Insight into vaccine development for Alpha-c Coronaviruses based on structural and immunological analyses of spike proteins

Running Title: Vaccine development for coronavirus spike protein

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Abstract word count: 228

Importance word count: 146

Text word count: 4262

Key words: coronavirus; spike protein; receptor binding domain; structural analysis; neutralizing antibody
Abstract

Coronaviruses that infect humans belong to the Alpha-coronavirus (including HCoV-229E) and Beta-coronavirus (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 Alpha-coronavirus (HCoV-229E) and Beta-coronavirus (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: 1.28×10^5; 2.75×10^5) 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; 5×10^5) than HCoV-229E (antibody titers: 1.125×10^3). 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 Alpha-coronavirus. Our findings will provide important implications for future development of coronavirus vaccines.
Importance

Outbreak of coronaviruses, especially SARS-CoV-2, poses 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 as a major target for vaccine development. Currently, structural studies have shown that Alpha-coronavirus (HCoV-229E) and Beta-coronavirus (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 Beta-coronavirus (SARS-CoV and SARS-CoV-2) with a RBD “standing” state. However, for Alpha-coronavirus (HCoV-229E) with a 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.

Introduction

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses with the largest genomes (26-32 kb) among known RNA viruses and are phylogenetically divided into four genera (Alpha-, Beta-, Gamma-, and Delta-CoV) (1, 2). To date, seven human-infecting coronaviruses (hCoVs) (3, 4) cause varying 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 (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 “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 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-coronaviruses spike proteins

To date, multiple S-trimer structures of coronaviruses have been resolved (12-14, 16-24). 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). Due to 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 (SARS-CoV and SARS-CoV-2). The predicted positive residues (the corresponding spatial epitope and linear epitope) are displayed on the structural surface (Fig. 2A, 2C and 2E), 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, 2C and 2E). Meanwhile, we analyzed the binding area of the RBD and receptors from SARS-CoV, SARS-CoV-2 and HCoV-229E (Fig. 2B, 2D and 2F). Among them, the interaction areas of SARS-CoV and SARS-CoV-2 were similar (approximately 829.7Å² and 843.5Å², respectively), which were larger than that of HCoV-229E (approximately 497Å²). Furthermore, surface area analysis also yielded consistent results (Fig. 2B, 2D and 2F). 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-EM structures of S-trimers in the prefusion conformation has 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 RBDs 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, 3E, 3I and 3K). Due to 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). In vitro binding measurements (Fig. 3B, 3F, 3J and 3L; Table 2), the equilibrium dissociation constant ($K_D$) of HCoV-229E spike proteins (S-trimer and RBD) and hAPN are $3.29\pm0.0129\text{nM}$ and $36.0\pm0.037\text{nM}$, respectively. Besides, the $K_D$ value of SARS-CoV spike proteins (S-trimer and RBD) and hACE2 are $246\pm21.1\text{nM}$ and $38.8\pm2.05\text{nM}$, 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 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: 1.36×10^5, 3.84×10^5; 1.625×10^6, 5×10^5, respectively. **Fig. 4A-4D**). Moreover, the S-trimers of both SARS-CoV and SARS-CoV-2 could induce high-titer RBD antibodies (antibody titers: 1.28×10^5; 2.75×10^5, respectively, **Fig. 4B and 4D**), and the RBD could induce high-titer S-trimer antibodies (antibody titers: 6.72×10^4; 5×10^5, respectively, **Fig. 4A and 4C**). Similar to SARS-CoV and SARS-CoV-2, the S-trimer and RBD of HCoV-229E both had good immunogenicity (antibody titers: 8.8×10^4; 7.68×10^5, respectively, **Fig. 4E and 4F**). 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 induce low-titer S-trimer antibodies (antibody titer: 1.125×10^3, **Fig. 4F and 4H**).

To confirm this finding, a higher immunization dose of the HCoV-229E RBD (50 µg) was used in the same manner. Nevertheless, no significant increase in the RBD-specific antibody titer (antibody titers: 1.792×10^5, 3.072×10^6, **Fig. 3G**) or antibody titer for the HCoV-229E S-trimer (antibody titers: 2.5×10^2, 2.5×10^3, **Fig. 3H**) was noted. Overall, our immune epitope analysis and biochemical tests consistently showed that the S-trimer with a “standing” RBD state that 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. HCoV-229E RBD induced fewer specific neutralizing antibodies than that of
SARS-CoV and SARS-CoV-2

Next, we tested the neutralizing ability of the sera using a vesicular stomatitis virus (VSV)-based pseudovirus (100 TCID_{50}/well). Both the S-trimer and RBD sera from SARS-CoV-1 and SARS-CoV-2-immunized mice had a good neutralizing ability (Fig. 4I and 4J). For HCoV-229E, the S-trimer sera had a comparable neutralizing ability to that of SARS-CoV or SARS-CoV-2, but the RBD sera had no detectable neutralizing ability (Fig. 4K). Based on the results, we speculate that the antibodies induced by HCoV-229E RBD contain only a small part of neutralizing antibodies, which may be the reason for the weak ability of RBD sera to neutralize viruses. In HCoV-229E, the distribution of the potential B cell epitopes in the RBD was lower than that of SARS-CoV or SARS-CoV-2 (Fig. 2A, 2C, and 2E; Table 1), which may cause fewer nAbs were induced by the RBD. Therefore, we believe that this finding illustrates the inherent difference between the RBDs of alpha- and beta-CoV. Besides, our results indicate that HCoV-229E S-trimer induces the production of very few antibodies targeting the RBD (antibody titer: <500, Fig. 4F), but the S-trimer still produces strong neutralizing antibody levels, the mechanism needs further clarification.

The intact and stable S1 subunit in HCoV-229E S-trimer is a prerequisite for the production of effective nAbs

Our experimental results showed that the HCoV-229E S-trimer can induce strong nAbs levels, while the RBD alone is less immunogenic. Then, we explore which functional domains of the S-trimer are involved in the generation of nAbs. To clarify
this issue, we immunized mice with the HCoV-229E S-trimer (10 µg), S1 (10 µg; Fig.
3C and 3D), NTD (10 µg; Fig. 5A), RBD (10 µg) and NTD+RBD (5 µg+ 5 µg).

Besides, the HCoV-229E strain VR740 was used for the neutralizing assay. Our
results indicated that the S-trimer sera had the best neutralizing ability, followed by
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 nAbs induction. To verify the importance of the
complete S1 structure for nAbs 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 (K_D value is
3.29±0.0129nM), in vitro binding measurements showed that S-trimer/ΔNTD has a
similar binding affinity with hAPN (K_D value is 2.12±0.15nM) and function well
(Figs. 3B, 5D and 5E). 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 indicate that the characteristics of RBD itself may lead to the generation of less
neutralizing antibodies than S-trimer in HCoV-229E (Fig. 4K). Hence, we screened
monoclonal antibodies using HCoV-229E S-trimer, and the results showed that 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 Alpha-coronavirus (HCoV-229E) and Betacoronavirus (SARS-CoV and SARS-CoV-2)

We compared the structures of S-trimers and RBDs among alpha-coronaviruses (Figs. 1B and 7A). We also predicted the potential B-cell epitopes for their RBDs (Fig. 7A; Table1). 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 RSMD 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 7C; Table1). Based on the above data, inherent differences exist in the RBDs between alpha- and beta-CoVs (Figs. 2,
7A and 7B). However, the alpha- and beta-CoVs show high similarity in their RBDs and similar potential immune characteristics within their respective genera (Figs. 2, 4, 7A and 7B). 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 that 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 1-1,116; NTD-deficient S-trimer: S-trimer/ΔNTD) and SARS-CoV S glycoprotein ectodomain with an R667A mutation (GenBank accession number NP_828851.1, residues 1-1,195) 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 66-967) (39) and Human ACE2 extracellular domain (residues 19-615) (40) were cloned into the pFast-bac1 vector with an N-terminal honeybee melittin signal peptide and a C-terminal 6×His-tag. Besides, HCoV-229E S1 (residues 1-536), S1-NTD (residues 1-258), S1-RBD (residues 295-428) and SARS-CoV S1-RBD (residues 306-527) containing an N-terminal honeybee melittin signal peptide and a C-terminal Fc-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). For the S-trimer (HCoV-229E and SARS-CoV) including strep-tag, which were captured using StrepTactin Sepharose High Performance resin (GE Healthcare) (21). For HCoV-229E S1, S1-NTD, S1-RBD and SARS-CoV S1-RBD including FC-tag, which 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, which were harvested using a HisTrap™ HP Columns (GE Healthcare, Uppsala, Sweden). Finally, the harvested proteins 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 (WT, △NTD mutant), S1, S1-NTD, S1-RBD and SARS-CoV S-trimer, S1-RBD were analyzed using a Superdex 200 10/300 GL column (GE Healthcare) with a buffer containing 10 mM HEPES pH 7.2 and 150 mM NaCl at a flow rate of 0.3 ml/min (4 °C). Then, these proteins were analyzed 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, △NTD mutant), S1,
S1-RBD samples with the hAPN and purified SARS-CoV S-trimer, S1-RBD samples with the hACE2 were measured by surface plasmon resonance (OpenSPR, Nicoyalife), as described previously (42-44). Briefly, the hAPN or hACE2 (200 µl, 5 µg) was immobilized on the OpenSPR™ COOH Sensor Chip (Nicoya # 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, and the running buffer was injected for blank measurement followed by successive injections of buffer matched HCoV-229E spike samples or SARS-CoV spike samples at 20 µl/min, and the binding time was approximately 240 s and the natural dissociation approximately 400 s was carried out. Response unit (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) diluted in HEPES-buffered saline (HBS; 10 mM HEPES and 150 mM NaCl) were mixed 1:1 with the 2×Sigma Adjuvant System. The mice were intramuscularly inoculated with 50 µl of this solution (25 µl into each hind leg). Two weeks after the final immunization, sera were collected for subsequent assays. For SARS-CoV-2, the corresponding rabbit polyclonal antibodies (pAbs) were purchased from Sino Biological, Inc.
Enzyme-linked immunosorbent assay (ELISA)

To measure the immune responses of different sera, ELISA plates were coated with purified protein at 0.1 µM/well in citrate-buffered saline (CBS, pH=9.6) overnight at 4°C and subsequently blocked with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST) containing 1% bovine serum albumin (BSA, w/v) at 37°C.
After standard washes, the plates were incubated with 2- or 10-fold serially diluted sera for 1 h at 37°C. Then, horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit IgG (1:10,000 diluted in PBST with 1% BSA (w/v), Boster) was used as the secondary antibody, and 3,3',5,5'-tetramethylbenzidine (TMB) (Beyotime) was used as the substrate for detection. Optical density (OD) was determined at 450 nm and 630 nm using a SPARK10M microplate reader (TECAN) after stopping the reaction with 2 M H₂SO₄. Sera from mice immunized with HBS 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 two-weeks intervals. Mice with the highest titers of antibodies against the HCoV-229E S-trimer or S1 protein were further boosted by intraperitoneal injection 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 subcloned 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 1μg/ml in CBS (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 PBST with 1% BSA (w/v), 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 HuH7 cells, and pseudotyped viruses were produced as previously described (41). The HEK-293T cells were transfected with 1 μg of HCoV-229E-S-Δ19-pcDNA3.1 (C-terminal deletion 19 aa), SARS-CoV-S-Δ22-pcDNA3.1 plasmids (C-terminal deletion 22 aa) and SARS-CoV-2-S-Δ18-pcDNA3.1 plasmids (C-terminal deletion 18 aa) using Exfect2000 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-G at 1 MOI. After 24 h, VSV-ΔG-HCoV-229E-S, VSV-ΔG-SARS-CoV-S and VSV-ΔG-SARS-CoV-2-S (culture supernatants) were harvested and stored at -80°C until use.

For titration of these three pseudovirus, 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 5xBuffer (Promega, Cat. # E4030) was added to each well. Then, the luciferase substrate (Promega, Cat. #E1500) was added, 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) was 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 \( \frac{(V-X)}{(V-C)} \times 100\% ; x: \text{sample reading, c: cell control reading, v: virus control reading; n=3} \), the neutralization protection rate (IC₉₀) was calculated. Besides, for virus neutralization assay, 2-fold serial dilutions of mouse sera (initially 1:8) were mixed with an equal volume of HCoV-229E (ATCC, VR-740™, 100 TCID₅₀ /well) at 37°C. After 5 days of incubation, the cytopathic effect (CPE) were observed by an inverted optical microscope. The highest serum dilution that protected more than the 90% of cells from CPE was taken as the neutralization titer (IC₉₀). Sera 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, http://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 IDs: 3KBH), the PEDV RBD (PDB ID: 6U7K), the FIPV RBD (PDB ID: 6JX7), the PRCoV RBD (PDB ID: 4F5C), and the transmissible gastroenteritis virus (TGEV) RBD (PDB ID: 4F2M). 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-CoVs RBDs were aligned using ClustalW2 (46). The NCBI accession numbers of the sequences used were as follows: HCoV-229E (AAQ90002.1), HCoV-NL63 (AVL25587.1), PRCoV (AAA46905.1), TGEV (CAB91145.1), PEDV (AIU98611.1) and FIPV (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. *p<0.05, and **p<0.01 were considered 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).

Conflict of Interest

The authors declare no competing interests.

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Figure legends

**Fig. 1** Structural analysis of S1-RBDs from coronaviruses S-trimer. (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 coronaviruses S-trimer. 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: 5X5B), SARS-CoV-2 (PDB ID: 6VSB), MERS-CoV (PDB ID: 5X5F), HCoV-HKU1 (PDB ID: 5I08), HCoV-OC43 (PDB ID: 60HW) and MHV (PDB ID: 3JCL) are shown. The S1-RBDs are colored in magenta. The lengths of the coronaviruses S-trimers are shown as previous reports.

**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.

**Fig. 3** Surface plasmon resonance-binding data for the interaction between the 
coronaviruses spike proteins and the receptors. (A, C, E, G, H, I, K) Oligomerization 
state analysis of spike proteins (HCoV-229E: S-trimer, S1 and RBD; SARS-CoV: 
S-trimer and RBD) and the receptors (hAPN and hACE2). Protein samples were 
eluted using a Superdex 200 10/300 GL column. The results of SDS-PAGE analysis 
are also shown. (B, D, F, J, L) Interactions of spike proteins and soluble hAPN and 
hACE2. Purified recombinant hAPN or hACE2 was covalently immobilized on the 
OpenSPR™ COOH Sensor Chip via their amine groups and purified recombinant 
spike proteins flowed over the hAPN or hACE2. Response unit (RU) values were 
measured at 298 K. Binding kinetics was evaluated using a 1:1 Langmuir binding 
model; binding constants are indicated. All injections were carried out in duplicate 
and gave essentially identical results. Only one of the duplicates is shown.

**Fig. 4** Immunological analysis of *Beta-CoV* (SARS-CoV and SARS-CoV-2) and 
*Alpha-CoV* (HCoV-229E) spike proteins. (A and B) Cross-reactivity of the 
SARS-CoV S-trimer and RBD-specific sera is measured using ELISA. Mice sera of 
SARS-CoV S-trimer (red) and SARS-CoV RBD (blue) were 2-fold serially diluted 
and reacted with the S-trimer (A) or RBD (B), respectively. (C and D) 
Cross-reactivity of the SARS-CoV-2 S-trimer and RBD-specific sera is measured
using ELISA. Mice sera of SARS-CoV-2 S-trimer (magenta) and SARS-CoV-2 RBD (cyan) were 10-fold diluted and reacted with SARS-CoV-2 S-trimer (C) and RBD (D).

(E and F) Cross-reactivity of the HCoV-229E S-trimer and RBD-specific sera is measured using ELISA. Mice sera of HCoV-229E S-trimer (orange) and HCoV-229E RBD (green) were 2-fold diluted and reacted with HCoV-229E S-trimer (E) and RBD (F). (G and H) The antibody titers of sera from mice immunized with of the HCoV-229E RBD (10 μg, brown; 50 μg, purple). Mice sera were reacted with the HCoV-229E RBD (G) or S-trimer (H). Mice sera was serially diluted from a 500-fold dilution. All the data are presented as the mean OD₄₅₀ +/- SD (n=3) and the IgG antibody titers of each serum were calculated as the maximum endpoint dilution that remained positive. (I, J and K) The neutralization assay of mouse sera from the S-trimer and RBD against SARS-CoV, SARS-CoV-2 and HCoV-229E pseudoviruses is determined. The limit of detection for the assay depends on the initial dilution and is represented by dotted lines, a reciprocal IC₉₀ titer of 40 was assigned. The data are presented as the mean reciprocal IC₉₀ titer +/- SD (n=3). All differences between means with p < 0.05 are indicated.

**Fig. 5** The intact and stable S1 subunit of HCoV-229E S-trimer is a prerequisite for the production of effective nAbs. (A, D) Oligomerization state analysis of spike proteins (HCoV-229E NTD and S-trimer/ΔNTD). Protein samples were eluted using a Superdex 200 10/300 GL column. The results of SDS-PAGE analysis are also shown. (B) The neutralization abilities of mice sera from the HCoV-229E S-trimer, S1, NTD+RBD, NTD and RBD against HCoV-229E strain VR740. (C) Structural model
of HCoV-229E S-trimer/△NTD. The RBD, SD1, SD2 and S2 are colored in magenta, green, cyan and gray, respectively. (E) Interactions of HCoV-229E S-trimer/△NTD and soluble hAPN. The detailed description is consistent with Fig.3B. (F) The neutralization ability of mice sera from HCoV-229E S-trimer/△NTD against HCoV-229E strain VR740. The data are presented as the mean reciprocal IC$_{90}$ titer +/- SD (n=3). The limit of detection for the assay depends on the initial dilution and is represented by dotted lines, a reciprocal IC$_{90}$ titer of 8 was assigned.

**Fig. 6** Monoclonal antibody epitope mapping of the HCoV-229E spike protein. Monoclonal antibody (MAb) epitope regions in the HCoV-229E spike protein (A) and S1 domain (B). Supernatants of positive hybridomas were reacted with the HCoV-229E spike protein, S1, NTD and RBD. Data are presented as the OD$_{450}$ (bottom). MAbs and their epitope regions are indicated below the schematic of the HCoV-229E spike protein.

**Fig. 7** B cell epitope analysis of alpha-coronaviruses S1-RBDs. (A) Structures of alpha-coronaviruses (HCoV-229E, HCoV-NL63, PEDV, FIPV, PRCoV and TGEV) S1-RBDs. The linear (red cartoon) and conformational (yellow sphere) B cell epitopes were predicted by Bepipred 2.0 or Discotope 2.0 and labeled into the corresponding structures by PyMOL. HCoV-229E (PDB IDs: 6ATK), HCoV-NL63 (PDB IDs: 3KBH), PEDV (PDB IDs: 6U7K), FIPV (PDB IDs: 6JX7), PRCoV (PDB IDs: 4F5C) and TGEV (PDB IDs: 4F2M). (B) Structural comparison of S1-RBDs between HCoV-229E and other alpha-CoVs (HCoV-NL63, PEDV, FIPV, PRCoV and TGEV). (C) Sequence alignment of alpha-coronaviruses S1-RBDs. The RBM or putative
RBM region is shown in cyan. The predicted amino acid residues for linear (purple) and conformational (red) B cell epitopes are also shown. 

**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 target the RBD, and the HCoV-229E RBDs also induce fewer nAbs than beta-CoVs (SARS-CoV, SARS-CoV-2); thus, their S-trimers can be an effective potential subunit vaccine. In beta-CoVs (SARS-CoV, 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.
Table 1 Distribution of residues predicted positive for B cell epitopes

|          | RBD     | RBM     | RBM/RBD ratio |
|----------|---------|---------|---------------|
|          | Conformational | Linear | Conformational | Linear | (%)   |
| SARS-CoV | 51      | 56      | 47           | 33   | 92.2/58.9 |
| SARS-CoV-2 | 26     | 59      | 25           | 35   | 96.2/59.3  |
| HCoV-229E | 3       | 14      | 3            | 9    | 100/64.3  |
| HCoV-NL63 | 4       | 33      | 4            | 19   | 100/57.6  |
| PRCoV    | 4       | 41      | 4            | 27   | 100/65.9  |
| TGEV     | 7       | 34      | 4            | 24   | 100/70.6  |
| PEDV     | 0       | 21      | 0            | 8    | 0/38.1    |
| FIPV     | 0       | 17      | 0            | 11   | 0/64.7    |
Table 2 Surface plasmon resonance-binding data for the interaction between the coronaviruses (HCoV-229E and SARS-CoV) spike proteins and the receptors (hAPN and hACE2).

| Spike protein | $K_{on}$ ($\times 10^4$ M$^{-1}$ s$^{-1}$) | $K_{off}$ ($\times 10^4$ s$^{-1}$) | $K_D$ (nM) |
|---------------|----------------------------------------|----------------------------------|------------|
| S-trimer      | 3.67 ± 0.0005                          | 1.21 ± 0.0046                    | 3.29 ± 0.0129 |
| HCoV-229E S-trimer/ΔNTD | 1.94 ± 0.0006                          | 0.41 ± 0.0289                    | 2.12 ± 0.15  |
| S1            | 0.0886 ± 0.0006                        | 2.26 ± 0.104                     | 255 ± 13.6  |
| S1-RBD        | 3.2 ± 0.00174                          | 11.5 ± 0.0056                    | 36.0 ± 0.037 |
| SARS-CoV S-trimer | 0.622 ± 0.0499                          | 15.3 ± 0.0755                    | 246 ± 21.1  |
| CoV S1-RBD    | 0.812 ± 0.0004                         | 3.15 ± 0.165                     | 38.8 ± 2.05  |

Values after ± correspond to the residual standard deviation. Each experiment was repeated independently twice with similar results.
Lying state for immune evasion
Receptor binding is blocked

HCoV-229E

Other alpha-CoVs (HCoV-NL63, TGEV, PEDV, FIPV)?

Standing state for inducing neutralizing antibodies
Receptor binding is promoted

beta-CoVs (SARS-CoV and SARS-CoV-2)