

# Identification of an immunogenic epitope and protective antibody against the furin cleavage site of SARS-CoV-2



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## Summary

**Background** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the global coronavirus disease 2019 (COVID-19) pandemic, contains a unique, four amino acid (aa) “PRRA” insertion in the spike (S) protein that creates a transmembrane protease serine 2 (TMPRSS2)/furin cleavage site and enhances viral infectivity. More research into immunogenic epitopes and protective antibodies against this SARS-CoV-2 furin cleavage site is needed.

**Methods** Combining computational and experimental methods, we identified and characterized an immunogenic epitope overlapping the furin cleavage site that detects antibodies in COVID-19 patients and elicits strong antibody responses in immunized mice. We also identified a high-affinity monoclonal antibody from COVID-19 patient peripheral blood mononuclear cells; the antibody directly binds the furin cleavage site and protects against SARS-CoV-2 infection in a mouse model.

**Findings** The presence of “PRRA” amino acids in the S protein of SARS-CoV-2 not only creates a furin cleavage site but also generates an immunogenic epitope that elicits an antibody response in COVID-19 patients. An antibody against this epitope protected against SARS-CoV-2 infection in mice.

**Interpretation** The immunogenic epitope and protective antibody we have identified may augment our strategy in handling COVID-19 epidemic.

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### Research in context

#### Evidence before this study

SARS-CoV-2 contains a unique, four aa “PRRA” insertion in the S protein, creating a TMPRSS2/furin cleavage site. The furin site of SARS-CoV-2 has been found to enhance viral infectivity,<sup>1</sup> to be required for transmission in ferrets,<sup>2</sup> and to mediate membrane fusion in either the presence or absence of trypsin.<sup>3</sup> However, relevant immunogenic epitopes and human antibody screening against this site are rarely reported.

#### Added value of this study

By bioinformatic prediction and experimental confirmation, we identified the S672-691 peptide as an immunogenic epitope that can be used for COVID-19 diagnosis with high

sensitivity and specificity. Further, mouse immunization experiments proved that the S672-691 peptide stimulated antibody generation in mice, and a monoclonal antibody identified from COVID-19 patient peripheral blood mononuclear cells using an scFv phage display library protected against SARS-CoV-2 infection.

#### Implications of all the available evidence

Our identification of an immunogenic epitope and protective antibody against the furin cleavage site indicates the ability of our immune system to react to the unique evolution of SARS-CoV-2 and also provides potential applications in the diagnosis and treatment of COVID-19.

## Introduction

Since the initial outbreak in December 2019, coronavirus disease 2019 (COVID-19) has quickly developed into a full-blown global pandemic.<sup>4-6</sup> New severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants are continuously emerging, posing sustained threats to global health and economic stability.<sup>7-10</sup> COVID-19 patients generate polyclonal antibodies that recognize various epitopes across the SARS-CoV-2 proteome.<sup>11,12</sup> These polyclonal antibodies can neutralize and provide humoral immunity, whereas CD8 T cells can provide cellular immunity against SARS-CoV-2 infection. Many previous efforts have tried to identify and isolate immunogenic T cell and B cell epitopes using single-cell sequencing, activation-induced marker (AIM) assay, degranulation, proliferation, ELISA, ELISpot, intracellular cytokine staining (ICS), cytotoxicity, and multimer-based assays.<sup>13-19</sup> However, many new epitopes exist among SARS-CoV-2 viral proteins, and which among these are immunogenic and have potential value in COVID-19 treatment requires investigation.

The SARS-CoV-2 viral genome consists of a 29.8 kb, positive-strand RNA molecule encoding proteins including 16 nonstructural proteins (NSPs), 4 structural proteins (Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N)) and several accessory proteins (ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10).<sup>20,21</sup> SARS-CoV-2 infects host cells through its S protein, which contains the S1 domain, which binds to cellular angiotensin-converting enzyme 2 (ACE2) receptors, and the S2 domain, which accomplishes membrane fusion.<sup>22-24</sup> As a member of the large coronavirus family that includes Alphacoronavirus (i.e., Human coronavirus 229E, Human coronavirus NL63), Betacoronavirus (SARS-CoV, SARS-CoV-2, Middle East respiratory syndrome-related coronavirus (MERS-CoV), human coronavirus OC43 and HKU1), Gammacoronavirus

(Avian coronavirus (IBV)), and Deltacoronavirus (Bulbul coronavirus HKU11 (Bulbul-CoV HKU11)),<sup>25,26</sup> SARS-CoV-2 has a unique four amino acid (aa) insertion between the S1 and S2 domains of the S protein, which creates a transmembrane protease serine 2 (TMPRSS2)/furin cleavage site and has been shown to increase viral infectivity.<sup>1</sup>

Most of the neutralization antibodies identified thus far bind to the receptor binding domain (RBD), while a few bind to the amino-terminal domain (NTD) or the S2 domain of the S protein. In this study, we have identified an immunogenic epitope at the furin cleavage site that can specifically and sensitively detect IgM and IgG in COVID-19 patients and can generate strong immune response in immunized mice. Most importantly, we have identified monoclonal antibody targeted to this furin cleavage site in COVID-19 patient's sera and shown to have immuno-protective activity against SARS-CoV-2 infection in a mouse model.

## Methods

### Viruses, cells, plasmids, and reagents

SARS-CoV-2 virus and SARS-CoV-2 original and Omicron strain pseudovirus with *Luciferase* coding sequence were described in our previous paper.<sup>27</sup> Huh7.5 (RRID: CVCL\_U443), HEK293T (RRID: CVCL\_4U22), and Vero (RRID: CVCL\_0059) cell lines were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 50 µg/mL streptomycin (37 °C, 5% CO<sup>2</sup>). Authentication of cell lines (Huh7.5, HEK293T, and Vero) with short tandem repeat DNA profiles were performed every year with Procell Life Science & Technology Co., Ltd. (Wuhan, China). Mycoplasma contamination was tested by PCR with

supernatant of cell culture (Beyotime, Cat No.: C0301S), and only cell lines which were negative for mycoplasma testing were used in this study. hACE2 expression plasmid was purchased from GenScript (Cat No.: OHU20260D). hTMPRSS2 expression plasmid was purchased from Neobioscience (Cat No.: puno1-htps2a). 2-(N-Morpholino) ethanesulfonic acid (MES) was purchased from Sigma (Cat No.: S908908), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from ThermoFisher Scientific (Cat No.: 22981 and 002023). Adjuvant MF59 was prepared according to the protocol described by Ott G.<sup>28</sup> S monomer and trimer proteins were purified and provided by AtaGenix Laboratories (Wuhan).

### Clinical investigations and data analysis

The clinical information on human serum samples was self-reported by participants and described in our previous work.<sup>27</sup> Clinical investigations of patients with SARS-CoV-2 infection and healthy individuals were approved by the Ethics Committee of Taihe hospital (2021XM001), and informed consent was obtained from the patients. All serum samples were collected from patients admitted to Taihe hospital (Shiyan, China) for COVID-19 diagnosis from December 01, 2019 to March 11, 2020. The patients' venous blood was collected and serum samples were obtained by centrifugation. Briefly, 43 COVID-19 patients' samples were collected from the patients with obvious SARS-CoV-2 infection symptoms (high temperature, radiologic evidence of pneumonia, low or normal white-cell or lymphocyte count), and PCR+; 117 COVID-19 suspected patients' samples were collected from patients who were PCR- at the time the sera were collected, but were admitted in hospital because of obvious SARS-CoV-2 infection symptoms like high temperature, radiologic evidence of pneumonia, low or normal white-cell or lymphocyte count, and probable SARS-CoV-2 infection; 46 healthy individuals' samples were collected from who were PCR- and without SARS-CoV-2 infection symptoms.

The participants' clinical information (sex, age, whether high temperature and cough, and CT scan results) was collected, statistically compared and analyzed to avoid potential confounders before serum ELISA data was analyzed.

### Mouse experiments

The animal study was carried out following the recommendations for the care and use of animals by the Office of Laboratory Animal Welfare, NIH. The Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch (UTMB) approved the animal studies (2103023). Sample sizes of mice utilized in each experiment are calculated based on the pre-experiment

results. The calculation formula is  $n = 2 (Z_{\alpha} + Z_{1-\beta})^2 \delta^2 / \Delta^2$ .  $Z_{\alpha}$  (1.96) and  $Z_{1-\beta}$  (0.8416) are constants set by convention according to the accepted  $\alpha$  error and power of the study,  $\delta$  is the standard deviation and  $\Delta$  is the difference in effect of two interventions which are calculated based on the results of pre-experiments. We referenced the calculation with animal ethnicity and animal welfare considered to evaluate how many mice should be used in each experiment. For R4P1-C2 prophylactic assay, when the mean of three mice per group in pre-experiments is used, the sample size  $n$  is  $2 \times (1.96 + 0.8416)^2 \times (0.5915)^2 / (1.12)^2 = 4.69$  mice/group. So, it would need approximately 5 mice/group for this assay.

Ten 8-week-old C57Bl/6 (For the immunization assay, S672-691 peptide group and control group,  $n = 5$ /group) and 10 10 to 12-week-old female Balb/c mice (For R4P1-C2 prophylactic assay, R4P1-C2 antibody group and IgG control group,  $n = 5$ /group) were purchased from Charles River Laboratories and maintained in Sealsafe HEPA-filtered air in/out units.

For the immunization assay, C57Bl/6 mice were randomly divided into two groups. Under sterile conditions, 50  $\mu$ g BSA-conjugated peptide dissolved in 50  $\mu$ L DNase/RNase-free H<sub>2</sub>O was mixed with 50  $\mu$ L adjuvant MF59. Each C57Bl/6 mouse was immunized with 50  $\mu$ g BSA-conjugated S672-691 peptide or control peptide. Eight days post immunization, 100  $\mu$ L orbital blood was collected. The sera were used for ELISA and SARS-CoV-2 pseudovirus neutralization assay.

For R4P1-C2 prophylactic assay, Balb/c mice were randomly divided into two groups, and administrated intraperitoneally with antibody R4P1-C2 (1.7 mg/kg) or IgG control. 6 h later, animals were challenged intranasally with  $10^4$  PFU of mouse-adapted SARS-CoV-2 (CMA3p20 strain).<sup>29</sup> Two days after infection, lung samples of infected mice were harvested and homogenized in 1 mL DPBS using the MagNA Lyser (Roche Diagnostics). The homogenates were clarified by centrifugation at 15,000 rpm for 5 min. The supernatants were collected for measuring infectious virus titers by standard plaque assay.

### B-cell epitope prediction and analysis

SARS-CoV-2 sequence data were obtained from NCBI GenBank (NC\_045512). The SARS-CoV-2 S protein sequence was extracted based on the whole genome for subsequent analysis. We used Bepipred Linear Epitope Prediction 2.0 method (<http://tools.immuneepitope.org/bcell/>) at the immune epitope database and analysis resource (IEDB, [https://www.iedb.org/home\\_v3.php](https://www.iedb.org/home_v3.php)) to predict the potential B cell epitopes in SARS-CoV-2 S protein. Based on the computer-guided homology modeling method, the structural models were constructed by SWISS-MODEL online server as described.<sup>30,31</sup> The model of SARS-CoV-2 S protein was based on PDB ID: 6M0J, and the epitope sequences are marked in PYMOL.<sup>32</sup>

### Viral sequence analysis

We obtained S protein sequences of representative human CoVs, bat SARS-like CoVs, and MERS from NCBI GenBank (The corresponding accession numbers are marked in Fig. 1d). Then the SARS-CoV-2 S protein sequences were extracted based on the whole genome for subsequent analysis. The multiple sequence alignment was conducted using the MegAlign Pro software in DNASTAR Lasergene package with default parameters.

### Enzyme-Linked ImmunoSorbent Assay

Enzyme-Linked ImmunoSorbent Assay (ELISA) was described in our previous paper.<sup>27</sup> Briefly, we synthesized peptides from GenScript (Nanjing, China) and diluted in MES buffer (0.1 M, PH = 6.0) before adding 10  $\mu$ L EDC and 50  $\mu$ L peptide to each well and incubating the plates either at 4 °C overnight, or at room temperature (RT) for more than 2 h. After washing three times and blocking the plates, 100  $\mu$ L/well diluted sera (1:500) was added to the plates and incubated at 37 °C for 2 h. The plates were washed three times before addition of 100  $\mu$ L/well diluted secondary antibody (HRP-goat anti human IgG) and incubation at 37 °C for 50 min. These plates were then washed 5 times before addition of 100  $\mu$ L/well TMB and incubation for 5–10 min with protection from light. The reactions were terminated by adding 50  $\mu$ L/well 2 M H<sub>2</sub>SO<sub>4</sub> before measuring absorbance at 450 nm with SpectraMax i3 (Molecular Devices) plate reader.

### Net OD<sub>450</sub> calculation

We used the net OD<sub>450</sub> values in statistical analysis of ELISA results and in sensitivity and specificity calculations for receiver operating characteristics (ROC) curves. The net OD<sub>450</sub> is equal to OD<sub>450</sub> in the experimental samples minus the background OD<sub>450</sub>, for which serum was added to wells without peptide.

### Virus neutralization assay

The neutralizing ability of S672-691 immunized mouse sera were determined in Huh7.5 cells via *Luciferase* reporter assay. Briefly, serially diluted sera were mixed with SARS-CoV-2 pseudovirus and incubated at 37 °C for 2 h. The mixtures were then added to Huh7.5 cells and incubated for 24 h. Luciferase activity was measured as described previously,<sup>33</sup> with the average activity in the control group set as 0, representing no neutralization.

### S672-691 peptide inhibition assay

Huh7.5 cells were seeded in 48-well plates and cultured overnight. BSA-conjugated 672–691 peptide was diluted as indicated and added to Huh7.5 cells. Then Huh7.5 cells were incubated for 2 h before infection with SARS-CoV-2 original or Omicron strain

pseudovirus. Quantity of pseudovirus was measured at approximately 24 h post infection by Luciferase reporter assay.

### In vitro enzyme cleavage assay

Approximately 2  $\mu$ g of full-length S protein was cleaved *in vitro* by 0.2  $\mu$ L furin enzyme in 10  $\mu$ L reaction system supplemented with PBS, 2  $\mu$ g BSA, BSA-S25-39, BSA-S672-691, BSA-S672-691 sequence scrambled (S672-691 (S), QTQAARSYTVASRSQNSPR) peptide or titrated BSA-672-691 peptide as indicated at 25 °C for 1 h. Then, the cleaved S protein was immunoblotted with anti-S2 antibody (MP Biomedicals, Cat No.: S201123, RRID: AB\_2920626), which detected both full-length S and cleaved S2.

### COVID-19 scFv phage display library construction, biopanning, and purification

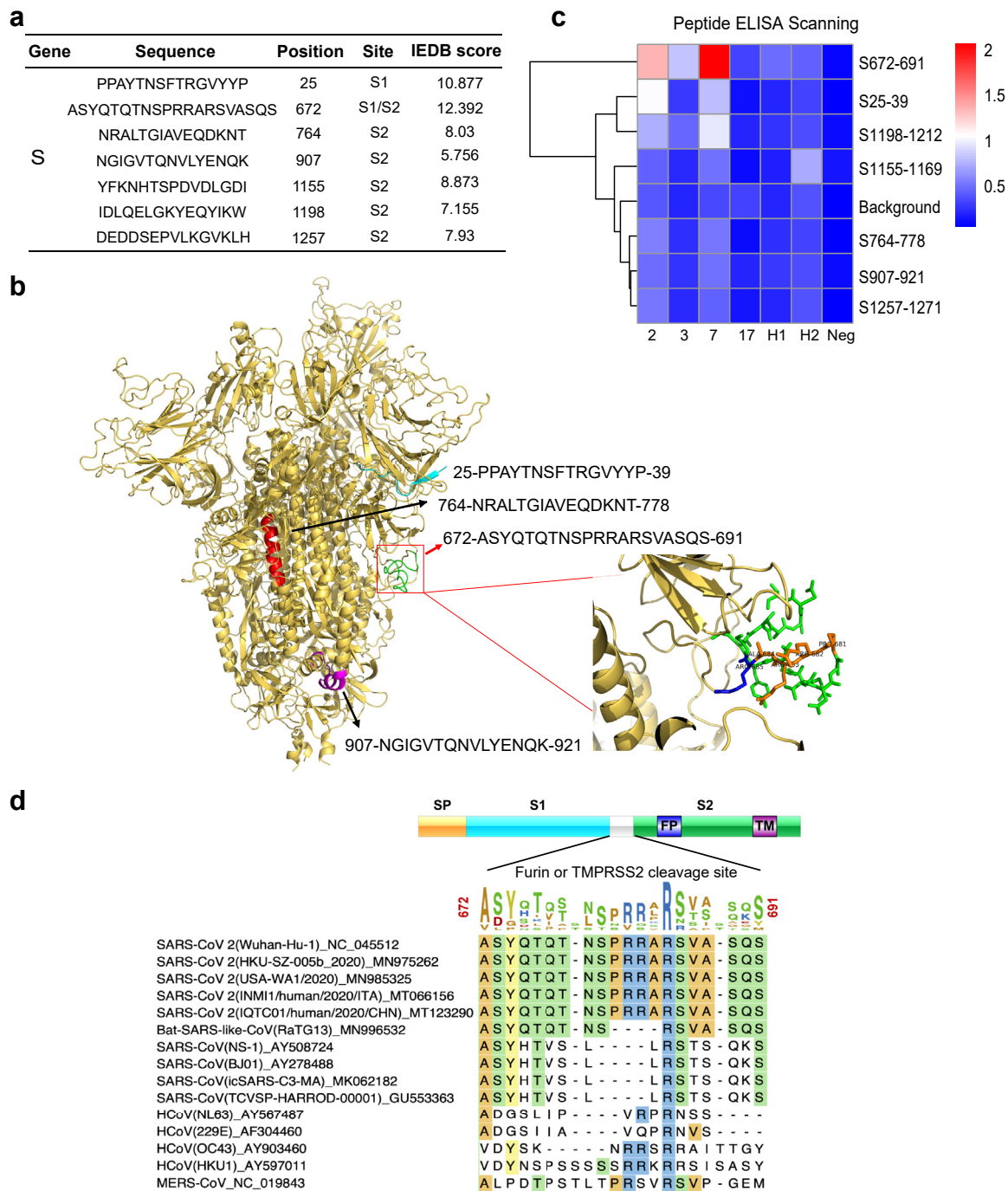
COVID-19 scFv phage display library was used in this study as previously described.<sup>27</sup> Specific phages against the S672-691 were affinity-enriched by 4 rounds of biopanning which cross-used biotin and BSA-S672-691 peptides as the capture antigens. When analyzing for S672-691 naked peptide-specific binding by phage ELISA, 2 unique positive antibodies were obtained by validated ELISA and sequencing analysis. R3P1-B9 and R4P1-C2 expression plasmids were transformed into cells and the expressed scFv were purified as described.<sup>27</sup>

### Surface plasmon resonance

The binding of R4P1-C2 antibody to S672-691 peptide and S protein under laminar flow was analyzed by surface plasmon resonance (SPR) using a BIAcore T200 system (GE Healthcare). The surface of a carboxymethylated dextran (CM5) sensor chip (GE Healthcare) was activated with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ThermoFisher Scientific, Cat No.: E2247) and 0.1 M N-hydroxysuccinimide (ThermoFisher Scientific, Cat No.: 24500). R4P1-C2 antibody was immobilized by amine coupling to one flow cell. All free reactive surface groups were blocked using 1 M ethanolamine (Merck, Cat No.: 398136). Different concentrations of antigen in HBS buffer containing 0.005% Tween-20 were injected over the flow cells at 30  $\mu$ L/min (contact time, 2 min). After each injection, any bound protein was stripped with 10 mM glycine (15 s). Data analysis was performed using the BIAcore T200 evaluation software 3.1 (GE Healthcare).

### Quantitative reverse transcription PCR

Vero cells were treated for 1 h with either ddH<sub>2</sub>O (vehicle control) or 1  $\mu$ g/mL BSA-conjugated S672-691, and then infected with 100  $\mu$ L TCID<sub>50</sub> SARS-CoV-2. The



**Fig. 1: Prediction and identification of B cell epitopes in the SARS-CoV-2 S protein.** (a) Prediction of specific B cell epitope sequences and sites of SARS-CoV-2 S protein. (b) The structure of SARS-CoV-2 S protein (Modelled by SWISS-MODEL). The aa 25–39 region is marked with blue, aa 672–691 is marked with green, aa 764–778 is marked with red, and aa 907–921 is marked with purple. (c) Antibody profile of randomly chosen patients with clinical SARS-CoV-2 infection (2, 3, 7, 17, n = 4) and healthy individuals (H1, H2, n = 2). Each row represents a predicted B cell epitope peptide, each column represents a serum sample. The color intensity of each cell indicates the ELISA OD<sub>450</sub>. (d) Coronavirus S protein structure and potential furin or TMPRSS2 cleavage sites at the S1/S2 junction. Insertion mutation at aa position 672 of S protein and sequence comparison with human and bat SARS-like CoVs and MERS.

SARS-CoV-2 RNA copies in supernatant were measured by quantitative reverse transcription-PCR (qRT-PCR).

### Statistics

Statistical analysis was performed with GraphPad Prism 8 software or R Studio version 3.6.3. For serum samples collected from individuals with normal distribution (Whether the data followed a normal distribution or not was analyzed by Normality and Lognormality analysis with D'Agostino & Pearson test method). Before the unpaired and paired Student's *t* test for continuous variables were used to compare IgM/IgG levels among different peptides or human serum samples, the demographics of healthy individuals, COVID-19 suspected and COVID-19 patients were statistically compared with Chi-square test or *t*-test, and the correlation of every confounder (age, sex, whether high temperature and cough, and CT scan results) and IgG/IgM levels against S25-39 and S672-691 peptides were respectively analyzed with Pearson correlation analysis to adjust for potential confounders. ELISA data from all the serum samples was used in *t*-test analysis. For some clinical information was not completely recorded, only recorded data was used in multiple variable analysis. For mice experiments and other detection results without supposed distribution model, Mann–Whitney U test was used for other analysis. The data were presented as mean ± SD. *P* values were indicated by ns, not significant, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001. ROC curve analysis and their corresponding areas under the curve (AUC) were calculated using the predicted values estimated by supervised machine learning random forest (RF) algorithm models, and AUC were calculated using MedCalc statistic software.

### Ethics

The clinical samples used in the study were approved by the Ethics Committee of Taihe hospital and informed written consent was obtained from the patients (2021XM001). The IACUC of the UTMB approved animal studies (2103023).

### Role of funders

The funders have no role in study design, data collection, data analyses, interpretation and writing of the manuscript or decision to publish it. Study, writing and review of the manuscript was completed by the authors.

## Results

### Prediction and identification of B cell epitopes in the SARS-CoV-2 S protein

Among the surface proteins shared by the coronavirus family, the S protein is the most divergent and consequently the best candidate for strain-specific epitope screening. To identify epitopes specific to SARS-CoV-2,

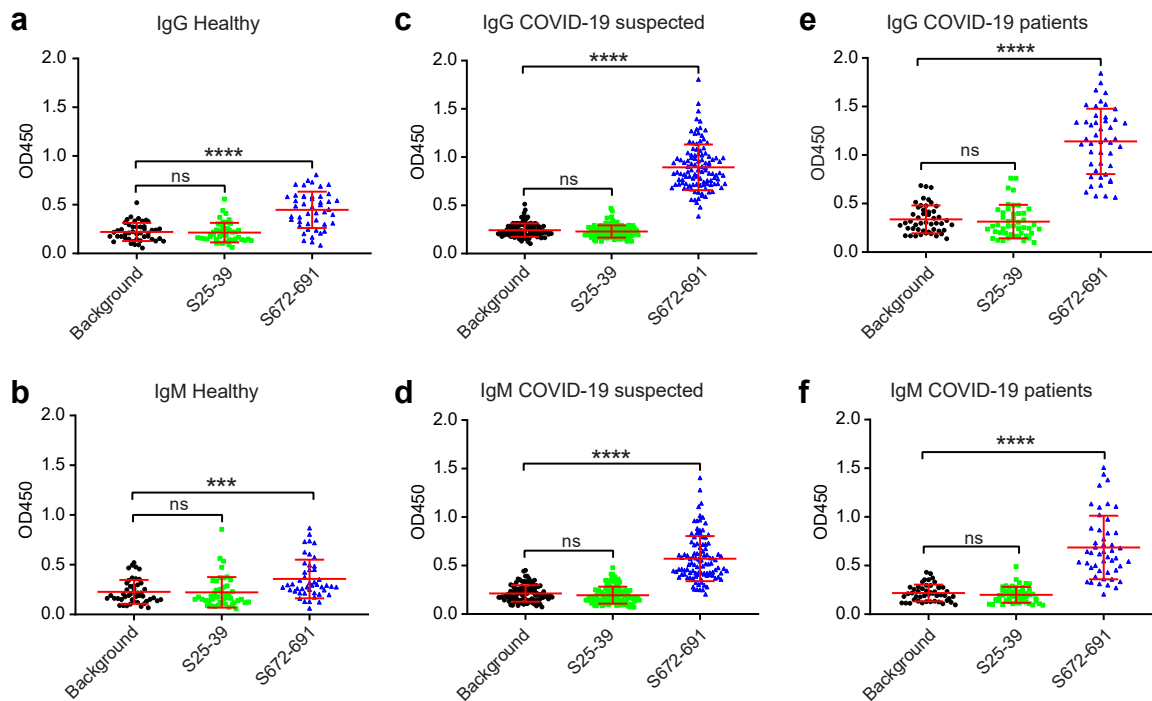
we used IEDB prediction to select seven peptides,<sup>34</sup> which structural modeling indicated are exposed, from the S protein of SARS-CoV-2 (Fig. 1a and b). Based on the computer-guided homology modeling method, structural models were constructed by SWISS-MODEL online server<sup>30,31</sup> (Fig. 1b). These peptides were chemically synthesized and used as bait in an ELISA to measure peptide-specific antibody levels in serum samples from healthy donors and COVID-19 patients. We found that the peptide located at the 672–691 aa position of the S protein (S672-691) had the strongest antibody reactivity in sera from COVID-19 patients, suggesting that this peptide contains at least one immunoreactive epitope (Fig. 1c). We then compared the aa sequence of the SARS-CoV-2 S672-691 peptide to the corresponding region of other human coronaviruses. We found that S672-691 peptide of SARS-CoV-2 possessed sequence differing from that of other coronaviruses (Fig. 1d). Interestingly, the S672-691 peptide has a unique, four aa “PRRA” insertion between the S1 and S2 domains of the S protein that does not exist in other coronaviruses. We therefore hypothesized that the S672-691 peptide contained an immunoreactive epitope specific for SARS-CoV-2 that could specifically detect COVID-19 patients.

### Antibodies against the S672-691 peptide were generated in both COVID-19 patients and suspected patients

To investigate the antibody profiles of different COVID-19 patients, we used serum samples from COVID-19 patients and suspected patients to measure both the IgM and IgG levels against the S672-691 peptide and a control peptide, S25-39. As shown in Fig. 2a–f, the IgM and IgG antibody levels specific for the S672-691 peptide were significantly higher than those for the S25-39 peptide and negative control (*P* < 0.0001). When compared among different individuals, IgG and IgM levels against S672-691 peptide in both COVID-19 patients (SARS-CoV-2 PCR test positive) were much higher than those in healthy controls. We also tested a group of COVID-19 suspected patients who were SARS-CoV-2 PCR test negative but showed COVID-19 like symptoms and/or abnormal lung CT scans at the time of serum sample collection. Interestingly, they also had higher IgG and IgM levels against the S672-691 peptide than those in healthy controls. Our heat map analysis presented similar results (Fig. S1a and b). These results showed that antibodies against the S672-691 peptide were generated in both COVID-19 patients and suspected patients.

### S672-691 peptide-based ELISA for COVID-19 diagnosis showed high sensitivity and specificity

To further explore the possibility of using the S672-691 peptide as an immunogenic antigen for the

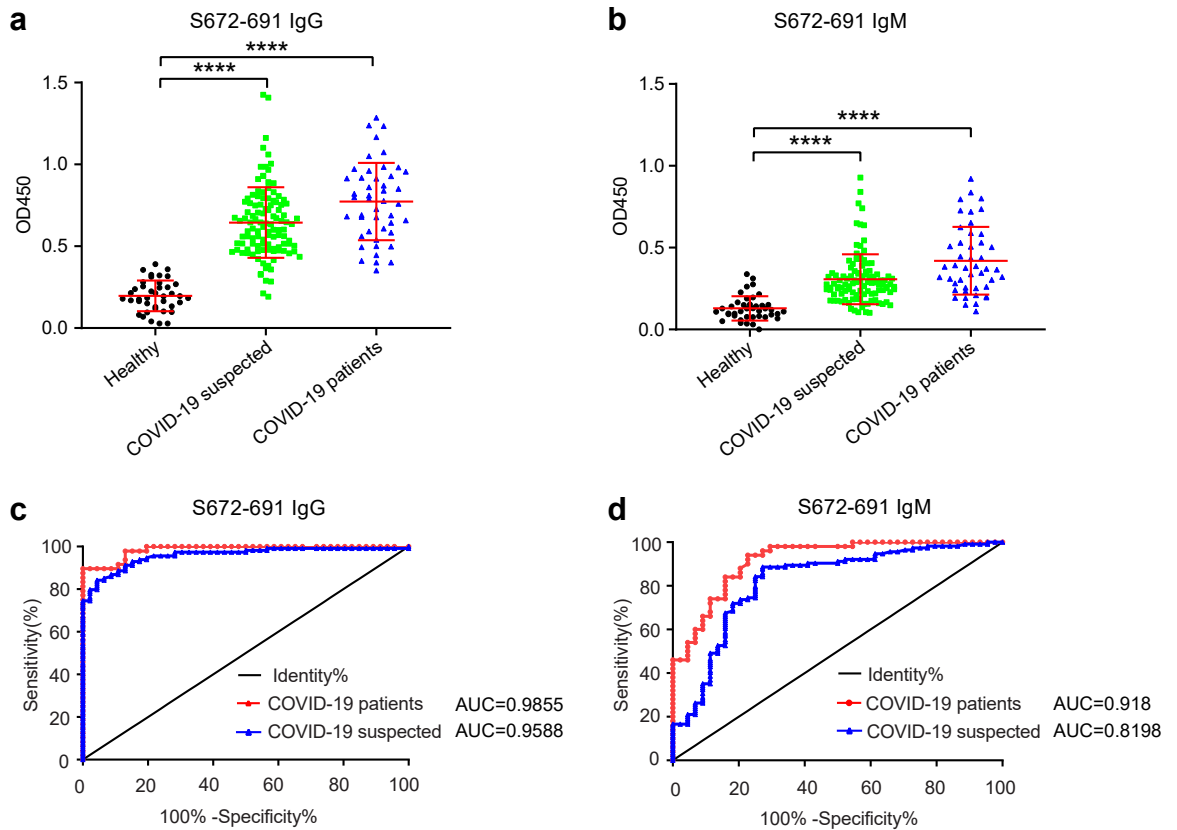


**Fig. 2: Antibodies against the S672-691 peptide were generated in both COVID-19 patients and suspected patients.** (a–f) S25-39 and S672-691 epitope-specific IgM/IgG antibody levels were detected by peptide-specific ELISA. Data are shown for serum samples from COVID-19 patients, suspected patients, and healthy individuals ( $n = 43:119:46$ ) (Paired Student *t* test, ns, not significant,  $P < 0.0001$  for panel a, c–f,  $P = 0.000285$  for panel b).

diagnosis of COVID-19 patients, IgM and IgG levels against the S672-691 peptide were compared among COVID-19 patients, suspected patients, and healthy individuals (Table S1). Before serum ELISA data was analyzed, the participants' clinical information was collected and compared with Chi-square test or *t*-test to avoid unnecessary variance of samples. Age and sex showed no obvious difference among healthy individuals, COVID-19 patients, and suspected patients. Although whether cough and lung CT scan results showed no difference between COVID-19 patients and suspected patients, more COVID-19 patients were high temperatures than COVID-19 suspected patients, which may result from viral replication in COVID-19 patients (Table S2). Pearson correlation analysis was also performed to check the correlation between IgG/IgM levels and potential confounders (sex, age, whether high temperature and cough, and CT scan results). The results suggested that age and sex did not correlate with IgG and IgM levels against S25-39 or S672-691 peptides among healthy individuals, COVID-19 patients and suspected patients, and whether high temperature and cough and lung CT scan results also did not correlate with IgG and IgM levels between COVID-19 patients and suspected patients (Table S3). These results showed that the

above demographics do not interfere with the serum ELISA detection results.

Presence of a furin site in the S protein is not uncommon in human coronaviruses; about half of human seasonal coronaviruses as well as MERS-CoV contain a furin site. A furin site is also present in H5N1 avian influenza virus. The healthy individuals in Fig. 2a and b were not infected by SAR-CoV-2, but they may have been infected by some other virus which also contained a furin site, resulting in the relatively higher IgG and IgM level against the S672-691 peptide than the S25-39 peptide or a negative control. However, when compared with COVID-19 patients and suspected patients, the IgG and IgM levels against the S672-691 peptide in healthy individuals were significantly lower (Fig. 3a and b,  $P < 0.0001$ ). The Fig. 3c and d represent the ROC curves for measuring both the sensitivity and specificity of the S672-691 peptide. The AUC for the S672-691 peptide-specific IgM was 0.918 and 0.8198 among COVID-19 patients and suspected patients, respectively. More strikingly, the AUC for the S672-691 peptide specific IgG was 0.9855 and 0.9588 among COVID-19 patients and suspected patients, respectively (Fig. 3c and d). These results suggest a possible use of the levels of antibodies (particularly IgG) specific for the S672-691 peptide as biomarkers for COVID-19 diagnosis.



**Fig. 3: S672-691 peptide-based ELISA for COVID-19 diagnosis showed high sensitivity and specificity.** (a and b) Scatter plot of S672-691 peptide IgG/IgM OD<sub>450</sub> readings detected in COVID-19 patients, COVID-19 suspected patients, and healthy individuals (n = 43:119:46) (Unpaired Student t test,  $P < 0.0001$ ). (c and d) ROC curves of (a) and (b).

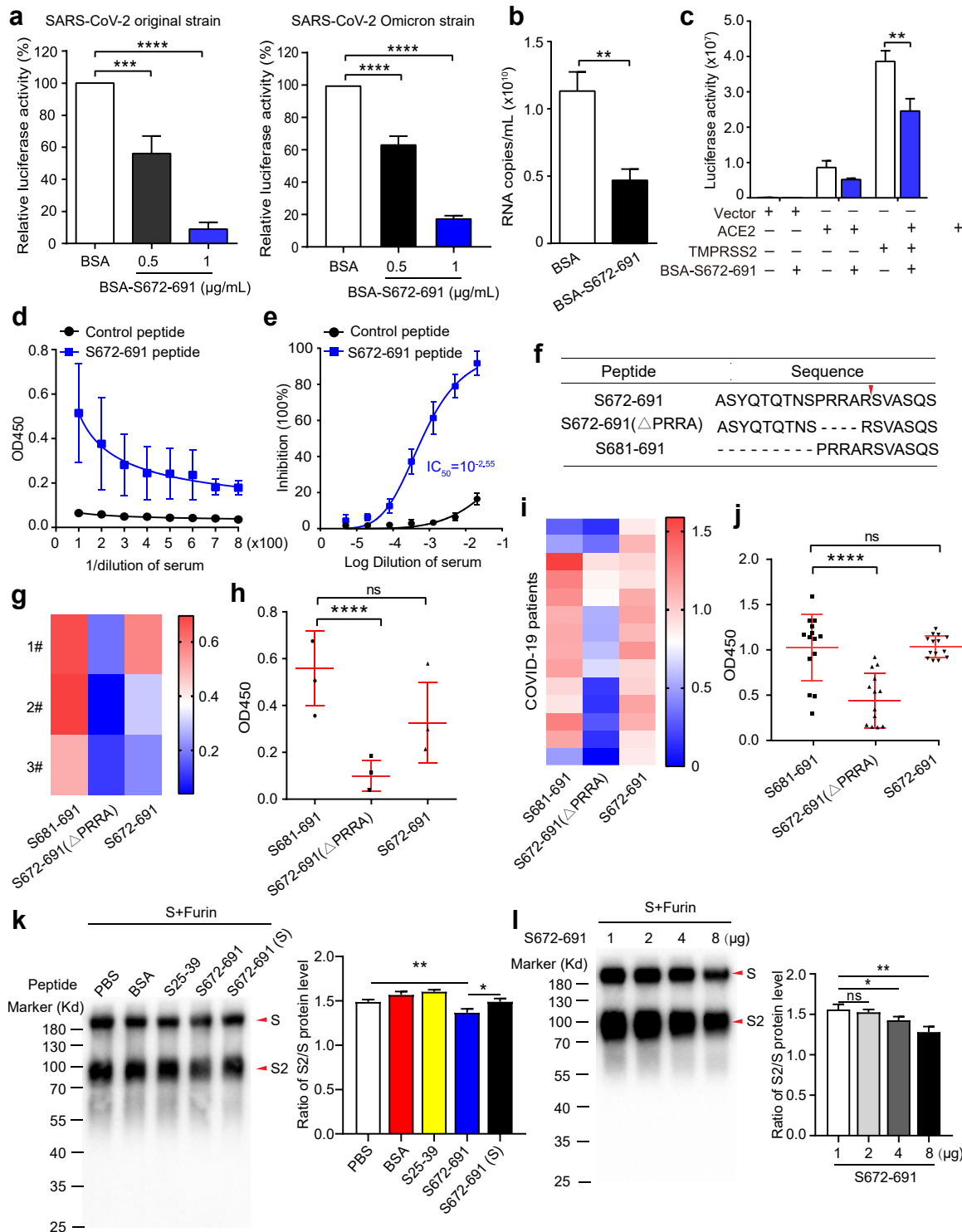
### S672-691 peptide and immunized mouse sera inhibit SARS-CoV-2 infection

To further determine the function of the S672-691 peptide in SARS-CoV-2 infection, we added BSA-conjugated synthetic S672-691 peptide to cultured cells and found that the S672-691 peptide significantly blocked the original ( $P = 0.0002$ ,  $P < 0.0001$ ) and Omicron ( $P < 0.0001$ ) strain SARS-CoV-2 pseudovirus infection and SARS-CoV-2 RNA replication (Fig. 4a and b,  $P = 0.0039$ ). More importantly, when hTMPRSS2 was co-transfected with hACE2 into HEK293T cells, it strongly enhanced SARS-CoV-2 pseudovirus infection, which could be inhibited in turn by the S672-691 peptide (Fig. 4c,  $P = 0.0035$ ). Overall, these results indicate that the synthetic S672-691 peptide inhibited SARS-CoV-2 infection.

To further determine if antibodies binding to the S672-691 peptide have a neutralization effect against SARS-CoV-2 infection, we immunized mice with the S672-691 peptide in the presence of MF59 adjuvant. We found high levels of S672-691 specific antibody presented in the sera of immunized mice, which again indicated that the S672-691 peptide contains an

immunogenic epitope (Fig. 4d). To test neutralization activity against SARS-CoV-2, serum samples from the immunized mice were used for neutralizing pseudovirus. As shown in Fig. 4e, sera from independently immunized mice, but not control peptide immunized mice, inhibited the infection of SARS-CoV-2 pseudovirus at  $IC_{50} = 10^{-2.55}$  (Sera inhibited SARS-CoV-2 infection by half at the dilution of  $10^{2.55}$ ). These results suggest that antibodies binding SARS-CoV-2 outside the RBD also had antiviral activity. The S672-691 peptide contains a unique four aa “PRRA” insertion, which creates a potential TMPRSS2 and/or furin cleavage site. In order to further understand the mechanism responsible for the humoral immunity against this region, we generated deletion mutants to map the antibody recognition epitope within the S672-691 peptide (Fig. 4f). The S681-691 peptide, but not the S672-691 peptide with deleted PRRA sequence ( $\Delta$ PRRA), strongly bound to antibodies in sera both from mice immunized with the S672-691 peptide (Fig. 4g and h,  $P < 0.0001$ ) and from multiple COVID-19 patients (Fig. 4i and j,  $P < 0.0001$ ). To prove the direct furin enzyme competition activity of the S672-691 peptide, we performed an *in vitro* S protein





**Fig. 4: S672-691 peptide and immunized mouse sera inhibit SARS-CoV-2 infection.** (a) The infection of original (left) and Omicron (right) strains of SARS-CoV-2 pseudovirus in control (1 μg/mL) and BSA-S672-691 peptide treated Huh7.5 cells was analyzed by Luciferase reporter assay ( $P = 0.0002$  and  $P < 0.0001$ ). (b) The replication of SARS-CoV-2 in control (2 μg/mL) and BSA-conjugated S672-691 peptide (2 μg/mL) treated Vero was analyzed by qRT-PCR ( $P = 0.0039$ ). (c) HEK293T cells were transfected with ACE2 and TMPRSS2 plasmids as indicated. Transfected cells were pre-treated with BSA-conjugated S672-691 peptide (2 μg/mL) before SARS-CoV-2 pseudovirus infection. The infection of

cleavage assay and found that the S672-691 peptide modestly but significantly inhibited S protein cleavage mediated by furin (Fig. 4k and l,  $P = 0.0035$  and  $P = 0.0184$  for panel k,  $P = 0.043$  and  $P = 0.0066$  for panel l). These data suggest that the immunogenic epitope of the S672-691 peptide overlaps with the four aa “PRRA” insertion, unique to SARS-CoV-2, that creates a TMPRSS2/furin cleavage site.

### Monoclonal antibody against the S672-691 peptide inhibits SARS-CoV-2 infection

We then used a scFv phage display library with surface expression of Ig heavy and light chain variable region pairs from 15 COVID-19 patients' peripheral blood mononuclear cell (PBMC) samples to screen for human monoclonal antibodies against the S672-691 peptide (Fig. 5a). From the library containing  $8.7 \times 10^9$  scFv phages, we obtained 192 phage clones after four rounds of enrichment for binding to the BSA/biotin-labeled S672-691 peptide. The four strongest clones were identified and two independent clones were revealed by sequencing analysis: R3P1-B9 and R4P1-C2, which represented three clones with identical sequences (Fig. 5b and Table S4). S672-691 peptide-dependent ELISA showed that R4P1-C2 scFv protein fragments were able to bind strongly to BSA-S672-691 peptide, while R3P1-B9 scFv bound weakly to BSA-S672-691 peptide (Fig. 5c). We subsequently cloned the R3P1-B9 and R4P1-C2 scFv cDNA fragments into expression constructs containing the human IgG1 backbone and analyzed the binding of R3P1-B9 and R4P1-C2 IgG1 antibodies with the S672-691 peptides of both SARS-CoV-2 original and Omicron strains. The results showed that R4P1-C2, but not R3P1-B9, bound to the S672-691 peptides from both SARS-CoV-2 original and Omicron strains (Fig. 5d). We further detected the binding affinity with S monomer and trimer proteins, finding that only R4P1-C2 bound well to both S monomer and trimer proteins (Fig. 5e). Surface plasmon resonance (SPR) assay also confirmed binding of the R4P1-C2 antibody to both the S672-691 peptide and S protein (Fig. 5f and g). Phage ELISA assay used S672-691 peptide

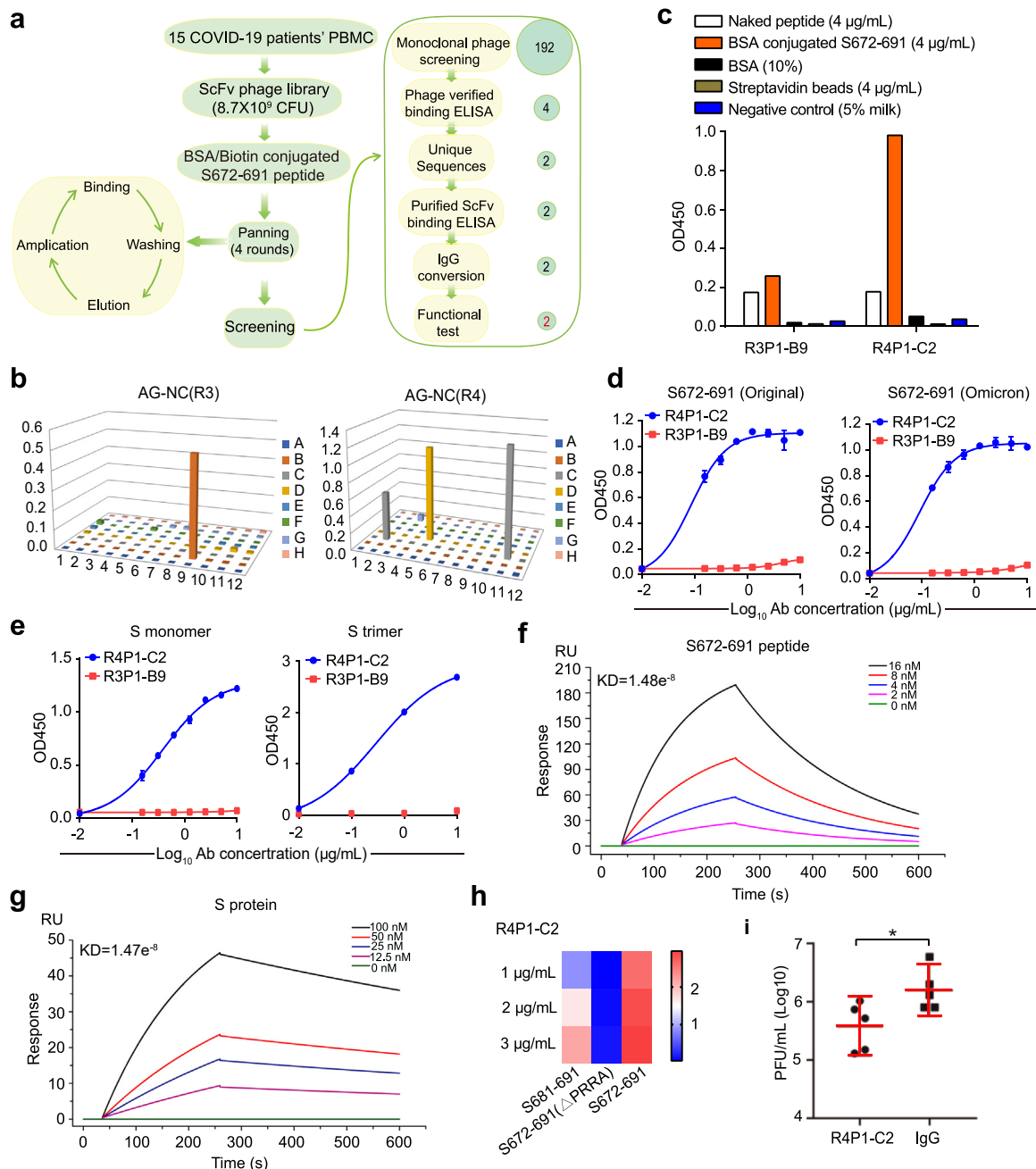
as bait. The screened R3P1-B9 and R4P1-C2 scFv thus bound to S672-691 peptide. Full-length S protein may display folding structure that disabled the binding of R3P1-B9 scFv with S protein. To observe the role of “PRRA” aa on the binding with R4P1-C2 antibody, S672-691 mutant peptide ELISA was performed. We found that while the S681-691 peptide had weak binding activity, the S672-691 peptide lacking the four aa “PRRA” had no binding activity to this monoclonal antibody, suggesting that the R4P1-C2 antibody binds at the furin cleavage site (Fig. 5h). More importantly, when we tested the antiviral effect of R4P1-C2 antibody in SARS-CoV-2 infected mice, we found that the R4P1-C2 antibody effectively decreased the viral titer in the lung of SARS-CoV-2 infected mice (Fig. 5i,  $P = 0.032$ ). These studies have therefore demonstrated that a monoclonal antibody against the S672-691 peptide, identified from COVID-19 patients' PBMC, inhibited SARS-CoV-2 infection.

### Discussion

COVID-19 is a worldwide pandemic disease caused by SARS-CoV-2 infection, with hundreds of millions of confirmed cases and several million deaths.<sup>7,35</sup> Through studying a unique peptide in the S protein of SARS-CoV-2, we have demonstrated that while SARS-CoV-2 has evolved to possess a four aa “PRRA” insertion to create a TMPRSS2/furin cleavage site that makes the virus more infectious, the host immune system recognizes this inserted sequence as an immunogenic epitope and generates antibodies against it to inhibit viral infection. Furthermore, our results suggest that a monoclonal antibody binding to this peptide may have therapeutic potential to neutralize SARS-CoV-2 infection.

S protein epitopes have been extensively screened by both computational and experimental methods.<sup>36–38</sup> Among these studies, Li et al.<sup>37</sup> used a microarray of 12 aa peptides with 6-aa overlap between adjacent peptides to generate a linear epitope landscape of the S protein by analyzing the serum IgG of COVID-19 patients. Wang et al.<sup>36</sup> used a proteome microarray of 15 aa peptides with 5-aa overlap to analyze IgG and IgM of COVID-19 patients. In this study, through bioinformatic analysis and

SARS-CoV-2 pseudovirus was analyzed by Luciferase reporter assay ( $P = 0.0035$ ). (d) S672-691 specific IgG levels were analyzed in S672-691, and control peptide immunized mice sera ( $n = 5:5$ ). (e) The SARS-CoV-2 pseudovirus neutralization activities of S672-691 immunized mouse sera were analyzed by Luciferase reporter assay ( $n = 5:5$ ). (f) Diagram of S672-691 deletion mutations. The red arrow indicates the furin cleavage site. (g and h) Specific antibodies against S672-691 and its deletion mutations in S672-691 immunized mouse sera were detected by ELISA. Data are shown in both heatmap (g) and scatter plot (h) (ns, not significant,  $P < 0.0001$ ). (i and j) Specific antibodies against S672-691 and its deletion mutations were detected by ELISA in COVID-19 patients' sera. Data are shown in both heatmap (i) and scatter plot (j) (ns, not significant,  $P < 0.0001$ ). (k) Full-length S protein was cleaved by furin enzyme in a reaction system supplemented with PBS, BSA, BSA-S25-39, BSA-S672-691 or BSA-S672-691 sequence scrambled (S672-691 (S), QTQAARSYTVASRSQSNSPR) peptide (2  $\mu\text{g}$ ) at 25 °C for 1 h. The cleaved S protein was detected by immunoblotting with anti-S2 antibody (left panel), and the immunoblot result was quantitated and normalized (right panel,  $P = 0.0035$  and  $P = 0.0184$ ). (l) Full-length S protein was cleaved by furin enzyme in reaction system supplemented with titrated BSA-S672-691 peptide at 25 °C for 1 h. The cleaved S protein was detected by immunoblotting with S2 antibody (left panel), and the immunoblot result was quantitated and normalized (right panel, ns, not significant,  $P = 0.043$  and  $P = 0.0066$ ). The statistical analysis was performed with Mann-Whitney U test, and  $P$  values were indicated. Data represent the cumulative results from two or three independent experiments.



**Fig. 5: Monoclonal antibody against the S672-691 peptide inhibits SARS-CoV-2 infection.** (a) Schematic diagram of monoclonal antibody screening. (b) Phage verified binding ELISA identified four strongest clones. (c) ELISA verified the binding of S672-691 peptide with R3P1-B9 and R4P1-C2 scFv monoclonal antibodies. (d) ELISA verified the binding of R3P1-B9 and R4P1-C2 antibodies with S672-691 peptide from SARS-CoV-2 original or Omicron strain. S672-691 peptide from SARS-CoV-2 original or Omicron strain were coated on the plates and serially diluted R3P1-B9 and R4P1-C2 antibodies were added into the plates to detect the binding. (e) ELISA verified the binding of R3P1-B9 and R4P1-C2 antibodies with S monomer and trimer protein. (f and g) Sensorgrams of the binding of R4P1-C2 IgG1 antibody with S672-691 peptide (f) and S protein (g). The antibody concentrations were used as indicated. (h) The binding of R4P1-C2 scFv monoclonal antibody with S672-691 and its deletion mutations in COVID-19 patient sera was detected by ELISA. (i) Mice were administrated intraperitoneally with R3P1-C2 Ab (1.7 mg/kg) or IgG control (n = 5:5) 6 h before intranasal challenged with SARS-CoV-2 mouse-adapted strain CMA3p20 (10<sup>4</sup> PFU). Lung viral loads were tested by plaque assay on day 2 post infection (Mann-Whitney U test, P = 0.032). Data (b-e) represent the cumulative results from two or three independent experiments.

structure-based prediction, we have tested several peptides in the S protein for their ability to detect antibodies in COVID-19 patients and suspected patients, and have identified the S672-691 peptide as a potential antigen (Fig. 1c). Though short peptides would be nicely presented, conformational epitopes may be lost. S672-691 peptide, which is 20 aa and located on the surface of S protein (based on the structural prediction), contains a four aa, positively charged peptide insertion that is unique to SARS-CoV-2. Upon the outbreak of the COVID-19 epidemic, some researchers have predicted the epitopes of SARS-CoV-2 with computational methods provided by the IEDB server.<sup>39-41</sup> However, those predictions were dependent on literature mining and lacked experimental confirmation.

Our study clearly identified the S672-691 peptide with complementary use of both computational and experimental methods. The S672-691 peptide is longer than peptides used in previously reported microarrays, which may be missed in those screens. When comparing the S672-691 peptide sequences of SARS-CoV-2 with the corresponding aa sequences of SARS, MERS and other human coronaviruses, we did not find any coronavirus that shared five or more consecutive aa with the S672-691 peptide sequences of SARS-CoV-2 (Fig. 1d). We therefore believe the S672-691 peptide can specifically detect COVID-19 patients, although more patients' sample studies are needed to confirm this conclusion. More importantly, the S672-691 peptide proved useful both for the inhibition of SARS-CoV-2 infection and for immunogenicity, triggering protective antibodies in mice that could suppress SARS-CoV-2 infection (Fig. 5i). The emergence and rapid spread of SARS-CoV-2 Omicron strain have challenged current SARS-CoV-2 vaccines and monoclonal antibodies. The serum samples from COVID-19 patients, COVID-19 suspected patients infected with SARS-CoV-2 original strain we used in this study may not be representative of the wider population infected with SARS-CoV-2 Omicron strain. However, our S672-691 peptide also showed suppression on Omicron strain (Fig. 4a), indicating the potential of S672-691 peptide for COVID-19 diagnosis of patients infected with SARS-CoV-2 Omicron strain and more work is needed to confirm this.

The immunodominant S672-691 peptide is located in the middle of the S protein between the S1 domain, responsible for ACE2 receptor binding, and the S2 domain, responsible for fusion between viral and cell membranes.<sup>3,42,43</sup> Interestingly, this peptide contains a unique, four aa "PRRA" insertion as the result of a 12 nucleotides insertion in the genome of SARS-CoV-2 during its evolution. This insertion creates a potential cleavage site for proteases such as TMPRSS2 and furin.<sup>1,44,45</sup> TMPRSS2 is a transmembrane protease that has been shown to play an essential role in facilitating cellular entry of many viruses, including influenza viruses and coronaviruses.<sup>46-48</sup> We found that the S672-

691 peptide may inhibit TMPRSS2-mediated cleavage of the S protein and suppress SARS-CoV-2 infection. Furthermore, sera from mice immunized with the S672-691 peptide strongly inhibited SARS-CoV-2 infection. More importantly, the monoclonal antibody we isolated from the COVID-19 patients directly targeted the four aa "PRRA" insertion and inhibited SARS-CoV-2 infection. Our studies therefore indicate that while SARS-CoV-2 creates a TMPRSS2 protease cleavage site through four aa insertion to enhance its infectivity, the immune system recognizes this inserted sequence as an immunogenic epitope and generates antibodies to protect cells against SARS-CoV-2 infection. ACE2 has a high affinity for binding the RBD domain, and antibodies against the RBD domain with higher affinity than ACE2 may inhibit SARS-CoV-2 viral entry.<sup>49,50</sup> We speculate that the antibody we identified may suppress SARS-CoV-2 by affecting the binding of TMPRSS2/furin with the S protein. Further structure analysis is needed to provide more details.

Our current studies have limitations because of a small number of samples without samples from asymptomatic infected patients or SARS-CoV-2 vaccinated individuals. The efficiency of this antigen in detecting recovered COVID-19 patients and vaccinated individuals who may gain protective immunity against SARS-CoV-2 infection will be needed to determine in future study. Meanwhile, part of the clinical information of human serum samples is not completely recorded, and the missing data in Table S1 is not missed at random. When performing statistical comparisons in Tables S2 and S3, we just used the available information and excluded the missing data. While analyzing ELISA data in Figs. 2 and 3, we used data from all the serum samples (Although some clinical information such as sex and age et al. is missed, the clinical symptoms and subsequent SARS-CoV-2 PCR test results suggest the probable infection of COVID-19 suspected patients). Potential bias exists in our conducting a complete-case analysis for data that cannot be assumed to be missing at random. Thus, the conclusion is conserved based on our existing data, other possible confounders are not excluded. More research work about the efficiency, safety, and potential of clinical application of S672-691 peptide and isolated R4P1-C2 antibody is also needed.

COVID-19 is still spreading, with continuously emerging mutant variants causing sustained global health and economic impacts. Identification of an immunogenic epitope that overlaps with the critical furin cleavage site may help our future understanding of virus-host interactions affecting viral infectivity and host immunity. As most of the currently available monoclonal neutralization antibodies bind to the RBD region, there remains further opportunity to develop monoclonal antibodies as potential therapeutic agents against COVID-19 by targeting them to an alternative yet critical site for SARS-CoV-2 infection.

**Contributors**

G.C., H.Y., P.-Y.S. and C.-F.Q. jointly designed this study. M.G., Y.W. and C.C. performed experiments concerning serum ELISA. L.L., H.Z., J.W., X.X., S.R.A. and S.Z. performed virus infection experiments. J.L. and J.Z. collected patient serum samples and clinical data analysis. L.L. and X.X. performed *in vitro* S protein cleavage assay. D.W. and J.D. performed the scFv phage library construction and panning experiments. X.X. (Xuping Xie) performed the mouse experiments. G.C. and L.L. wrote the manuscript. S.G. revised and edited the manuscript. All authors read and approved the final version of the manuscript, and ensured it was the case. G.C. and H.Y. have verified the underlying data.

**Data sharing statement**

Datasets and additional documents generated, analyzed, or used during the study are available upon reasonable request to the corresponding author.

**Declaration of interests**

H.Y. have filed a patent related to the antiviral activity of R3P1-B9 and R4P1-C2 antibodies. The other authors declared that no conflict of interest exists.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2022.104401>.

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