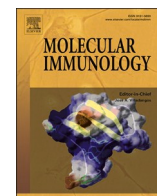




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Identification of virus-specific B-cell epitopes by convalescent plasma from COVID-19 patients

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ABSTRACT

Identification of immunologic epitopes against SARS-CoV-2 is crucial for the discovery of diagnostic, therapeutic, and preventive targets. In this study, we used a pan-coronavirus peptide microarray to screen for potential B-cell epitopes and validated the results with peptide-based ELISA. Specifically, we identified three linear B-cell epitopes on the SARS-CoV-2 proteome, which were recognized by convalescent plasma from COVID-19 patients. Interestingly, two epitopes (S 809–823 and R1ab 909–923) strongly reacted to convalescent plasma collected at the early phase (< 90 days) of COVID-19 symptom onset, whereas one epitope (M 5–19) reacted to convalescent plasma collected > 90 days after COVID-19 symptom onset. Neutralization assays using antibody depletion with the identified spike (S) peptides revealed that three S epitopes (S 557–571, S 789–803, and S 809–823) elicited neutralizing antibodies in COVID-19 patients. However, the levels of virus-specific antibody targeting S 789–803 only positively correlated with the neutralizing rates at the early phase (<60 days) after disease onset, and the antibody titers diminished quickly with no correlation to the neutralizing activity beyond two months after recovery from COVID-19. Importantly, stimulation of peripheral blood mononuclear cells from COVID-19-recovered patients with these SARS-CoV-2 S peptides resulted in poor virus-specific B cell activation, proliferation, differentiation into memory B cells, and production of immunoglobulin G (IgG) antibodies, despite the B-cells being functionally competent as demonstrated by their response to non-specific stimulation. Taken together, these findings indicate that these newly identified SARS-CoV-2-specific B-cell epitopes can elicit neutralizing antibodies, with titers and/or neutralizing activities declining significantly within 2–3 months in the convalescent plasma of COVID-19 patients.

1. Introduction

Historically, human coronavirus (HCoV) infections have led to global pandemics every decade, including severe acute respiratory syndrome (SARS) in 2003, Middle East respiratory syndrome (MERS) in 2010, and the most recent novel coronavirus infectious disease-2019 (COVID-19, caused by SARS-CoV-2) in late 2019. To date, there are nearly 253 million confirmed cases of COVID-19 worldwide, with more than 5 million deaths recorded by WHO (Organization, 2021). In the United

States, more than 46.6 million people have been infected with SARS-CoV-2, resulting in 755,950 deaths, surpassing the approximately 675,000 deaths that occurred in the 1918 Spanish flu pandemic (Johnson and Mueller, 2002). Therefore, a better understanding of the host immune responses to SARS-CoV-2 is critical for combating future HCoV pandemics.

Immunological memory is an essential aspect of the host immune system, as it can respond rapidly and effectively to previously encountered immunogenic epitopes of different pathogens (Janeway et al.,

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2001). As part of the adaptive immune system, memory T cells (MTCs) and memory B cells (MBCs) are long-lived lymphocytes able to memorize the characteristics of the antigenic epitopes during initial infection. Upon re-infection by the same pathogen, MTCs and MBCs trigger an accelerated and robust secondary immune response via direct killing or secreting inflammatory cytokines/chemokines and protective immunoglobulin G (IgG) antibodies to quickly clear the pathogenic infection (Crotty et al., 2003; Weisel and Shlomchik, 2017; Seifert and Küppers, 2016).

There is an urgent need to identify SARS-CoV-2-specific epitopes and to determine if COVID-19-recovered patients can develop sustained antibody and functional MTC/MBC responses that protect against subsequent SARS-CoV-2 infections. Thus far, data on SARS-CoV-2 immunodominant epitopes remain mostly limited to bioinformatic predictions of human T- and B-cell epitopes using computerized tools (Grifoni et al., 2020; Zheng and Song, 2020; Ahmed et al., 2020). In addition, limited studies (Wang et al., 2020; Poh et al., 2020) have analyzed virus-specific epitopes in COVID-19 patients using proteome microarrays, which need to be further validated. We have recently reported that COVID-19 convalescent plasma can block SARS-CoV-2 as well as SARS-CoV spike (S) protein-mediated cell-cell fusion and virus entry (Wang et al., 2021). Moreover, we identified SARS-CoV-2 MTC epitopes through microarray screening and ELISpot using peripheral blood mononuclear cells (PBMCs) from COVID-19-recovered subjects (Zhao et al., 2021).

In the present study, we further identified and characterized SARS-CoV-2-specific B cell epitopes using COVID-19 convalescent plasma. We found that, despite the occurrence of antibodies against these epitopes in the plasma of COVID-19-recovered patients, these antibodies were not long-lasting, lost their neutralizing titers and/or activities within 2–3 months after recovery from the infection. These findings support the notion that the COVID-19 vaccine boosters may be necessary to provide extended protection from re-infection and developing severe symptoms in SARS-CoV-2-recovered patients and COVID-19-vaccinated individuals.

2. Materials and methods

2.1. Study subjects

The study protocol was approved by the joint Institutional Review Board (IRB) of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB# 0519.24 s). Written informed consent was obtained from all participants. The study was comprised two populations: 76 COVID-19-recovered subjects and 44 control subjects, which included 35 healthy subjects (HS), 4 Influenza patients, 1 HIV patient, and 4 HCV patients. All control subjects were all confirmed seronegative for SARS-CoV-2, and all COVID-19 subjects were diagnosed by a positive nuclear acid amplification test (NAAT) and reported recovery from symptoms. Blood samples were collected at least two weeks after the diagnosis. Blood from HS was obtained from BioIVT (Gray, TN). Fresh blood samples were aliquoted and stored at -80°C . The characteristics of these COVID-19 subjects are shown in Supplemental Table 1.

2.2. Peptide microarrays

To map the dominant linear B-cell epitopes recognized in COVID-19 patients, RepliTope Antigen Collection Pan-Coronavirus Peptide Microarrays (Product code: RT-HD-CoV2) were performed by JPT Peptide technologies (Berlin, Germany), using unheated fresh plasma from six COVID-19 patients (S19, S23, S25, S26, S28, and S30) and one HS. The plasma was diluted (1:200) with T20 buffer and incubated for 2 h at 30°C on a microarray slide using the TECAN HS4 \times 00 microarray processing station. Following sample incubation, a secondary, fluorescently labeled antibody specific to human IgG was added at a concentration of 0.1 $\mu\text{g}/\text{ml}$ and incubated for 45 min. After washing and drying, the slides

Table 1

The sequences of selected peptides.

Name	Sequence
SARS-CoV-2 SPIKE glycoprotein (S) 557–571	KKFLPFQQFGRDIAD
SARS-CoV-2 SPIKE glycoprotein (S) 789–803	YKTPPIKDFGGFNFS
SARS-CoV-2 SPIKE glycoprotein (S) 809–823	PSKPSKRSFIEDLLF
SARS-CoV-2 Membrane protein (M) 5–19	NGTITVEELKKLEQ
SARS-CoV-2 Nucleoprotein (N) 161–175	LPQGTTLPGFYAEG
SARS-CoV-2 Nucleoprotein (N) 233–247	KMSGKGQQQGGQTVT
SARS-CoV-2 Nucleoprotein (N) 397–411	AADLDDFSKQLQQSM
SARS-CoV-2 Replicase polyprotein 1a (R1a) 649–663	VKFISTCAEIVGQ
SARS-CoV-2 Replicase polyprotein 1ab (R1ab) 573–587	ISQYSLRLIDAMMFT
SARS-CoV-2 Replicase polyprotein 1ab (R1ab) 909–923	DESGEFLKASHMYCS
SARS-CoV-2 Replicase polyprotein 1ab (R1ab) 2349–2363	LFFSYFAVHFISNSW
SARS-CoV-2 Replicase polyprotein 1ab (R1ab) 3853–3867	IKVATVQSKMSDVKC
SARS-CoV-2 Replicase polyprotein 1ab (R1ab) 4781–4795	LDKRTTCFSVAALTN

were scanned with a high-resolution laser scanner at 635 nm to obtain fluorescence intensity profiles. To enhance weak interactions, the slides were scanned with a receiver gain of 650 V. Finally, images were acquired and quantified to yield a mean pixel value for each peptide. Control incubation with the secondary antibody only (no plasma) was performed in parallel to assess non-specific binding to the peptides and to determine assay performance.

2.3. Peptide-based enzyme-linked immunosorbent assay (Peptide-ELISA)

A total of 13 peptides (Table 1) - that were recognized more often by plasma from COVID-19 patients than HS control - were synthesized by GenScript (Piscataway, NJ) to verify and validate the peptide-antibody interactions using Peptide-ELISA. Next, Invitrogen Nunc MaxiSorp flat-bottom 96-well ELISA plates (Waltham, MA) were coated with 5 $\mu\text{g}/\text{ml}$ of these synthetic peptides in 50 mM carbonate-bicarbonate buffer (Bioworld, Dublin, OH) by incubating overnight at 4°C . The plates were washed three times with PBS buffer containing 0.05 % Tween 20 (PBSTw, 250 $\mu\text{l}/\text{well}$), and then blocked with 3 % BSA/PBSTw for 1 h at 37°C . Plasma from 66 COVID-19-recovered subjects and 26 control subjects (21 HS, 1 HIV, and 4 HCV patients) was diluted (1:100) and incubated with the peptide-coated plates for 1 h at 37°C . After washing three times, the plates were incubated with diluted (1:4000) horseradish peroxidase (HRP)-conjugated mouse anti-human IgG antibody (Fisher, Pittsburgh, PA) for 1 h at 37°C , followed by washing. The plates were developed using ABTS (ThermoFisher, Waltham, MA) and read at 410 nm with a BioTek SYNERGY H1 microplate reader.

2.4. Detection of SARS-CoV-2 NAbs

To assess neutralizing antibodies (NAbs) in COVID-19 patients' plasma that inhibited SARS-CoV-2 S RBD-ACE2 interactions, the SARS-CoV-2 Surrogate Virus Neutralization Test kit was used per the manufacturer's protocol (GenScript). A neutralizing antibody against SARS-CoV-2 S protein was used as a positive control. First, the diluted (1:10) positive and negative controls and patient plasma were, respectively, pre-incubated with HRP conjugated RBD, to allow the nAbs in the positive control and patient plasma to conjugate with HRP-RBD, and then the mixture was added to a 96-well plate pre-coated with human ACE2 protein (hACE2). After washing and incubating with TMB solution, stop solution was added and the absorbance was read at 450 nm using a BioTek SYNERGY H1 microplate reader. The NAB levels were calculated based on their inhibition extents according to the following equation: $[(1-\text{OD value of samples})/(\text{OD value of negative control}) \times 100 \text{ \%}]$.

2.5. Peptide affinity depletion of COVID-19 patient plasma

Invitrogen Nunc MaxiSorp flat-bottom 96-well ELISA plates were pre-coated with 5 $\mu\text{g}/\text{ml}$ of BSA (control), SARS-CoV-2 S protein

(Biolegend, San Diego, CA), S 557–571, S 789–803, or S 809–823 peptide overnight at 4 °C, followed by washing three times with PBS buffer. Plasma from 15 COVID-19 recovered patients (S2, S4, S11, S14, S19, S23, S26, S29, S77, S87, S88, S89, S94, S102, and S157) were diluted (1:100) with Dulbecco's Modified Eagle's Medium (DMEM, Corning, Manassas, VA). 100 µl of diluted plasma was added to each well in the pre-coated 96-well plates. The plates were incubated for 20 min at room temperature, which allowed the adsorption of the antibodies in the plasma to the protein/peptides coated on the plates. After 24 rounds of adsorption, the unbound fraction was collected and used for pseudovirus neutralization assays.

2.6. Pseudovirus neutralization assay

SARS-CoV-2-S Δ19 Pseudotyped Luciferase-EGFP lentivirus was produced as described previously (Wang et al., 2021). The 293T/ACE2 target cells were seeded in a 96-well plate (10^4 cells in 100 µl medium per well) and cultured overnight in a 5 % CO₂ incubator at 37 °C. The depleted patient plasma was mixed with the pseudovirus. Each 100 µl of the mixture contained 30 µl depleted plasma, 40 µl pseudovirus, 15 µl DMEM, 5 µl Fetal Bovine Serum (FBS), 1 % Gibco Penicillin-Streptomycin, and 5 µg/ml polybrene (ThermoFisher). After incubating for 1 h at 37 °C, the mixture was added to the cultured 293T/ACE2 cells. The fluorescent images were captured at 72 h post-infection with an EVOS FL Image System (ThermoFisher). The infected 293 T/ACE2 cells were lysed and the luciferase activities were measured using the Nano-Glo Luciferase Assay System (Promega, Madison, WI) and a BioTek SYNERGY H1 microplate reader.

2.7. B cell activation by SARS-CoV-2 peptides

PBMCs from 8 COVID-19-recovered subjects (collected > 90 days post-infection) and 8 healthy subjects were seeded in 24-well plates ($3-4 \times 10^6$ cells/well), and stimulated without or with 5 µg/ml SARS-CoV-2 S protein, mixed peptides S 557–571, S 789–803 and S 809–823 (1.67 µg/ml each), or 1 µg/ml R848 plus 10 ng/ml IL-2 for 72 h. To measure the expression levels of the following cell surface markers, the cells were harvested and stained with anti-human CD19-FITC, anti-human CD86-PE, anti-human PDL1-APC antibodies (Biolegend); or stained with anti-human CD19-FITC, anti-human CD27-BV421 (Biolegend), anti-human CD20-PerCP-Cy5.5, anti-human CD38-AF700, anti-human IgD-PE-Cy7, anti-human IgG-PE, anti-human IgM-APC (BD Biosciences), and anti-human CD21-SB600 (ThermoFisher) antibodies at RT for 20 min. To measure Ki67 and T-bet expression levels, the cells were pre-stained with anti-human CD19-FITC, anti-human CD20-PerCP-Cy5.5, anti-human CD38-AF700, anti-human CD21-SB600, anti-human CD27-BV421, or anti-human IgD-PE-Cy7 antibodies for 20 min at RT, followed by fixation and permeabilization using the eBioscience Intracellular Fixation and Permeabilization Buffer for 45 min at RT. Next, the cells were stained with anti-human Ki67-PE and anti-human T-bet-APC antibodies (Biolegend) for 1 h at RT. The stained cells were analyzed using a BD FACSymphony A3 cytometer (BD, Franklin Lakes, NJ), and the data were analyzed by FlowJo (Tree Star, Ashland, OR). Unstained cells and cells stained with isotypes and single antibody were used as controls to determine staining background levels and to adjust for multicolor compensation as a gating strategy.

2.8. Statistical analysis

Data were analyzed using Prism 7 software (Irvine, CA) and are presented as mean ± SD or SEM. Comparisons between two groups were made using a parametric paired or unpaired t-test (with or without Welch's correction for unequal or equal SD, respectively) for normally distributed data or non-parametric Wilcoxon paired t-test or Mann-Whitney U-test for non-normal distributions. Comparisons among multiple groups were made using a one-way ANOVA at a 95% confidence

level (Tukey's honest significance test). The magnitude of correlation was analyzed with Pearson correlation coefficient (parametric approach) or nonparametric Spearman correlation. P-values < 0.05 (*) were considered statistically significant, and p-values < 0.01 (**), < 0.001 (***) or < 0.0001 (****) were considered very significant.

3. Results

3.1. Identification of potential B-cell epitopes on the SARS-CoV-2 proteome

The pan-coronavirus microarrays are specifically designed to screen for linear epitopes by measuring plasma antibody reactivity against coronaviral peptides. These microarrays contain 5513 peptides stemming from different HCoV proteins, including 3546 peptides for SARS-CoV-2, 484 peptides for SARS-CoV-1, 506 peptides for MERS, and 457 or 520 peptides for the common colds HCoV 229E or OC43, respectively. As shown in Fig. 1A, each peptide microarray comprises three identical subarrays, and each spot in the microarray represents a single individual peptide. To identify SARS-CoV-2-specific B-cell epitopes, we used convalescent plasma from COVID-19-recovered patients (n = 6) versus HS control (n = 1). After incubating the unheated plasma with the peptide arrays, the antibodies bound with peptides were detected using fluorescently labeled secondary antibodies. As shown in Fig. 1B, among the 5513 peptides stemming from different HCoV proteomes, 13 peptides were identified as strongly reactive (> 2-fold) to the plasma IgG antibodies in at least 3 out of 6 COVID-19-recovered patients compared with the HS control. These 13 peptides were all from the SARS-CoV-2 proteome, including 3 spike protein peptides (S 557–571, S 789–803, and S 809–823), 1 membrane protein peptide (M 5–19), 3 nucleoprotein peptides (N 161–175, N 233–247, and N 397–411), and 6 replicase polyprotein 1 peptides (R1a 649–663, R1ab 573–587, R1ab 909–923, R1ab 2349–2363, R1ab 3853–3867, and R1ab4187–4175). These virus-specific peptides, recognized by antibodies in the COVID-19 convalescent plasma, represent potential B-cell epitopes in the SARS-CoV-2 proteome that warrant further validation and characterization.

3.2. Validation of SARS-CoV-2-specific B-cell epitopes

To validate these potential B-cell epitopes on SARS-CoV-2, we synthesized these 13 peptides (by GeneScript) and assessed their reactivity to both COVID-19 convalescent plasma (n = 66) and control plasma (n = 26) using peptide-based ELISA. As shown in Fig. 2A, the peptides S 809–823, M 5–19, and R1ab 909–923 strongly reacted with convalescent plasma from COVID-19 patients compared with those from controls. We have recently reported that SARS-CoV-2 antibodies decline rapidly in the plasma of COVID-19-recovered patients, with IgG titers decreasing to non-significant levels after 3 months post-infection (Wang et al., 2021). To determine the durability of these antibodies against our identified epitopes, we divided the patient cohort into several subgroups (i.e., <20 days, <60 days, < 90 days, and > 90 days) based on the time of plasma collection after COVID-19 symptom onset. Interestingly, peptides S 809–823 (Fig. 2D) and R1ab 909–923 (Fig. 2G) significantly reacted with convalescent plasma collected at < 20, < 60, and < 90 days after COVID-19 symptom onset, but not with the plasma collected > 90 days after COVID-19 symptom onset. Peptide M 5–19 (Fig. 2E) strongly reacted with convalescent plasma collected at > 90 days of COVID-19 symptom onset, but not with plasma from the other subgroups. We did not observe any significant differences in reactivity of peptide S 557–571 (Fig. 2B), S 789–803 (Fig. 2C), and N 161–175 (Fig. 2F) with convalescent plasma from all subgroups of COVID-19-recovered subjects. These results confirmed our peptide microarray data, validating the reactivity of SARS-CoV-2-specific B-cell immunodominant epitopes and the durability of their specific antibodies in the convalescent plasma of COVID-19 patients.

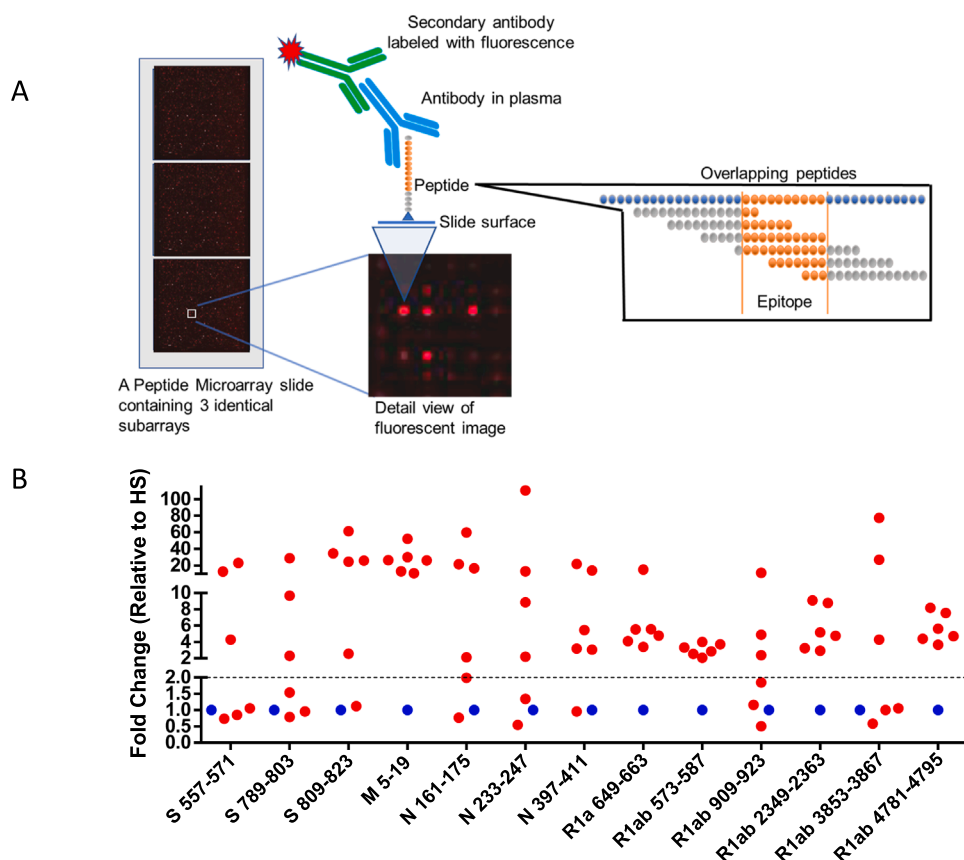


Fig. 1. Identification of SARS-CoV-2 B-cell epitopes using peptide microarray. A) Schematic representation of the RepliTope antigen collection pan-coronavirus microarrays. Each peptide microarray slide contains 3 identical subarrays. In each subarray, antibody reactivities against 5513 overlapping peptides (each peptide has 15 amino acids with 11 amino acid overlaps) were measured by red fluorescently labeled anti-human IgG antibody. B) Peptides strongly reactive (> 2 folds) to the anti-human IgG in at least 3 out of 6 plasma samples from COVID-19-recovered patients (red) compared with HS control (blue).

3.3. Correlation between SARS-CoV-2 peptide-specific antibody titers and neutralization rates

nAbs against SARS-CoV-2 block the interaction between the receptor-binding domain (RBD) of the S protein and the angiotensin-converting enzyme-2 (ACE2) cell surface receptor. To determine the presence of protective nAbs recognized by S1-RBD, 1:10 diluted COVID-19 convalescent plasma was incubated with HRP-conjugated, recombinant SARS-CoV-2-S1 RBD for 30 min at 37 °C before being added to an ACE2 pre-coated ELISA plates. The neutralization rates for blocking SARS-CoV-2 S RBD interaction with the ACE2 receptor were calculated as described in the Materials and Methods. The correlations between the neutralization rates and antibody titers measured by the peptide-based ELISA were analyzed with the Pearson correlation coefficient or nonparametric Spearman correlation, depending on if the data followed a normal distribution. As shown in Fig. 3A–B, E–F, and I–J, there were no correlations between the neutralization rates and antibody titers measured by peptide-based ELISA using the three S peptides (S 557–571, S 789–803, and S 809–823) as coating antigens and plasma from both < 90 days and > 90 days subgroups. Given the rapid decline of the SARS-CoV-2 antibody titers in COVID-19 convalescent plasma, we further analyzed the antibody titers and neutralization rates in samples collected from the < 60 days and < 20 days subgroups. The neutralization rates positively correlated with the S 789–803-reactive antibodies in the plasma collected at < 20 days ($p = 0.0371$, $r = 0.7113$) and < 60 days ($p = 0.0225$, $r = 0.3772$) (Fig. 3G–H), suggesting that S 789–803 peptide is a neutralizing epitope. There were no correlations between the neutralization rates and antibodies targeted with S 557–571 and S 809–823 in the plasma collected at both < 60 days and < 20 days subgroups (Fig. 3C–D, K–L). To compare the titers of S1-specific antibodies and S peptide-reactive antibodies, their correlations were analyzed and the patterns were very similar with those between the neutralization rates and S peptide-specific antibody titers (Data not

shown). Notably, the S 789–803-reactive antibodies had positive correlation with the S1-specific antibodies in the patient plasma collected at < 60 days ($p = 0.0236$, $r = 0.3819$), which further indicates that S 789–803 peptide is a neutralizing epitope. Taken together, these data suggest that the levels of antibodies in COVID-19 convalescent plasma that target the S 789–803 peptide positively correlate with their neutralizing activities at the early phase of COVID-19 recovery, however, the titers of these SARS-CoV-2 B-cell epitope-reactive antibodies diminish quickly, with no correlation to the nAb activity beyond 2 months of recovery.

3.4. Characterization of SARS-CoV-2 neutralizing epitopes

A pseudotyped lentivirus system expressing SARS-CoV-2 S glycoprotein tagged with green fluorescent protein (GFP) and a luciferase reporter allow rapid and safe assessment of nAbs in convalescent plasma of COVID-19 patients (Wang et al., 2021; Schmidt et al., 2020). To further assess if the antibodies against SARS-CoV-2 S epitopes could interfere with the interaction between SARS-CoV-2 S RBD and host cell ACE2 receptor, we performed a modified neutralization assay using Ab-depleted plasma and the identified S peptides and compared it with the corresponding non-depleted plasma that has relatively high titers of virus-specific antibodies as blocking agents. The Ab depletion was carried out by incubating the plasma with SARS-CoV-2 S protein or B-cell epitope-coated plates for 24 rounds, as described in the Materials and Methods. Depletion efficiency was validated by peptide-based ELISA, and the results showed that the levels of the antibodies targeting S protein, S peptides or M peptide were significantly and dramatically dropped compared with non-depleted controls (Fig. 4A). The non-depleted and Ab-depleted plasma from COVID-19-recovered patients were then incubated with the SARS-CoV-2-S Δ 19 pseudotyped luciferase-EGFP lentivirus for 1 h at 37 °C, followed by infection of 293 T/ACE2 cells for 3 days as we reported previously (Wang et al.,

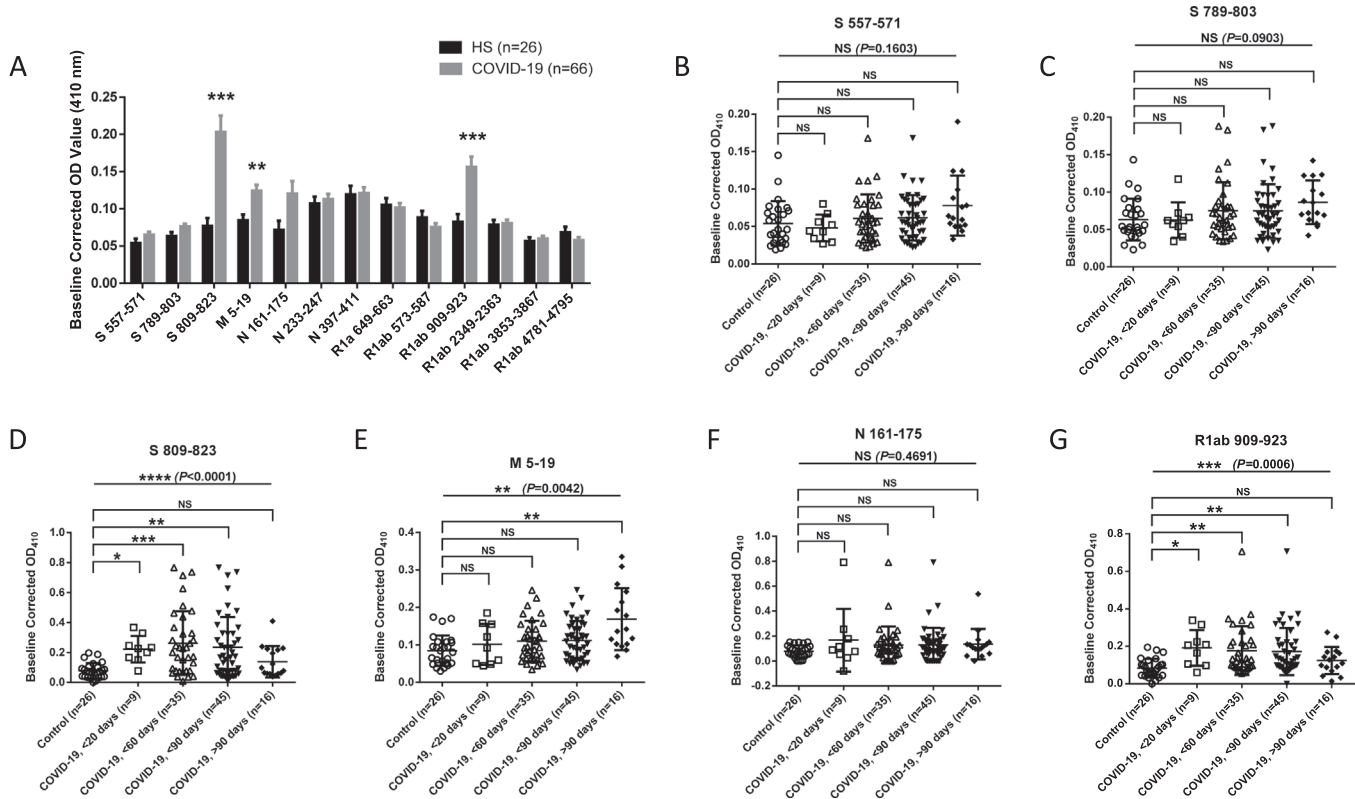


Fig. 2. Validation of SARS-CoV-2 B-cell epitopes using convalescent plasma from COVID-19-recovered patients using peptide-based ELISA. A) Identification of SARS-CoV-2 B-cell epitopes reactive to plasma from 66 COVID-19-recovered patients versus 26 controls by peptide-based ELISA using 13 peptides selected from the peptide microarray. * $P < 0.01$; *** $P < 0.001$, as determined by paired t-test, and mean \pm SEM. B–G) Time-dependent Ab responses to the selected epitopes (shown on the top of each figure) were assessed by peptide-based ELISA. The patients were divided into subgroups based on the time plasma was collected in relation to COVID-19 symptom onset: > 90 days, < 90 days, < 60 days, and < 20 days. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS = not significance, as determined by one-way ANOVA, and mean \pm SD.

2021). The fluorescent images were captured before measuring luciferase activities, and the representative images are shown in Fig. 4B. Compared with the cells treated with the non-depleted or BSA-depleted patient plasma, we observed significantly more EGFP (green) positive cells in the infected 293 T cells treated with S 557–571-, S 789–803-, or S 809–823-depleted plasma - similar to those treated with S protein-depleted plasma. There was no much difference on EGFP green cells between the infected 293T cells treated with M5–19-depleted and BSA-depleted plasma. These data indicated that the inhibition of the interaction between S protein on the pseudovirus and ACE2 on 293T cells was attenuated due to the depletion of nAbs in COVID-19 convalescent plasma by these three S peptides (i.e., neutralization epitopes), resulting in an increased pseudovirus infection. Consistent with the EGFP images, data from the luciferase assay (Fig. 4C) showed that the nAbs of COVID-19 convalescent plasma were significantly suppressed by the depletion of antibodies that target the S protein and these three S peptides, but not M 5–19, compared with the control (BSA)-depleted plasma. Taken together, these results demonstrate that the specific antibodies targeting the S 557–571, S 789–803, and S 809–823 peptides play an important role in neutralizing SARS-CoV-2 infection and that these virus-specific peptides represent SARS-CoV-2 neutralizing epitopes capable of eliciting B-cell nAb responses.

3.5. B cell activation in response to identified B-cell epitopes of SARS-CoV-2

To measure B-cell responses to virus-specific and non-specific stimulations, we cultured PBMCs from COVID-19-recovered patients and HS in the presence or absence of SARS-CoV-2 S protein, mixed S peptides (S 557–571, S 789–803 and S 809–823), or the rhIL-2 + R848 for 24 h,

followed by flow cytometry analysis to determine the levels of B-cell activation, proliferation, and differentiation markers.

CD86, CD25 and CD30 are transmembrane glycoproteins expressed in activated B cells. CD86 is rapidly upregulated on B cells following activation by the cross-linking of the Ig receptor or a variety of cytokines (Lenschow et al., 1993). B cells expressing CD25 display a mature and activated phenotype belonging to the memory B-cell subset and have a more robust proliferative and antigen-presenting capacity (Amu and Brisslert, 2011). CD30 has been recognized as a unique marker in many lymphomas and as an activation molecule on B cells (Muta and Podack, 2013). We first measured the expression of CD86 on CD19⁺ cells in PBMCs of COVID-19-recovered patients. As shown in Fig. 5A, while COVID-19 patients' B cells responded to the rhIL-2 and R848 non-specific stimulation as robustly as those of HS, they didn't exhibit any increase in CD86 expression in response to SARS-CoV-2 S protein or mixed S peptide-specific stimulation compared with B cells from HS or unstimulated B cells. The expression of CD25 and CD30 was extremely low, with no significant differences amongst all groups (data not shown).

PDL-1 is the ligand for the PD-1 inhibitory receptor and serves as an activation marker on antigen-presenting cells, including B-cells. Similar to CD86 expression, we found that PDL-1 level was remarkably and equally upregulated by the non-specific stimulation (rhIL-2/R848) of B cells from both HS and COVID-19-recovered patients, indicating their potent responsiveness. However, while PDL-1 expression was significantly upregulated in COVID-19 patients' B cells stimulated with SARS-CoV-2 S protein compared with HS or unstimulated B cells, this antigen-specific upregulation was not observed in B cells stimulated by the mixed S peptides (Fig. 5B).

To determine memory B cell differentiation and function in response

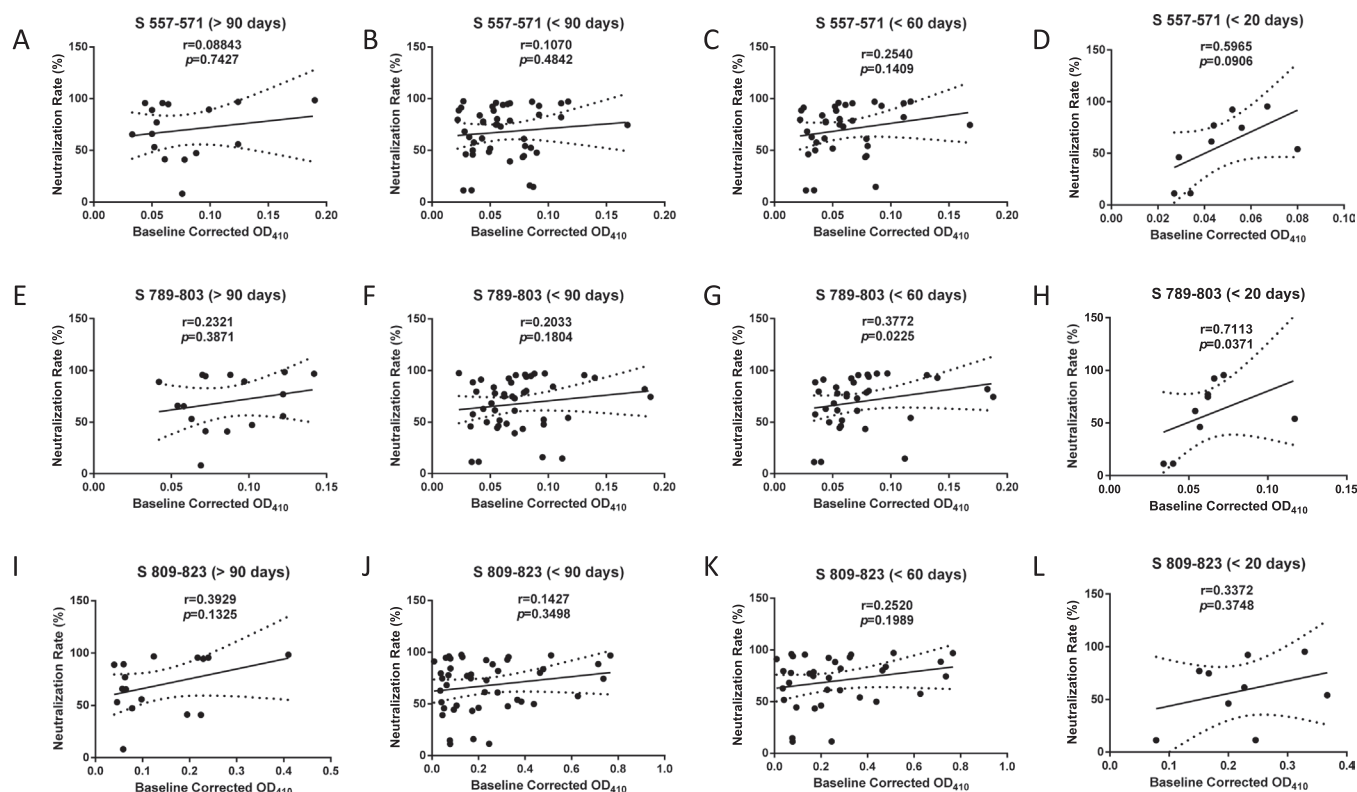


Fig. 3. Correlation analysis of antibody levels measured by neutralization assay and peptide-based ELISA. The magnitude of the correlation was analyzed by either Pearson correlation coefficient (parametric, Fig. 3D, E, and L) or nonparametric Spearman correlation (Figs. 3A–C and 3F–K), depending on if the data followed a normal distribution.

to this antigenic stimulation, we treated PBMCs as described above and stained them with anti-human CD19, CD20, CD38, CD21, CD27, and Ki67, IgG, or IgM antibodies for flow cytometry analysis. The gating strategy for naïve ($CD19^+CD20^+CD38^-CD21^+CD27^-$) and memory B cells ($CD19^+CD20^+CD38^+CD21^+CD27^-/CD21^+CD27^+/CD21^+CD27^+$) are shown in Fig. 5C. While the overall frequencies of memory B cells were slightly higher in COVID-19-recovered patients compared with HS, we did not observe increases in the memory B cells stimulated with SARS-CoV-2 S protein or mixed S peptides (data not shown). Similar to the pattern of PDL-1 expression, IgG production by memory B cells was significantly upregulated when PBMCs from COVID-19 patients were stimulated with SARS-CoV-2 S protein but not S peptides when compared with unstimulated control or HS (Fig. 5D). There were no significant differences in IgM⁺ memory B cell numbers amongst all groups when PBMCs were subjected to virus-specific stimulation (data not shown). Also, there was no significant difference in Ki67 (a proliferation marker) expression in memory B cells amongst all groups following virus-specific stimulation of PBMCs, although the Ki67 level was remarkably and equally upregulated in memory B cells in response to rHL-2/R848 stimulation (data not shown).

Previous studies showed that antigen-specific memory B cells expressing transcription factor T-bet persisted in circulation over time after primary infection or vaccination, and their number correlates with long-lived antibody responses. T-bet⁺ memory B cells reactivated by reinfection rapidly proliferate and then differentiate into protective plasmablasts to produce high-affinity and viral-specific IgG antibodies (Nellore et al., 2019; Knox et al., 2019, 2017). We thus measured T-bet expression in memory B cells within PBMCs in response to virus-specific stimulation. As shown in Fig. 5E, there was no significant difference in T-bet expression in memory B cells in the absence of stimulation. Importantly, stimulation with both S peptides and S protein dramatically expanded the T-bet⁺ memory B cell population within PBMCs from COVID-19-recovered patients compared with HS.

Taken together, these results suggest that virus-specific B-cell activation in response to the identified SARS-CoV-2-specific epitopes is attenuated in COVID-19-recovered patients, albeit T-bet expression remains responsive to virus-specific stimulation.

4. Discussion

In this study, we identified virus-specific B-cell epitopes of the SARS-CoV-2 proteome and characterized both the durability of their neutralizing antibodies in convalescent plasma and the function of memory B cells from COVID-19-recovered patients. We demonstrated that while these SARS-CoV-2 B-cell epitopes can elicit virus-specific antibodies, these antibodies are not durable in the convalescent plasma and lose their neutralizing activities within a few months after recovery from COVID-19 disease. In addition, we found that B-cell activation in response to the identified virus-specific epitopes is attenuated in COVID-19-recovered patients. These findings potentially support the notion that COVID-19-recovered individuals might require booster dosing of the COVID-19 vaccine to extend protection from severe disease and detrimental outcomes.

The PepStar Peptide Microarrays (by JPT Peptide technologies) are an attractive tool for identifying potential biomarkers for infectious diseases, autoimmune diseases, cancer, and allergies, and for uncovering protein-protein interactions. Because of the high cost of the Peptide Microarrays, only 6 COVID-19 patient plasma samples were used for initial microarray screen. To avoid data bias due to the small number of patients, the selected patients had high diversities on their ages, genders, IgG antibody titers, and neutralization rates (Supplemental Table 1). Using this powerful microarray, we identified the B-cell epitopes on SARS-CoV-2 and then validated their reactivity by peptide-based ELISA using convalescent plasma from COVID-19-recovered patients. Notably, the antibody titers measured by the peptide-based ELISA positively correlated with the antibody neutralizing rates in a time-dependent

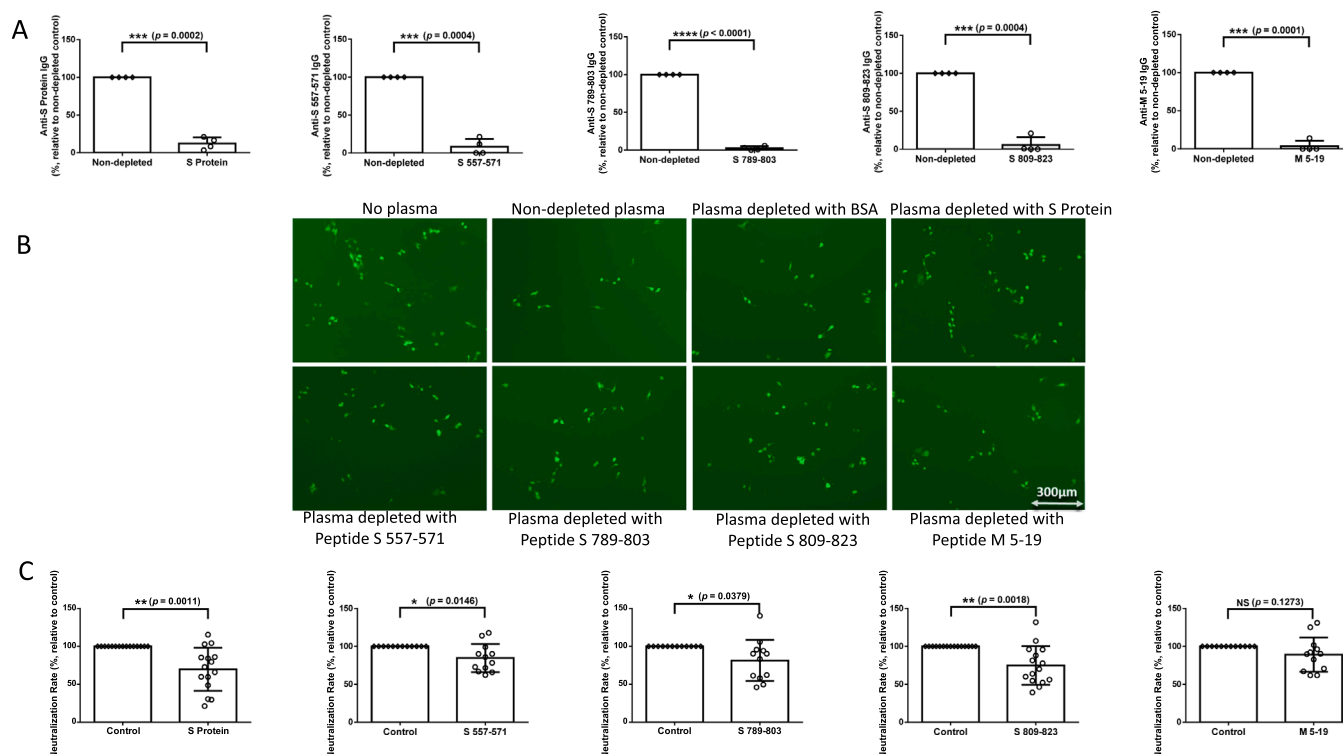


Fig. 4. Identification of neutralizing epitopes using Ab-depletion and neutralization assays. A) Ab-depletion efficiency validated by the peptide-based ELISA. Values were normalized to the non-depleted control. The data are summarized using 4 plasma samples from COVID-19-recovered patients. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS = not significance, as determined by paired t-test, and mean \pm SD. B) Representative fluorescent images for neutralization assays using SARS-CoV-2 S Δ 19 pseudotyped luciferase-EGFP lentivirus infection of 293 T/ACE2 cells in the presence of blocking plasma depleted with the indicated peptides. C) The percentage (%) of neutralization by plasma antibodies depleted of the respective peptides. Values were normalized to the BSA control. The data are summarized from the antibody-depletion assays using 12 (S 557–571, S789–803, and M 5–19) or 15 (S protein and S 809–823) plasma samples from COVID-19-recovered patients. * $P < 0.05$; ** $P < 0.01$; NS = not significance, as determined by paired t-test, and mean \pm SD.

manner. We determined the function of these immunodominant epitopes using a lentivirus system expressing SARS-CoV-2 S glycoprotein that allows for the rapid and safe assessment of nAbs in convalescent plasma of COVID-19 patients in a BSL-2 laboratory. Functionally, antibody depletion with the identified S peptides (S557–571, S789–803, and S809–823) showed that antibodies targeting these three epitopes significantly altered viral neutralization capacity, suggesting that these epitopes are indeed neutralizing sites on the S protein of SARS-CoV-2. Notably, the S557–571 epitope is located on the S1 subunit of the S protein proximal to the RBD. It is highly likely that the antibodies that target this site interfere with SARS-CoV-2 S glycoprotein binding to the ACE2 receptor, thereby abolishing virus entry and infection. The other two epitopes identified in this study (S789–803 and S809–823) are localized on the S2 subunit and cover part of the fusion domain, which is relatively conservative amongst HCoV (Bangaru et al., 2020). Previous studies showed targeting the SARS-CoV, SARS-CoV-2, and MERS-CoV fusion domain suppressed HCoV infection with a pan-HCoV peptide inhibitor (Xia et al., 2019, 2021). Importantly, our peptides S557–571 and S809–823 overlap with the S14P5 and S21P2 peptides that were previously described (Poh et al., 2020; Amrun et al., 2020). These two independent studies showed consistent and significant recognition of S809–823 by plasma from recovered SARS-CoV-2 patients, which further support our findings.

The expression of T-bet - a reliable marker of memory B cell antiviral function - is increased during SARS-CoV-2 infection, especially in symptomatic patients (Notarbartolo et al., 2021; Reyes et al., 2021; Rodda et al., 2021), and is maintained at high levels for a few months after disease onset (Rodda et al., 2021). At this point, the frequency of virus-specific T-bet⁺ IgG⁺ memory B cells decreases to a baseline level (Reyes et al., 2021). Our data further demonstrated that T-bet⁺ memory

B cells rapidly declined in COVID-19 patients after 3 months of recovery. Significantly, we found that their levels can be upregulated upon re-exposure to SARS-CoV-2 S protein or S peptides, suggesting that protective immune responses could be re-induced in these recovered patients.

Remarkably, we didn't detect a robust or universal B-cell response (other than T-bet expression) in PBMCs from COVID-19-recovered patients following stimulation with a mixture of these linear neutralizing peptides, despite memory B cell response to the S protein or non-specific stimulation. It is likely that these B-cell epitopes only represent a portion of the total anti-S antibody response (Lv et al., 2020; Wu et al., 2020). It is also possible that these synthetic linear B-cell peptides lack the structural conformation and post-translational modifications of the whole S glycoprotein, which are essential for efficient antigenic stimulation. Future studies are needed to fully understand the role and neutralization capacity of antibodies targeting these epitopes and the durability of memory B cells in the blood of COVID-19-recovered patients. Nevertheless, our studies provide a guide for the design and evaluation of sensitive and specific serology tests against these virus-specific B-cell epitopes and may help in prioritizing and improving vaccine and therapeutic targets against this devastating infectious disease.

CRedit authorship contribution statement

Ling Wang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – original draft, Writing – review & editing. **Juan Zhao:** Investigation, Writing – review & editing. **Madison Schank:** Investigation, Writing – review & editing. **Sushant Khanal:** Investigation. **Xindi Dang:** Investigation. **Dechao Cao:**

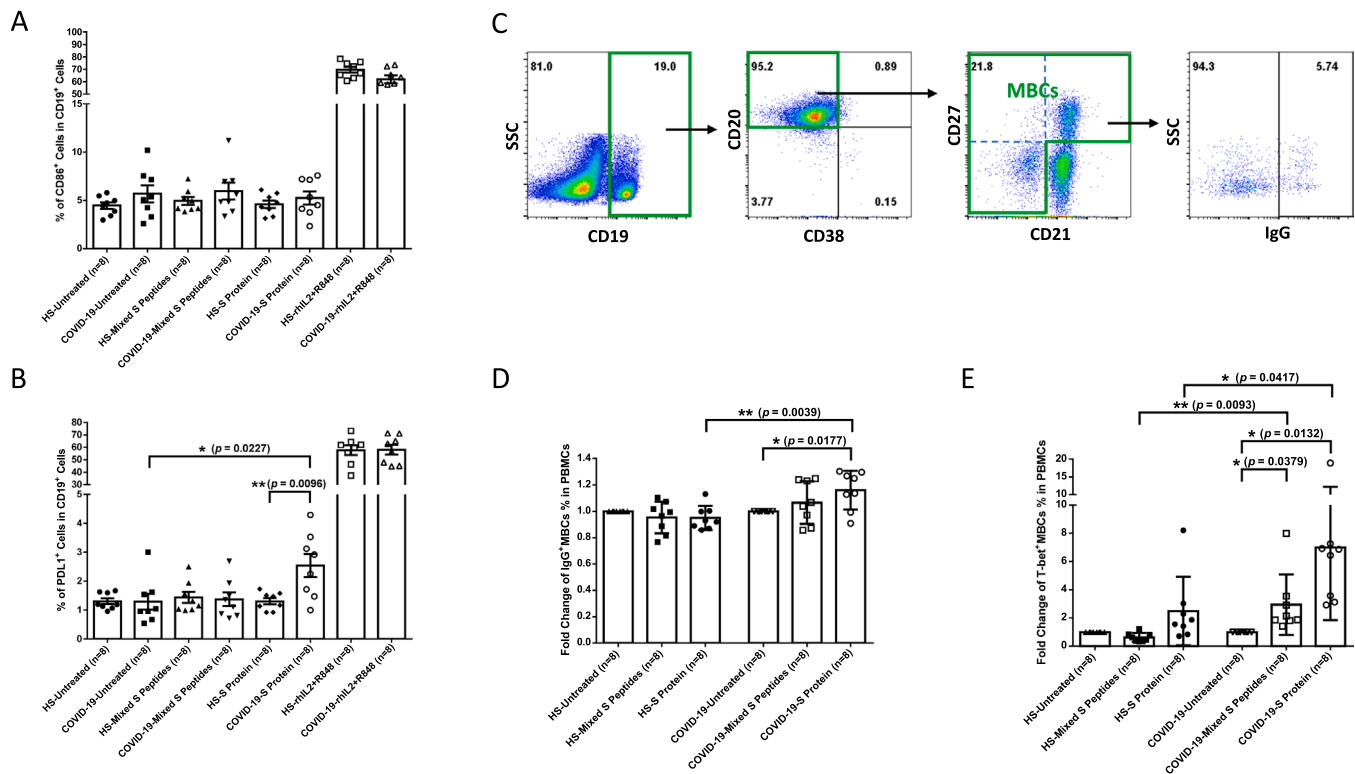


Fig. 5. Characterization of B-cell responses in PBMCs from COVID-19-recovered patients and HS. A) Assessment of activation marker CD86 expression on B-cells from COVID-19 patients and HS by flow cytometry. The percentages (%) of CD86⁺ cells in CD19⁺ B cells within PBMCs without or with virus-specific (S protein or peptides) and non-specific (rhIL-2 + R848) stimulation are shown. Each symbol represents one individual. B) Assessment of activation marker PDL-1 expression on B-cells from COVID-19 patients and HS by flow cytometry. The percentages of PDL-1⁺ in CD19⁺ B cells within PBMCs without or with virus-specific (S protein or peptides) and non-specific (rhIL-2 + R848) stimulation are shown. Each symbol represents one individual. C) Gating strategy of flow cytometry for IgG⁺ memory B cells. Memory B cells were defined as CD19⁺CD20⁺CD38⁺CD21⁺CD27⁺/CD21⁺CD27⁺/CD21⁺CD27⁺. D) Fold-change in the percentage of IgG⁺ memory B cells within PBMCs from COVID-19 patients and HS without or with virus-specific (S protein or peptides) stimulation. E) Fold-change in the percentage of T-bet⁺ memory B cells within PBMCs from COVID-19 patients and HS without or with virus-specific (S protein or peptides) stimulation. *P < 0.05; **P < 0.01; NS = not significance, as determined by paired and unpaired t-test, and mean ± SD.

Investigation. **Lam N.T. Nguyen:** Investigation. **Yi Zhang:** Investigation. **Xiao Y. Wu:** Project administration. **James L. Adkins:** Resources. **Justin Brueggeman:** Investigation. **Jinyu Zhang:** Writing – review & editing. **Shunbin Ning:** Writing – review & editing. **Mohamed El Gazzar:** Writing – review & editing. **Jonathan P. Moorman:** Writing – review & editing, Funding acquisition. **Zhi Q. Yao:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Competing interests

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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Author Contributions

L.W. performed most of the experiments, J.Z., M.S., S.K., X.D., D.C., L.N.T.N., Y.Z., J.L.A and J.B. performed some experiments. J.P.M. coordinated human subject recruitment. X.Y.W provided technical support. M.E., J.Y.Z. S.N., and J.P.M. provided intellectual input for troubleshooting and discussed the results. Z.Q.Y. supervised the research and wrote the manuscript, with the help of all co-authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.molimm.2022.10.016](https://doi.org/10.1016/j.molimm.2022.10.016).

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