Insight into Vaccine Development for Alphacoronaviruses Based on Structural and Immunological Analyses of Spike Proteins

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ABSTRACT Coronaviruses that infect humans belong to the Alphacoronavirus (including HCoV-229E) and Betacoronavirus (including SARS-CoV and SARS-CoV-2) genera. In particular, SARS-CoV-2 is currently a major threat to public health worldwide. The spike (S) homotrimers bind to their receptors via the receptor-binding domain (RBD), which is a major target to block viral entry. In this study, we selected Alphacoronavirus (HCoV-229E) and Betacoronavirus (SARS-CoV and SARS-CoV-2) as models. Their RBDs exist two different conformational states (“lying” or “standing”) in the prefusion S-trimer structure. Then, the differences in the immune responses to RBDs from these coronaviruses were analyzed structurally and immunologically. Our results showed that more RBD-specific antibodies (antibody titers: 1.28 × 10^2 and 2.75 × 10^5, respectively) were induced by the S-trimer with the RBD in the standing state (SARS-CoV and SARS-CoV-2) than the S-trimer with the RBD in the lying state (HCoV-229E; antibody titers: <500), and more S-trimer-specific antibodies were induced by the RBD in the SARS-CoV and SARS-CoV-2 (antibody titers: 6.72 × 10^5 and 5 × 10^5, respectively) than HCoV-229E (antibody titers: 1.125 × 10^5). Besides, we found that the ability of the HCoV-229E RBD to induce neutralizing antibodies was lower than S-trimer, and the intact and stable S1 subunit was essential for producing efficient neutralizing antibodies against HCoV-229E. Importantly, our results reveal different vaccine strategies for coronaviruses, and S-trimer is better than RBD as a target for vaccine development in Alphacoronavirus. Our findings will provide important implications for future development of coronavirus vaccines.

IMPORTANCE Outbreaks of coronaviruses, especially SARS-CoV-2, pose a serious threat to global public health. Development of vaccines to prevent the coronaviruses that can infect humans has always been a top priority. Coronavirus spike (S) protein is considered a major target for vaccine development. Currently, structural studies have shown that Alphacoronavirus (HCoV-229E) and Betacoronavirus (SARS-CoV and SARS-CoV-2) RBDs are in “lying” and “standing” states in the prefusion S-trimer structure. Here, we evaluated the ability of S-trimer and RBD to induce neutralizing antibodies among these coronaviruses. Our results showed that the S-trimer and RBD are both candidates for subunit vaccines in Betacoronavirus (SARS-CoV and SARS-CoV-2) with an RBD standing state. However, for Alphacoronavirus (HCoV-229E) with an RBD lying state, the S-trimer may be more suitable for subunit vaccines than the RBD. Our results will provide novel ideas for the development of vaccines targeting S protein in the future.

KEYWORDS coronavirus, spike protein, receptor-binding domain, structural analysis, neutralizing antibody


Editor Tom Gallagher, Loyola University Chicago

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Received 30 November 2020
Accepted 21 December 2020
Accepted manuscript posted online 7 January 2021
Published 10 March 2021
Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses with the largest genomes (26 to 32 kb) among known RNA viruses and are phylogenetically divided into four genera (Alpha-, Beta-, Gamma-, and Deltacoronavirus) (1, 2). To date, seven human-infecting coronaviruses (hCoVs) (3, 4) cause various degrees of symptoms: HCoV-229E, HCoV-NL63, HCoV-HKU1, HCoV-OC43, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, which caused the current epidemic of COVID-19. Among them, alpha-CoVs (HCoV-229E and HCoV-NL63) and beta-CoVs (HCoV-OC43 and HCoV-HKU1) are well adapted to humans and widely circulate in the human population, with most infections causing mild disease in immunocompetent adults (3, 5, 6). In addition, SARS-CoV, SARS-CoV-2, and MERS-CoV belong to Beta-CoV and are highly pathogenic (7–9). SARS-CoV emerged in 2002 and spread worldwide, resulting in 8,273 infections and nearly 775 deaths, with an approximately 9% case fatality rate (CFR) (9). MERS-CoV emerged in 2012 and caused numerous outbreaks in humans, with an approximately 36% CFR (10). SARS-CoV-2 is a newly emerged coronavirus strain (4), which has resulted in more than 70 million infections worldwide and more than 1 million deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019).

As the primary glycoprotein on the surface of the viral envelope, the spike (S) glycoprotein contains two subunits responsible for receptor binding (S1 subunit) and membrane fusion (S2 subunit) (11). In particular, the S1 subunit of the prefusion S protein is structurally organized into four distinct domains: the N-terminal domain (NTD), the C-terminal domain (CTD), subdomain 1 (SD1), and subdomain 2 (SD2) (12–24). The receptor-binding domain (RBD) in the S protein mediates the binding of the virus to host cells, which is a critical step for the virus to enter target cells (11, 25). The CTDs of alpha-CoVs HCoV-NL63 and HCoV-229E are used as RBDS, which bind to angiotensin-converting enzyme 2 (ACE2) and aminopeptidase (APN), respectively (26, 27). The CTDs of beta-CoVs (SARS-CoV, SARS-CoV-2, and MERS-CoV) are similar in their core structures but are markedly different in their receptor-binding motifs (RBMs), leading to different receptor specificities; SARS-CoV and SARS-CoV-2 recognize ACE2 (28, 29), whereas MERS-CoV recognizes dipeptidyl peptidase-4 (DPP4) (30).

Currently, the S-trimer structures in the prefusion state have been reported for members of alpha-CoVs (HCoV-NL63, HCoV-229E, porcine epidemic diarrhea virus [PEDV], and feline infectious peritonitis [FIPV]) (12, 14, 16, 22), Beta-CoVs (mouse hepatitis virus [MHV], HCoV-HKU1, HCoV-OC43, SARS-CoV, SARS-CoV-2, and MERS-CoV) (13, 15, 20, 21, 23, 24), gamma-CoV (avian coronavirus [infectious bronchitis coronavirus [IBV]]) (19), and delta-CoV (porcine deltacoronavirus [PDCoV]) (17, 18). The S1 subunits of Beta- and Gamma-CoV strains utilize the cross-subunit packing mode, reducing the conformational conflict of the RBD in a “standing” state (13, 19–21). In contrast, Alpha- and Delta-CoV strains both utilize an intrasubunit packing mode, and the S1-CTD is limited by the conformational conflict with surrounding domains (12, 14, 16–19, 22). Hence, the S1-RBD in the S-trimer was captured in two different states among different coronaviruses. In the beta-CoVs (SARS-CoV, SARS-CoV-2, and MERS-CoV), the S1-CTD adopts a standing state, which is believed to be a prerequisite for receptor binding and RBM-specific antibody binding (13, 20, 21). Nevertheless, the S1-CTDs of alpha-CoVs all adopt the “lying” state, which is considered more conducive to evading antibody recognition (12, 14, 16, 22).

Currently, a number of subunit vaccines targeting S protein or RBD have been developed against SARS-CoV, SARS-CoV-2, and MERS-CoV (31–36). Compared with beta-CoVs, relatively few studies have investigated two alpha-hCoVs: HCoV-229E and HCoV-NL63. Besides, their S1 subunit structure and receptor recognition pattern, especially the structure of the RBD and its state in the S-trimer, differ substantially from those of beta-CoVs, suggesting different S protein immune responses between alpha- and beta-CoVs. Importantly, considering the low homology between different coronavirus genera, related research on alpha-CoVs can not only help to elucidate the differences between S proteins that adopt different RBD conformational states but can also
facilitate the development of coronavirus vaccines. In this study, we selected SARS-CoV, SARS-CoV-2, and HCoV-229E as models, which exist in two RBD conformational states, and evaluated the ability of S-trimer and RBD to induce neutralizing antibodies. Moreover, we provide possible vaccine strategies for alpha- and beta-CoVs, which may facilitate the design and development of coronavirus vaccines in the future.

RESULTS AND DISCUSSION

Structural and immunological analyses of alpha- and beta-coronavirus spike proteins. To date, multiple S-trimer structures of coronaviruses have been resolved (12–24). Through structural comparison, we found that although the amino acid sequences are quite different, the compositions and structures of different functional domains of coronaviruses are consistent (Fig. 1A). All the alpha-CoVs bind to protein receptors with CTDs (as receptor-binding domains [RBDs]), and the RBDs are in a lying state (12, 14, 16, 22, 26, 27) (Fig. 1B). However, beta-CoVs (SARS-CoV, SARS-CoV-2, and MERS-CoV) bind receptors with CTDs (as RBDs), and the RBDs are in a standing state (13, 20, 21) (Fig. 1B). The RBD directly binds to receptors, which is an important target for the induction of neutralizing antibody (NAb).

Then, we performed B-cell epitope predictions for the S-trimers and RBDs of alpha-CoV (HCoV-229E) and beta-CoVs, which may facilitate the design and development of coronavirus vaccines in the future.

FIG 1 Structural analysis of S1-RBDs from coronavirus S-trimers. (A) Schematic diagram of coronavirus spike protein organization. S1, receptor-binding subunit; S2, membrane fusion subunit; NTD, N-terminal domain; RBD, receptor-binding domain; FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. (B) Overall structure comparison of coronavirus S-trimers. The S-trimer structures of HCoV-229E (PDB ID: 6U7H), HCoV-NL63 (PDB ID: 5SZS), PEDV (PDB ID: 6U7K), FIPV (PDB ID: 6JX7), PDCoV (PDB ID: 6BFU), IBV (PDB ID: 6CV0), SARS-CoV (PDB ID: 5X5F), SARS-CoV-2 (PDB ID: 6VSB), MERS-CoV (PDB ID: 5X5F), HCoV-HKU1 (PDB ID: 5I08), HCoV-OC43 (PDB ID: 6OHW), and mouse hepatitis virus (MHV) (PDB ID: 3JCL) are shown. The S1-RBDs are colored in magenta. The lengths of the coronavirus S-trimers are shown as previously reported.
residues (the corresponding spatial epitope and linear epitope) are displayed on the structural surface (Fig. 2A, C, and E), and the distribution of positive residues on the RBD is summarized in Table 1. A total of 51 and 26 amino acid residues located on the RBD were predicted to be conformational epitopes for SARS-CoV and SARS-CoV-2, respectively. Of these, 47 and 25 residues were located in the RBM subdomains of SARS-CoV and SARS-CoV-2, respectively. The linear B-cell epitope prediction results were similar in SARS-CoV and SARS-CoV-2. However, in HCoV-229E, only 3 residues located in the RBM subdomain were predicted to be conformational epitopes, and 9 residues were predicted to be linear epitopes. The same results also appeared in the HCoV-229E S-trimer; fewer positive residues were located in the RBD than in the SARS-CoV or SARS-CoV-2 RBM subdomain (Fig. 2A, C and E). Meanwhile, we analyzed the

![Image](https://example.com/image.png)

**FIG 2** Structure-based B-cell epitope predictions of Beta-CoV (SARS-CoV and SARS-CoV-2) and Alpha-CoV (HCoV-229E). (A, C, and E) The predicted B-cell epitopes of SARS-CoV, SARS-CoV-2, and HCoV-229E are shown. The linear (red cartoon) and conformational (yellow sphere) B-cell epitopes were predicted using Bepipred 2.0 or DiscoTope 2.0 and labeled into the corresponding structure via PyMOL. (B, D, and F) The complex structures of the RBDs of SARS-CoV (PDB ID: 2AJF), SARS-CoV-2 (PDB ID: 6M0J), and HCoV-229E (PDB ID: 6ATK) with the receptors (hACE2 and hAPN) are shown. The interface area and the surface area were calculated via PDBePISA. The RBM region of the RBD and the receptors (hACE2 and hAPN) are shown in red and cyan, respectively.

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of residues</th>
<th>RBD/M/RBD ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SARS-CoV</td>
<td>51</td>
<td>56/47/33</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>26</td>
<td>59/25/35</td>
</tr>
<tr>
<td>HCoV-229E</td>
<td>3</td>
<td>14/3/9</td>
</tr>
<tr>
<td>HCoV-NL63</td>
<td>4</td>
<td>33/4/19</td>
</tr>
<tr>
<td>PRCov</td>
<td>4</td>
<td>41/4/27</td>
</tr>
<tr>
<td>TGEV</td>
<td>7</td>
<td>34/4/24</td>
</tr>
<tr>
<td>PEDV</td>
<td>0</td>
<td>21/0/8</td>
</tr>
<tr>
<td>FIPV</td>
<td>0</td>
<td>17/0/11</td>
</tr>
</tbody>
</table>

**Table 1** Distribution of residues predicted positive for B-cell epitopes
binding area of the RBD and receptors from SARS-CoV, SARS-CoV-2, and HCoV-229E (Fig. 2B, D, and F). Among them, the interaction areas of SARS-CoV and SARS-CoV-2 were similar (approximately 829.7 Å² and 843.5 Å², respectively) and were larger than that of HCoV-229E (approximately 497 Å²). Furthermore, surface area analysis also yielded consistent results (Fig. 2B, D, and F). Compared with HCoV-229E, the larger surface areas and binding areas of the SARS-CoV and SARS-CoV-2 RBDs to the receptor may induce more NAbs. The cryo-electron microscopy (cryo-EM) structures of S-trimers in the prefusion conformation have shown that the SARS-CoV and SARS-CoV-2 RBDs are in a standing state (13, 20, 21); however, HCoV-229E RBDs are in a lying state (16) (Fig. 1B). Hence, compared with HCoV-229E, this may make it difficult for SARS-CoV and SARS-CoV-2 to evade neutralizing antibodies that target RBD recognition.

Distinct immunogenicity of the RBDs in Alpha-CoV (HCoV-229E) and Beta-CoV (SARS-CoV and SARS-CoV-2). To evaluate the immunogenicity of the S-trimers and RBDs between Alpha-CoV (HCoV-229E) and Beta-CoV (SARS-CoV and SARS-CoV-2), the recombinant S proteins were expressed and purified (Fig. 3A, E, I, and K). Because the prefusion-stabilized S proteins could influence RBD standing or lying conformational states, two proline substitutions in the loop between the heptad repeat (HR1) and the central helix (CH) have not been introduced (31, 37). For in vitro binding measurements (Fig. 3B, F, J, and L; Table 2), the equilibrium dissociation constants (Kₐ) of HCoV-229E spike proteins (S-trimer and RBD) and hAPN are 3.29 ± 0.0129 nM and 36.0 ± 0.037 nM, respectively. Besides, the Kₐ values of SARS-CoV spike proteins (S-trimer and RBD) and hACE2 are 246 ± 21.1 nM and 38.8 ± 2.05 nM, respectively. Compared with previous research (26), our results showed that all the spike proteins function well in vitro. Then, the S-trimer (10 μg) and RBD (10 μg) were used as antigens to immunize mice. The antibody response was measured via enzyme-linked immunosorbent assay (ELISA) using collected sera. The data showed that the S-trimer and RBD of SARS-CoV and
SARS-CoV-2 could induce high levels of protein-specific antibodies (antibody titers: $1.36 \times 10^5$ and $3.84 \times 10^5$, and $1.625 \times 10^6$ and $5 \times 10^5$, respectively) (Fig. 4A to D). Moreover, the S-trimers of both SARS-CoV and SARS-CoV-2 could induce high-titer RBD antibodies (antibody titers: $1.28 \times 10^6$ and $2.75 \times 10^5$, respectively) (Fig. 4B and D), and the RBD could induce high-titer S-trimer antibodies (antibody titers: $6.72 \times 10^4$ and $5 \times 10^4$, respectively) (Fig. 4A and C). Similar to SARS-CoV and SARS-CoV-2, the S-trimer and RBD of HCoV-229E both had good immunogenicity (antibody titers: $8.8 \times 10^4$ and $7.68 \times 10^5$, respectively) (Fig. 4E and F). However, the HCoV-229E S-trimer induced fewer RBD-specific antibodies than those of SARS-CoV and SARS-CoV-2 (antibody titer: $<500$) (Fig. 4F), and the HCoV-229E RBD induced low-titer S-trimer antibodies (antibody titer: $1.125 \times 10^4$) (Fig. 4E and H). To confirm this finding, a higher immunization dose of the HCoV-229E RBD (50 $\mu$g) was used in the same manner. Nevertheless, no significant increase in the RBD-specific antibody titer (antibody titers: $1.792 \times 10^6$ and $3.072 \times 10^6$) (Fig. 3G) or antibody titer for the HCoV-229E S-trimer (antibody titers: $2.5 \times 10^2$ and $2.5 \times 10^2$) (Fig. 3H) was noted. Overall, our immune epitope analysis and biochemical tests consistently showed that the S-trimer with a standing RBD state is more conducive to inducing RBD-specific antibodies in SARS-CoV and SARS-CoV-2. However, the S-trimer with a lying RBD state induces fewer RBD-specific antibodies in HCoV-229E.

**Table 2** Surface plasmon resonance binding data for the interaction between the coronavirus (HCoV-229E and SARS-CoV) spike proteins and the receptors (hAPN and hACE2)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Spike protein</th>
<th>$K_D$ ($10^4$ M$^{-1}$ s$^{-1}$)</th>
<th>$K_{12}$ ($10^{-4}$ s$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCoV-229E</td>
<td>S-trimer</td>
<td>$3.67 \pm 0.0005$</td>
<td>$1.21 \pm 0.0046$</td>
<td>$3.29 \pm 0.0129$</td>
</tr>
<tr>
<td></td>
<td>S-trimer/NTD</td>
<td>$1.94 \pm 0.0006$</td>
<td>$0.41 \pm 0.0289$</td>
<td>$2.12 \pm 0.15$</td>
</tr>
<tr>
<td></td>
<td>S1</td>
<td>$0.0886 \pm 0.0006$</td>
<td>$2.26 \pm 0.104$</td>
<td>$255 \pm 13.6$</td>
</tr>
<tr>
<td></td>
<td>S1-RBD</td>
<td>$3.2 \pm 0.00174$</td>
<td>$11.5 \pm 0.0056$</td>
<td>$36.0 \pm 0.037$</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>S-trimer</td>
<td>$0.622 \pm 0.0499$</td>
<td>$15.3 \pm 0.0755$</td>
<td>$246 \pm 21.1$</td>
</tr>
<tr>
<td></td>
<td>S1-RBD</td>
<td>$0.812 \pm 0.0004$</td>
<td>$3.15 \pm 0.165$</td>
<td>$38.8 \pm 2.05$</td>
</tr>
</tbody>
</table>

*Values after ± correspond to the residual standard deviation. Each experiment was repeated independently twice with similar results.*
the S1 and NTD+RBD sera, while the NTD and RBD sera alone had no detectable neutralizing effects (Fig. 5B). Hence, our results indicate that the S1 region in the S-trimer should be the key region for NAb induction. To verify the importance of the complete S1 structure for NAb induction, we designed S-trimer mutants, namely, an NTD-deficient S-trimer (S-trimer/ΔNTD), the S1 subunit integrity of which was destroyed (Fig. 5C). Compared with HCoV-229E S-trimer (KD value is 3.29 ± 0.0129 nM), in vitro binding measurements showed that S-trimer/ΔNTD has a similar binding affinity with hAPN (KD value is 2.12 ± 0.15 nM) and functions well (Fig. 3B and Fig. 5D and E). However, the incomplete S1 conformation significantly reduces the level of NAbs induced by the S-trimer (Fig. 5F). Taken together, these results showed that the intact and stable S1 subunit in HCoV-229E S-trimer is a prerequisite for the production of effective NAbs.

Furthermore, our results showed that RBD functioned well in vitro (Fig. 3F), which indicates that the characteristics of RBD itself may lead to the generation of fewer neutralizing antibodies than S-trimer in HCoV-229E (Fig. 4K). Hence, we screened monoclonal antibodies using HCoV-229E S-trimer, and the results showed few antibodies targeting S1-RBD (Fig. 6A). To further determine the ability of RBD to induce antibodies itself, we screened monoclonal antibodies targeting the S1 region and found that the
proportion of antibodies targeting RBD was approximately 20% (Fig. 6B). Since the S1 protein was expressed in a monomeric form, RBD is not restricted by the conformation of the surrounding domains and should be in a standing state. Therefore, our results indicate that HCoV-229E RBD may induce weaker levels of neutralizing antibodies,
which may be related to its own characteristics. Furthermore, the RBD in the HCoV-229E S-trimer is in the lying state. Its own characteristics and conformational states may prompt the HCoV-229E to escape the host’s immune surveillance, thereby allowing itself to circulate in the population for a long time.

Potential vaccine strategies for Alphacoronavirus (HCoV-229E) and Betacoronavirus (SARS-CoV and SARS-CoV-2).

We compared the structures of S-trimers and RBDs among alphacoronaviruses (Fig. 1B and Fig. 7A). We also predicted the potential B-cell epitopes for their RBDs (Fig. 7A; Table 1). In Alpha-CoV, the S-trimer had a closed S1 subunit with three lying RBDs (Fig. 1B). Moreover, the RBDs consist of a standard β-sandwich fold core and three short discontinuous loops in the same spatial region (12, 14, 16, 22, 26, 27, 38) (Fig. 7A). Meanwhile, we performed a structural conservative analysis and the results showed that the RBD structures of HCoV-NL63, PEDV, and FIPV were similar to HCoV-229E, with root mean square deviation (RMSD) values of 1.7, 1.8, and 2.4, respectively (Fig. 7B). Besides, the distribution of potential B-cell epitopes in the RBDs of alpha-CoVs was also similar to that of HCoV-229E (Fig. 7A and C; Table 1). Based on the above data, inherent differences exist in the RBDs between alpha- and betacoronaviruses.

![Diagram of B-cell epitope analysis of alphacoronavirus S1-RBDs.](image-url)
beta-CoVs (Fig. 2 and Fig. 7A and B). However, the alpha- and beta-CoVs show high similarity in their RBDs and similar potential immune characteristics within their respective genera (Fig. 2, 4, and 7A and B). Accordingly, we speculate that in alpha-CoVs such as HCoV-229E, subunit vaccines should prioritize the S-trimer rather than the RBD; in beta-CoVs such as SARS-CoV and SARS-CoV-2, the S-trimer and RBD are both good candidates for subunit vaccines (Fig. 8).

In summary, we systematically analyzed the immunogenicity of the S-trimers and RBDs of Alpha-CoV (HCoV-229E) and Beta-CoV (SARS-CoV and SARS-CoV-2). Our results showed the inherent differences between the RBDs of alpha- and beta-CoVs and revealed potential identical immune characteristics in alpha- and beta-CoVs. Based on these findings, we provide potential vaccine strategies for alpha- and beta-CoVs. For alpha-CoVs, the S-trimer may be more suitable for subunit vaccines than the RBD. For beta-CoVs, SARS-CoV and SARS-CoV-2, the S-trimer and RBD are both candidates for subunit vaccines. Considering the potential influence of conformational states on immunogenicity, we will further evaluate the immunogenicity difference among the different aggregation states of S-trimer and RBD in the future. Although our inference requires more experimental data for further confirmation, our results will provide novel ideas for the development of coronavirus vaccines in the future.

**MATERIALS AND METHODS**

**Plasmid construction.** According to previous research, insect codon-optimized sequences encoding the HCoV-229E S glycoprotein ectodomain (GenBank accession number NP_073551.1, residues 1 to 1116; NTD-deficient S-trimer: S-trimer/ΔNTD) and SARS-CoV S glycoprotein ectodomain with an R667A mutation (GenBank accession number NP_828851.1, residues 1 to 1195) were cloned into the baculovirus transfer vector pFastbac1 (Invitrogen) with a gene fragment encoding the GCN4 trimerization motif (LIKRMKQIEDKIEEIESKQKKIENEIARIKKIK) and an eight-residue Strep tag (WSHPQFEK) (21, 23). The human aminopeptidase N (hAPN) (GenBank accession number JX869059, residues 66 to 967) (39) and human

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**FIG 8** Potential vaccine strategies for alpha- and beta-CoVs. The model showed that the RBDs of the alpha-CoV S-trimers are in a lying state. In this state, the S protein cannot bind to the receptor, which is conducive to escaping the immune response targeting the RBD, and the HCoV-229E RBDs also induce fewer NAbs than beta-CoVs (SARS-CoV and SARS-CoV-2); thus, their S-trimers can be an effective potential subunit vaccine. In beta-CoVs (SARS-CoV and SARS-CoV-2), the RBDs of the S-trimer are in a standing state, which is conducive to binding receptors, and the RBD can induce more antibodies than HCoV-229E; thus, their S-trimers and RBDs can produce more NAbs. Hence, their S-trimers and RBDs can be an effective potential subunit vaccine.
ACE2 extracellular domain (residues 19 to 615) (40) were cloned into the pFastbac1 vector with an N-terminal honeybee melittin signal peptide and a C-terminal 6×His tag. Besides, HCoV-229E S1 (residues 1 to 536), S1-NTD (residues 1 to 258), S1-RBD (residues 295 to 428), and SARS-CoV S1-RBD (residues 306 to 527) containing an N-terminal honeybee melittin signal peptide and a C-terminal His tag were constructed using the same method (29). Besides, the S fragments of HCoV-229E, SARS-CoV, and SARS-CoV-2 were cloned into the pcDNA3.1 (+) vector with a C-terminal His tag using a previously described protocol (41). All constructs were validated using DNA sequencing. The sequences of S proteins (HCoV-229E, SARS-CoV, and SARS-CoV-2), hAPN, and hACE2 were synthesized by GenScript Corporation (GenScript, Nanjing, China).

Protein expression and purification. The spike protein ectodomain was expressed and purified using a previously described protocol (21, 29). Briefly, the construct was transformed into bacterial DH10Bac competent cells (Invitrogen); then, the extracted bacmid was transfected into Sf9 cells (American Type Culture Collection). The supernatant of the cell culture containing the secreted S glycoprotein was harvested at 60 h after infection and concentrated, and the buffer was changed to binding buffer (10 mM HEPES, pH 7.2, and 500 mM NaCl). The S-trimer (HCoV-229E and SARS-CoV) including Strep tag was captured using StrepTactin Sepharose high-performance resin (GE Healthcare) (21). HCoV-229E S1, S1-NTD, S1-RBD, and SARS-CoV S1-RBD including FC tag were harvested using a HiTrap Protein G HP column (GE Healthcare, Uppsala, Sweden). Besides, for SARS-CoV-2, the S-trimer (item no. 40589-V08B1) and S1-RBD (item no. 40592-V05H) were purchased from Sino Biological, Inc. In addition, the hAPN and hACE2 including His tag were harvested using HisTrap HP columns (GE Healthcare, Uppsala, Sweden). Finally, the harvested proteins were equilibrated with buffer (10 mM HEPES, pH 7.2, and 150 mM NaCl).

In vitro gel filtration analysis. Oligomerization of the receptors (hAPN and hACE2), HCoV-229E S-trimer (wild type [WT], ΔNTD mutant), S1, S1-NTD, and S1-RBD, and SARS-CoV S-trimer and S1-RBD was measured by SDS-PAGE. The gel filtration results were analyzed using Origin 8.5 software.

Surface plasmon resonance. Binding kinetics of purified HCoV-229E S-trimer (WT, ΔNTD mutant), S1, and S1-RBD samples with the hAPN and purified SARS-CoV S-trimer and S1-RBD samples with the hACE2 were measured by surface plasmon resonance (OpenSPR; Nicoya Life), as described previously (42–44). Briefly, the hAPN or hACE2 (200 μg, 5 μg/ml) was immobilized on the OpenSPR COOH sensor chip (Nicoya no. SEN-AU-100-12-COOH). Free activated carboxyl groups were deactivated with the addition of 100 μl blocking buffer (Nicoya). The immobilized protein was washed, the running buffer was injected for binding measurement by successive injections of buffer-matched HCoV-229E spike samples or SARS-CoV spike samples at 20 μl/min, the binding time was approximately 240 s, and the natural dissociation of approximately 400 s was carried out. Response units (RU) values were measured at 298 K. Binding kinetic parameters were obtained by fitting the curve to a one-to-one binding model using the TraceDrawer software package (Ridgeview Instruments, Uppsala, Sweden). All injections were carried out in duplicate and gave essentially identical results. Only one of the duplicates is shown.

Animal immunization. Female BALB/c mice aged 6 weeks were immunized with different proteins at 0 and 3 weeks. Proteins (10 μg) in PBS were intramuscularly injected into mice (American Type Culture Collection). The supernatant of the cell culture containing the secreted S glycoprotein was harvested at 60 h after infection and concentrated, and the buffer was changed to binding buffer (10 mM HEPES, pH 7.2, and 500 mM NaCl) were mixed 1:1 with the 2×-bovine serum (CBS). The mixture was used as the substrate for detection. Optical density (OD) was determined at 450 nm and 630 nm using a Spark 10M microplate reader (Tecan) after stopping the reaction with 2 M H2SO4. Serum from mice immunized with PBS was used as a control.

Generation of HCoV-229E S protein MAbs and epitope mapping. Six-week-old female BALB/c mice were immunized with 100 μg of purified HCoV-229E S-trimer or S1 protein. Antigens were emulsified in Freund’s complete adjuvant (Sigma-Aldrich; F5881) for the first immunization or Freund’s incomplete adjuvant (Sigma-Aldrich; F5506) for the subsequent boost. Each mouse received three subcutaneous injections at 2-week intervals. Mice with the highest titer of antibodies against the HCoV-229E S-trimer or S1 protein were furtherboosted by intraperitoneal injection of 200 μg of purified HCoV-229E S-trimer or S1 protein diluted in PBS buffer. Three days after the last injection, spleen cells were collected and fused with SP2/0 cells with PEG1450 (Sigma-Aldrich; P7181) to generate hybridoma cells. Antigen-specific ELISA was used for the hybridoma screening. Positive hybridomas were further cloned and used for epitope mapping. Finally, ELISA plates were coated with different proteins (the HCoV-229E S-trimer, S1, S1-NTD, and S1-RBD) at 1 μg/ml in PBS (pH 9.6) overnight at 4°C and subsequently blocked and washed. Then, the plates were reacted with the hybridoma culture supernatants at 37°C for 1 h. HRP-conjugated goat anti-mouse IgG (1:5,000 diluted in PBS) with 1% [wt/vol] BSA; Boster) was used for detection. Signal reading was carried out in the manner described above. Hybridoma culturing medium was used as a control.
Pseudotyped-virus neutralization assay. In this study, HCoV-229E (ATCC, VR-740) was amplified using HuH-7 cells, and pseudoviruses were produced as previously described (41). The HEK-293T cells were transfected with 1 μg of HCoV-229E-SΔ19-pcDNA3.1 (C-terminal deletion 19 amino acids [aa]), SARS-CoV-5-SΔ22-pcDNA3.1 (C-terminal deletion 22 aa), and SARS-CoV-2-SΔ18-pcDNA3.1 (C-terminal deletion 18 aa) plasmids using Effect2000 transfection reagent (Vazyme). Besides, VSV-G-pcDNA3.1 and pcDNA3.1 were transfected as positive and negative controls, respectively. Then, the transfected cells were infected with VSV-ΔG-HCoV-229E-S, VSV-ΔG-SARS-CoV-5, and VSV-ΔG-SARS-CoV-2-S (culture supernatants) were harvested and stored at −80°C until use.

For titration of these three pseudoviruses, 10-fold dilution was performed in 96-well culture plates. The cell with added pcDNA3.1-transfected supernatant was used as a control. After 48 h, the culture supernatant was removed and washed using PBS, and 20 μl of reporter lysis S⩾ Buffer (Promega; catalog no. E4030) was added to each well. Then, the luciferase substrate (Promega; catalog no. E1500) was added, and the luminescence activity was measured using a multimode microplate reader (Tecan Spark 10M). The viral titers were calculated using the Reed-Muench method.

For pseudovirus neutralization assay, 2-fold serial dilutions of mouse sera (initially 1:40) were mixed with the pseudovirus strains (HCoV-229E, SARS-CoV, and SARS-CoV-2; 100 TCID50/well). After 48 h, the luminescence activity was measured. According to the formula (V − X)/(V − C) × 100% (where X is sample reading, C is cell control reading, and V is virus control reading; n = 3), the neutralization protection rate (90% inhibitory concentration [IC90]) was calculated. Besides, for virus neutralization assay, 2-fold serial dilutions of mouse sera (initially 1:16) were mixed with an equal volume of HCoV-229E (ATCC, VR-7540F), HCoV-NL63 (ATCC, VR-7401F), and HBS. The IC90 was observed by an inverted optical microscope. The highest serum dilution that protected more than 90% of cells from CPE was taken as the neutralization titer (IC90). Serum from mice immunized with HBS was used as a control.

B-cell epitope prediction analysis. According to previous research, B-cell epitopes were predicted and analyzed (IEDB, https://www.iedb.org/) (45). Briefly, structure-based B-cell epitope prediction was performed by DiscoTope 2.0 with a positive cutoff greater than −3.7 (corresponding to a specificity greater than or equal to 0.75 and a sensitivity less than 0.47) using the following protein structures: the HCoV-229E S-trimer and RBD (PDB IDs: 6U7H and 6ATK, respectively), the SARS-CoV S-trimer and RBD (PDB IDs: 5X5B and 2AJF, respectively), the SARS-CoV-2 S-trimer and RBD (PDB IDs: 6VYB and 6M0J, respectively), the HCoV-NL63 (PDB ID: 3KBH), the PEDV RBD (PDB ID: 6UTK), the PIVF RBD (PDB ID: 6JX7), the porcine respiratory coronavirus (PRCoV) RBD (PDB ID: 4FSC), and the transmissible gastroenteritis virus (TGEV) RBD (PDB ID: 4FM2). For linear B-cell epitope prediction, the corresponding amino acid sequences from the above structures were used. The BepiPred 2.0 algorithm was applied with a cutoff of 0.55 (corresponding to a specificity greater than 0.81 and a sensitivity less than 0.3). All the predicted residues were labeled in corresponding structures using PyMOL (Schrödinger). Besides, the interaction area and surface area were analyzed via PDBePISA (https://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Additionally, the amino acid sequences of alpha-CoV RBDs were aligned using ClustalW2 (46). The NCBI accession numbers of the sequences used were as follows: HCoV-229E (AAQ00002.1), HCoV-NL63 (AVL25587.1), PRCoV (AAA46903.1), TGEV (CAB91145.1), PEDV (AIU98611.1), and PIVF (ACT10887.1).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 7.0. Statistical analysis was conducted on data from three independent experimental replicates. Statistical significance was determined using an unpaired two-tailed Student’s t test. Data are presented as mean values ± SD (95% confidence interval). All differences between means with P < 0.05 are indicated. * indicates P < 0.05, which was considered significant, and ** indicates P < 0.01, which was considered highly significant.

Ethics statement. All the mice used in this study were maintained in compliance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals established by the Ministry of Science and Technology of China. The experiments were carried out using the protocols approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit number: HZAUSW-2018-009).

ACKNOWLEDGMENTS This work was supported by National Key R&D Plan of China grant 2018YFD0500100, National Natural Science Foundation of China grants 31722056 and 31702249, China Postdoctoral Science Foundation grant 2019M662674, and the Huazhong Agricultural University Scientific and Technological Self-innovation Foundation (program no. 2662017PY028).

We declare no competing interests.


