	Discharged	A*01:01	A*02:01	B*35:01	B*35:02
COVID-002	88	None	ID	N/A	Died	A*03:02	A*32:01	B*18:01	B*44:02
COVID-004	36	None	ID	N/A	Discharged	A*03:01	A*24:02	B*07:02	B*35:01
COVID-009	60	Tocilizumab	ID	3	Discharged	A*02:05	A*32:01	B*13:02	B*14:02
COVID-033	75	Tocilizumab	ID	0	Discharged	A*02:01	A*68:01	B*35:01	B*49:01
COVID-087	52	Tocilizumab	ID	8	Discharged	A*02:01	A*03:02	B*39:01	B*50:01
COVID-116	80	Tocilizumab	ID	1	Discharged	A*01:01	A*26:01	B*13:02	B*44:02
COVID-117	67	Tocilizumab	ID	1	Discharged	A*01:01	A*31:01	B*15:17	B*38:01
COVID-121	34	Tocilizumab	ID	8	Discharged	A*03:01	A*74:01	B*07:02	B*42:01
COVID-127	72	Tocilizumab	ID	2	Died	A*29:02	A*31:01	B*35:01	B*44:03
COVID-152	66	Tocilizumab	ID	1	Died	A*11:01	A*30:01	B*13:02	B*53:01
COVID-094	75	Tocilizumab	ICU	13	Died	A*02:01	A*32:01	B*51:08	B*56:01
COVID-042	50	Anakinra	ICU	2	Transferred	A*03:01	A*24:02	B*35:02	B*35:02
COVID-112	70	Tocilizumab	ICU	10	Discharged	A*01:01	A*26:01	B*35:02	B*35:03
COVID-123	70	Tocilizumab	ICU	13	Died	A*03:01	A*32:01	B*27:05	B*51:01
COVID-129	76	Tocilizumab	ICU	15	N/A	A*02:01	A*29:02	B*15:01	B*44:03
COVID-140	56	Tocilizumab	ICU	6	Died	A*11:01	A*68:02	B*35:01	B*53:01
COVID-141	77	Tocilizumab	ICU	6	N/A	A*11:01	A*68:01	B*35:03	B*50:01
COVID-147	71	Tocilizumab	ICU	6	N/A	A*02:01	A*26:01	B*38:01	B*44:02
COVID-150	74	Anakinra	ICU	6	Died	A*24:02	A*29:01	B*13:02	B*35:03
HD1	73	N/A	N/A	N/A	N/A	A*02:01	A*03:01	B*15:01	B*35:01
HD2	77	N/A	N/A	N/A	N/A	A*01:01	A*03:01	B*07:02	B*40:01
HD3	73	N/A	N/A	N/A	N/A	A*03:01	A*03:01	B*35:01	B*35:01
HD5	54	N/A	N/A	N/A	N/A	A*01:01	A*11:01	B*08:01	B*38:01

Figures

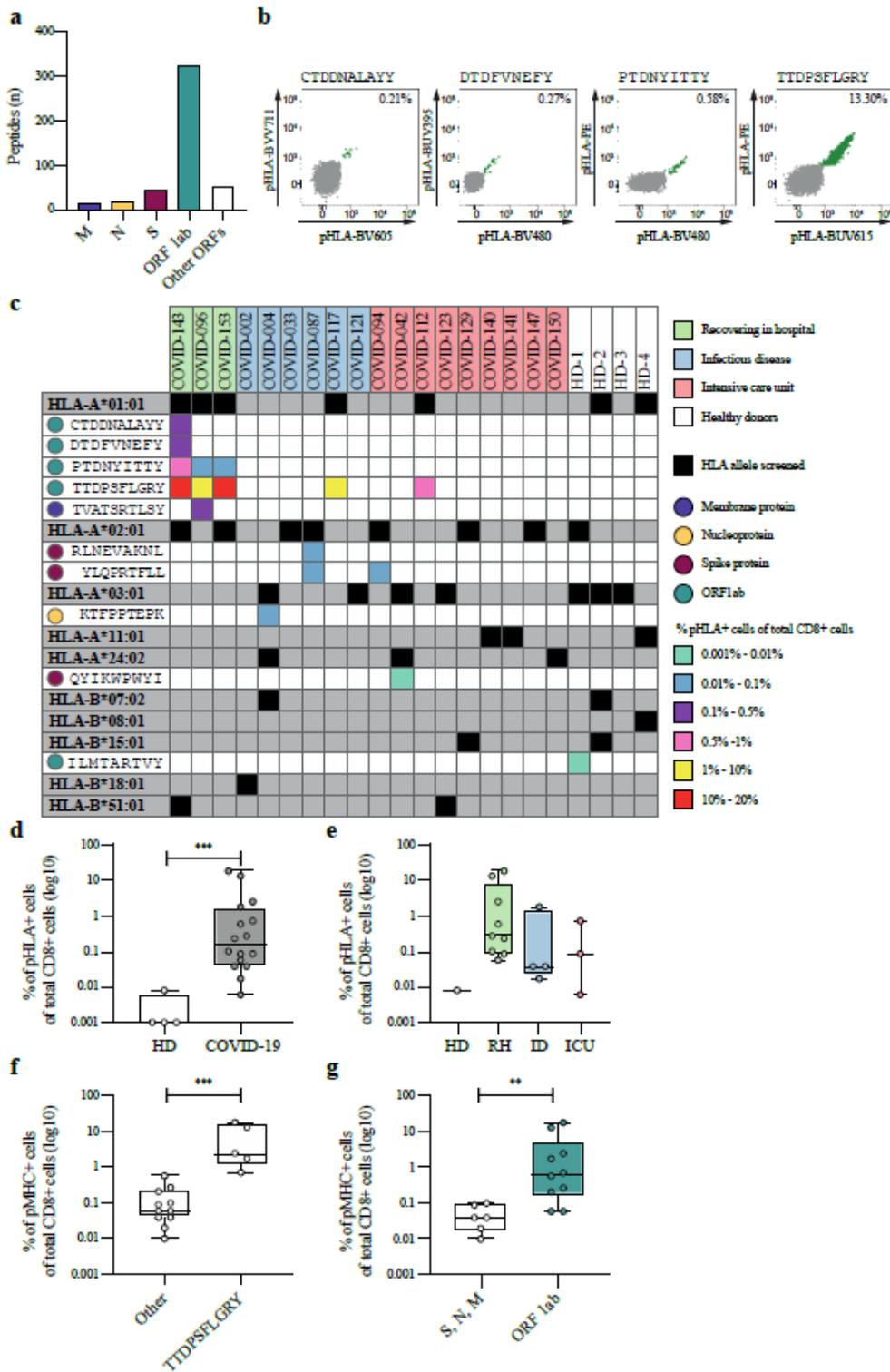


Figure 1

The T cell recognized SARS-CoV-2 derived epitopes. a) Number of selected epitopes derived from different proteins of the SARS-CoV-2 virus. M: Membrane protein, N: Nucleoprotein, S: Spike protein, ORF1ab: ORF1ab polyprotein, Other proteins (For details see Table S. 1). b) Representative flow cytometry plots of four different SARS-CoV-2-specific T cell responses (green) detected in patient COVID-143. Magnitude of SARS-CoV-2-specific T cell responses represents the percentage of double-positive pHLA+

cells of total CD8+ cells (grey). A representative example of the full gating strategy is provided in Figure S. 2. c) Heatmap of all detected SARS-CoV-2-specific CD8 T cell responses. Each column represents a patient. The colour-coding of the patients corresponds to disease status. Green are patients recovering in hospital after being admitted to the infectious disease clinic, blue indicates patients admitted to the infectious disease clinic, and red patients admitted to ICU. The black boxes in the grey bars indicate the HLA-alleles covered in the analysis. All the T cell recognized SARS-CoV-2 derived epitopes are listed on the left with colour-coding indicated which viral protein they were derived from. Each coloured square indicates a detected T cell response and the colour-coding represents the magnitude of the antigen-specific T cell response in pHLA+ CD8+ cells of total CD8+ cells. d) Difference in the magnitude of SARS-CoV-2-specific CD8 T cell responses detected in COVID-19 patients (n = 9) compared to healthy donors (n = 4). Boxplot represents the median and interquartile ranges, and the whiskers represent the full range. Statistical significance was tested with a two-tailed Mann-Whitney U-test. HD, Healthy donors. e) Differences in the magnitude of SARS-CoV-2-specific T cell responses detected in healthy donors (n = 1) and different subgroups of COVID-19 patients (RH: n = 9, ID: n = 4, ICU: n = 3). Statistical significance between groups was tested with an ordinary one-way ANOVA. No statistical significance was found. RH: recovering in hospital, ID: infectious disease, ICU: intensive care unit. f) Profound differences in the magnitude of T cell responses specific for the immune dominant epitope TTDPSFLGRY (n = 5) compared to other detected SARS-CoV-2-specific CD8 T cell responses in COVID-19 patients (n = 11). Statistical significance was tested with a two-tailed Mann-Whitney U-test. P value = 0.0002. g) The magnitude of SARS-CoV-2-specific T cell responses detected in COVID-19 patients directed towards epitopes from ORF1ab (n = 10) and towards other SARS-CoV-2 proteins (n = 6) combined. Statistical significance was tested with a two-tailed Mann-Whitney U-test. P = 0.0027. M: Membrane protein, N: Nucleoprotein, S: Spike Protein, ORF1ab: ORF1ab polyprotein.

- [Supplementaryfiguresandtables.pdf](#)