

1 Potent neutralizing antibodies in the sera of convalescent COVID-19 patients **2 are directed against conserved linear epitopes on the SARS-CoV-2 spike** **3 protein**

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46 **Abstract (69 words)**

47 The ongoing SARS-CoV-2 pandemic demands rapid identification of immunogenic
48 targets for the design of efficient vaccines and serological detection tools. In this
49 report, using pools of overlapping linear peptides and functional assays, we present
50 two immunodominant regions on the spike glycoprotein that were highly recognized
51 by neutralizing antibodies in the sera of COVID-19 convalescent patients. One is
52 highly specific to SARS-CoV-2, and the other is a potential pan-coronavirus target.

53 **Main**

54 In December 2019, a cluster of pneumonia cases of unknown etiology was reported
 55 in the city of Wuhan in the province of Hubei. The previously unidentified pathogen,
 56 which induces symptoms resembling an infection by the Severe Acute Respiratory
 57 Syndrome Coronavirus (SARS-CoV), was later identified as a novel coronavirus,
 58 SARS-CoV-2 [1]. Within a span of four months, there are more than 750,000
 59 laboratory-confirmed cases of human Coronavirus Disease 2019 (COVID-19), with
 60 over 35,000 deaths across 199 countries and territories (For up to date information
 61 consult [https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports/)
 62 [reports/](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports/)). After being declared a pandemic by World Health Organization (WHO) on
 63 11th March 2020, there is a compelling need to understand and develop effective
 64 therapeutic interventions against SARS-CoV-2.

65 SARS-CoV-2 uses the spike (S) glycoprotein to bind to the angiotensin-
 66 converting enzyme 2 (ACE2) receptor with a better affinity than SARS-CoV S
 67 glycoprotein for entry [2]. Thus, blocking the binding to ACE2, or blocking host
 68 protease cleavage to release the fusion peptide is an efficient strategy to prevent
 69 coronavirus entry [3-5]. To date, one study has assessed the immunogenicity of
 70 structural domains of recombinant SARS-CoV-2 S protein [6]. At the time of writing,
 71 findings on SARS-CoV-2 linear epitopes remain limited to bioinformatics prediction of
 72 human B and T-cell epitopes using SARS-CoV as a model [7-9]. Five regions on the
 73 S glycoprotein of SARS-CoV (residues 274-306, 510-586, 587-628, 784-803 and
 74 870-893) were predicted to be associated with a robust immune response [7], while
 75 other studies reported candidate epitopes [8, 9] that require validation with human
 76 patient samples.

77 In this brief communication, we report the antibody profiles of COVID-19
 78 patients, and the identification of two immunodominant linear B-cell epitopes on the
 79 S glycoprotein of SARS-CoV-2 that are crucial in controlling infection. A total of 25
 80 convalescence serum samples collected during the current COVID-19 outbreak in
 81 Singapore were screened at 1:1000 dilution for neutralizing antibodies against a
 82 pseudotyped lentivirus expressing SARS-CoV-2 S glycoprotein tagged with a
 83 luciferase reporter (Figure 1a). Of the 25 patients tested, six patients (2, 4, 6, 7, 11,
 84 20) with sufficient amount of serum samples that displayed a good neutralizing
 85 activity were selected for further functional characterization. Sera from all patients
 86 showed similar IC_{50} , ranging from a titre of 694 to 836, except patient 20, who
 87 showed the strongest neutralizing activity with an IC_{50} of 1603 (Figure 1b).

88 Next, we assessed the antigenic targets of these sera using a linear B-cell
 89 peptide library spanning the entire S protein of either SARS-CoV or SARS-CoV-2
 90 with pools of five overlapping peptides (Figure 1c, Supplementary Figure 1b).
 91 Interestingly, two distinct peptide pools from SARS-CoV-2 S library, pools S14 and
 92 S21, were strongly detected by sera from COVID-19 patients, and not by recovered
 93 SARS patients (17 years post disease recovery) (Figure 1c, Supplementary Figure
 94 1b). Sera from recalled SARS patients could neutralize SARS-CoV, but not the
 95 SARS-CoV-2 pseudotyped lentiviruses (Supplementary Figure 1c). Moreover,
 96 COVID-19 patients sera could strongly detect SARS-CoV S library pool S51, which
 97 partially overlaps with SARS-CoV-2 pool S21 (Figure 1c, Supplementary Figure 1b).
 98 This region encompasses the fusion peptide, which is highly conserved among
 99 coronaviruses [10, 11], suggesting a potential pan-coronavirus epitope at this
 100 location. Interestingly, targeting this region was also demonstrated to neutralize
 101 infection with a pan-coronavirus fusion inhibitor peptide [12]. Surprisingly, no linear

epitope was identified in the receptor binding domain (RBD) which suggest that antibodies targeting that region are mostly conformational epitopes. Further assessment of individual peptides within pools S14 and S21 narrowed down the specific region of interest to peptides S14P5 and S21P2, respectively (Figure 1d). Recognition of these regions was stronger for the peptides of SARS-CoV-2 than SARS-CoV (Figure 1e). Together, these findings suggest that these linear B-cell epitopes are dominant antigenic regions, which corroborated previous bioinformatics predictions [7].

Using a recently published structure of SARS-CoV-2 S protein in prefusion conformation [2], peptide S14P5 is localized in proximity to the RBD (Figure 2a). As such, it is plausible that antibodies binding to this region may sterically hinder binding to ACE2 receptor, thereby abolishing virus infection [13]. Another possibility could be an allosteric effect on ACE2 binding. Similarly, peptide S21P2 contains a part of the fusion peptide sequence, which may potentially affect virus fusion (Figure 2b). In order to assess the importance of these regions in controlling SARS-CoV-2 infection, antibody depletion assays were performed against S14P5 and S21P2 (Figure 2c). Depletion efficiency and specificity was validated by ELISA, showing that only antibodies against the respective peptides were depleted but not other unrelated antibodies (Figure 2d). Interestingly, sera that were depleted for antibodies targeting either peptides S14P5, S21P2, or S14P5+S21P2 led to a significantly reduced ability to neutralize SARS-CoV-2 pseudovirus infection, as compared to the non-depleted sera controls (Figure 2e).

Our results demonstrate that the two B-cell linear epitopes identified in this study are immunodominant. Depletion assays functionally validated that antibodies targeting S14P5 and S21P2 peptides possess significant neutralizing roles against

127 SARS-CoV-2 pseudotyped lentiviruses. Importantly, we also assessed the potential
128 presence of mutations in the peptide regions and found a low rate of mutations for
129 S14P5 and S21P2 with low to moderate impact on the viral sequence (2 and 24
130 mutations out of 2596 viral genome sequences respectively, supplementary table 3)
131 [14]. These results are essential to guide the design and evaluation of efficient and
132 specific serological assays, as well as help prioritize vaccine target designs during
133 this unprecedented crisis.

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Author contributions

CMP, GC, SNA, CYPL conceptualized, designed, acquired, analyzed, interpreted the data and wrote the manuscript. BW acquired, analyzed, interpreted the data and wrote the manuscript. RSLC, WHL and NKWY acquired and analyzed the data. YSL, MICC, SYT, LC, SK, SYT, BEY, and DCL designed and supervised sample collection. CIW, LR, LFPN conceptualized, designed, analyzed and wrote the manuscript. All authors revised and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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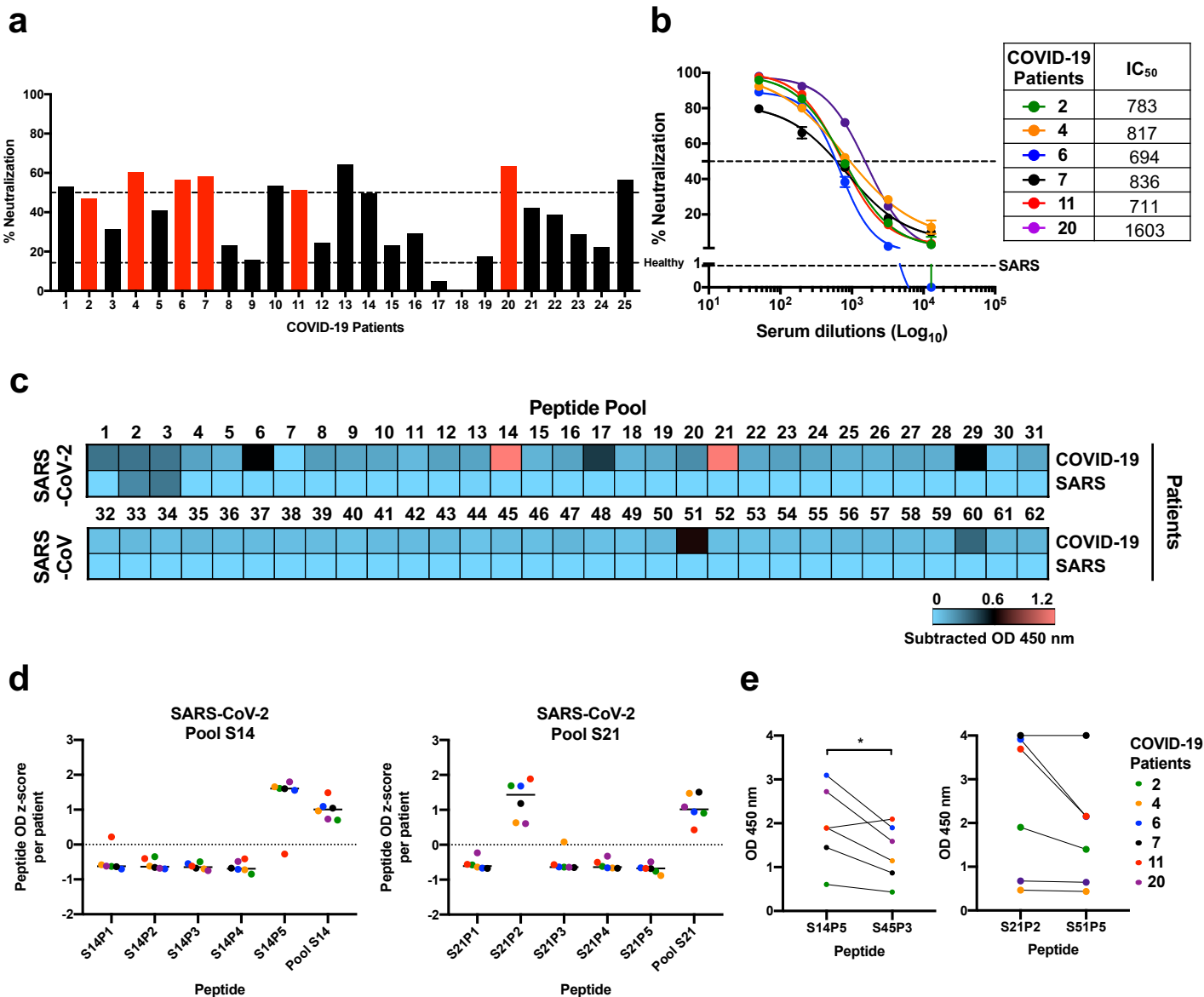


Figure 1. COVID-19 patients elicit neutralizing and specific antibody response against SARS-CoV-2. (a-b) Sera of COVID-19 patients (n=25) were mixed with pseudotyped lentiviruses expressing SARS-CoV-2 spike (S) glycoprotein protein, prior to incubation with CHO-ACE2 cells for 48 h. Infection levels were determined by luciferase assay, and percentage of infected cells were analyzed. Healthy control and recovered SARS patients (n=5) were also conducted in parallel. **(a)** Percentage of virus neutralization at 1:1000 sera dilution. IC₅₀ titer is the reciprocal of sera dilution at which half-maximal neutralization was observed (dotted lines). **(b)** Dose-response neutralization kinetics from sera (1:50 to 1:12800 dilutions) of selected COVID-19 patients (n=6). Dotted lines indicate 50% neutralization or percentage neutralization of SARS patients (n=5) at 1:100 sera dilution. **(c)** Preliminary mapping of linear B cell epitopes within SARS-CoV-2 and SARS-CoV S protein. Sera of COVID-19 (n=6) and SARS patients (n=5) at 1:1000 dilution were subjected to peptide-based ELISA for IgG using peptide pools of the S protein of SARS-CoV-2 (pools S1-31) and SARS-CoV (pools S32-62) in duplicates. Sera of pooled healthy donors (n=13) were carried out in parallel. Data were normalized against healthy controls and the subtracted OD values are presented in a heat-map. Blue and pink indicate low and high OD values, respectively. **(d-e)** Determination of SARS-CoV-2 specific and pan-CoV linear B cell epitopes on S protein. **(d)** Sera of COVID-19 patients (n=6) were subjected to peptide-based ELISA for IgG detection using individual peptides of SARS-CoV-2 S peptide pools 14 and 21. The z-score values of patient were calculated using the formula [value – average (OD value of patient) / standard deviation (OD value of patient)]. Data shown are from two independent experiments and presented as mean. **(e)** Peptide binding response of COVID-19 patients on SARS-CoV-2 peptides S14P5 and S21P2, and the corresponding regions on SARS-CoV peptides S45P3 and S51P5, respectively. Statistical analysis was carried out with paired parametric two-tailed t-test (*p<0.05).

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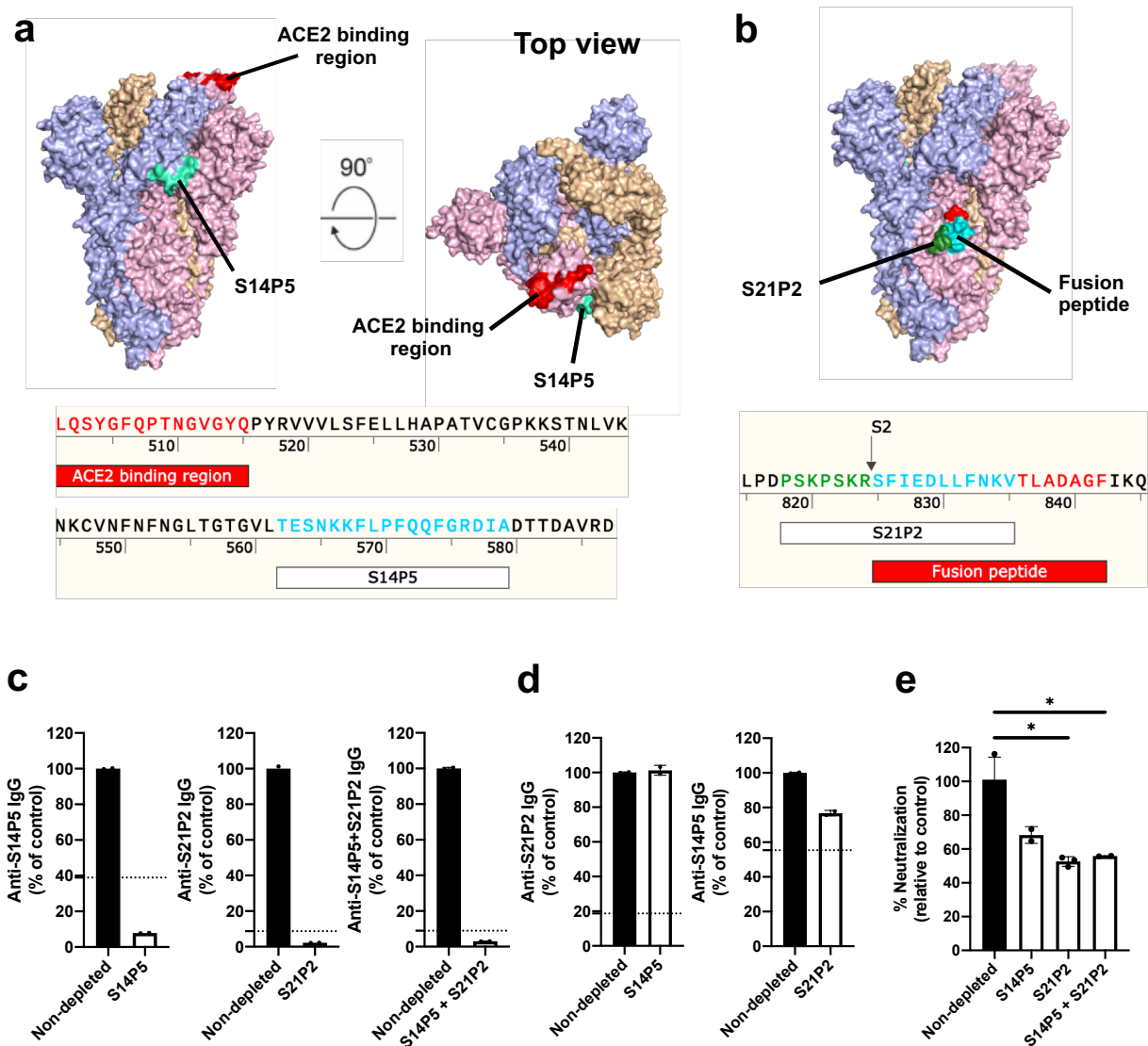


Figure 2. Antibodies against S14P5 and S21P2 linear B-cell epitopes neutralize SARS-CoV-2. (a-b) Localization and sequences of (a) SAR-CoV-2 specific S14P5 and (b) pan-CoV S21P2 epitopes on spike (S) protein (PDB: 6VSB) are shown. Each S monomer is denoted as either pink, blue or orange. (c-e) Pooled sera of COVID-19 patients (n=6) were added to plates coated with the corresponding peptides to deplete specific antibodies. (c) Non-depleted and peptide-specific antibody-depleted sera were then subjected to peptide-based ELISA of the peptide (d) or were cross-assayed. Data of depleted sera (white) were normalized to percentages of the non-depleted sera (black). Signal from pooled healthy donor sera were displayed as black dotted lines and data are shown as mean $\pm$ SD. (e) Non-depleted and peptide-specific antibody-depleted pooled sera were then mixed with SARS-CoV-2 pseudovirus before incubating with CHO-ACE2 cells for 48 h. The percentage of neutralization against pseudovirus infection, relative to the non-depleted sera, are shown. Data are presented as mean $\pm$ SD, and signal from pooled healthy donor sera were displayed as black dotted lines. Statistical analysis was carried out with one-sample t test (* p <0.05, ** p <0.01).

Supplemental Information

Methods

Ethics statement

Written informed consent was obtained from participants in accordance with the tenets of the Declaration of Helsinki. For COVID-19 serum collection “A Multi-centred Prospective Study to Detect Novel Pathogens and Characterize Emerging Infections (The PROTECT study group)”, a domain specific review board (DSRB) evaluated the study design and protocol, which was approved under study number 2012/00917. Serum collection of SARS recall “Comparison of host immune responses to coronavirus infections” (ref) was approved by DSRB under study number 2020/00091. Sera from healthy volunteers “Study of blood cell subsets and their products in models of infection, inflammation and immune regulation” was approved under study number 2017/2512.

Patient Serum

Serum was collected in BD Vacutainer SST II Advance tubes (Fisher Scientific, #12927696). After clotting, serum was separated using centrifugation for 10 minutes at 1000 rcf, and aliquoted before storing at -80 °C. Patient serum was heat-inactivated for 30 minutes at 56°C before usage for this study. Patient information is provided in table 1.

Table 1: Details of patients individually tested for the library screen

Patient ID	ICU	Sex	Age	Serum collection time (days post disease onset)
2	no	M	35	17
4	no	M	41	15

6	no	F	47	15
7	no	F	53	27
11	no	M	51	23
20	yes	M	56	30

Linear peptide library

The sequence used for the design of biotinylated linear peptides of the S glycoprotein of SARS-CoV and SARS-CoV-2 are under GenBank accession numbers [NC_004718.3](#) and [MN908947.3](#). Preliminary epitope screening was used with a library of peptides (Mimotopes, Mulgrave, VIC, Australia) consisting of 18-mer overlapping sequences. Peptides were used individually or as pooled sets. Five peptides were combined to form one pooled peptide set. Lyophilized individual peptides were dissolved in 200 μ L of DMSO (Sigma-Aldrich, #D8418-100ML) to obtain a stock solution.

Peptide-based ELISA

B-cell linear library ELISA was performed as previously described (1). Briefly, streptavidin-coated plates (Thermo Fisher Scientific, #15125) were blocked with 0.1% PBST (0.1% v/v Tween-20, Sigma-Aldrich, #P1379-500ML, in PBS, Gibco, #20012-043) containing 1% w/v sodium caseinate (Sigma-Aldrich, #C8654-500G, lot BCBP6469) and 1% w/v bovine serum albumin (BSA; Sigma-Aldrich, #A7030-500G, lot SLBW5033) overnight at 4°C, before addition of pooled biotinylated peptides (1:1000 dilution in 0.1% PBST), followed by heat-inactivated patient serum samples (1:1000 dilution in 0.1% PBST). HRP-conjugated goat anti-human IgG (H+L) antibody (Jackson ImmunoResearch, # 109-035-088, lot 139159) prepared in 0.1% blocking buffer was used for detection of peptide-bound antibodies. 100 μ L of TMB substrate

(Sigma-Aldrich, # T8665, lot SLCB5343) was used for a 5 min development and was stopped by addition of 100 μ L of 0.16 M sulfuric acid prepared from 95-97% Sulfuric Acid stock solution (Merck, #1.00731.1000), prior to absorbance measurements. Absorbance was measured with the following parameters: 450 nm minus 690 nm (bandwidth of 9nm) in 5 flashes after a 10 sec shaking at 1 mm amplitude on an Infinite M200 plate reader (Tecan, firmware V_2.02_11/06).

Peptide-specific antibody depletion of pooled SARS-CoV-2 sera

Peptide-specific antibody depletion was performed as previously described (2) with some slight modifications. Briefly, selected synthetic biotinylated peptides were added at 1:1000 dilution in 0.1% PBST to pre-blocked streptavidin-coated plates and incubated at room temperature for 1 hour. Plates were washed 3 times with 0.1% PBST followed by PBS wash to remove traces of tween. Pooled patient sera were prepared at a dilution of 1:100 in DMEM (HyClone, #SH30243.01, lot AE29431634), and 50 μ l per well of samples were added and incubated for 20 mins at room temperature for adsorption. The unbound portion was collected after 24 rounds of adsorption. ELISA analysis was performed as described before but at 1:2000 dilution, to assess the levels of selected antibodies before and after affinity depletion. Adsorbed samples were then mixed with lentiviruses pseudotyped with SARS-CoV-2 S protein as described below. Selected peptide sequences are given in table 2.

Table 2: List of individual peptide sequences tested

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Cell lines and cell culture

The human embryonic kidney epithelial cell 293T (ATCC, CRL-3216) was cultured in Dulbecco's modified Eagle's medium (Hyclone, SH30022.01) supplemented with 10% heat-inactivated FBS (Gibco, 10270-106). A stable cell line expressing human ACE2, CHO-ACE2 (a kind gift from Professor Yee-Joo Tan, Department of Microbiology, NUS, Singapore)(3) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 1% MEM Non-Essential Amino Acids Solution (Gibco, 11140-050) and 0.5mg/ml of GeneticinTM Selective Antibiotic (Gibco, 10131-027). Every 2-3 days, cells were passaged by dissociating the cells with StemProTM AccutaseTM Cell Dissociation Reagent (Gibco, A1110501). ACE2 surface expression on CHO-ACE2 cells was verified using anti-human ACE2 AF647 (Santa Cruz Biotech, sc-390851, lot B0320).

Generation of pseudotyped viral particles expressing SARS-CoV-2 S glycoprotein

Based on the third-generation lentivirus system, pseudotyped viral particles expressing SARS-CoV-2 S protein was produced by reverse transfection of 30×10^6 of 293T cells with 12µg pMDLg/pRRE (Addgene #12251), 6µg pRSV-Rev (Addgene #12253), 12µg pTT5LnX-coV-SP (a kind gift from DSO National Laboratories) and 24µg pHIV-Luc-ZsGreen (Addgene #39196) using Lipofectamine 2000 transfection reagent (Invitrogen, #11668-019) and cultured at 37°C incubator for 3 days. Viral supernatant was harvested, spun down by centrifugation to remove cell debris and filtered through a 0.45µm filter unit (Sartorius, #16555). Lenti-X p24 rapid titer kit (Takara Bio, #632200) was used to quantify the viral titres following the manufacturer instructions.

Neutralization assay against SARS-CoV-2 S glycoprotein pseudotyped lentiviruses

CHO-ACE2 cells were seeded at a density of 2.5×10^4 cells in 100 μL of complete medium without Geneticin in 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Corning, #3904). After heat-inactivation at 56°C for 30 minutes, serially diluted patient serum were incubated in a 96-well flat-bottom cell culture plate (Costar, #3596) with an equal volume of pseudotyped virus (12ng of p24) at the final volume of 50 μL at 37°C for 1 hour, and the mixture was added to the monolayer of pre-seeded CHO-ACE2 cells. After 1 hour of pseudotyped viral infection at 37°C , 150 μL of culture medium was added to each well and the cells were further incubated for another 48 hours. Upon removal of culture medium, cells were washed twice with sterile PBS, and then lysed in 20 μL of 1x Passive lysis buffer (Promega, E1941) with gentle shaking at 400rpm at 37°C for 30 minutes. Luciferase activity was then assessed using Luciferase Assay System (Promega, E1510) on a Promega GloMax Luminometer. IC_{50} titer is the reciprocal of sera dilution at which half-maximal neutralization was calculated using GraphPad Prism (version 8.0.0).

Data visualization and statistical analysis

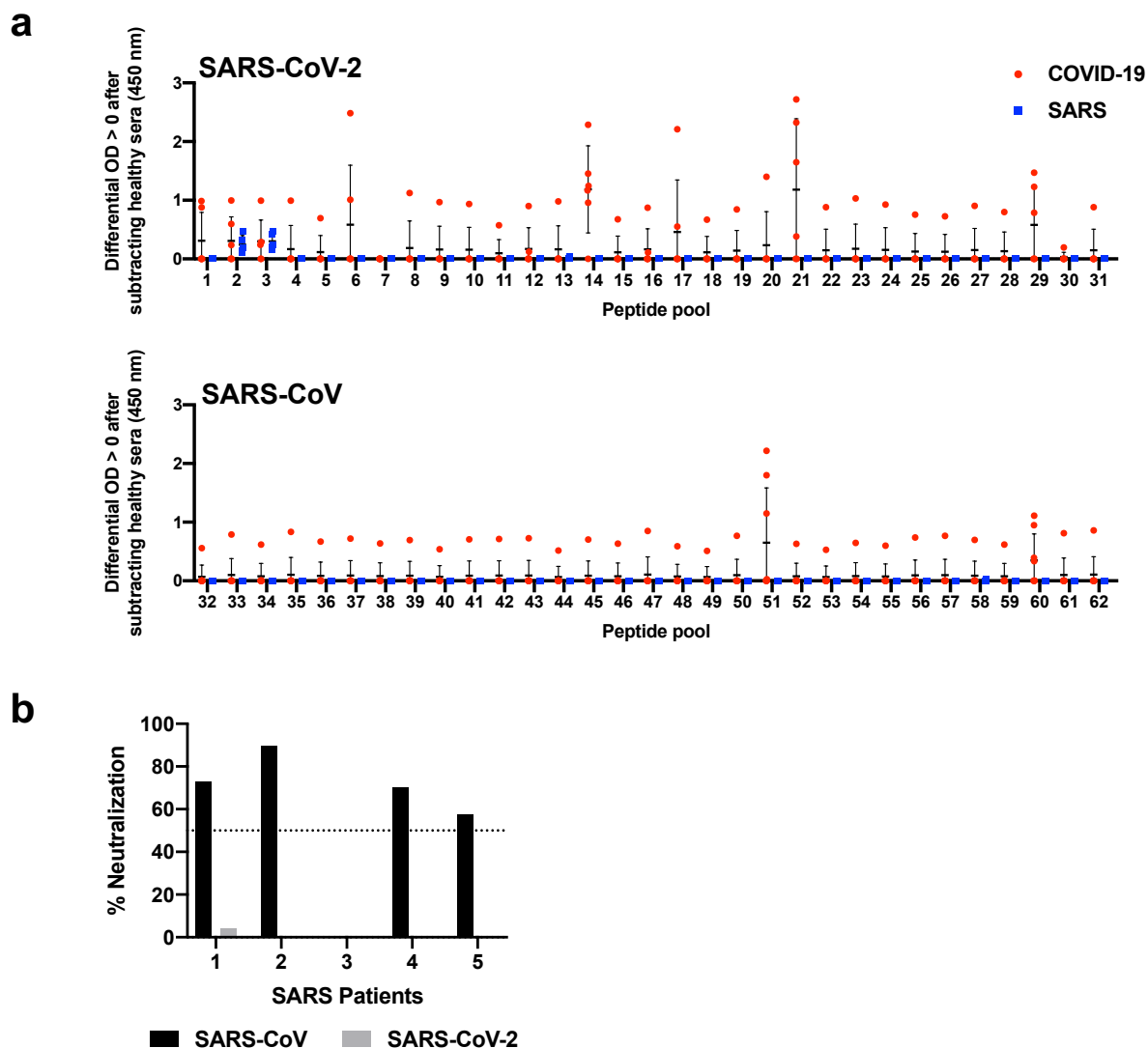
Structural data of SARS-CoV-2 S protein was retrieved from Protein Databank (PDB ID 6VSB) in homotrimeric prefusion conformation and visualized using PyMOL (Schrodinger, version 2.2.0). Heat-maps were generated using GraphPad Prism (version 8.0.0.).

Table 3: List of mutations that appear in from a list of 2596 sequences that were curated by China National Center for Bioinformation (CNCB) extracted on 30 March 2020 <https://bigd.big.ac.cn/ncov> (4)

Peptide	Genome position	Virus number with variation	Annotation Type	Protein.Position.Amino acids change	Gene.Position.Codons	Impact Ensembl Variation - Calculated variant consequences"	Last Update
	23202	1	missense_variant	QHD43416.1:p.547T>I	gene-S:c.1640aCa>aTa	MODERATE	2020-03-29 20:27:38
S14P5	23242	1	synonymous_variant	QHD43416.1:p.560L	gene-S:c.1680ctG>ctT	LOW	2020-03-29 20:27:38
	23347	1	synonymous_variant	QHD43416.1:p.595V	gene-S:c.1785gtC>gtT	LOW	2020-03-29 20:27:38
	23403	717	coding_sequence_variant; missense_variant	QHD43416.1:p.614-; QHD43416.1:p.614D>G	gene-S:c.1841gAt>gRt; gene-S:c.1841gAt>gGt	MODIFIER; MODERATE	2020-03-29 20:27:38
	23986	1	synonymous_variant	QHD43416.1:p.808D	gene-S:c.2424gaT>gaC	LOW	2020-03-29 20:27:38
S21P2	23987	2	missense_variant	QHD43416.1:p.809P>S	gene-S:c.2425Cca>Tca	MODERATE	2020-03-29 20:27:38
	23989	1	synonymous_variant	QHD43416.1:p.809P	gene-S:c.2427ccA>ccG	LOW	2020-03-29 20:27:38
	23996	1	missense_variant	QHD43416.1:p.812P>S	gene-S:c.2434Cca>Tca	MODERATE	2020-03-29 20:27:38
	24011	1	missense_variant	QHD43416.1:p.817F>L	gene-S:c.2449Ttt>Ctt	MODERATE	2020-03-29 20:27:38
	24023	2	missense_variant; synonymous_variant	QHD43416.1:p.821L>I; QHD43416.1:p.821L	gene-S:c.2461Cta>Ata; gene-S:c.2461Cta>Tta	MODERATE; LOW	2020-03-29 20:27:38
	24034	17	synonymous_variant; coding_sequence_variant	QHD43416.1:p.824N; QHD43416.1:p.824-	gene-S:c.2472aaC>aaT; gene-S:c.2472aaC>aaY	LOW; MODIFIER	2020-03-29 20:27:38
	24054	9	missense_variant	QHD43416.1:p.831A>V	gene-S:c.2492gCt>gTt	MODERATE	2020-03-29 20:27:38

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Supplementary Figure 1. Antibody responses of COVID-19 and recovered SARS patients. (a) Preliminary mapping of linear B-cell epitopes within SARS-CoV-2 and SARS-CoV spike (S) protein. Sera of COVID-19 (n=6) and SARS patients (n=5) at 1:1000 dilution were subjected to peptide-based ELISA for IgG using peptide pools from the S protein of SARS-CoV-2 (pools S1-31) and SARS-CoV (pools S32-62) in duplicates. Sera of pooled healthy donors (n=13) were carried out in parallel. Subtracted values are presented after normalization against healthy controls. Data is shown as mean $\pm$ SD. **(b)** Sera of recovered SARS patients (n=5; 1:100 dilution) were mixed with pseudotyped lentiviruses expressing SARS-CoV or SARS-CoV-2 S glycoprotein protein, prior to incubation with CHO-ACE2 cells for 48 h. Infection levels were determined by luciferase assay, and percentage neutralization was analyzed. Dotted line indicates 50% neutralization efficiency.