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Dominant and cross-reactive S1-specific memory B cell response induced by primary SARS-CoV-2 infection

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The protective component of specific memory B cells (MBCs) response relative to serum antibody response in primary SARS-CoV-2 infection is not well understood. Using a relatively unbiased B-cell culture method with a limited number of MBCs in each well (100 cells/well), we characterized the fine specificity of MBC responses against SARS-CoV-2 infection. While serum spike antibody is predominantly against S2 domain, the memory B cells mainly recognize S1 domain. The 44.4–85.3% of S-binding MBCs are specific to S1 domain. High frequency of MBCs (30–62% of SARS-CoV-2 S-specific MBCs) cross-reacting with SARS-CoV S has also been demonstrated. 22–33% of S1-binding MBCs were cross-reactive with the SARS-CoV RBD. In addition, a panel of human monoclonal Ab was derived from S1-binding MBCs recognizing six group epitopes (groups 1–6). Among them, RBD-specific Ab (826) in group 4 and cross-reactive Ab (808) could resist the neutralizing escape of omicron. Herein, we demonstrated that a dominant S1-directed MBC response was generated during primary SARS-CoV-2 infection. More importantly, the cross-reactive RBD-directed MBCs against SARS-CoV may protect against emerging SARS-CoV-2 variants.

Several studies have shown that an antibody response to SARS-CoV-2 is elicited during natural infection, although it varies widely among individuals[1–3]. As with other pathogens, specific antibodies (Abs) are first secreted into the serum by plasmablast cells during SARS-CoV-2 infection. These Ab-secreting cells are differentiated from the rapidly activated B cells outside the follicle of the lymph nodes, this process is known as the extrafollicular antibody response. Serum Ab levels are maintained by the long-lived plasma cells that originate from the follicular germinal center (GC) response[1,2,4]. During the two phases of the humoral immune response, specific memory B cells (MBCs) are generated[1,2,4].

Typically, MBCs circulate in the peripheral blood to monitor invading pathogens. Thus, individuals recovering from SARS-CoV-2 infection usually develop specific MBCs that provide a rapid response upon re-exposure. These specific MBCs can persist for years after primary SARS-CoV-2 infection, even when specific Abs are not detectable in serum[5–7]. Furthermore, specific MBC immunity is more robust in corona virus disease 2019 (COVID-19) convalescents than in vaccinees[6,8]. These MBCs can rapidly proliferate and differentiate into Ab-secreting cells upon activation by antigens from re-exposure to the virus or vaccination[4,9]. New antigenic variants of SARS-CoV-2 are constantly emerging[10], and it is of great importance to know whether an MBC recall response can protect from the new variants. This reinforces the need better to understand the specific MBC response, compared to circulating serum response, after SARS-CoV-2 infection.

Several studies suggest that consecutive antigen exposures resulted in high affinity and cross-reactive neutralizing antibodies (nAbs)[2,11–13]. Hybrid immunity was generated in individuals who were vaccinated after

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SARS-CoV-2 infection or who had breakthrough infections after vaccination. Such hybrid immunity tends to produce cross-reactive nAbs that can target more conserved parts of the S protein and effectively neutralize the variants of concern (VOCs)[14,15]. A broad-spectrum SARS-CoV nAb (SA55) has been isolated from a SARS-CoV-2 vaccinated SARS convalescent and can neutralize a broad spectrum of SARS-CoV-2 variants, including the newly emerging JN.1 variant[16]. What about the cross-reactive antibody response generated by primary SARS-CoV-2 infection? The MBC-derived human monoclonal Abs (hmAbs) that exhibited cross-reactivity with SARS-CoV and neutralized most of the VOCs except the omicron variant and its sub-lineages were reported[17]. Sakharkar et al. found that 37–62% of MBC-derived mAbs from COVID-19 convalescent donors were cross-reactive with SARS-CoV spike (S) protein, of which only 0.6% (3/540) had potent cross-neutralizing activity, and all of these targeted the RBD region[18]. A detailed study of the composition of the MBC and the circulating serum antibody would help researchers and clinicians to better understand the picture of antibody response upon infection.

Here, using a relatively unbiased B-cell stimulation-based method with a limited number of MBCs in each well (100 cells/well), we characterized the specific MBC response in comparison to serum antibody response of convalescent individuals with primary SARS-CoV-2 infection. In addition, functional analysis of specific MBCs was performed through MBC-derived mAbs. Our study observed a dominant S1-directed MBC response during primary SARS-CoV-2 infection. Moreover, the cross-reactive RBD-directed MBCs against SARS-CoV may be protective against the emerging SARS-CoV-2 variants.

Materials and methods
Plasma and peripheral blood mononuclear cell (PBMC) samples
All experimental protocols were approved by the Human Ethics Committee of Guangzhou Eighth People's Hospital (202020153) and carried out in accordance with relevant guidelines and regulations. Blood samples were collected in May 2020 from three convalescent individuals infected with the primary SARS-CoV-2 virus during the first epidemic in Wuhan, China. The patients all had a history of close virus contact, positive RNA results for SARS-CoV-2 infection, and were admitted to Guangzhou Eighth People's Hospital, Guangzhou Medical University, China. Each participant provided written informed consent. Plasma samples were clarified, aliquoted, and stored at −80 °C until use. Fresh PBMCs were obtained from the patients after four months of SARS-CoV-2 infection and were used for B-cell isolation by fluorescence-activated cell sorting (FACS).

Expression of S proteins and their subdomains
Specific proteins were expressed in mammalian cells. The coding sequence of the S ectodomain of SARS-CoV-2 (NC_045512.2) or SARS-CoV (NC_004718.3) was inserted into the pcDNA3.1 expression vector (Invitrogen, Waltham, MA) with a D7 tag at the C-terminus of each S protein gene as previously reported[22]. A vector expressing the full-length SARS-CoV-2 nucleocapsid protein (N) (NC_045512.2) was also constructed. The expression vectors for the S subdomain proteins (S1, S2, and RBD for SARS-CoV-2 and RBD for SARS-CoV) were constructed using the strategy described above. The recombinant plasmid was transfected into 293T mammalian cells. After culturing, protein-containing supernatants were collected, cleared by centrifugation, and stored at −80 °C until use. Prior to be used in the ELISA tests, antigen-containing supernatants were verified by WB using specific Abs and the D7-tag Ab to ensure that the same amounts of antigen were added to the plates.

Ab detection in plasma samples by ELISA
Plasma samples for Ab detection were collected from three convalescent COVID-19 patients (Pt1, Pt2, and Pt3) at two or four-time points between day 27 and day 122 post-infection. Specific IgG Abs against SARS-CoV-2 S, S1, S2, RBD, and N proteins were tested using capture ELISA[20]. An anti-D7 tag Ab[19] was precoated on the ELISA microplates and captured the specific antigen (S, S1, S2, RBD, or N protein) in the 293T cells transfected with the expression vector supernatant. Plasma samples were diluted at 1:100 dilution and followed by a 3-fold serial dilution. The diluted plasma samples were then added to the plates. The subsequent procedures followed the standard ELISA experiment. Each dilution was tested in duplicate. The supernatant of 293T cells transfected with the empty vector was used as a negative control. The Area Under the Curve (AUC) was calculated using GraphPad Prism 7. Cross-reactive Abs in plasma samples against SARS-CoV S and RBD were also tested.

B-cell activation in vitro
Memory B cells obtained from convalescent individuals after four months of SARS-CoV-2 infection were activated and expanded into Ab-secreting cells using a B-cell culture approach as previously reported[21]. First, MBCs in fresh PBMCs were isolated by flow cytometry (MoFlo Astrios EQ, Beckman Coulter) using a panel of fluorochrome-conjugated anti-human monoclonal antibodies specific for the identification of B-cell subsets (DuraClone IM B cells Tube, B53318, Beckman Coulter). IgG MBCs were designated CD19$^+$IgD$^-$IgM$^-$CD27$^+$CD38^{low}. Next, IgG MBCs were sorted into 96-well culture plates at 100 cells per well and cultured with EBV (cultures of B95.8 cells, ATCC VR-1492) and CpG (ODN 2006, Invivogen tlrl-hodnb-5) in the presence of irradiated PBMCs from healthy blood donors as feeder cells. Finally, after 10 days of culture, ELISA was used to determine specific Abs in the supernatants of the culture plates.

Analysis of memory B-cell specificity and cross-reactivity
SARS-CoV-2 S, S1, and N proteins were used to detect specific Abs in the supernatants of the culture plates by capturing ELISA (described above). The B-cell culture supernatant from the same well was also tested for cross-reactive Abs against SARS-CoV S and RBD proteins using the same ELISA procedure.

Generation of HmAbs from MBCs

Cell pellets in the wells of B-cell culture plates were used for mAb isolation when culture supernatants showed positive binding to a SARS-CoV-2 S protein. RNA was extracted from cell pellets and reverse transcribed to cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Waltham, MA). As previously reported, the cDNA was used as a template for the nested PCR with gene-specific primers or primer mixes to amplify the transcripts of Ab heavy and light variable genes[22]. Purified PCR products extracted from agarose gel were digested with the restriction enzymes AgeI/SalI, AgeI/BsiWI, or AgeI/XhoI (ThermoFisher Scientific, Waltham, MA) and then inserted into human IgG1, Igκ, or Igλ expression vectors through ligation[22]. The recombinant Ab-expressing constructs were sequenced and analyzed using a sequence alignment software for immunoglobulin variable region sequences, IMGT/V-QUEST (IMGT, France).

Ab competition assay by BLI

Bio-layer interferometry (BLI) was used for the Ab competition assay on the Octet K2 system (ForteBio, Fremont, CA). The biotin-labeled (EZ-LinkTM NHS-PEG12-Biotin, ThermoFisher Scientific, A35389) anti-D7 Ab was immobilized on the streptavidin (SA) sensor (Sartorius, 18-5019) at 40 µg/ml. After equilibration in PBS, SARS-CoV-2 S1 protein in the supernatant of recombinant vector-transfected 293 T cells was captured by anti-D7 Ab. After equilibration, Ab1 and Ab2 or Ab2 and Ab1 were added in tandem. As a control, the response was also determined with Ab1 or Ab2 alone (400 nM). Residual binding was calculated as the percentage binding of the second antibody in the first antibody's presence relative to the second antibody's binding alone.

Pseudotyped virus neutralization assay

A flow cytometry-based neutralization assay detected SARS-CoV-2 neutralizing antibody activity. Pseudotyped lentiviral particles were prepared by transfecting HEK 293T cells with three plasmids (psPAX2, pLVX-eGFP and p19eH-IgG1-WTS or pcDNA3. 1(+)-Omicron S, provided by Jia Runqing, Beijing University of Technology, and Peng Tao, Guangzhou Medical University) encoding human immunodeficiency virus type 1 (HIV-1) backbone proteins, eGFP, and SARS-CoV-2 wildtype S or omicron S protein (B.1. 1.529). The generated HIV-1-based pseudovirus carries the enhanced green fluorescent protein (eGFP).

The neutralization assay was performed in HEK293T-hACE2 cells (Biofragon, China). HEK-293T-hACE 2 cells ($1–2 \times 10^4$ cells/well) were seeded into 96-well plates one day before inoculation. Pseudovirus wildtype or omicron (B.1. 1.529) was mixed with a serial dilution of anti-S mAb, incubated at 37 °C for 1 h, and added to each well. Cell-only and virus-control wells were included in each plate.

After 48 h, HEK293T-ACE2 cells were harvested, and eGFP-positive cells were quantified by flow cytometry using a Guava easyCyte (Luminex). Results were analyzed using GuavaSoft 5.0 (Luminex). IC_{50} was determined using a four-parameter logistic regression model (GraphPad 7.0). At least two independent experiments were performed for the neutralization assay.

Authentic virus neutralization assay

The neutralization potential of the Ab to authentic virus was evaluated using a focus reduction neutralization test (FRNT)[23]. Briefly, a serially diluted Ab was mixed with authentic SARS-CoV-2 (wild type) and incubated at 37 °C for 1 h. The mixtures were then added to prepared Vero E6 cells in 96-well plates. One hour later, each well was covered with 1.6% semisolid carboxymethylcellulose (CMC) to limit the spread of virus. After 24 h, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. A foci formation assay was then performed by staining Vero cells with a rabbit anti-SARS-CoV-2 nucleocapsid protein antibody (Cat. No.: 40143-T62, Sino Biological, Inc. Beijing) followed by an HRP-conjugated goat anti-rabbit secondary antibody (Cat. No.: 109-035-088, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Finally, foci (infected cells) were visualized by adding TrueBlue peroxidase substrate (KPL, Gaithersburg, MD) and read using an ImmunoSpot S6 Ultra reader (Cellular Technology Limited, USA). IC_{50} values were calculated using GraphPad Prism 7.0. At least two independent experiments were performed for the neutralization assay.

Prophylactic protection of HmAbs against SARS-CoV-2 virus in a mouse model

Animal study was approved by the Experimental Animal Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (Approval No.: 202092). All methods were performed in accordance with the relevant guidelines and regulations. The recombinant adenoviral vectors expressing hACE2 (Ad5-ACE2) transduced BALB/c mice[23] were used in this experiment. Mice were purchased from gempharmatech (Guangdong Province). Five days prior to Ab injection, 2.5×10^8 FFU of Ad5-ACE2 were transduced intranasally into mice. Mice were treated intraperitoneally with the hmAbs one day before SARS-CoV-2 (wild type) challenge, and four doses (20 µg, 50 µg, 100 µg, and 200 µg) were used for each hmAb. The group of mice treated with PBS or mock antibody (4B8, a Zika virus NS1-hmAb)[24] was used as a negative control. Three days after virus challenge, mice were sacrificed, and lungs were harvested. For virus challenge and tissue harvest, mice were anesthetized and euthanased by isoflurane. Virus titers in lung tissue were detected by FRNT and calculated as the number of FFU per gram of tissue.

Data analysis

GraphPad Prism 7.0 was used to generate graphs and for statistical analysis. Results are presented as mean ± SEM. Each group was compared with the control group using a t test. P values < 0.05 were considered statistically significant. (*, ≤ 0.05; **, ≤ 0.005; ***, ≤ 0.0005; ****, ≤ 0.0001).
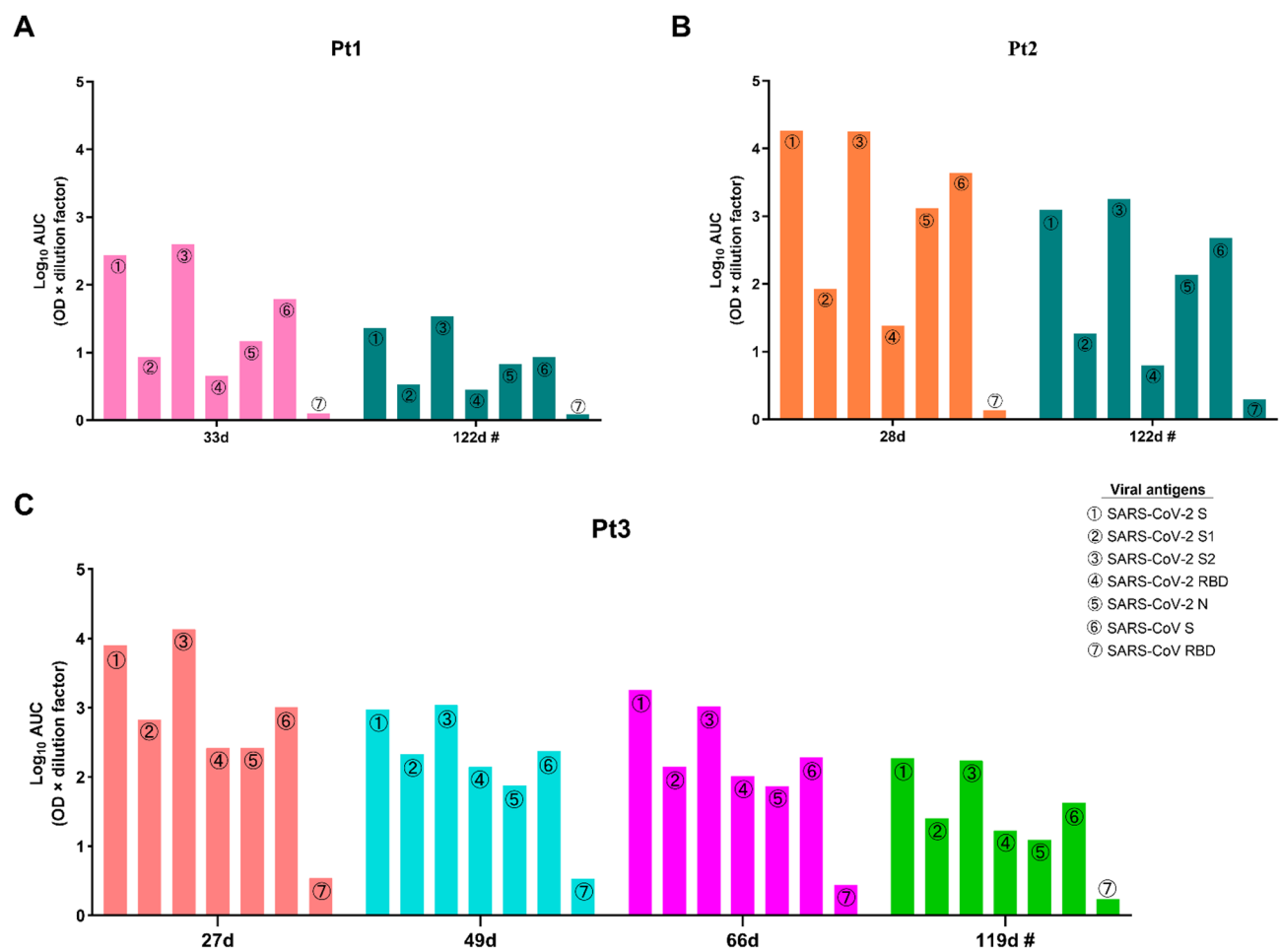
Fig. 1. Specificity and cross-reactivity of plasma Ab response. IgG Abs against SARS-CoV-2 S, S1, S2, RBD and N proteins in plasma samples from three infected individuals, Pt1, Pt2 and Pt3 (**A**, **B** and **C**), were detected using ELISA. Cross-reactive Abs in plasma samples against SARS-CoV S and RBD were also tested. Each plasma sample was initially diluted 1:100, followed by 3-fold serial dilution and tested in duplicate. AUC was calculated from the background-subtracted OD value using GraphPad Prism 7. AUC, area under curve;#, PBMCs at these time points were used for memory B-cell analysis.

Viral antigens		Pt1		Pt2		Pt3			
		33d	122d	28d	122d	27d	49d	66d	119d
SARS-CoV-2	S	2.44	1.36	4.26	3.09	3.90	2.97	3.26	2.27
	S1	0.94	0.53	1.93	1.27	2.83	2.33	2.15	1.41
	S2	2.60	1.53	4.25	3.25	4.13	3.04	3.02	2.24
	S1/S2	0.36	0.35	0.45	0.39	0.68	0.77	0.71	0.63
	RBD	0.66	0.45	1.38	0.80	2.42	2.15	2.01	1.23
	N	1.17	0.82	3.12	2.14	2.42	1.87	1.87	1.09
SARS-CoV	S	1.79	0.93	3.64	2.68	3.01	2.37	2.28	1.62
	RBD	0.10	0.08	0.13	0.29	0.54	0.53	0.44	0.24

Table 1. IgG-Ab titers in plasma samples (Log_{10} AUC, OD × Dilution factor).

Results

Dominant S2-IgG Ab response in plasma

Specific IgG Abs against SARS-CoV-2 were detected by capturing ELISA. Pt2 and Pt3 had higher levels of Abs to SARS-CoV-2 S and N proteins than Pt1 (Fig. 1; Table 1). The characteristics of plasma Ab response to SARS-CoV-2 among these individuals were observed as follows (Fig. 1; Table 1, and Fig. S1): First, some level of Abs to SARS-CoV-2 S and N proteins remained in the plasma of COVID-19 patients during convalescence (day

27 to day 122) and decreased significantly from one month to four months post-infection. Second, when the Ab response to SARS-CoV-2 S was dissected by probing S1-, S2-, and RBD-specific IgG Abs, the titer of S2-Abs was higher than that of S1-Abs (1.53–4.25 vs. 0.53–2.83, Table 1). Third, cross-reactive Abs to SARS-CoV S were detected in all plasma samples with titers ranging from 0.93 to 3.64. The level of cross-reactive Abs recognizing SARS-CoV RBD domain was low (titers between 0.08 and 0.54), which suggested that the cross-reactive Abs mainly targeted the non-RBD region of SARS-CoV S. Our results showed that S2-specific IgG Abs were dominant in plasma relative to S1-Abs during primary SARS-CoV-2 infection, although the total amount of specific Abs decreased significantly during convalescence.

Dominant S1-specific MBC response in blood

The plasma Ab repertoire may underestimate the breadth of humoral immunity[19]. To better understand the protective immunity against SARS-CoV-2, we investigated the specific MBC response using an in vitro B-cell stimulation method (Fig. 2A). A total of 274,200 MBCs (100 cells per culture well) were cultured with CpG2006, EBV, and growth factors in the presence of feeder cells. After 10 days of incubation, the supernatants of the culture wells were collected for detection of SARS-CoV-2 specific Abs (Fig. 2A).

S and N proteins are the main targets of specific Ab responses and the S1 subunit of S protein is recognized by the majority of neutralizing Abs, therefore, the three recombinant proteins S, S1, and N were used to identify SARS-CoV-2 specific MBCs. A total of 426 S-binding (0.16%), 297 S1-binding (0.11%) and 145 N-binding (0.05%) MBCs were identified (Table 2). Among these three infected individuals, Pt2 showed robust S- (0.46% positive MBCs) and N-specific MBC responses (0.25% positive MBCs (Table 2). The results also revealed that more MBCs targeted S (total 0.16%) than N protein (total 0.05%).
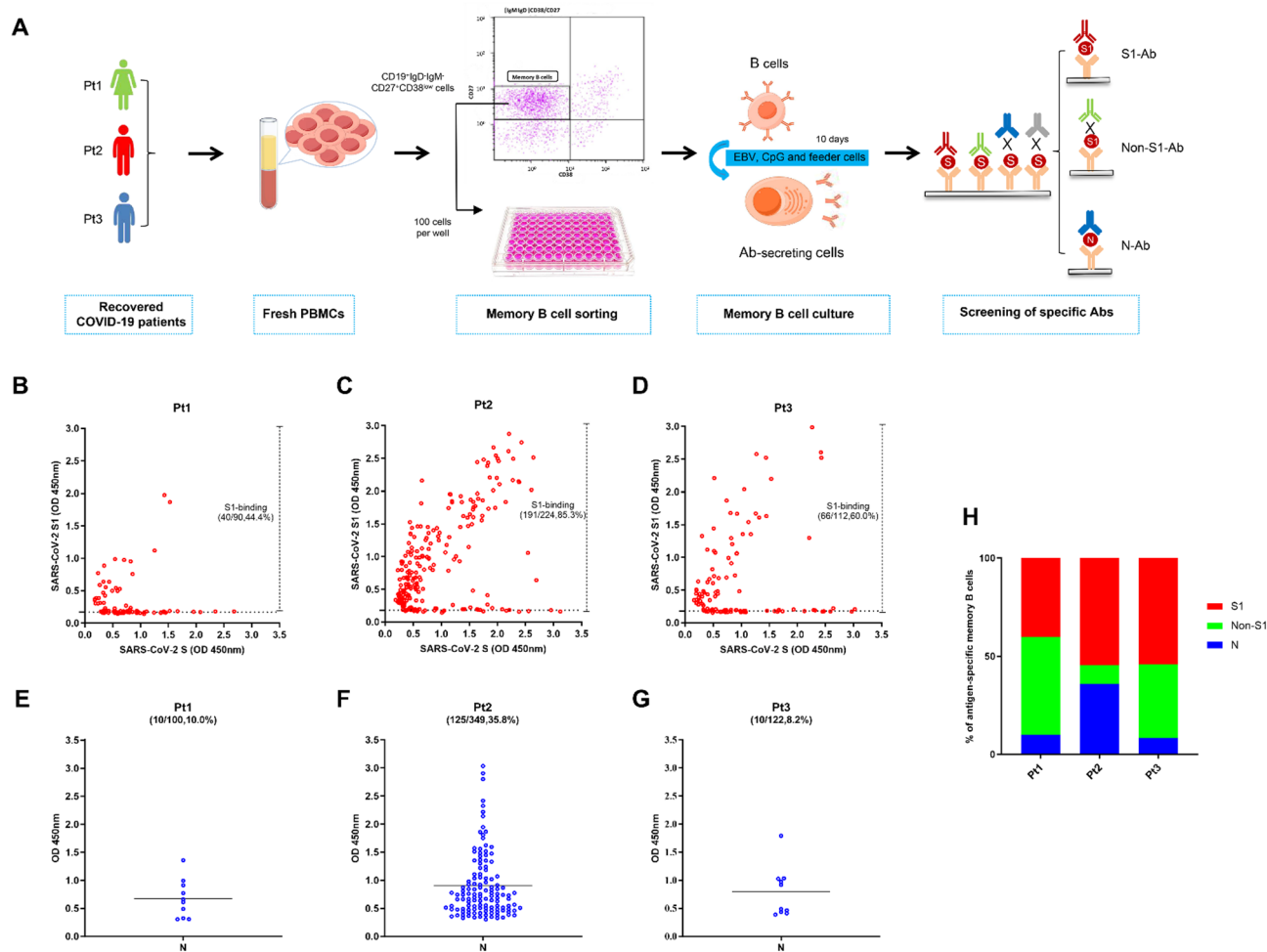

Fig. 2. Profiling of specific MBC responses. (**A**) Three recombinant proteins S, S1 and N were used to identify SARS-CoV-2-specific MBCs. S-, S1-, and N-specific binding Abs in supernatants collected from MBC incubation wells were tested using ELISA. (**B-D**) Percentage of S1-binding MBCs in the total number of S-binding MBCs. The dotted line represents the threshold for S1-binding positive. (**E-G**) Percentage of N-binding MBCs in the total number of N- and S-binding MBCs. (**H**) Proportion of S1, non-S1, and N-specific MBCs for each infected individual.

Patient #	Culture well (100 cells/well)	Positive well					N (%)
		S					
		S1-binding	Non-S1-binding	(% S1 in S)	Total S (%)		
Pt1	1300	40	50	44.4	90 (0.07) [a]		10 (0.01)
Pt2	492	191	33	85.3	224 (0.46)		125 (0.25)
Pt3	950	66	46	60.0	112 (0.12)		10 (0.01)
Total (%)	2742	297 (0.11)	129 (0.05)	69.7	426 (0.16)		145 (0.05)

Table 2. Identification of SARS-CoV-2-specific MBCs using in vitro B-cell stimulation and ELISA. a, Percentage in parentheses was calculated by dividing positive wells by total MBC cultured of each individual.

The proportion of S1-binding MBCs that could contribute to SARS-CoV-2 neutralization varied significantly among these three individuals (Fig. 2B–D; Table 2). The percentage of S1 binding in S-positive wells ranged from 44.4 to 85.3%. Notably, the proportion of S1-binding MBCs was higher than that of non-S1-binding MBCs (0.11% vs. 0.05%) (Table 2). Furthermore, a higher proportion of N-specific B-cell response was induced in Pt2 (Fig. 2E–H) than in Pt1 and Pt3.

Collectively, the results show that the SARS-CoV-2-specific MBC response was characterized by a dominant S1-directed MBC response after natural infection.

Cross-reactive MBCs recognizing the SARS-CoV RBD subdomain

Because of the high sequence similarity between SARS-CoV-2 and SARS-CoV S proteins[25]–[26], we sought to detect cross-reactive MBCs against conserved epitopes in the S protein between the two viruses. Abs in the supernatants of SARS-CoV-2 S-positive wells were further tested for the binding to SARS-CoV S (Fig. 3A-C) and RBD (Fig. 3D–F) by ELISA method. Cross-reactivity of SARS-CoV-2 S-specific MBCs with SARS-CoV was observed in all three individuals. The cross-reactive Abs to SARS-CoV S were detected with the percentage ranging from 30 to 62% in SARS-CoV-2 S-specific MBCs (Fig. 3A-C). Notably, 22–33% of S1-directed MBCs were cross-reactive with SARS-CoV RBD (Fig. 3D–F). Non-S1-directed MBCs showed higher cross-reactivity than S1-directed MBCs (67–86% vs. 22–33%) (Fig. 3D–I). Our results demonstrate that conserved epitopes exist between SARS-CoV-2 and SARS-CoV S proteins, not only in the S2 region, but also in the S1 region. These cross-reactive RBD-directed MBCs may provide protection against SARS-CoV-2 variants as well as SARS-CoV.

Neutralizing HmAbs derived from S1-directed MBCs targeting various RBD epitopes and the NTD domain

To determine whether specific MBCs identified in the MBC pool could exert their protective function upon re-infection with SARS-CoV-2, particularly omicron, the hmAbs were isolated from MBCs with a focus on S1-directed B cells. According to the binding specificity to S and its subdomains in the primary screening, 24 hmAbs were selectively cloned and expressed (Table S1). These 24 hmAbs were also submitted to the coronavirus immunotherapeutics consortium (CoVIC) for identification (Table S1). BLI technology was used to test how the hmAbs compete against each other to bind RBD or S1 protein. The 10 hmAbs that we isolated from S1-directed MBCs can be clearly divided into six groups: group 1, 825 and 824; group 2, 843; group 3, 818; group 4, 826, 816, and 855; group 5, 808; and group 6, 806 and 846 (Fig. 4A). Among the eight RBD-specific mAbs, 818 competed completely with group 1 and group 2, while 808 competed partially with group 1 and group 4 (Fig. 4A–B). 806 and 846 in group 6 did not bind the RBD domain.

The cross-reactivity of these S1-directed hmAbs with SARS-CoV S was also analyzed using ELISA. Recombinant S proteins and their subdomains of SARS-CoV-2 and SARS-CoV were used for binding specificity analysis (Fig. 2). As shown in Tables 3, 818, 825, 826 and 843, and 808 from groups 1–5 were RBD-directed hmAbs, which was consistent with the results obtained using BLI (Fig. 4A). All five group hmAbs did not cross-react with SARS-CoV S and RBD except for 808 in group 5 (Table 3), which could recognize the conserved epitope in the RBD domain shared by SARS-CoV-2 and SARS-CoV. The results also showed that 846 in group 6 is an NTD-directed Ab (Table 3). It bound only to SARS-CoV-2 S, but not to SARS-CoV S. Furthermore, 872 is an hmAb derived from non-S1 specific MBCs. As shown in Tables 3 and 872 is an S2- directed Ab and could cross-react with SARS-CoV S as expected.

The neutralizing activity of hmAbs with diverse epitopes was measured using flow-based S-pseudotyped HIV-1. HmAbs obtained from MBCs of these three infected individuals with wildtype SARS-CoV-2 neutralized wildtype S pseudovirus well (Fig. 4C). The most potent antibodies were 825 and 843 from group1 and group2, with an IC_{50} of 21.5 ng/ml and 15.2 ng/ml, respectively. The second most potent Abs were 818 and 826 from group3 and group4, with an IC_{50} of 74.9 ng/ml–198.6 ng/ml. The cross-reactive Ab 808 can neutralize both of SARS-CoV-2 and SARS-CoV with IC_{50} of 0.78 μg/ml and 15.5 μg/ml, respectively (Fig. 4C and Fig. S3). Since 846 and 872 did not show any neutralizing activity, the rank order of neutralization was 843 > 825 > 818 > 826 > 808 > 846; except for the position of 846, this finding is consistent with the CoVIC data (Table S1). As shown above, 846 is an NTD-specific Ab that showed potent neutralizing activity on the CoVIC pseudovirus detection system with an IC_{50} of 39.4 ng/ml, but no neutralization in our pseudovirus detection system (Fig. 4C). The omicron variant of the virus escapes the neutralization by most neutralizing Abs, therefore, we also evaluated the neutralizing activity of these hmAbs on omicron. Interestingly, 826 retained the same neutralizing activity on omicron as on wildtype virus (249.5 ng/ml vs. 198.6 ng/ml for IC_{50} comparison of omicron and wildtype, Fig. 4C–D), suggesting that the epitope in the RBD domain recognized by 826 is conserved. The cross-reactive Ab 808 was
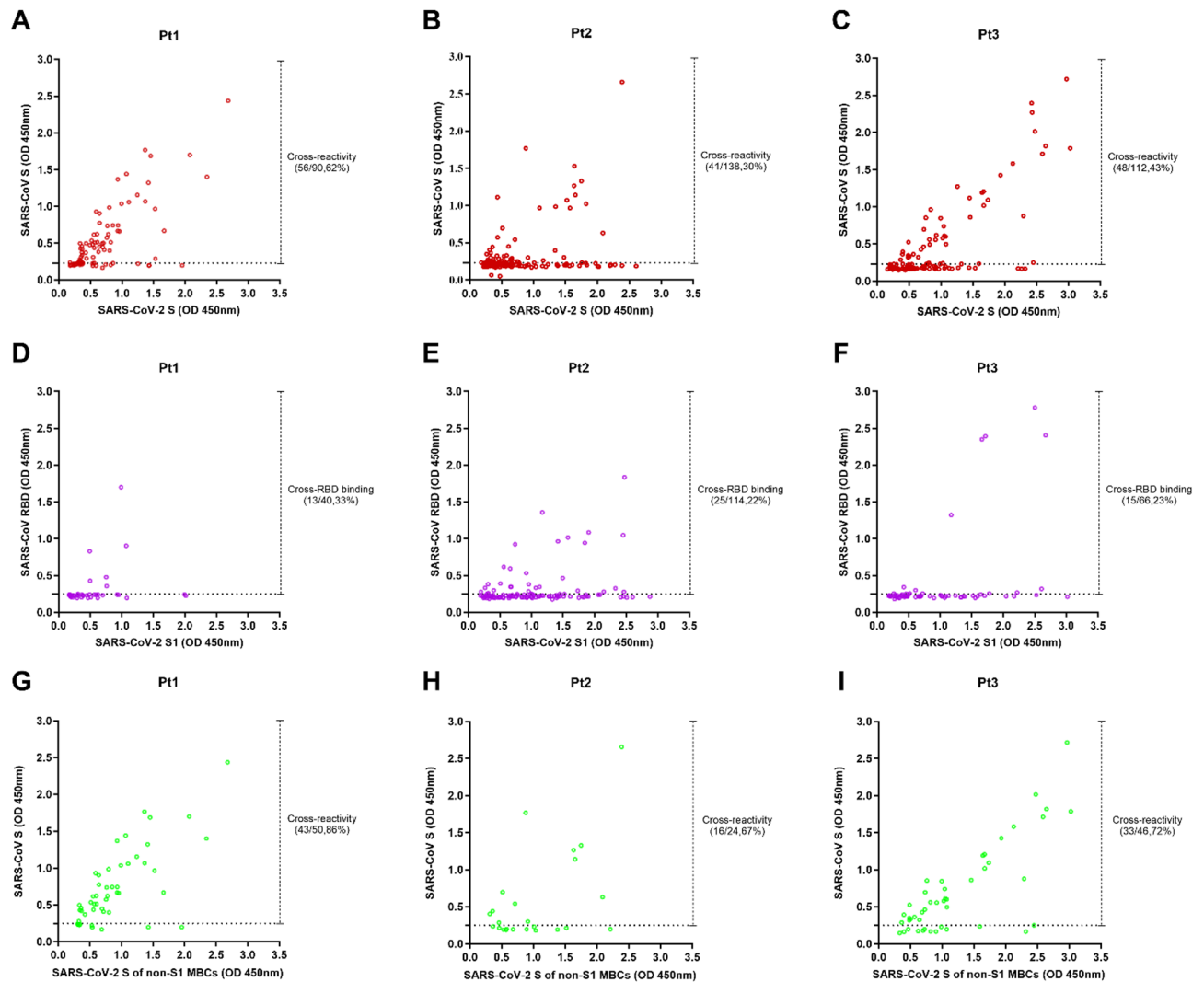
Fig. 3. Cross-reactivity of SARS-CoV-2 S-specific MBCs with SARS-CoV. Cross-reactive Abs to SARS-CoV S and RBD in supernatants collected from MBC incubation wells were tested using ELISA. (**A-C**) Percentage of SARS-CoV S-binding MBCs among total of SARS-CoV-2 S-binding MBCs. (**D-F**) Percentage of SARS-CoV RBD-binding MBCs among SARS-CoV-2 S1-binding MBCs. (**G, H**) Percentage of SARS-CoV S-binding MBCs among SARS-CoV-2 non-S1-binding MBCs. The dotted line represents the threshold for SARS-CoV-1 S- or RBD-binding positive.

able to neutralize omicron with an IC$_{50}$ of 3.4 µg/ml (Fig. 4D). Among these three highly neutralizing hmAbs 825, 843, and 818 from groups 1–3, the neutralizing capacity of 825 on omicron decreased significantly (4.2 µg/ml vs. 0.02 µg/ml for omicron vs. wildtype), while 843 and 818 almost lost the ability to neutralize when test on the omicron variant.

We have successfully isolated a panel of hmAbs from SARS-CoV-2 specific MBCs. These MBC-derived hmAbs from wildtype-virus-infected individuals can neutralize wildtype virus well and recognized different epitopes in the RBD and NTD subdomains of SARS-CoV-2 S. Some conserved epitopes in RBD were revealed by hmAbs 826 and 808, which could neutralize wildtype virus and omicron variant with compatible potency or cross-neutralize SARS-CoV. Our findings clearly demonstrate that functional SARS-CoV-2-specific MBCs existing in the MBC pool can exert their protective role against SARS-CoV-2.

Protection of HmAbs with diverse epitopes against SARS-CoV-2 in vitro and in vivo

Among the eight RBD-specific mAbs we identified in this study, 825 (group 1), 843 (group 2), and 826 (group 4) did not compete with each other (Fig. 4A–B), suggesting that the epitopes recognized by these three hmAbs do not overlap. Thus, three RBD-Abs (825, 843, and 826), together with one NTD-Ab (846) and one S2-Ab (872), were selected for further investigation of their protective role in vitro and in vivo. FRNT was used to measure the neutralizing potential of these Abs. The RBD Abs 825, 843, and 826 efficiently neutralized live SARS-CoV-2 (wildtype) virus with an IC$_{50}$ of 0.307 µg/ml, 0.162 µg/ml and 2.535 µg/ml, respectively (Fig. 5A, Fig. S4). The order of neutralization was 843 > 825 > 826, and is consistent with the neutralization results on pseudovirus
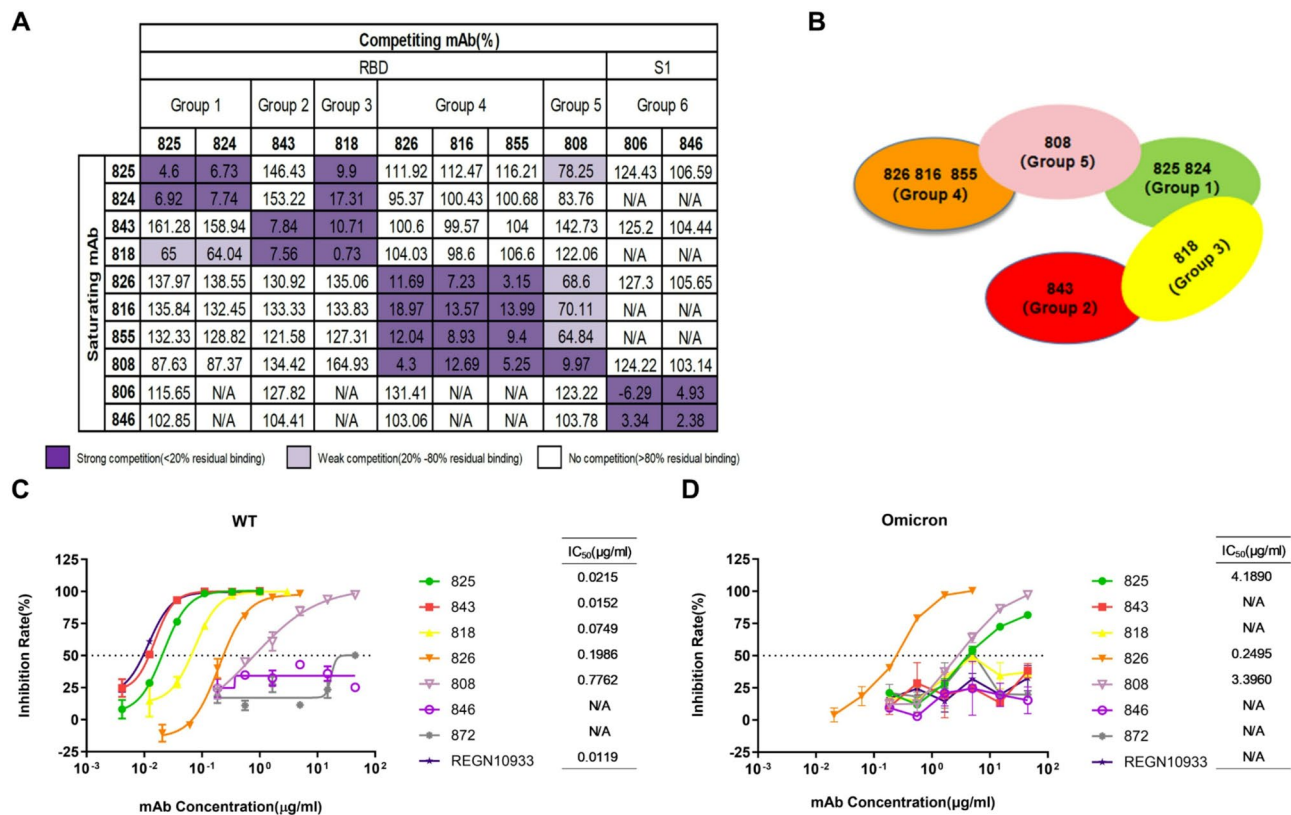
Fig. 4. Epitope mapping and neutralizing activities of hmAbs derived from SARS-CoV-2 S1-specific MBCs. (**A**) The six distinct epitope groups of S1-directed hmAbs identified by epitope binning using BLI. The percentage of residual binding was calculated as the binding value of the second antibody in the presence of the first antibody divided by the binding value of the second antibody alone. Percentage of residual binding < 20% is highlighted in dark purple; 20–80% is highlighted in purple. (**B**) The scheme of five epitope groups of RBD-directed mAbs showing epitope overlap among them. (**C** and **D**) Neutralizing activities of S1-directed hmAbs tested on SARS-CoV-2 S-pseudotyped HIV-1. REGN10933, a commercial SARS-CoV-2 specific hmAb; 872, an S2-hmAb.

Ab ID	SARS-CoV-2 (EC_{50}, ng/ml)					SARS-CoV (EC_{50}, ng/ml)	
	S	S1	S2	RBD	NTD	S	RBD
825	12.22	39.26	N/A	27.74	N/A	N/A	N/A
843	2.897	6.181	N/A	5.05	N/A	N/A	N/A
818	5.95	7.527	N/A	5.57	N/A	N/A	N/A
826	8.021	30.23	N/A	22.29	N/A	N/A	N/A
808	11.76	26.31	N/A	18.71	N/A	25.32	41.24
846	136.7	49.35	N/A	N/A	29.44	N/A	N/A
872	21.27	N/A	19.58	N/A	N/A	47.24	N/A

Table 3. EC_{50s} of HmAbs binding to S proteins and their subdomains of SARS-CoV-2 and SARS-CoV.

(Fig. 4C). Notably, NTD-Ab 846 neutralized authentic SARS-CoV-2 with an IC_{50} of 1.155 μg/ml (Fig. 5A, Fig. S4), but showed no neutralizing activity measured in our HIV-1 lentivirus assay (Fig. 4C). The S2-Ab 872 had no detectable neutralizing activity against either pseudo- or authentic viruses.

The prophylactic efficacy of Ab 825,843, 826 or 846 was evaluated in an Ad5-hACE2 transduced mouse model (Fig. 5B). Ad5-hACE2 mice were treated with each Ab at a dose of 20 μg, 50 μg, 100 μg, or 200 μg per mouse 24-hours prior to SARS-CoV-2 challenge. Mice treated with an isotype Ab (4B8, a Zika virus NS1-specific hmAb)[27] or PBS were used as controls (Fig. 5C). The Abs 825, 843, 826, and 846 could significantly reduce the viral load in the lung with an approximately two log reduction (Fig. 5C) at the dose of 20 μg (~ 1 mg/kg)
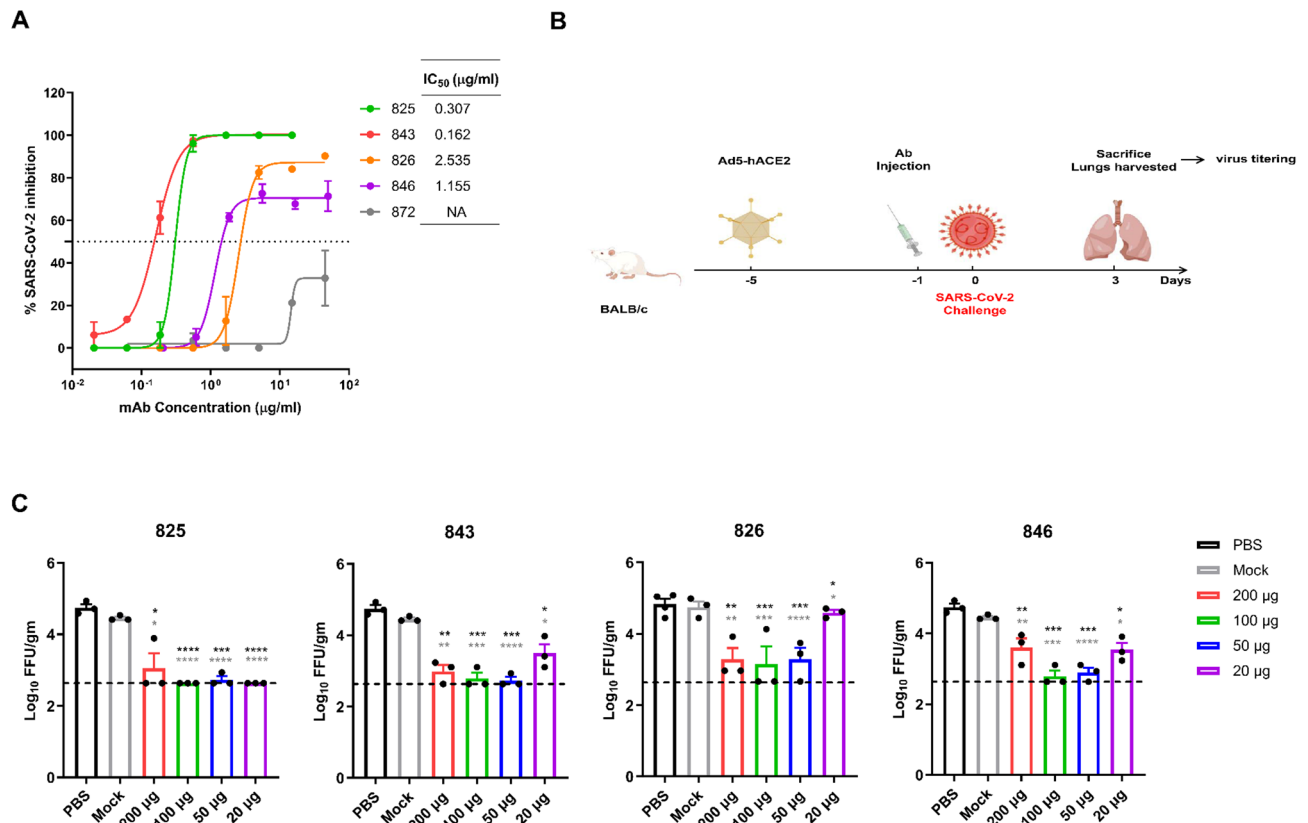
Fig. 5. Neutralization of hmAbs with diverse epitopes on authentic virus and their protection in mouse model. (**A**) Neutralizing activities of hmAbs against wildtype SARS-CoV-2 were measured by FRNT. (**B**) Experimental design of hmAbs to protect mice from SARS-CoV-2 wildtype virus infection by prophylactic application. The Ad5-ACE2 transduced BALB/c mice were injected intraperitoneally with hmAbs one day before the SARS-CoV-2 challenge. Three days after the challenge, virus titers in lung tissues were detected by FRNT. (**C**) The virus titers in lung tissues after hmAbs administration are shown. For each hmAb, 20 μg, 50 μg, 100 μg, and 200 μg were used per mouse ($n = 3$ mice per dose group). Groups treated with PBS or mock antibody (4B8, a Zika virus NS1-hmAb) were used as controls. FFU/gm, focus forming unit/gram. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

per mouse compared to the isotype Ab ($p < 0.05$) or PBS ($p < 0.05$). The order of protective efficacy of Abs was $825 > 843 > 846 > 826$.

Our results showed that Abs 825, 843, 826, and 846 targeted non-overlapping epitopes on SARS-CoV-2 S and protected against SARS-CoV-2 infection in vitro and in vivo.

Discussion

MBCs are an important component of SARS-CoV-2-specific humoral immunity upon re-exposure to the virus. The ability to culture and expand B cells in vitro has been a useful tool in the study of human immunity. For SARS-CoV-2 infection, MBCs have been studied for their ability to bind fluorescently labeled RBD or S protein and their ability to produce specific antibodies in the enzyme-linked immunosorbent assay (ELISpot assay)[6–8,27–29]. Moreover, functional analysis of MBCs has been performed using MBC-derived hmAbs[6,29]–[30]. In the present study, we investigated the MBC responses to SARS-CoV-2 S and N proteins in an MBC cell pool activated by B-cell culture with 100 cells per culture well.

To date, most of the population in China has been vaccinated with 3 to 4 doses of inactive vaccine and infected or re-infected with omicron variants. To better understand the human immune response to SARS-CoV-2, especially MBCs, we returned to the primary infected individuals who were not vaccinated and were confirmed to have been infected with the wildtype SARS-CoV-2. Among the COVID-19 convalescents enrolled in our study, two were diagnosed with moderate COVID-19, and one was diagnosed as mild. All were infected with wild-type SARS-CoV-2 during the early outbreak in January and February 2020, when a vaccine was not available.

Both S protein, including S1 and S2 subunits, and N protein were immunogenic and induced significant levels of specific Abs in serum during the course of SARS-CoV-2 infection[13]. Serum anti-S1/S2 responses differ between infection, vaccination, and hybrid immunity[31]. Infection generated IgG Abs predominantly against the S2 domain, whereas vaccination generated IgG Abs predominantly against the S1 domain. Hybrid immunity induced strong anti-S1 and anti-S2 Abs. In our study, the level of serum Abs (S1- and S2-IgGs) from each patient

decreased during the infection (day 27 to day 122). However, the proportion of S1- and S2-IgGs did not change, with S2-IgGs predominating. This is consistent with what was previously reported[31]. SARS-CoV-2-specific MBCs could circulate in the blood for years after symptom onset[6,32]. In the present study, using an unbiased detection method, the frequency of S- and N-specific MBCs was measured to be 0.16% and 0.05%, respectively, in blood samples collected four months after infection. Notably, S1-binding MBCs were dominant with a ratio of 44.4–85.3% of S-binding MBCs, which was different from the antigen specificity recognized by the serum Abs. One explanation for this finding is that serum Abs are secreted by terminally differentiated plasmablast cells or long-lived plasma cells, whereas MBCs undergo the process of affinity maturation[13,33], that may represent different pathways. B-cell clones are preferentially selected for specific epitopes during B-cell maturation[34]. Studies on recovered patients with severe COVID-19 infection suggest that the proportion of RBD-specific MBCs continues to increase over the course of the disease[29]. Our finding that S1-specific MBCs were favored over S2-specific MBCs in mild or moderate patients is consistent with previous reports[29]. The mechanism by which certain epitope-specific B cells are selected remains unclear. Given that most neutralizing antibodies recognize the RBD and NTD domains in the S protein[35], an S1-dominant MBC response in COVID-19 patients may be favored in the memory pool during the convalescence and suggests a protective mechanism of the host immune response against the virus. The difference in antigen specificity recognized by MBCs and circulating plasma Abs has been reported in HIV infection by a research group[19]. To our knowledge, MBCs specific for SARS-CoV-2 S epitopes discordant with those recognized by contemporaneous plasma were first reported by our research group and will inform the understanding of protective immunity after SARS-CoV-2 infection.

The repeated emergence of new SARS-CoV-2 variants poses a challenge to vaccine development and hinders the use of neutralizing antibodies in the prevention and treatment of SARS-CoV-2 infection, especially in immunocompromised individuals[36]. Because of the high similarity in the coding sequences of SARS-CoV-2 S and SARS-CoV S proteins (~ 76% identity)[25], the researchers aimed to obtain cross-reactive antibodies that target the conserved epitopes in the S proteins of SARS-CoV-2 and SARS-CoV and resist the escape of variants[16,17,37,38]. In this study, we found that 30–62% of SARS-CoV-2 S-binding MBCs were cross-reactive to SARS-CoV S from blood samples collected four months after SARS-CoV-2 infection. Since the S2 domain of the S protein is much more conserved than the S1 domain[26], the cross-reactivity may mainly originate from the S2-binding MBCs[18]. All potent cross-neutralizing hmAbs derived from S-specific MBCs targeted epitopes within the RBD domain[18,39,40]. The next step is, therefore, to focus on SARS-CoV RBD cross-binding MBCs. Our findings showed that a significant proportion of S1-binding MBCs (22–23%) were cross-reactive with SARS-CoV RBD. In other words, a specific memory B-cell response to the conserved RBD epitopes between SARS-CoV-2 and SARS-CoV could be efficiently induced by a single wildtype SARS-CoV-2 infection. The conserved RBD epitopes of SARS-CoV-2 VOCs and other sarbecoviruses exist[41,42]. The antibodies targeting these conserved epitopes could be induced by infection and vaccination[42–44]. In addition, several broadly reactive mAbs have been identified that can bind to conserved RBD epitopes from SARS-CoV or SARS-CoV-2 convalescent donors[16,35,45,46]. Our findings show that a cross-reactive SARS-CoV MBC response, particularly cross-RBD binding MBCs, was induced by primary wildtype SARS-CoV-2 infection. Antibodies derived from these MBCs may be directed against the conserved epitopes of SARS-CoV-2 VoCs and other sarbecoviruses and exert their protective functions against SARS-CoV-2, SARS-CoV, or other sarbecoviruses.

SARS-CoV-2 RBD, S1 or S-specific MBCs have been quantified during the course of infection[6–8,28]. Several groups of neutralizing epitopes in the regions of RBD, NTD or S2 have been mapped by numbers of isolated SARS-CoV-2 specific hmAbs[47–52]. In the present study, a panel of hmAbs was also derived from S1- binding MBCs. The neutralizing epitopes recognized by the identified hmAbs could be classified into six groups, including five epitopes in RBD and one in NTD. Among them, one RBD epitope recognized by neutralizing Ab (808) is conserved between SARS-CoV and SARS-CoV-2. The cross-reactive Ab (808) retains neutralizing activity against the omicron variant, supporting the strategy of screening cross-binding Abs for nAbs. The group 4 RBD epitope recognized by the representative Ab (826) could resist the neutralizing escape of omicron, although its neutralizing activity was not as potent as the other group Abs (Abs in groups 1, 2, and 3). Ab 826 also showed a protective role against SARS-CoV-2 in an animal model ($p < 0.0001$ at a dose of 50 μg per mouse), suggesting that the 826-like Ab may be a candidate for anti-SARS-CoV-2 variants. NTD-specific Ab (846) showed potent neutralizing activity against authentic virus (IC_{50},1.155 μg/ml), but not against pseudovirus of our lentiviral-based neutralization assay in this study. The protective role of Ab 846 against SARS-CoV-2 in an animal model ($p < 0.0001$ at a dose of 50 μg per mouse) was further demonstrated. A similar NTD-specific Ab (0304-3H3) was reported, which showed neutralizing activity with an EC_{50} of 0.04 μg/mL against authentic SARS-CoV-2 but failed to neutralize pseudotyped virus[50]. One possible explanation is that the 846-like Ab targeting the NTD domain does not compete to inhibit RBD binding to ACE2. Instead, it may indirectly inhibit the binding of the virus to ACE2 or its fusion with the cell membrane by preventing the conformational change of the S protein. Antibodies inhibiting the virus by this mechanism may not function properly in the pseudovirus system[53]. 846-like Ab can be used as a candidate component of an Ab combination to combat the variants. Our findings demonstrate that some conserved epitopes in the RBD recognized by 826- or 808-like Abs are antigenic and that the corresponding Abs could be elicited by a single wildtype SARS-CoV-2 infection.

The main limitation of this study is the relatively small number of analyzed MBCs from three donors. Additional studies that include more donors and higher numbers of cultured MBCs are required to confirm our findings. Furthermore, antibodies such as 826 and 808 targeting the conserved RBD epitopes may resist the neutralizing escape of new variants. A detailed analysis of these conserved RBD epitopes will be conducted in the future using cryogenic electron microscopy technology. Finally, the representative cross-reactive Ab (808) can neutralize both SARS-CoV-2 omicron and SARS-CoV, but the neutralizing capacity is weak, with IC_{50} values of 3.4 and 15.5 μg/mL, respectively. To combat the newly emerging variants, more high potency and broad-spectrum Abs are required.

Overall, we characterized the MBC responses to S and N proteins in the MBC pool of SARS-CoV-2 convalescents using an unbiased method (without pre-selection of antigen-specific MBCs) and compared with that of serum Ab responses. Our findings that an S1-dominant and cross-reactive SARS-CoV MBC response, particularly cross-RBD binding MBCs, was induced by primary wild-type SARS-CoV-2 infection with an S2-dominant serum Ab response, suggests that MBCs and serum Ab may represent different pathways. Our findings have implications for the design of immunogens that preferentially stimulate protective B-cell responses. In addition, we provided a useful method to isolate cross-reactive mAbs from memory B cells for the development of antibody-based therapies and effective prophylaxes needed by millions of immunocompromised persons[54].

Data availability

The raw data and reagents used in this study can be made available to interested researchers upon reasonable request to the corresponding authors.

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Author contributions

L.Y. and Y. G. conceptualized and designed the study, interpreted the data, and wrote the manuscript. Y.W. and J. Z. supervised the experiments in the Biosafety Level 3 laboratory. X.X., Y.W., W.G., X.Z., and K.Z. performed the experiments. Z.Z., L.Z., P.W., and S.Z. performed the experiments in the Biosafety Level 3 laboratory. Y.M. and Y.P. managed the patients and collected their clinical samples. All authors reviewed the results and approved the final version of the manuscript. L.Y. directed the entire research activities.

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Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

L.Y. and Y.G. have filed patent applications for 825, 843, 826, and 846 antibodies against SARS-CoV-2 (PCT/CN2021/135785 and 202180104547.4).

Ethics approval

This study was approved by the Human Ethics Committee of Guangzhou Eighth People's Hospital (202020153) and was conducted in accordance with Chinese rules and regulations for the protection of human subjects. All subjects provided written informed consent for the research use of their blood samples. Animal study was approved by the Experimental Animal Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (Approval No.: 202092). The study is reported in accordance with ARRIVE guidelines.

Additional information

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