



Longitudinal and proteome-wide analyses of antibodies in COVID-19 patients reveal features of the humoral immune response to SARS-CoV-2



Jianxin Wang^{a,1}, Yongfei Yang^{d,1}, Te Liang^{b,1}, Ning Yang^{c,1}, Tao Li^{a,1}, Chang Zheng^{b,1}, Nianzhi Ning^a, Deyan Luo^a, Xiaolan Yang^a, Zhili He^a, Guang Yang^c, Bo Li^c, Jie Gao^a, Wenjing Yu^a, Saisai Gong^a, Yanyu Huang^a, Jiajia Li^a, Hongye Wang^b, Hao Zhang^c, Tian Zhang^c, Peiran Li^c, Yongli Li^c, Jiayu Dai^b, Xiaomei Zhang^b, Boan Li^{c,*}, Xiaobo Yu^{b,*}, Hui Wang^{a,2,*}

^a State Key Laboratory of Pathogens and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China

^b State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Sciences-Beijing (PHOENIX Center), Beijing Institute of Lifeomics, Beijing 102206, China

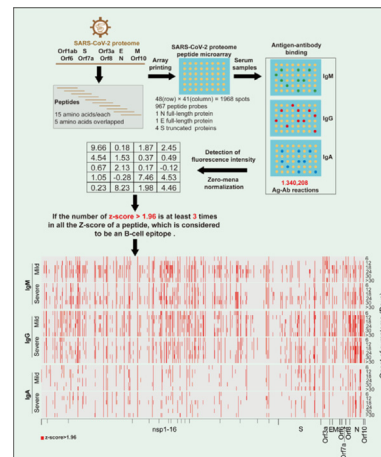
^c Department of Clinical Laboratory, The Fifth Medical Center of PLA General Hospital, No.100 West Fourth Ring Road, Beijing 100039, China

^d School of Life Science, Beijing Institute of Technology, Beijing 100081, China

HIGHLIGHTS

- We performed a longitudinal and proteome-wide analysis of antibodies in the COVID-19 patients using a SARS-CoV-2 proteome microarray (1,340,208 Ag-Ab reactions). As far as we know, this is the first systematic analysis of antibodies in the COVID-19 patients through the whole viral proteome of the SARS-CoV-2, the whole course of the patient, and different antibody isotypes (IgM, IgG, and IgA).
- We profiled a B-cell epitope landscape of SARS-CoV-2 and identified specific epitopes recognized by IgM, IgG, or IgA.
- We identified 12 dominant B-cell epitopes eliciting antibodies in most COVID-19 patients and identified the key sequence of epitopes at the amino acid resolution (five key amino acids).
- We found epitope S-82 and S-15 are perfect immunogenic peptides and should be considered in vaccine design.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 24 May 2021

Peer review under responsibility of Cairo University.

* Corresponding authors.

E-mail addresses: lba@263.net (B. Li), xiaobo.yu@hotmail.com (X. Yu), geno0109@vip.sina.com (H. Wang).

¹ These authors contributed equally to this work.

² Lead contact.

ABSTRACT

Introduction: The SARS-CoV-2 pandemic has endangered global health, the world economy, and societal values. Despite intensive measures taken around the world, morbidity and mortality remain high as

<https://doi.org/10.1016/j.jare.2021.07.008>

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Revised 12 July 2021
Accepted 23 July 2021
Available online 27 July 2021

Keywords:
SARS-CoV-2
COVID-19
Proteomics
Longitudinal
Epitope
Antibodies

many countries face new waves of infection and the spread of new variants. Worryingly, more and more variants are now being identified, such as 501Y.V1 (B.1.1.7) in the UK, 501Y.V2 (B.1.351) in South Africa, 501Y.V3 in Manaus, Brazil, and B.1.617/B.1.618 in India, which could lead to a severe epidemic rebound. Moreover, some variants have a stronger immune escape ability. To control the new SARS-CoV-2 variant, we may need to develop and redesign new vaccines repeatedly. So it is important to investigate how our immune system combats and responds to SARS-CoV-2 infection to develop safe and effective medical interventions.

Objectives: In this study, we performed a longitudinal and proteome-wide analysis of antibodies in the COVID-19 patients to revealed some immune processes of COVID-19 patients against SARS-CoV-2 and found some dominant epitopes of a potential vaccine.

Methods: Microarray assay, Antibody depletion assays, Neutralization assay.

Results: We profiled a B-cell linear epitope landscape of SARS-CoV-2 and identified the epitopes specifically recognized by either IgM, IgG, or IgA. We found that epitopes more frequently recognized by IgM are enriched in non-structural proteins. We further identified epitopes with different immune responses in severe and mild patients. Moreover, we identified 12 dominant epitopes eliciting antibodies in most COVID-19 patients and identified five key amino acids of epitopes. Furthermore, we found epitope S-82 and S-15 are perfect immunogenic peptides and should be considered in vaccine design.

Conclusion: This data provide useful information and rich resources for improving our understanding of viral infection and developing a novel vaccine/neutralizing antibodies for the treatment of SARS-CoV-2.

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Introduction

Since December 2019, the coronavirus disease 2019 (COVID-19) caused by the infection of a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a devastating worldwide pandemic [1–3]. As of May 18, 2021, there have been 163.6 million COVID-19 cases and 3.39 million deaths, and the cases continue to grow (<https://coronavirus.jhu.edu/map.html>). Despite intensive measures taken around the world, morbidity and mortality remain high as many countries face new waves of infection and the spread of new variants [4,5]. There is an urgent need for a safe and effective medical intervention to respond to the new variants of SARS-CoV-2 and reduce the burden on healthcare systems, the world economy, and society.

In December 2020, the new SARS-CoV-2 variants 501Y.V1 (B.1.1.7) in the UK and 501Y.V2 (B.1.351) in South Africa led to a significant increase in COVID-19 cases [6,7]. Both variants have significantly increased transmissibility, with estimates of increased transmission ranging from 40% to 70% [7]. Moreover, the 501Y.V2 variant was able to evade immunity through two additional mutations (E484K and K417N) [8]. Preliminary clinical trial results of COVID-19 vaccine ChAdOx1 nCoV-19 showed 74% efficacy in the 501Y.V1 variant but only 22% in the 501Y.V2 variant, whereas NVX-CoV2373, a protein-based COVID-19 vaccine, showed 89% efficacy in the 501Y.V1 variant but only 49% efficacy in the 501Y.V2 variant. Worryingly, more and more variants are now being identified, such as 501Y.V3/P.1 in Brazil and B.1.617/B.1.618 in India [9,10], which also could reduce the effectiveness of neutralizing antibodies generated by the vaccine [11,12]. To control the new SARS-CoV-2 variant, we may need to develop and redesign new vaccines repeatedly. Detailed knowledge of the humoral immune response to SARS-CoV-2 infection is critical for improving our understanding of viral infection and inform the development of vaccines and antibody-based therapies.

Currently, a few studies have revealed some information on humoral immune responses to SARS-CoV-2 infection in patients. Zhang baozhong, et. al. profiled IgG/IgM/IgA levels against the S and N proteins in the sera of COVID-19 patients, which provided serological evidence of immune responses in vivo, and offered insight into the use of specific antigenic epitopes of S protein for vaccine design against SARS-CoV-2 [13]. Pooled plasma samples from PCR positive COVID-19 patients were used to identify linear

B-cell epitopes from a SARS-CoV-2 peptide library of the spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins by peptide-based ELISA [14]. By analyzing the dynamic changes of serum IgM and IgG specifically against SARS-CoV-2 in 97 hospitalized patients Zhou Wei, et al. found that the decline of IgM might be an indicator of virus clearance, and the IgG was essential for the robustness and sustainability of humoral immune response [15]. By analyzing epitopes in the SARS-CoV-2 using VirScan, Ellen Shrock, et al. revealed cross-reactivity and correlates of the severity of SARS-CoV-2 [16]. All these works provide valuable information for developing vaccines and antibodies with better safety and efficacy against SARS-CoV-2 infection. Given the timeline of the COVID-19 pandemic, these studies are operating with only a partial picture of the intricate humoral immune response to SARS-CoV-2. There is no analysis of the humoral immune response of the COVID-19 patients encompassing the complete viral proteome and the entirety of the humoral immune response.

In this study, we collected 227 serum samples from 41 COVID-19 patients (24 mild COVID-19 patients and 17 severe COVID-19 patients) from the onset to the end of the disease, and 20 pre-2020 negative serum samples were collected as healthy controls. We performed a longitudinal and proteome-wide analysis of antibodies in the COVID-19 patients using a SARS-CoV-2 proteome microarray (1,340,208 Ag-Ab reactions). We analyzed data from the complete B-cell linear epitope of SARS-CoV-2, epitopes frequency in different proteins, epitopes recognized by different antibody isotypes, the difference of immune responses between mild and severe patients, the longitudinal changes of antibodies elicited by each epitope, and frequency of epitopes eliciting antibodies in patients. These results provide useful information and rich resources for improving our understanding of viral infection and developing effective and safe vaccines, and neutralizing antibodies to treat SARS-CoV-2.

Materials and methods

Subjects and specimen collection

We collected blood from 41 patients enrolled in The Fifth Medical Center of PLA General Hospital from March 2020 to April 2020

after obtaining written informed consent from patients or their surrogates (The Fifth Medical Center of PLA General Hospital IRB approval #2020069D). Eligibility criteria are admission to The Fifth Medical Center of PLA General Hospital with a positive SARS-CoV-2 nasopharyngeal swab by RT-PCR. Patients were divided into two groups, including severe patients and mild patients. All mild and severe COVID-19 patients were diagnosed according to the Diagnosis and Management Plan of Pneumonia with New Coronavirus Infection (trial version 7). 24 males and 17 females comprised the 41 total patients with their ages ranging from 15 to 79 years (median 50). For controls, blood was collected from 20 healthy adult donors as part of the Profiling Healthy Immunity study after obtaining written informed consent (The Fifth Medical Center of PLA General Hospital IRB approval #2020069D). The study was approved by the Ethics Committee of The Fifth Medical Center of General Hospital of PLA, China.

For patients, blood drawing occurred in concert with usual care. Blood was collected into heparin tubes (Becton, Dickinson, and Co.), and serum was isolated by high-speed centrifugation using Ficoll-Paque PLUS (GE Healthcare). The processing of blood was started within four hours of collection for all samples.

Detection of serological antibodies in mild and severe COVID-19 patients using SARS-CoV-2 proteome microarray

The proteome microarrays containing full-length N, full-length E, and truncated S proteins of SARS-CoV-2 and 966 peptides representing SARS-CoV-2 proteins were prepared as described previously [17]. The proteome microarrays were assembled in an incubation tray and blocked with 5% (w/v) milk in PBS containing 0.05% (v/v) Tween-20 (PBST) for 10 min at room temperature before antibody detection. After aspirating the blocking solution, 1:101 diluted serum was added to the array and incubated at room temperature for 30 min. After washing three times with PBST, the array was then incubated for 20 min with a mixture containing Alexa Fluor[®]647 Affinipure goat anti-human IgM (Jackson ImmunoResearch, USA, CAT#709-165-149) and Cy[™]3 Affinipure donkey anti-human IgG(H + L) antibody (Jackson ImmunoResearch, USA, CAT#109-605-043) (4 µg/mL) or only Cy[™]3 AffiniPure goat anti-human serum IgA antibody (Jackson ImmunoResearch, USA, CAT#109-165-011) (4 µg/mL). Finally, the array was washed with PBST and deionized water, disassembled from the tray, and dried with a vacuum pump. The proteome microarray was scanned at 532 and 635 nm using a GenePix 4300A microarray scanner (Molecular Devices). The median of fluorescent signal intensity with the deduction of the background was extracted using GenePix Pro7 software.

Identification of B-cell linear epitopes

To determine whether the peptide is a B-cell linear epitope, all microarray (including full-length N protein, full-length E protein, five S truncated proteins, and 966 tiled peptides) signal data were normalized with a z-score before statistical analysis. If the number of z-score > 1.96 is at least three times in all the z-score of a peptide, which is considered to be a B-cell epitope. The analysis of serological antibody response to peptides was performed using the R heatmap.

Analysis of epitopes that are more frequently recognized by IgM, IgG, or IgA antibodies

To screen epitopes that are more frequently recognized by a certain Ig type, we used an unpaired *t*-test to analyze the difference of the z-score of each determined B-cell linear epitope between IgM/IgG/IgA. The selection criteria include that the average value

of the z-score of one epitope in a certain Ig type is twice that of each of the other two Ig types, and the P-value is both <0.05.

Analysis of epitopes with different response levels in mild and severe patients

In this analysis, we used an unpaired *t*-test to compare the z-score differences of all identified B cell epitopes among all serum samples of healthy donors, mild and severe patients. If the P-value of a certain epitope between mild and severe patients is <0.05, and there is a P < 0.05 between at least one group of mild and severe patients and healthy donors, this epitope is considered to be an epitope with a difference in response level between mild and severe patients. We further analyzed the differences of epitopes with different response levels between mild and severe patients at different time points after the onset of symptoms. An unpaired *t*-test was used in this analysis.

Analysis of the frequency of epitopes elicited antibodies in the patients

In this analysis, we analyzed the z-score value of antibody level elicited by each epitope in each patient. As long as the z-score in one serum was >1.96, we determined that the epitope elicited antibody in the patient. The ratio of the total number of patients with the antibody elicited by an epitope to the total number of patients is the frequency of this epitope elicited antibody in the patients.

Analysis of the dominant epitopes

We used an unpaired *t*-test to compare the z-score differences of all identified B cell epitopes between healthy donors and severe patients. In the end, 12 dominant B-cell linear epitopes were identified as the COVID-19 specific epitope (vs. healthy donors, *p* < 0.01) and epitope widely eliciting antibodies in diverse patients (frequency > 50%).

Peptide affinity depletion of sera

Using principles similar to previous work, we performed affinity depletion as follows. Magnetic beads conjugated peptide S-15, and 50 µL sera were incubated for 4 h at room temperature for adsorption. The unbound fraction was collected after 3 rounds of adsorption.

SARS-CoV-2 neutralization assay

Using principles similar to previous work [18], we performed a SARS-CoV-2 neutralization assay as follows. Vero-E6 cells were inoculated in 96-well cell culture plates (20,000 cells per well) with DMEM (Gibco) supplemented with 10% fetal bovine serum at 37 °C. Sera or Adsorbed samples were mixed with 100 TCID50 SARS-CoV-2. The mixture was moved to the wells containing Vero-E6 cells and incubated at 37 °C for 1 h. Following removing the supernatants, 200 µL cell culture medium was added, and the plates were then incubated at 37°C with 5% CO₂ for three days. Cells were stained with crystal violet, and absorbance at 570 nm/630 nm was measured.

Ethics statement

All experiments involving human patients were conducted according to the ethical policies and procedures approved by the IRB of The Fifth Medical Center of PLA General Hospital (#2020069D). Written informed consent was obtained from all study patients or their surrogates prior to their inclusion in the study.

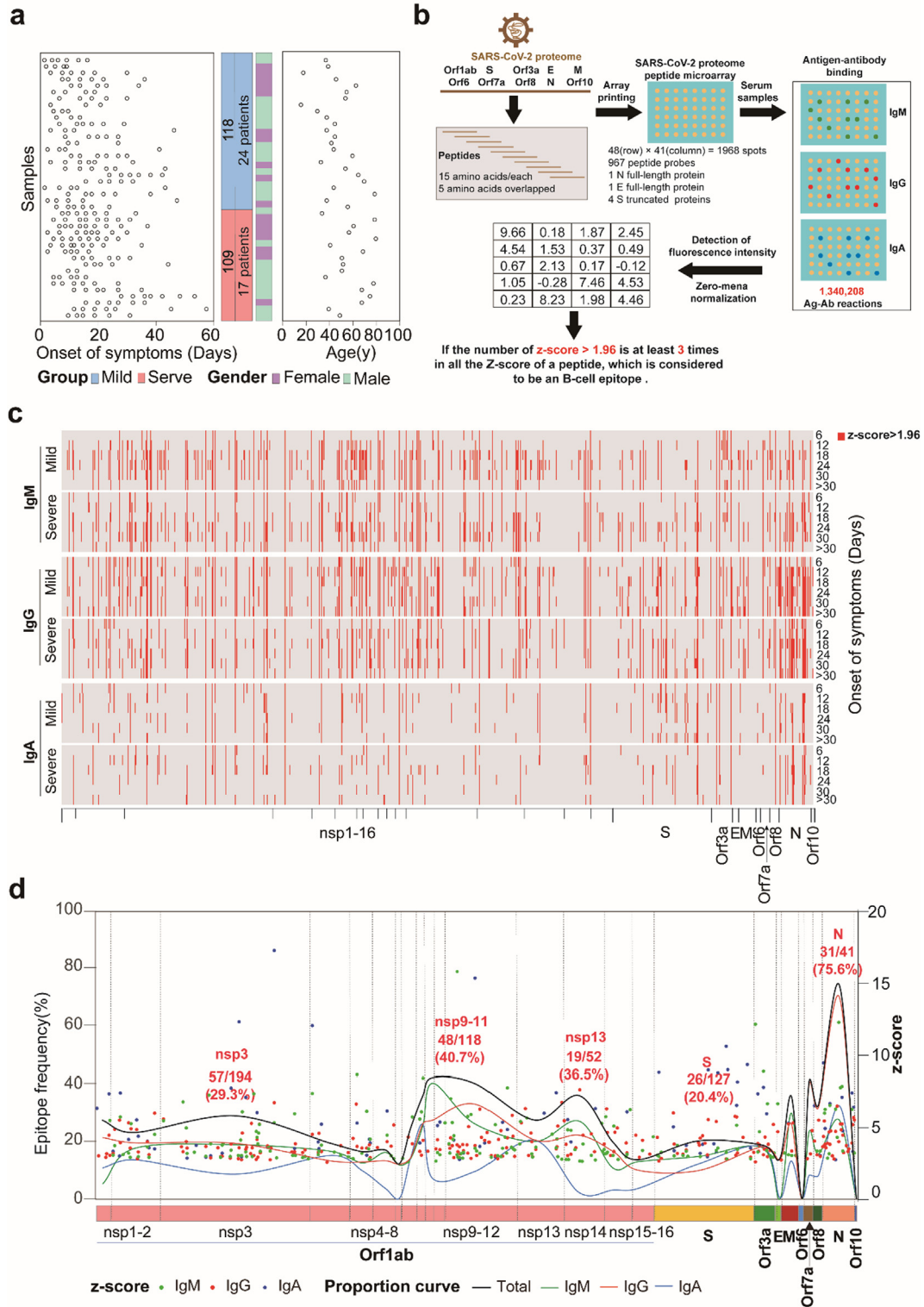


Fig. 1. Longitudinal and proteome-wide study of SARS-CoV-2 antibodies in the mild and severe COVID-19 patients. (a) Scheme of longitudinal sample collection. A total of 227 serum samples were collected from 24 mild and 17 severe patients, including 24 males and 17 females aged 15 to 78 years, within 1–60 days after diagnosis. (b) The workflow of serum samples analyzed by the SARS-CoV-2 proteome microarray. The microarray contained all structural and non-structural proteins of SARS-CoV-2, and three kinds of antibodies were analyzed. The positive binding is selected with a z-score > 1.96 for at least three times. (c) The distribution of human IgG, IgM, and IgA antibodies against SARS-CoV-2 proteins. The x-axis represents SARS-CoV-2 proteins, the left y-axis represents three isotypes of antibodies in serum samples from mild and severe COVID-19 patients, and the right y-axis represents the time points of longitudinal sample collection after patient’s diagnosis. Red indicates positive binding signals between antibodies and SARS-CoV-2 proteins (z-score > 1.96). (d) The distribution of positive epitopes in SARS-CoV-2 proteins. Each spot represents an epitope that is specifically binding to antibodies. Green represents the epitopes recognized by IgM antibodies, red represents the epitopes recognized by IgG antibodies, and blue represents the epitopes recognized by IgA antibodies. The curve represents the frequency of positive epitopes in each protein. Specially, green, red, blue, and black curve represents the frequency of the IgM-, IgG-, IgA-, total Ig-recognized epitopes on each protein. The x-axis represents SARS-CoV-2 proteins, the left y-axis represents the positive epitope frequency, and the right y-axis represents the z-score of each positive epitope. The frequency of epitopes on different proteins is also shown, such as S protein is 20.4%, and N protein is 75.6%.

Table 1
Basic information of 41 COVID-19 patients.

	Mild patients (n = 24)	Severe patients (n = 17)	P
Median ages (IQR)	44.5(37.0–54.8)	56.0(44.0–71.0)	0.02
Gender			
Female	10(42%)	7(41%)	>0.99
Male	14(58%)	10(59%)	–
Blood group			
A	6(25%)	4(23.53%)	>0.99
B	7(29.17%)	4(23.53%)	0.74
O	4(16.67%)	6(35.29%)	0.29
AB	7(29.17%)	3(17.65%)	0.46
Median hospital Stay days (IQR)	19.0(17.0–27.0)	26.0(20.5–26.75)	0.03
Presenting symptoms			
Fever	21(88%)	14(82%)	0.68
Diarrhoea	1(4%)	0(0%)	>0.99
Weak	4(17%)	7(41%)	0.15
Cough	12(50%)	13(76%)	0.11
Dyspnoea	0(0%)	8(47%)	0.00
Chronic comorbidities			
None	15(63%)	7(41%)	0.22
Hypertension	6(25%)	6(35%)	0.51
Thalassemia	1(4%)	0(0%)	>0.99
Subacute thyroiditis	1(4%)	0(0%)	>0.99
Diabetes	3(13%)	2(12%)	>0.99
asthma	1(4%)	1(6%)	>0.99
Cerebral infarction	0(0%)	1(6%)	0.41
asthma	0(0%)	1(6%)	0.41
Hepatitis	0(0%)	1(6%)	0.41

IQR: interquartile range.

Table 2
Basic information of 20 healthy donors.

	Healthy donors (n = 20)
Median ages(IQR)	34(31.25–43.25)
Gender	
Female	9(45%)
Male	11(55%)
Blood group	
A	4(20%)
B	5(25%)
O	7(35%)
AB	4(20%)

IQR: interquartile range.

Results

Longitudinal and proteome-wide study of SARS-CoV-2 antibodies in the mild and severe COVID-19 patients

To investigate the dynamic antibody responses against SARS-CoV-2, we collected 247 longitudinal serum samples from 24 mild and 17 severe COVID-19 patients (15–79 years of age) between 1 and 57 days after diagnosis (Fig. 1a and Table 1), and 20 pre-2020 negative serum samples were collected as healthy controls (Table 2). To investigate the immunogenic linear epitopes of SARS-CoV-2 proteins, we assayed the samples using a proteome microarray that contains full-length N protein, full-length E protein, five S truncated proteins and 966 tiled peptides[17]. Each peptide was 15 amino acids long with a five amino acid overlap (Fig. 1b). We profiled IgM/IgG/IgA levels against both the structural and non-structural proteins in the samples of COVID-19 patients (1,340,208 Ag-Ab reactions) and the representative images of serum antibody detection are shown in Fig. S1a. All microarray data were normalized using z-score, and immunoreactive peptides were selected using a z-score higher than 1.96 (95% confidence interval) in ≥ 3 independent serum samples [19].

We then constructed a linear epitope landscape aligned to the sequence of SARS-CoV-2 proteins and plotted all samples' signals on this landscape (Fig. 1c). The B-cell epitopes were identified by sequence alignment of immune reactive peptides with neighboring peptides[17], including 65 epitopes on the structural proteins and 214 epitopes on the non-structure proteins (Fig. S1b). The identified epitopes were relatively evenly distributed on both structural and non-structural proteins (Fig. S1b). In the different proteins of the SARS-CoV-2 proteome, the epitopes frequency of structural protein N are the highest, reaching 75.6%; the epitopes frequency of non-structural proteins nsp13 and nsp3 reached 36.5% and 29.3%, while the epitope frequency of structural protein S is only 20.4% (Fig. 1d). Further analysis of S protein showed the epitopes of NTD and RBD domains were relatively high, while the epitopes of N protein were relatively uniform within different domains (Fig. S1c).

Identification and longitudinal analysis of IgM-, IgG- and IgA-recognized epitopes

To investigate whether the different epitopes are recognized by particular isotypes of antibodies, we analyzed the epitopes of IgM, IgG, and IgA antibodies, respectively. As shown in Fig. 2a, IgM can recognize 187 epitopes, IgG recognizes up to 224 epitopes, and IgA recognizes only 86 epitopes. Although the number of epitopes is not the same between different types of antibodies, the epitopes they recognized are present in both structural and non-structural proteins (Fig. 2a). Curiously, only 19.25% of the epitopes recognized by IgM were located in the structural proteins, while the proportions of the epitopes recognized by IgG and IgA were 26.34% and 30.23%, respectively (Fig. 2a). For B-cell epitopes, the immunogenicity of SARS-CoV-2 proteins for different antibody isotypes can be ranked in the following order: (IgM) Orf1ab(nsp1-16) > S > N > M > Orf3a/Orf8 > Orf7a, (IgG) Orf1ab(nsp1-16) > S > N > Orf3a > Orf8/Orf7a > M > E/Orf10 and (IgA) Orf1ab(nsp1-16) > S > N > Orf3a > Orf7a / Orf8 protein. Interestingly, Orf6 and Orf10 did not show any reactivity to all IgM, IgG, and IgA antibodies in the serum, and thus they are assigned as non-immunogenic proteins (Fig. 2a).

Next, we sought to analyze which epitopes are common to all three kinds of antibodies and which are specific to a particular type of antibody. As shown in Fig. 2b, there are 64 common epitopes recognized by IgM, IgG, and IgA antibodies. The longitudinal changes of antibodies recognizing these common epitopes in the serum of COVID-19 patients show that some epitopes remain throughout the course of the disease after a diagnosis, while others are present only for certain time periods (Fig. S2). In non-structural proteins, nsp3 and nsp12 had more distinct epitopes, 14 and 8 epitopes, respectively; In structural proteins, there were relatively more epitopes, 8 and 7 epitopes, respectively, on S and N (Fig. S2).

We also performed detailed epitope analysis on these epitope varieties to determine which epitopes are more frequently recognized by IgM, IgG, or IgA antibodies. As shown in Fig. 2c, 33 epitopes are more frequently recognized by IgM, and these epitopes are mainly concentrated on non-structural proteins, with only one each on structural proteins S and N. These results suggest that non-structural proteins are more frequently recognized in the immune system during the early stages of virus invasion. Although the number of epitopes that are more frequently recognized by IgG is less than IgM (29 vs. 33), there are 7 and 6 epitopes on structural proteins S and N, respectively (Fig. 2c and 2d). This result indicates that functional IgG antibodies continually recognize structural protein epitopes, such as S-15, S-46, etc., with the immune process (Fig. 2c). More importantly, among these identified specific epitopes, some epitopes have been proved to be effective for neutralizing antibody recognition. For example, S-15 is the epitope recognized by 4A8, S-46 is the epitope recognized by B-38. Moreover, the longitudinal analysis showed that compared with S-46,

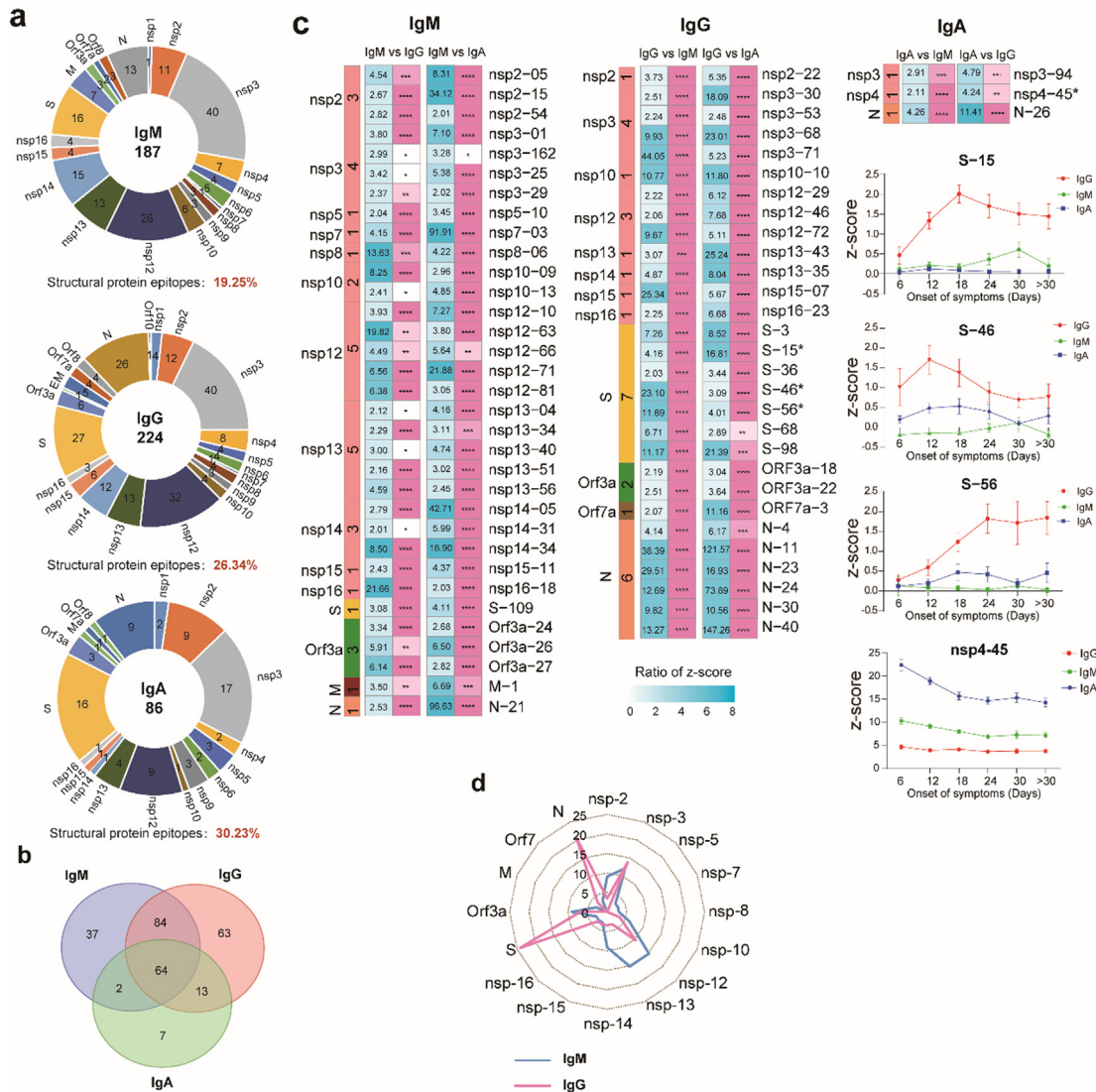


Fig. 2. Identification and analysis of IgM-, IgG- and IgA-recognized epitopes. (a) Distribution of IgM-, IgG- and IgA-recognized epitopes on each structural and non-structural protein. (b) Epitope overlap analysis, as depicted in a Venn diagram, showing overlaps between IgM-, IgG- and IgA-recognized epitopes. (c) Detailed epitopes analysis revealed the epitopes that are more frequently recognized by IgM, IgG, or IgA antibodies. Longitudinal changes of antibodies recognizing the specific epitopes (S-15, S-46, S-56, and nsp4-45) are shown. The number of epitopes in different proteins are also shown. The blue rainbow represents the difference of z-score between the two isotypes of antibodies that recognize a certain epitope. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. (d) The epitopes that are more frequently recognized by IgM antibodies are enriched on non-structural proteins, but epitopes that are more frequently recognized by IgG antibodies are enriched on S and N proteins.

the IgG antibody levels elicited by S-15 and S-56 increased with time, suggesting that S-15 and S-56 epitopes have more significant application potential in vaccine development.

Identification and longitudinal analysis of specific epitopes in the mild and severe COVID-19 patients

To investigate whether the immune responses of mild and severe patients to the SARS-COV-2 virus are different, we first analyzed the variety of epitopes recognized in mild and severe patients. We found no difference in the number of epitopes recognized by different antibodies in mild and severe patients (data not shown). We then analyzed differences in epitope-elicited antibody levels between mild and severe patients. As shown in Fig. 3a, the expression level of 23 epitope-elicited IgM antibodies was higher in mild patients than in severe patients, and the expression level of 5 epitope-elicited IgM antibodies was higher in patients with severe symptoms than in patients with mild symptoms, indicating that the immune response of mild patients was stronger than that of severe patients in the early stage of virus invasion. However, the

number of epitopes eliciting higher IgG expression was similar in severe patients and mild patients (13 vs 14), indicating that the immune response of severe and mild patients was similar in late-stage viral invasion (Fig. 3a). The number of epitopes eliciting higher IgA expression was less than IgM and IgG, but the number of epitopes was similar in severe and mild patients (Fig. 3a). And these epitopes are present in both structural and non-structural proteins, but the proportion of epitopes in non-structural proteins elicited a higher response IgM, IgG, or IgA in mild patients than in severe patients (Fig. S3a). We also analyzed the common epitopes of all three kinds of antibodies. As shown in Fig. S3b, there is only one common epitope recognized by IgM, IgG, and IgA antibodies in either mild or severe patients.

To explore the longitudinal changes of these specific epitopes, we longitudinally analyzed the changes in the numbers of IgM, IgG, and IgA-related specific epitopes in severe and mild patients. The number of IgM and IgG-related epitopes increased along with virus infection, only dropping slightly at 30 days, but the number of IgA-related epitopes was relatively stable, with an increase in severe patients at 30 days (Fig. 3b). However, investigation of the

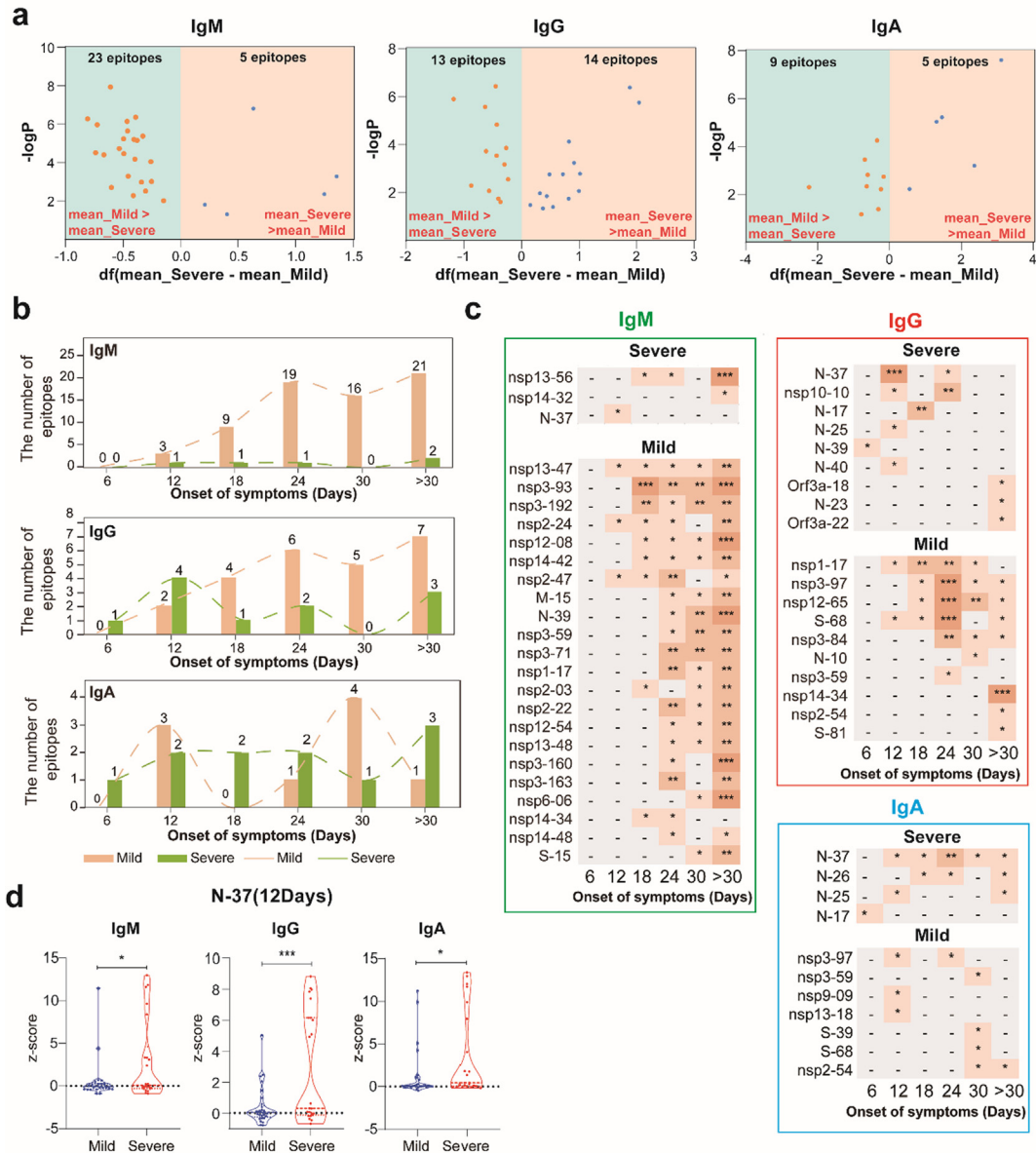


Fig. 3. Identification and longitudinal analysis of specific epitopes in patients with severe or mild disease. (a) Epitopes analysis revealed the specific epitopes in patients with severe or mild disease. The x-axis represents the $df(\text{mean_Severe} - \text{mean_Mild})$, the y-axis represents the $-\log p$. (b) Longitudinal changes in the number of specific epitopes in patients with severe or mild disease. (c) Longitudinal analyses of serum samples from patients with severe and mild disease showed differences in the expression of antibodies that recognized each specific epitope. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. (d) The N-37 epitope can bind to the IgM, IgG, and IgA antibodies in serum samples collected at day 12, and be used as a biomarker to distinguish severe and mild patients.

longitudinal changes of each epitope indicated some epitopes were continuous, while others are discontinuous. For example, the N-37 epitope of IgM only exists in the sample of 12 days but not in the other time points (Fig. 3c). Similarly, the N-37 epitope of IgG only exists in the sample of 12 days and 24 days, but not in the other time points (Fig. 3c). In contrast, the N-37 epitope of IgA exists in the samples only after 12 days (Fig. 3c). Interestingly, the N-37 peptide antibodies are significantly expressed higher in the severe patients than in the mild patients for all three isotype antibodies, indicating that this epitope may be used as a marker to distinguish the mild from severe disease (Fig. 3d).

Identification of dominant epitopes against SARS-CoV-2 in COVID-19 patients

We then analyzed the frequency of all epitopes elicited antibodies in the patients and found that some epitopes were abundant,

while others were relatively infrequent (Fig. 4a). The frequency of 21 IgM-related epitopes, 23 IgG-related epitopes, and 10 IgA-related epitopes is $>50\%$, while the frequency of other epitopes is $<50\%$ (Fig. 4a). To identify the specific epitopes of COVID-19 patients, we compared the serum of patients with healthy controls ($n = 20$). As shown in Fig. 4b, there are 12 dominant epitopes eliciting antibodies in >25 patients (frequency $> 50\%$, vs. healthy donors, $p < 0.01$). In addition, we found that two epitopes associated with higher serum levels of antibodies in older patients (>50 years) than in younger patients (<50 years) (Fig. S4a).

We then mapped the linear sequences of these epitopes using the SARS-CoV-2 proteome microarray at the amino acid resolution. Among these immunogenic peptides, the S-15 (146-HKNNK-150) epitope is located in the N terminal domain (NTD) of S1 protein, which has been reported as a partial target of a neutralizing antibody [18]. In our study, we identified epitope S-15 as linear. In addition, epitope S-82 (816-SFIED-820) was located in the fusion

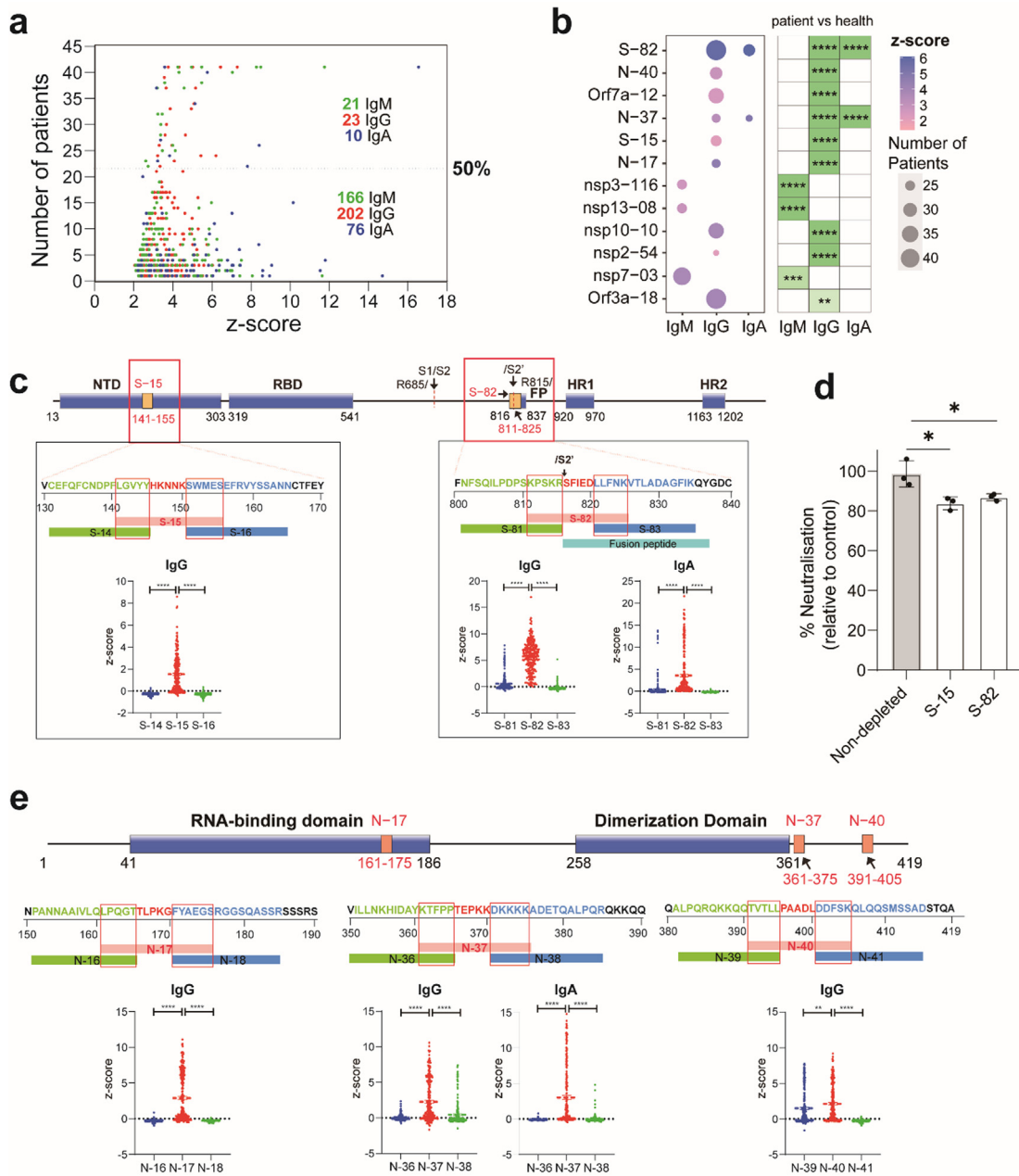


Fig. 4. Identification and analysis of dominant epitopes in COVID-19 patients. (a) The number of patients with antibodies elicited by each epitope in the serum. The x-axis represents the z-score of each epitope; the y-axis represents the number of patients. The rainbow color represents z-score, the circle size represents the number of patients, and the significance of the difference between patients and healthy people are represented by asterisks. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. (c) Identification of the minimum sequences of antibody binding epitopes (HKNNK, SFIED) on S protein through sequence alignment. (d) Non-depleted and S-15 or S-82-depleted sera were mixed with 100 TCID50 SARS-CoV-2. The mixture was moved to the wells containing Vero-E6 cells and incubated at 37 °C for 1 h. Following removing the supernatants, 200 μ L cell culture medium was added, and the plates were then incubated at 37°C with 5% CO₂ for three days. The percentage of neutralization relative to the non-depleted sera are shown. Data are presented as mean \pm SD in triplicates. *, $p < 0.05$. (e) Identification of the minimum sequences of antibody binding epitopes (TLCFT, TSPIS, KLWAQ) on N protein through sequence alignment. Identification of the minimum sequences of antibody binding epitopes (TLCFT, TSPIS, KLWAQ) on N protein through sequence alignment.

peptide (FP) of S2 protein (Fig. 4c), indicating that this epitope may be used as a target for neutralization antibodies. We further compared the levels of IgG elicited by S-82 and S-15 and found that the expression level of IgG elicited by S-82 was significantly higher than that induced by S-15 (Fig. S4b), indicating that S-82 may have higher application potential as an epitope for vaccine design. To assess the importance of antibodies elicited by S-15 or S-82 in controlling SARS-CoV-2 infection, antibody depletion assays were performed against S-15 and S-82. Interestingly, sera depleted for antibodies targeting either peptides significantly reduced the abil-

ity to neutralize SARS-CoV-2 infection compared with the non-depleted sera controls (Fig. 4d).

We also identified a linear sequence of three epitopes on N protein. The N-17 epitope (166-TLPKG-170) is located in the RNA-binding domain of the N protein. While the N-37 (366-TLPKG-370) and N-40 (396-PAADL-400) epitopes are in the C terminal of N protein (Fig. 4d). In addition, we also identified the linear sequence of seven epitopes on the non-structural proteins, including Orf3a-18 (176-PAADL-180), nsp7-03 (3886-PAADL-3890), nsp2-54 (716-LYRKC-720), nsp3-116 (1176-TPSKF-1180), nsp13-08 (5396-

CKSHK-5400), nsp10-10 (4346-KGKYV-4350), and Orf7a-12 (116-TLCFT-121).

Discussion

A systematic understanding of the host immune response against SARS-CoV-2 proteome epitopes in COVID-19 patients is of paramount importance to improve our understanding of viral infection and inform the development of improved diagnostics, vaccines, and antibody-based therapies. By analyzing the antibodies in the serum of patients, several research groups have revealed some immune processes of COVID-19 patients against SARS-CoV-2 and found some neutralizing antibodies or dominant epitopes of a potential vaccine [2,20–23]. However, there is still a lack of more systematic and comprehensive information on the immune response to SARS-CoV-2 to help us cope with this pandemic. Here, we overviewed a complete analysis of the humoral immune response of COVID-19 patients expanding the whole proteome of the virus and the complete process of humoral immunity.

Several papers have been published in the last several months to study the epitopes of SARS-CoV-2 S protein [2,13]. However, few studies have focused on the role of the other 26 proteins in the clinical immune response. In particular, by analyzing epitopes in the SARS-CoV-2 using VirScan, Ellen Shrock. et al. revealed cross-reactivity and correlates of the severity of SARS-CoV-2 [16], but in addition to the differences in the methodology, there were also differences in the population sampled and the SARS-CoV-2 strain infected by the population. To address this challenge, we measured the expression of SARS-CoV-2 IgM, IgG, and IgA antibodies in the serum of COVID-19 patients by using the SARS-CoV-2 proteome microarray containing all four structural and 23 non-structural proteins (1,340,208 Ag-Ab reactions). The B-cell epitopes were identified by sequence alignment of immune reactive peptides with neighboring peptides (Table S1-3). Moreover, because there are the most studies on the IgG-recognized epitopes in Spike protein, enough data on IgG-recognized epitopes in Spike protein for statistical analysis. We then analyzed and compared the data of IgG-recognized dominant epitope of S protein from seven different research teams (Table S4). We found our some immunogenic epitopes are partially consistent with these studies. However, some immunogenic epitopes with a high consistency were also observed, for example, S-56, S-82, and S-115/116 (Table S4). We found that the identified epitopes were relatively evenly distributed on both structural and non-structural proteins, which shows that non-structural proteins also play an important role in the process of humoral immunity against viral infection. In structural proteins, Protein S has received most of the attention thus far; however, surprisingly, M and N protein show a higher proportion of epitopes coverage in protein. This suggests that the presence of large numbers of these epitopes may induce serious ADE (antibody-dependent enhancement) risks. Given these non-structural proteins do not exist on the surface of the virus, they are not normally thought to activate the production of large numbers of antibodies, but our study found that the ability of unstructured proteins to induce antibody production is equal to that of structured proteins. Although the epitopes of non-structural proteins cannot directly guide the development of neutralizing antibodies and vaccines against the SARS-CoV-2, they can be used in the detection of virus infection to some extent.

Unlike most of the research to study the epitopes recognized by IgG or IgM [24–26], we analyzed the production and the longitudinal changes of SARS-CoV-2 proteome epitopes for three important isotype antibodies (IgM, IgG, and IgA). The IgM is the first antibody to be produced during infection, and its efficient activation of the complement system is important in controlling the acute infection.

IgG is produced by B cells and the most common antibody in the blood that protects against bacterial and viral infections. While IgA antibodies are produced by B cells located in the mucous membranes of the body and the main class of antibodies in body secretions, including tears, saliva, respiratory and intestinal secretions, colostrum, and serum. Furthermore, we found that the proportion of structural proteins in the epitopes recognized by IgM was 19.25%, which was lower than that in the epitopes recognized by IgG (26.34%). Moreover, we observed the differences in epitope recognition by IgM, IgG, or IgA antibodies. There are 33 epitopes that are more frequently recognized by IgM, and these epitopes are mainly concentrated in non-structural proteins, with only one each on structural proteins S, N, and M. These results suggest that the “cunning SARS-CoV-2” may induce a smokescreen of non-structural proteins, in which the immune response creates a large number of ineffective antibodies against non-structural proteins in the early stage, so the virus can replicate and proliferate in the body in the early stage. This may be an evolutionary adaptation of the SARS-CoV-2 against the host immune system, given the high proportion of non-structural proteins in the whole proteome.

We then investigated whether mild and severe patients have different immune responses to the SARS-CoV-2 virus. Interestingly, we identified some specific epitopes eliciting expression differences of IgM, IgG, or IgA. Moreover, we found that the epitopes that elicited higher antibody expression in mild patients are more distributed on non-structural proteins, but more epitopes that elicited higher antibody expression in severe patients are distributed on structural proteins. Considering that the immune response is a dynamic process, we further performed the longitudinal analysis of these specific epitopes. We first longitudinally compared the number of changes of IgM, IgG, and IgA-related specific epitopes between the two groups of patients. The number of IgM and IgG-related specific epitopes increased along with virus infection in mild patients, whereas the number of IgA-related specific epitopes was relatively stable. Moreover, some epitopes inducing differential expression of antibody at an early stage (6 days to 12 days) may be useful in the early detection of COVID-19 disease and the prediction of disease progression from mild to severe.

The ability of epitopes to elicit neutralizing antibodies in most vaccinated populations is an essential precondition as a vaccine candidate. Therefore, we analyzed the frequency of epitopes eliciting antibodies in 41 COVID-19 patients. Considering the possibility of non-specific epitopes cross-recognized by non-coronavirus antibodies and other coronavirus antibodies [27,28], we analyzed the antibody recognizing these epitopes in serum samples of 20 healthy donors. In the end, 12 dominant B-cell linear epitopes were identified as the COVID-19 specific epitope (vs. healthy donors, $p < 0.01$) and epitope widely eliciting antibodies in diverse patients (frequency > 50%). Among the 12 dominant epitopes, there were two epitopes on S protein and three epitopes on N protein. Therefore, these epitopes on structural proteins have great potential for vaccine design. In addition, the epitope of S-15 is located in the N terminal domain (NTD) of the S1 protein, which is the part of the target region previously reported for neutralizing antibodies [18]. Compared to previous reports, we identified the epitope of S-15 as linear and identified the frequency of S-15 eliciting antibodies in COVID-19 patients was 63.4%, indicating that S-15 is suitable as an epitope for vaccine design. We then identified five key amino acids of S-15 (146-HKNNK-150) in our study that may be used for the precise development of a vaccine.

We also identified epitopes of S-82 as a dominant epitope and identified five key amino acids of the S-82 (816-SFIED-820) epitope. In our follow-up analysis, we found that S-82 (816-SFIED-820) is located in the fusion peptide (FP) domain of the S2 protein (Fig. S5a). We know that in the fusion phase of SARS-CoV-2 after the S1 subunit is cut, the FP on the S2 subunit is inserted into

the host cell membrane to induce conformational changes of the S2 subunit for SARS-CoV-2 to invade into host cells [29,30]. Therefore, antibodies targeting the FP domain may prevent virus fusion, and S-82 epitope eliciting antibodies can be neutralizing. In addition, We analyzed the amino acid sequence of S-82 (816-SFIED-820) and found that S-82 was highly conserved among several human pathogenic coronaviruses (Fig. 5b). This highly conserved epitope sequence usually means that these amino acids are crucial to the protein's function and are the "life gate" of these viruses, so it is not easy to produce "escape mutation." Therefore, the antibody elicited by S-82 is rare broad-spectrum antibody. Broad-spectrum antibodies, such as S-82, can not only help us through the current crisis but also be a powerful weapon to deal with the future unknown coronavirus epidemic. It's also surprising that we found the level of antibody expression elicited by S-82 was similar to that of the S2ECD region of S protein (Fig. S5c), which indicated that S-82 is a very good immunogen. To assess the effectiveness of antibodies elicited by S-15 or S-82 in controlling SARS-CoV-2 variants, we compared and analyzed amino acid sequences of the S-15 and S-18 of five "Variants of Concern" (including B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta) and B.1.617.1 (Kappa)). We found amino acid variation of S-15 in B.1.1.7 (Alpha) and B.1.617.1 (Kappa) variants, suggesting that the effectiveness of antibody elicited by S-15 may be decreased to these variants. However, the amino acids of S-82 were highly conserved in five "Variants of Concern", indicating that antibody elicited by S-82 may still maintain a good effectiveness in different variants (Fig. S6).

Conclusion

In our study, we performed a longitudinal and proteome-wide analysis of antibodies in the COVID-19 patients using a SARS-CoV-2 proteome microarray (1,340,208 Ag-Ab reactions). As far as we know, this is the first systematic analysis of antibodies in the COVID-19 patients through the whole viral proteome of the SARS-CoV-2, the whole course of the patient, and different antibody isotypes (IgM, IgG, and IgA). In our findings, we profiled a B-cell epitope landscape of SARS-CoV-2 and identified specific epitopes recognized by IgM, IgG, or IgA. We found that epitopes more frequently recognized by IgM are enriched in non-structural proteins, which may be an evolutionary adaption of the SARS-CoV-2 against the host immune system. We further identified epitopes with different immune responses in severe and mild patients. Moreover, we identified 12 dominant B-cell epitopes eliciting antibodies in most COVID-19 patients and identified the key sequence of epitopes at the amino acid resolution (five key amino acids). Excitingly, epitope S-82 (fusion peptide domain of the S2 protein) and S-15 (N terminal domain of S1 protein) are perfect immunogenic peptides and should be considered in vaccine design. This data provides useful information and rich resources for developing a novel vaccine and neutralizing antibodies for the treatment of SARS-CoV-2.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported by the National Key R&D Program of China-Vaccine design (2020YFC0860100), National Natural Science Foundation of China (31870156), Beijing Municipal Science & Tech-

nology Commission (Z211100002521021), and National Key R&D Program of China (2020YFE0202200).

Data availability

All main and Supplementary Figures have associated raw data. Raw data will be available in the controlled access repository Mendeley (<https://data.mendeley.com/datasets/s6smvttgdr/draft?fa=55df2640-400e-4efb-ae6a-be668dc08b4e>).

Compliance with ethics requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

The study was approved by the IRB of The Fifth Medical Center of PLA General Hospital (#2020069D). Written informed consent was obtained from all study patients or their surrogates prior to their inclusion in the study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2021.07.008>.

References

- [1] Wu F, Zhao Su, Yu B, Chen Y-M, Wang W, Song Z-G, et al. A new coronavirus associated with human respiratory disease in China. *Nature* 2020;579(7798):265–9.
- [2] Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020;579(7798):270–3.
- [3] Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses. *J Adv Res* 2020;24:91–8.
- [4] Tegally H, Wilkinson E, Lessells RJ, Giandhari J, Pillay S, Msomi N, et al. Sixteen novel lineages of SARS-CoV-2 in South Africa. *Nat Med* 2021;27(3):440–6.
- [5] Wise J. Covid-19: New coronavirus variant is identified in UK. *BMJ (Clinical research ed)* 2020;371:m4857.
- [6] Volz E, Mishra S, Chand M, Barrett JC, Johnson R, Geidelberg L, et al. Transmission of SARS-CoV-2 Lineage B.1.1.7 in England: Insights from linking epidemiological and genetic data. *medRxiv*. 2021:2020.12.30.20249034.
- [7] Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, et al. Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. *medRxiv*. 2020:2020.12.21.20248640.
- [8] Wibmer CK, Ayres F, Hermanus T, Madzivhandila M, Kgagudi P, Lambson BE, et al. SARS-CoV-2 501Y.V2 escapes neutralization by South African COVID-19 donor plasma. *bioRxiv*. 2021:2021.01.18.427166.
- [9] Maggi F, Novazzi F, Genoni A, Baj A, Spezia PG, Focosi D, et al. Imported SARS-CoV-2 variant P.1 detected in traveler returning from Brazil to Italy. *Emerg Infect Dis* 2021;27(4).
- [10] Cherian S, Potdar V, Jadhav S, Yadav P, Gupta N, Das M, et al. Convergent evolution of SARS-CoV-2 spike mutations, L452R, E484Q and P681R, in the second wave of COVID-19 in Maharashtra, India. *bioRxiv*. 2021:2021.04.22.440932.
- [11] Hoffmann M, Hofmann-Winkler H, Krüger N, Kempf A, Nehlmeier I, Graichen L, et al. SARS-CoV-2 variant B.1.617 is resistant to Bamlanivimab and evades antibodies induced by infection and vaccination. *bioRxiv*. 2021:2021.05.04.442663.
- [12] Yadav PD, Sapkal GN, Abraham P, Ella R, Deshpande G, Patil DY, et al. Neutralization of variant under investigation B.1.617 with sera of BBV152 vaccinees. *Clin Infect Dis* 2021.
- [13] Zhang B-Z, Hu Y-F, Chen L-L, Yau T, Tong Y-G, Hu J-C, et al. Mining of epitopes on spike protein of SARS-CoV-2 from COVID-19 patients. *Cell Res* 2020;30(8):702–4.
- [14] Amrun SN, Lee C-P, Lee B, Fong S-W, Young BE, Chee R-L, et al. Linear B-cell epitopes in the spike and nucleocapsid proteins as markers of SARS-CoV-2 exposure and disease severity. *EBioMedicine* 2020;58:102911. doi: <https://doi.org/10.1016/j.ebiom.2020.102911>.
- [15] Zhou W, Xu X, Chang Z, Wang He, Zhong X, Tong X, et al. The dynamic changes of serum IgM and IgG against SARS-CoV-2 in patients with COVID-19. *J Med Virol* 2021;93(2):924–33.

- [16] Shrock E, Fujimura E, Kula T, Timms RT, Lee IH, Leng Y, et al. Viral epitope profiling of COVID-19 patients reveals cross-reactivity and correlates of severity. *Science* 2020.
- [17] Wang H, Wu X, Zhang X, Hou X, Liang T, Wang D, et al. SARS-CoV-2 proteome microarray for mapping COVID-19 antibody interactions at amino acid resolution. *ACS Central Science* 2020.
- [18] Chi X, Yan R, Zhang J, Zhang G, Zhang Y, Hao M, et al. A neutralizing human antibody binds to the N-terminal domain of the Spike protein of SARS-CoV-2. *Science* 2020;369(6504):650–5.
- [19] Radspieler M, Schindeldecker M, Stenzel P, Frsch S, Tagscherer K, Herpel E, et al. Lamin-B1 is a senescence-associated biomarker in clear-cell renal cell carcinoma. *Oncol Lett* 2019. doi: <https://doi.org/10.3892/ol.2019.10593>.
- [20] Guo L, Ren L, Yang S, Xiao M, Chang D, Yang F, et al. Profiling early humoral response to diagnose novel coronavirus disease (COVID-19). *Clin Infect Dis* 2020;71(15):778–85.
- [21] Liu Y, Yan L-M, Wan L, Xiang T-X, Le A, Liu J-M, et al. Viral dynamics in mild and severe cases of COVID-19. *Lancet Infect Dis* 2020;20(6):656–7.
- [22] Liu ZL, Liu Y, Wan LG, Xiang TX, Le AP, Liu P, et al. Antibody profiles in mild and severe cases of COVID-19. *Clin Chem*. 2020;66(8):1102–4.
- [23] Long QX, Liu BZ, Deng HJ, Wu GC, Deng K, Chen YK, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med*. 2020;26(6):845–8.
- [24] Sun B, Feng Y, Mo X, Zheng P, Wang Q, Li P, et al. Kinetics of SARS-CoV-2 specific IgM and IgG responses in COVID-19 patients. *Emerg Microbes Infect* 2020;9(1):940–8.
- [25] Qu J, Wu C, Li X, Zhang G, Jiang Z, Li X, et al. Profile of IgG and IgM antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *Clin Infect Dis* 2020.
- [26] Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis* 2020.
- [27] Ehrenfeld M, Tincani A, Andreoli L, Cattalini M, Greenbaum A, Kanduc D, et al. Covid-19 and autoimmunity. *Autoimmun Rev* 2020;19(8):102597. doi: <https://doi.org/10.1016/j.autrev.2020.102597>.
- [28] Zhang Y, Xiao M, Zhang S, Xia P, Cao W, Jiang W, et al. Coagulopathy and antiphospholipid antibodies in patients with covid-19. *N Engl J Med* 2020;382(17):e38. doi: <https://doi.org/10.1056/NEJMc2007575>.
- [29] Tang T, Bidon M, Jaimes JA, Whittaker GR, Daniel S. Coronavirus membrane fusion mechanism offers a potential target for antiviral development. *Antiviral Res* 2020;178:104792. doi: <https://doi.org/10.1016/j.antiviral.2020.104792>.
- [30] Mitra P. Inhibiting fusion with cellular membrane system: therapeutic options to prevent severe acute respiratory syndrome coronavirus-2 infection. *Am J Physiol Cell Physiol* 2020;319(3):C500–9.