Identification of T Cell Epitopes in the Spike Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus 2 in Rhesus Macaques

Xiaojuan Liu, Yuzhong Li, Hongjian Xiao, Yanwei Bi, Yue Gong, Zhengrong Hu, Yaxin Zeng, Ming Sun, Zhanlong He, Shan Lu, Qihan Li and Wei Cun

J Immunol 2021; 206:2527-2535; Prepublished online 12 May 2021;
doi: 10.4049/jimmunol.2000922
http://www.jimmunol.org/content/206/11/2527

Supplementary Material
http://www.jimmunol.org/content/suppl/2021/05/10/jimmunol.2000922.DCSupplemental

References
This article cites 34 articles, 3 of which you can access for free at:
http://www.jimmunol.org/content/206/11/2527.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Identification of T Cell Epitopes in the Spike Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus 2 in Rhesus Macaques

Xiaojuan Liu,*† Yuzhong Li,*† Hongjian Xiao,*† Yanwei Bi,*† Yue Gong,*† Zhengrong Hu,*† Yaxin Zeng,*† Ming Sun,*† Zhanlong He,*† Shan Lu,‡ Qihan Li,*† and Wei Cun*†

The T cell response is an important detection index in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine development. The present study was undertaken to determine the T cell epitopes in the spike (S) protein of SARS-CoV-2 that dominate the T cell responses in SARS-CoV-2–infected patients. PBMCs from rhesus macaques vaccinated with a DNA vaccine encoding the full-length S protein were isolated, and an ELISPOT assay was used to identify the recognized T cell epitopes among a total of 158 18-mer and 10-aa-overlapping peptides spanning the full-length S protein. Six multipeptide-based epitopes located in the receptor-binding domain, which were identified in the receptor-binding domain, were defined as the most frequently recognized epitopes in macaques. The conservation of the epitopes across species was also verified, and peptide mixtures for T cell response detection were established. Six newly defined T cell epitopes were found in the current study, which may provide a novel potential target for T cell response detection and the diagnosis and vaccine design of SARS-CoV-2 based on multipeptide subunit-based epitopes. The Journal of Immunology, 2021, 206: 2527–2535.

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a worldwide pandemic (1). People infected with SARS-CoV-2 suffer from fever, cough, dyspnea, or even acute respiratory distress, which may ultimately lead to death (2, 3). There were 10,185,374 people infected with SARS-CoV-2 globally, including 503,862 deaths, by June 30, 2020, according to the World Health Organization (4). However, effective antiviral drugs against SARS-CoV-2 and protective vaccines are still under development.

The entrance of a virus into host cells or tissue and initiation of virus infection are usually mediated by the recognition via the virus surface glycoprotein of the receptor located on the cell surface. Angiotensin-converting enzyme II (ACE2), as the cell entry receptor, is recognized by the spike glycoprotein (S protein) located on the virus surface of both severe acute respiratory syndrome coronavirus (SARS-CoV), which emerged and spread to five countries during 2002–2003, and SARS-CoV-2 (5–7). The Ag of the S protein dominates the T cell responses in COVID-19 patients, accounting for 27% of the total CD4+ T cell response and ~26% of CD8+ T cell response, respectively (8). In the case of CD4+ T cell responses, the S protein codominated with other Ags recognized in 100% of COVID-19 cases, despite a relatively narrow CD8+ T cell response elicited by the S protein (8).

Given that the S protein plays an important role in the regulation of SARS-CoV-2 entering host cells and serves as the key target for Abs (9), several vaccines under development based on mRNA (10), DNA (11, 12), and viral vectors (van Doremalen, N., T. Lamba, A. Spencer, S. Belij-Rammerstorfer, J. N. Purushotham, J. R. Port, V. Avanzato, T. Buschmaker, A. Flaxman, M. Ulaszewska, et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.05.13.093195) were designed to encode the S protein of SARS-CoV-2. When evaluating the protective effect of a vaccine, close attention should be paid to the T cell response, as an early study found that the T cell response elicited by SARS-CoV-2 persisted longer than the humoral response (13), even lasting for 11 y (14). In addition, cytokine dysregulation and Th1/Th2 imbalance occur in helper T lymphocytes following vaccination, such as with formalin-inactivated respiratory syncytial virus, leading to an Ab-dependent enhancement effect (15). In consideration of the important role of T cell response in immunological studies on SARS-CoV-2, recent studies employed overlapping peptide pools spanning the S protein of SARS-CoV-2 to detect the T cell response (11, 12). Although bioinformatics and algorithms have been used in several studies to predict the T cell epitopes of the S protein (16), the precise T cell epitopes of the S protein are still unknown because of a lack of experimental verification.

*Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming, China; †Yunnan Key Laboratory of Vaccine Research and Development of Severe Infectious Disease, Kunming, China; and ‡Department of Medicine, University of Massachusetts Medical School, Worcester, MA

ORCID IDs: 0000-0002-3450-8349 (Y.L.); 0000-0003-0451-575X (Y.Z.).

Received for publication August 5, 2020. Accepted for publication March 31, 2021.

This work was supported by a grant from the Fundamental Research Funds for the Central Universities (3332020064), a grant from the Chinese Academy of Medical Sciences Initiative for Innovative Medicine (2016-I2M-1-013, 2016-I2M-1-019), a grant from the National Natural Science Foundation of China (81971947), and a grant from the Major Science and Technology Projects of Yunnan Province (2019ZF001).

W.C. designed and coordinated the research. W.C. and X.L. interpreted the results, analyzed the data, and wrote the manuscript. X.L. carried out most of the experiments. S.L. provided the vector of the DNA vaccine and participated in the vaccine design. M.S., Z. He, and Q.L. provided the animals and coordinated the animal experiments. Y.L. and Y.B. conducted the procedure of animal vaccination. H.X. purified the DNA vaccine. Y.Z., Y.G., and Z. Hu participated in the experiment of ELISPOT assay. All authors in the study have read and agreed to the final version of the manuscript.

Address correspondence and reprint requests to Dr. Wei Cun, Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 935 Jiaoling Road, Kunming, Yunnan 650118, China. E-mail address: cumwei@foxmail.com

The online version of this article contains supplemental material.

Abbreviations used in this article: COVID-19, coronavirus disease 2019; RBBD, receptor-binding domain; S protein, spike glycoprotein; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SFU, spot-forming unit; SPLs, spleen lymphocyte; TeSma, T cell epitope in S protein in macaques; TeSmo, T cell epitope in S protein in mice.

Copyright © 2021 by The American Association of Immunologists, Inc. 0022-1767/21/37.50

www.jimmunol.org/cgi/doi/10.4049/jimmunol.2000922
The present study aimed to identify the T cell epitopes for the S protein of SARS-CoV-2. Rhesus macaques, the animal model most similar to human-like pathology postinfection with SARS-CoV-2 (17–19, Lu, S., Y. Zhao, W. Yu, Y. Yang, J. Gao, J. Wang, D. Kuang, M. Yang, J. Yang, C. Ma, et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.04.08.031807) were injected with a DNA vaccine encoding the full-length S protein to induce T cell immunity, and Th1 and Th2 responses were detected by ELISPOT assay using overlapping peptide pools spanning the whole S protein in macaque PBMCs. The T cell–recognized epitopes in the S protein were determined by the number of immunospots induced by the relative peptides. The conservation of the T cell epitope across species was also verified.

Materials and Methods

Animals and vaccination

All animals, including female 0- to 2-y-old and 1.6- to 2.5-kg rhesus macaques and 6- to 8-week-old female C57BL/6N mice, were obtained from the Primate Center at the Institute of Medical Biology, Chinese Academy of Medical Sciences. All animal care, feeding, and treatment were approved by the Ethical Review System of Experimental Animals at the Institute of Medical Biology, Chinese Academy of Medical Sciences, and followed the guidelines of the United States National Institutes of Health. Seven rhesus macaques were vaccinated with 2 mg of DNA vaccines (pCW1993) encoding the full-length S protein of SARS-CoV-2 without adjuvant by i.m. injection at 0, 2, and 8 wk, with macaques immunized with 2 mg of empty plasmid (pSW3891) as the control group. Peripheral blood of monkeys was obtained between 9 and 12 wk to perform the ELISPOT assay and neutralizing Ab detection. Three mice were injected with 1.6 mg of the DNA vaccine pCW1993 by i.m. injection, and three mice were immunized with 1.6 mg of the empty plasmid pSW3891 as the control group. Mouse ELISPOT assays were conducted on days 2 wk after vaccination. The plasmid pCW1993 was constructed in our previous study, and the empty plasmid pSW3891 was a gift from Shan Lu (20).

Dissolution of peptides

In total, 158 18-mer-long and 10-aa-overlapping peptides spanning the full-length S protein were synthesized by GL Biochem (Shanghai) (Shanghai, China). The sequences of the S protein were obtained from the National Center for Biotechnology Information, and its gene identifier is 43740568. All single peptides were dissolved at a concentration of 1 μg/mL. Peptide pools or peptide mixtures were dissolved at a concentration of 0.1 μg/μL or 0.17 μg/μL for each peptide, respectively. The dissolved peptides were stored at −80°C.

Isolation of monkey PBMCs and mouse spleen lymphocytes

Monkey PBMCs were isolated using a mouse PBL separation kit (Solarbio) according to the manufacturer’s instructions. In general, 2–4 ml of monkey peripheral blood with anticoagulant, such as EDTA-K2, was obtained and diluted by an equal volume of cell and tissue diluent. Then, diluted blood was added to an equal volume (the volume of blood and diluent) of separation medium and centrifuged at 1000 × g for 30 min at room temperature. After centrifugation, a milky white lymphocyte layer was present between the diluent layer and separation medium layer. The lymphocyte layer was isolated and washed with 10 ml of PBS once and with 5 ml of PBS twice. The wash steps were performed by centrifugation at 300 × g for 10 min at room temperature. The isolated monkey PBMCs were resuspended in serum-free medium for ELISPOT assay (Universal) (Dakewei) and counted for use.

Mouse spleen lymphocytes (SPLs) were isolated using mouse lymphocyte separation medium (Dakewei) according to the manufacturer’s instructions. In general, mouse spleens were obtained and ground into 4 ml of separation medium, and the cell suspension was filtered through a 100-μm cell strainer. The filtered cell suspension was transferred into a 15-ml centrifuge tube and covered with 1 ml of 1640 medium. After centrifugation at 800 × g for 30 min at room temperature, the milky white lymphocyte layer was isolated and washed with 10 ml of 1640 medium by centrifugation at 300 × g for 10 min. The isolated mouse SPLs were resuspended in serum-free medium for ELISPOT assay (Universal) and counted for use.

IFN-γ, IL-4, and IL-2 ELISPOT assays

Monkey IFN-γ ELISPOTPLUS (ALP) kits (Mabtech), Human IL-4 ELI- SPOTPLUS (ALP) kits (Mabtech), which can cross-react with rhesus monkeys according to the instructions of the kit; and Monkey IL-2 ELISPOTPLUS (ALP) kits (Mabtech) were used to detect IFN-γ, IL-4, and IL-2 secretion, respectively, in monkey PBMCs. Mouse IFN-γ ELISPOTPLUS (ALP) kits (Mabtech) were used to detect IFN-γ secretion in mouse SPLs. The anti-monkey–IFN-γ, anti-human–IL-4, anti-human–IL-2, or anti-mouse–IFN-γ Ab precoated strip plates were all washed four times with 200 μl of sterile PBS and incubated with 200 μl of serum-free medium for ELISPOT assay at room temperature for at least 30 min. After removing the medium, 50 μl of the same serum-free medium containing stimuli was added to the wells, followed by 50 μl of medium containing 3–6 × 10^6 monkey-isolated PBMCs or 3 × 10^5 mouse-isolated SPLs. The final concentrations of the stimuli were as follows: 5 μg/ml for each peptide in the peptide pool or peptide mixture, 40 μg/ml for a single peptide, and 20 μg/ml for the SI 1 protein. Cells were incubated at 37°C for 16 h. After removing the medium, the lysis of residual cells was performed under low-osmotic conditions by ice-cold deionized water and incubation at 4°C for 2 min. The plates were washed with PBS five times, with 2-min incubation at room temperature for every wash. The biotinylated detection anti-monkey–IFN-γ mAb (1:1000), biotinylated detection anti-mouse–IL-2 mAb (1:2000), biotinylated detection anti-mouse–IFN-γ mAb (1:1000), or alkaline phosphate-conjugated anti-human–IL-4 (1:300) mAbs were diluted in PBS containing 1 μg/ml of bovine serum albumin (BSA). After incubation at room temperature for 2 h, the PBS containing Abs was removed, and the plates were washed with PBS five times. A 2-min incubation for each wash was necessary. The plastic tray was removed, and the underside of the membrane was also washed during the last two washes.

For the plates for detection of monkey IL-4, the substrate solution BCIP/NBT-plus was filtered through a 0.2-μm filter, and 100 μl was added to the wells and incubated for ~15 min at 37°C in the dark or until distinct spots emerged. Then, the solution was removed, and the membrane as well as the underside of the membrane was washed extensively with deionized water.

For the detection of monkey IFN-γ, monkey IL-2, or mouse IFN-γ, the plates were incubated with 100 μl of PBS containing 0.5% FCS and streptavidin–alkaline phosphatase conjugate in the presence of 10 μl of 50 mM Tris-HCl (pH 7.4) at room temperature for 1 h. Then, the plates were washed with PBS five times, with a 2-min incubation for each wash, and the underside of the membrane was also washed at the last two washes. The substrate solution BCIP/NBT-plus was used, and the membrane was washed in the same way as for the detection of monkey IL-4.

After drying the plates for detection of monkey IFN-γ, monkey IL-4, monkey IL-2, or mouse IFN-γ overnight, the spots were scanned and counted by an ELISPOT reader (Cellular Technology Limited). Spot-forming units (SFUs) formed in the group of PBMCs or SPLs treated with stimuli were subtracted by the SFUs formed in the negative control (without stimulus) of PBMCs or SPLs to eliminate the influence of the background. Then, the data are presented as the IFN-γ, IL-4, or IL-2 SFUs per 10^6 PBMCs for monkeys and presented as the IFN-γ SFUs per 10^6 SPLs for mice. The plates were stored at room temperature in the dark.

Neutralizing Ab detection

Peripheral blood from monkeys or mice was centrifuged at 3000 × g for 10 min at room temperature to isolate the serum. After incubation of the serum at 56°C for 30 min, the serum was diluted with cell culture medium at ratios of 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. Fifty microliters of diluted serum and 50 μl of SARS-CoV-2 virus at a concentration of 2000 TCID50/ml were added to 96-well plates and incubated in a 37°C, 5% CO2 incubator for 1 h. Then, 100 μl of Vero cell suspension at a concentration of 2 × 10^4 cells/ml was added to the wells and incubated for 7 d to observe the pathological changes in the cells. The neutralizing Ab detection experiment was performed in a biological safety protection third-level laboratory at the Institute of Medical Biology, Chinese Academy of Medical Sciences.

MHC expression detection

MHC expression detection was performed at Beijing Bofurui Gene Diagnosis Technology Co. (Beijing, China). The total RNA of the spleen from the six vaccinated monkeys was extracted by a Direct-zol RNA miniprep kit (Zymo Research) and reverse transcribed by PrimeScript RT Master Mix (Takara). cDNA libraries were constructed according to the one-step cDNA library preparation method developed by Fluidigm Corporation (in the United States) or a Phusion high-fidelity PCR master mix kit (New England Biolabs). The primers used in the tests were eight pairs of primers (four pairs for MHC class I SBT195 and four pairs for MHC class II DRB283) developed by the University of Wisconsin

Downloaded from http://www.jimmunol.org/ by guest on January 20, 2022
FIGURE 1. Screening the peptide pools recognized by the T cell response. (A and B) Detection of the effect of the S1 protein in inducing IFN-γ (A) or IL-4 (B) secretion. (C) The structure of the S protein and a diagram of the total 158 peptides spanning the whole S protein. These peptides were 18-mer long with 10 aas overlapping. Those peptides were pooled into 16 peptide pools, each containing 10 peptides except peptide pool P. (Figure legend continues)
System in the United States. The amplification products were purified twice by magnetic beads using an Agencourt AMPure XP kit (Beckman Coulter). The samples were sequenced by MiSeq sequencing (Illumina) and analyzed by MHC Genotyping pipeline software developed by the University of Wisconsin System in the United States.

Statistical analysis
All data are presented as the mean ± SD. The statistical analysis was performed using GraphPad Prism 8.0.2. An unpaired t test was used to detect significant differences between the control and vaccination groups. A p value < 0.05 was considered significant.

Data availability
The source data involved in the paper were proved as a source data file. Other data can be required in reasonable request from the corresponding author.

Results
Screening the peptide pools recognized by Th1 and Th2 responses
Rhesus macaques were vaccinated with DNA vaccines (pCW1093) encoding the full-length S protein of SARS-CoV-2 or empty plasmid [pSW3891 (20)] as a control. PBMCs of macaques were isolated, and T cell responses to S protein and peptide pools recognized by T cells were tested by ELISPOT assay through detecting Th1-secreted IFN-γ and Th2-secreted IL-4. The results showed that the DNA vaccine induced Th1 cells, which secrete IFN-γ, and Th2 cells, which secrete IL-4, in S DNA–vaccinated macaques (Fig. 1A, 1B). To investigate the epitopes recognized by T cells, a total of 158 peptides were designed to span the whole S protein, and each peptide was 18 mer in length, with 10 aa overlapping. The 158 peptides were pooled into 16 groups, which were named groups A–P, and each peptide pool contained 10 peptides except peptide pool P, which contained eight peptides (Fig. 1C). Among the peptide pools A–P, the treatments with the peptide pools C, D, F, and G increased the numbers of both IFN-γ– and IL-4–secreting PBMCs compared with those in the control group, and the secretion of IL-4 was also increased by peptide pool H (Fig. 1D, 1E). The results indicate that the T cell epitopes in the S protein may be included in peptide pools C, D, F, G, or H.

Identification of the T cell epitopes in the S protein
The number of PBMCs secreting IFN-γ or IL-4 was tested under the treatment of each peptide contained in peptide pools C, D, F, and G using the mixed PBMCs from three macaques vaccinated with S DNA vaccines (pCW1093). In addition, the effect of each peptide contained in peptide pool H in inducing the secretion of IL-4 was also detected. ELISPOT results showed that peptides 40, 51, 59, 61, and 62 remarkably increased the number of both IFN-γ– and IL-4–secreting PBMCs compared with those in the control group, and the secretion of IL-4 was also increased by peptide pool H (Fig. 1D, 1E). The results indicate that the T cell epitopes in the S protein may be included in peptide pools C, D, F, G, or H.

Identification of specific T cell epitopes recognized by the Th1 and Th2 pathways. (A) IFN-γ SFUs detected after treatment with the single peptides 21–40 and 51–70 contained in the peptide pools C, D, F, or G. (B) IL-4 SFUs detected after treatment with the single peptides 21–40 and 51–80 contained in the peptide pools C, D, F, G, or H. PBMCs from three S DNA–vaccinated macaques were mixed and detected in (A) and (B). (C and D) The detection of IFN-γ (C) or IL-4 (D) secretion by PBMCs. The treatments of macaques are described in Fig. 1. Three macaques were involved in each group in (C) and (D). (A)–(D) were all detected by ELISPOT assay. An unpaired t test was used to detect significant differences between the control and S DNA–vaccinated groups. All data are presented as the mean ± SD. *p < 0.05, significantly different from the control group.
macaques, which induced mixed PBMCs to produce immunospots, as shown in Fig. 2A–D. Thus, the T cell epitopes in the S protein recognized by both Th1 and Th2 cells were defined as follows: peptide 38 (SETKCTLKSFTEKGQY, 297–314), peptide 40 (YQTNSFVRQPTESIVRFP, 313–330), peptide 51 (VIRGDEVQRQAPQGTGKI, 401–418), peptide 59 (ERDSTIEYQAGSTPCNG, 465–482), peptide 61 (NGVEGFCNYFPLQSYGFQ, 481–498), and peptide 62 (YFPLQSYGFQPTNGVYQ, 489–506).

The binding affinity between HLA-I or HLA-II alleles from humans and the T cell epitopes verified by the current study was apparently high, according to two early studies, in which the T cell epitopes showing high affinity with HLA-I or HLA-II alleles were predicted by bioinformatics approaches (21) or the Immune Epitope Database and Analysis Resource (22). As shown in Supplemental Table I, all six peptides overlapped or partly overlapped with the high-affinity T cell epitopes predicted in those two studies, especially peptides 40 and 59, which showed a high possibility of recognition by both HLA-I and HLA-II in both studies (21, 22).

The polymorphism of MHC expression in macaques

To investigate the causes of peptides 31, 33, 39, 78, and 79 being recognized by different individuals, as shown in Fig. 2, transcript-based MHC haplotypes in the six vaccinated monkeys, which were used in Fig. 2, were detected, as MHC polymorphisms play an important role in the recognition frequency of T cell epitopes. The data obtained from the gene loci of DR involved in MHC class II in the current study cannot match the existing database because of the current incompleteness of the MHC class II information in macaques, the current study cannot match the existing database because of the current incompleteness of the MHC class II information in macaques, but the haplotypes of loci A and B involved in MHC class I were analyzed in the two homologous chromosomes in each individual and are shown in Table I. The names of the haplotypes in macaques were defined according to a previous study (23). The haplotype A019 in Mamu-A was defined in two monkeys, and the haplotypes B090 and B039 in Mamu-B were defined in four and two monkeys, respectively, with other haplotypes of Mamu-A or Mamu-B in individuals all different from each other. Among those haplotypes, B083a, B083b, and B083c were newly observed and defined in the current study. The polymorphism of MHC expression in macaques may lead to the different recognition of T cell epitopes by different individuals.

Establishment of a peptide mixture for T cell response detection in macaques

The six T cell epitopes were all located in the S1 region, with peptides 31 (297–314), 40 (313–330), 51 (401–418), 59 (465–482), 61 (481–498), and 62 (489–506), were mixed into a peptide mixture as the T cell response detection stimuli for monkeys, named peptide mixture T cell epitopes in S protein in macaques (TeSma), and its effect in eliciting monkey PBMC IFN-γ or IL-4 secretion was detected by ELISPOT assay. Compared with that in the control group, the number of PBMCs secreting IFN-γ or IL-4 was increased upon treatment with the peptide mixture TeSma, consistent with the effect of the S1 protein, which contains the main T cell epitopes in the S protein (Fig. 3B, 3C). The results indicate that the peptide mixture TeSma can replace S1 to some extent in eliciting IFN-γ or IL-4 secretion in T cell response detection.

The IL-4 production response was obviously detectable in the current study, and IL-4 can be secreted by the polarized phenotype of T cells induced by CD3, CD28, IL-2, and IL-4. An IL-2 ELISPOT assay was performed, as IL-2 is secreted by both CD4+ and

Table I. The polymorphism of MHC class I expression in macaques

<table>
<thead>
<tr>
<th>Monkey ID</th>
<th>Mamu-A Haplotype: Major Alleles, Minor Alleles</th>
<th>Mamu-B Haplotype: Major Alleles, Minor Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>A009: A<em>009, A3</em>13</td>
<td>B039: B<em>019, B</em>007, B<em>102, B</em>031, B<em>046, B</em>051, B<em>070, B</em>088, B<em>082, B</em>105</td>
</tr>
<tr>
<td></td>
<td>A028: A<em>028, A2</em>05, A4*14</td>
<td>B076: B<em>076, B</em>044, B<em>036, B</em>046, B<em>050, B</em>072, B<em>109, B</em>060</td>
</tr>
<tr>
<td>C3</td>
<td>A051: A<em>051, A2</em>05, A4*14</td>
<td>B083b: B<em>083, B</em>069, B<em>100, B</em>072, B<em>079, B</em>098, B<em>051, B</em>046</td>
</tr>
<tr>
<td></td>
<td>A074: A<em>074, A2</em>05, A4*14</td>
<td>B090: B<em>090, B</em>065, B<em>045, B</em>046, B<em>051, B</em>072, B<em>082, B</em>088, B<em>109, B</em>142, B*151</td>
</tr>
<tr>
<td>A9</td>
<td>A019: A<em>019, A2</em>05, A3<em>13, A4</em>14</td>
<td>B047: B<em>047, B</em>027, B<em>038, B</em>046, B<em>072, B</em>082, B<em>060, B</em>114</td>
</tr>
<tr>
<td></td>
<td>A032: A<em>032, A2</em>05, A4*14</td>
<td>B090: B<em>090, B</em>065, B<em>045, B</em>046, B<em>051, B</em>072, B<em>082, B</em>088, B<em>098, B</em>109, B<em>142, B</em>151</td>
</tr>
<tr>
<td>C6</td>
<td>A004: A<em>004, A2</em>05, A4*14</td>
<td>B033a: B<em>083, B</em>056, B<em>081, B</em>018, B<em>017, B</em>072, B<em>193, B</em>098</td>
</tr>
<tr>
<td></td>
<td>A033: A<em>033, A2</em>05, A4*14</td>
<td>B090: B<em>090, B</em>065, B<em>045, B</em>046, B<em>051, B</em>072, B<em>082, B</em>088, B<em>098, B</em>109, B<em>142, B</em>151</td>
</tr>
<tr>
<td>G6</td>
<td>A074: A<em>074, A2</em>05, A4*14</td>
<td>B033c: B<em>083, B</em>056, B<em>034, B</em>074, B<em>109, B</em>051, B<em>098, B</em>078, B*046</td>
</tr>
<tr>
<td></td>
<td>A117: A<em>117, A2</em>05</td>
<td>B090: B<em>090, B</em>065, B<em>045, B</em>046, B<em>051, B</em>072, B<em>082, B</em>088, B<em>098, B</em>109, B<em>142, B</em>151</td>
</tr>
<tr>
<td>A0</td>
<td>A001: A<em>001, A2</em>05</td>
<td>B106: B<em>106, B</em>063, B<em>050, B</em>060, B<em>079, B</em>082</td>
</tr>
<tr>
<td></td>
<td>A019: A<em>019, A2</em>05, A3<em>13, A4</em>14</td>
<td>B039: B<em>019, B</em>007, B<em>102, B</em>031, B<em>046, B</em>051, B<em>070, B</em>088, B<em>082, B</em>105</td>
</tr>
</tbody>
</table>

The MHC class I Mamu-A and Mamu-B haplotypes from the six S DNA–vaccinated monkeys were detected by sequencing. The name of the haplotypes and the major and minor alleles included in it were defined in an early study (22), except B083a, B083b, and B083c, which were observed and defined in the current study. B083a, major: B*083, B*056, B*081, B*018, and B*017, minor: B*072, B*193, and B*098. B083b, major: B*083 and B*069; minor: B*100, B*072, B*079, B*098, B*051, and B*046. B083c, major: B*083, B*056, and B*034; minor: B*074, B*109, B*051, B*098, B*078, and B*046. According to the previous study (23), the major alleles (black) define the haplotypes; minor alleles (gray) are potentially variable and may not always be detected because of generally low transcript abundance.

ID, identifier.
CD8+ T cells to maintain T cell growth and promote T cell differentiation to a Th2-polarized phenotype. The results showed that the peptide mixture TeSma could also elicit monkey PBMC secretion of IL-2. The IL-2/C159 producing T cell responses under treatment with both the S1 protein and peptide mixture TeSma were all consistent with the IL-4/C159 producing T cell responses (Fig. 3D), suggesting that T cell epitopes recognized by the IL-4 response are also recognized in IL-2 production.

In addition, after monkeys were vaccinated with the DNA vaccine, neutralizing Abs against SARS-CoV-2 were also significantly produced at Ab titers of 1:16 to 1:64 (Fig. 3E), suggesting that both humoral and cellular immunity were induced by the DNA vaccine.

Identification of the T cell epitopes in the S protein in mice

To investigate the conservation of the T cell epitopes across species, the specific epitopes in the S protein frequently recognized by mice were identified. The DNA vaccine pCW1093 encoding the full-length S protein or the empty plasmid pSW3891 was injected into the mice, and the T cell responses were detected through IFN-γ ELISPOT assay. According to the results, peptide pools B, D, and G were recognized by T cells and induced IFN-γ secretion (Fig. 4A). Among the three peptide pools, peptides 14, 20, 33, and 39 remarkably increased the number of cells secreting IFN-γ in mixed mouse SPLs (Fig. 4B), and the recognition ability of those five peptides by T cells was also confirmed by the three individual mice (Fig. 4C). Thus, the T cell epitopes in the S protein in mice were defined as follows: peptide 14 (IFGTTLDSKTQSLLIVNN, 105–122), peptide 20 (MESEFRVYSSANNCTFEY, 153–170), peptide 33 (GWTAAGAAAYVGYLQPRT, 257–274), peptide 39 (SFTVEKGYQTSNFRVQP, 305–322), and peptide 67 (KSTNLVKNKCVNFNFNGL, 529–546). All specific T cell epitopes recognized by Th1 cells were located in the S1 region, with peptide 67 involved in the RBD. Compared with that in monkeys, the T cell epitope peptide 39 (305–322) identified in mice was completely consistent with the epitope region involved in peptides 38 (297–314) and 40 (313–330) defined in monkeys. Peptide 40 also exhibited high affinity for HLA-I or HLA-II in humans, as shown in Supplemental Table I, indicating that the T cell epitope peptides 39 and 40 may be conserved across species.

The five epitope peptides 14, 20, 33, 39, and 67 identified in mice were mixed into peptide mixture T cell epitopes in S protein in mouse (TeSmo), and the effect of TeSmo in the detection of the T cell response in mice was also confirmed (Fig. 4D). Furthermore, the neutralizing Ab titer against SARS-CoV-2 was also increased in vaccinated mice at 1:24 to 1:48 (Fig. 4E). The humoral immune response induced by the DNA vaccine was correlated with the T cell responses observed in both monkeys and mice, and the correlation between the T cell response and Ab production was also verified in humans (8).

Discussion

The well-defined T cell epitopes in the S protein play an important role in T cell response detection in vaccine development and...
FIGURE 4. Identification of the T cell epitopes in the S protein in mice. (A) The number of IFN-α-secreting mouse SPLs upon treatment with peptide pools A–P. (B) IFN-γ SFUs detected after treatment with the single peptides 11–20, 31–40, and 61–70 contained in peptide pools B, D, or G. (C) Detection of IFN-γ secretion after treatment with peptides 13, 14, 20, 33, 39, and 67. (D) The effect of the S1 protein and peptide mixture TeSmo on T cell IFN-γ secretion. (E) Detection of the titer of neutralizing Abs against SARS-CoV-2 in mice. ELISPOT assay was used in (A), (B), (C), and (D). Mice were vaccinated with 1.6 mg of full-length S protein–encoded DNA (S DNA vaccinated) or empty vector (control), spleens were collected at 14 d after vaccination, and SPLs were isolated. The results from three mice were included in each group in (C), (D), and (E) and in the S DNA–vaccinated group in (A); one mouse was used in the control group in (A). SPLs from three S DNA–vaccinated mice were mixed and detected in (B). An unpaired t test was used to detect significant differences between the control and S DNA–vaccinated groups. All data are presented as the mean ± SD. *p < 0.05, significantly different from the control group.

diagnosis in patients with COVID-19. Additionally, they may contribute to vaccine studies based on the immunogenicity of the virus. Compared with the use of the whole virus or protein as Ags, more advantages exist for peptides, including their high stability and specificity, the conservation of their sequence, and their low side effect risk due to their certain constitution. Multipptide subunit-based epitopes have been applied in several studies for the development of diagnostic systems and vaccines, such as the diagnosis of swine hepatitis E virus and common viral infections in India (24, 25), and vaccine development for influenza A viruses and some coronaviridae family members, including human coronavirus and SARS-CoV-2 (26–28). Furthermore, a recombinant vaccine against SARS-CoV-2 consisting of 33 antigenic epitopes from the nucleocapsid protein, membrane glycoprotein, and surface S glycoprotein showed the potential capability to induce cellular and humoral immune responses in hosts (28). The effective use of the computationally analyzed specific epitopes recognized by cytotoxic T lymphocytes, helper T lymphocytes, and B cells in this research strongly demonstrated the application potential of the T cell epitopes identified in the current study for vaccine design.

Based on the practical application value of specific epitopes, previous studies employed an immunological approach, computational method, or algorithm analysis to predict the B cell or T cell epitopes in the S protein of SARS-CoV-2 according to the binding affinities between target proteins and the MHC (16, 22, 29). The present study identified T cell epitopes through testing Th1 or Th2 cell-secreted IFN-γ or IL-4 to access recognized peptides among peptide pools spanning the whole S protein in macaques, whose anatomy, immune, and metabolic systems are most similar to those of humans. Six peptides were experimentally verified as the most representative T cell epitopes: peptides 38 (297–314), 40 (313–330), 51 (401–418), 59 (465–482), 61 (481–498), and 62 (489–506).

The conservation of the T cell epitopes across species was further detected in the current study between monkeys and mice. All the specifically recognized T cell epitopes were located in the S1 region in both monkeys and mice, with a peptide region (305–322) recognized by T cells in both kinds of animals. The conserved epitope region also showed high affinity for HLA-I or HLA-II alleles in humans, according to two previous studies that predicted the specific T cell epitopes by computational analyses (21, 22). The homogeneity of the T cell epitopes across species may increase the application of the identified peptides in the current study for the detection of T cell responses or diagnosis in patients with COVID-19 in further clinical research. Furthermore, the monkeys and mice used in the current study vaccinated with DNA vaccines all exhibited positive T cell responses or diagnosis in patients with COVID-19 in further clinical research. Additionally, they may contribute to vaccine studies based on the immunogenicity of the virus.
Owing to the critical role of the T cell reaction in response to SARS-CoV-2, the well-established T cell response detection system in the current study would provide a more-convenient method for human studies.

According to the T cell response results post-DNA vaccine administration in the current study, positive reactions were detected for IL-4 production in response to S1 or peptide pools in five monkeys among the seven monkeys vaccinated with the DNA vaccine, which may suggest the relevance of IL-4 production in the responses to the SARS-CoV-2 DNA vaccine, and it may be related to the features of the DNA vaccine that can induce immune response through multiple pathways. Although the details of its function need to be further studied, the increase of IL-4 production was also detected in other pre-clinical studies of DNA vaccines, such as DNA vaccines against acinetobacter baumannii, HIV, or legionella pneumophila (30–32).

Comparing the sequences of the S protein among coronaviridae family members, SARS-CoV-2 shares 76.0 and 29.4% homologous sequences with SARS-CoV and Middle East respiratory syndrome coronavirus, respectively (33). Despite the high similarity observed between SARS-CoV and SARS-CoV-2, discordance in T cell epitopes in the S protein of the two coronaviruses exists. Two of the five most frequently recognized T cell epitopes in the S protein of SARS-CoV-2 were located in the RBD, with another three located in the S2 region (34). However, for SARS-CoV-2, all defined peptide epitopes in monkeys are located in the S1 region, and most of them (four of six) are located in the RBD, with no peptides detected in the S2 region in the current study. The results were consistent with a previous study that showed that RBD Ag induced higher Ab titer and Ab affinity to native S protein than other Ags from the S protein in rabbits (35). SARS-CoV-2 entry into host cells is mediated by the interaction of the RBD and ACE2 receptor, and the central-ized appearance of the T cell epitopes in the RBD in SARS-CoV-2 may show some relationship with the immunogenicity and infection ability of the virus.

Previous studies have also found different effects of the RBD in regulating the immune route of SARS-CoV and SARS-CoV-2. The substitutions of amino acid residues in the RBD of S protein of SARS-CoV-2 strengthen the interaction and aff.

References


17. Munster, V. J., F. Feldmann, B. N. Williamson, N. van Doremalen, L. P. current study. The results were consistent with a previous study that showed that RBD Ag induced higher Ab titer and Ab affinity to native S protein than other Ags from the S protein in rabbits (35). SARS-CoV-2 entry into host cells is mediated by the interaction of the RBD and ACE2 receptor, and the central-ized appearance of the T cell epitopes in the RBD in SARS-CoV-2 may show some relationship with the immunogenicity and infection ability of the virus.

Previous studies have also found different effects of the RBD in regulating the immune route of SARS-CoV and SARS-CoV-2. The substitutions of amino acid residues in the RBD of the S protein of SARS-CoV-2 strengthen the interaction and affinity between SARS-CoV-2 and host cells compared with those between SARS-CoV and host cells (36, 37). Moreover, polyclonal Abs against the RBD of SARS-CoV-2 were not applicable to SARS-CoV-3 (36, 37). The differences between the antigenicity of those two viruses indicate that the vaccines currently under development against SARS-CoV-2 based on its RBD may not be effective against SARS-CoV-2. The newly verified T cell epitopes in the RBD and S1 region in SARS-CoV-2 by the current study may provide a new target for the study of vaccines against SARS-CoV-2.

In summary, the current study defined peptides 38 (297–314), 40 (313–330), 51 (401–418), 59 (465–482), 61 (481–498), and 62 (489–506) as the most frequently recognized T cell epitopes, and these peptides overlapped and produced three main long epitopes: cluster peptides 297–330, 401–418, and 465–506. This finding may provide a convenient pattern in the detection of T cell responses for the development of new vaccines and provides a novel (to our knowledge) target in the diagnosis of patients with COVID-19.

Acknowledgments

We thank Quan Liu and Yang for the animal care and feeding.

Disclosures

The authors declare that they have no conflicts of interest with the contents of this article.


