Viral targets for vaccines against COVID-19

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Abstract | Vaccines are urgently needed to control the coronavirus disease 2019 (COVID-19) pandemic and to help the return to pre-pandemic normalcy. A great many vaccine candidates are being developed, several of which have completed late-stage clinical trials and are reporting positive results. In this Progress article, we discuss which viral elements are used in COVID-19 vaccine candidates, why they might act as good targets for the immune system and the implications for protective immunity.

As of 3 December 2020, the coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has spread to 220 countries, areas or territories with more than 63 million laboratory-confirmed cases and more than 1.4 million deaths (World Health Organization (WHO)), leading to widespread social and economic disruption. The development of a safe and effective vaccine is urgently needed to help bring an end to this pandemic.

SARS-CoV-2 is a member of the Coronaviridae family, which comprises many virulent strains that infect humans and animals, including SARS-CoV and Middle East respiratory syndrome CoV (MERS-CoV)\(^1\). To date, a number of CoV vaccines have been licensed for use in domestic animals against canine CoV, feline CoV, bovine CoV (BCoV), porcine epidemic diarrhoea virus, transmissible gastroenteritis virus (TGEV) and infectious bronchitis virus (IBV)\(^2\); however, until now, none has been licensed for use in humans. Two vaccine candidates for SARS-CoV and three for MERS-CoV are in phase I clinical trials (WHO). These prior experiences of vaccine development for animal and human CoVs have provided important insights into the development of vaccines for SARS-CoV-2 infection.

To develop a vaccine against a newly emerged virus, it is important to understand the immune correlates of protection. Although much remains to be determined regarding immune correlates of protection for SARS-CoV-2 infection, emerging data have demonstrated the importance of both humoral and cellular immunity in protection. A strong correlation has been found between vaccine-induced neutralizing antibodies (nAbs) and a reduction of viral loads in non-human primates (NHPs) after SARS-CoV-2 infection\(^3–5\). In humans, passive administration of convalescent plasma\(^6–8\), purified IgG\(^9,10\) or monoclonal antibodies\(^11\) have been reported to show benefit for the treatment and prevention of infection by SARS-CoV-2. In particular, a nAb recently received authorization by the US Food and Drug Administration for emergency use as a treatment for COVID-19 (REF.14). Moreover, analysis of a COVID-19 outbreak aboard a fishery vessel with high infection rates supported the correlation of nAbs with protection\(^15\). In addition to nAbs, T cell responses also play critical protective roles in CoV infections. The depletion of T cells in mice has been shown to impair virus clearance in SARS-CoV, MERS-CoV and SARS-CoV-2 infections\(^16–18\). In patients, virus-specific CD4\(^+\) and CD8\(^+\) T cell responses are associated with milder disease, suggesting an involvement in protective immunity against COVID-19 (REFS\(^19–22\)). Therefore, an ideal vaccine is expected to evoke both the humoral and cellular arms of the immune system. However, an important safety concern for the development of a SARS-CoV-2 vaccine or of antibody-based therapies is the potential risk of vaccine enhancement of the disease, also known as antibody-dependent enhancement (ADE) and enhanced respiratory disease (ERD)\(^23\). Antibodies that can bind to a virus without neutralizing activities can cause ADE via Fcγ receptor-mediated virus uptake, allowing subsequent replication of the virus or Fc-mediated effector functions of the antibody–virus immune complex, allowing immunopathology\(^24,25\). This effect is typically associated with flaviviruses, such as dengue virus\(^26,27\) and Zika virus\(^28\), but it has also been described in CoV infection. Cats immunized with vaccinia virus expressing a viral protein of feline infectious peritonitis virus (FIPV; a feline CoV) or passively administered with anti-FIPV antibodies showed early mortality when challenged with the live virus\(^29–31\). ADE was also observed for SARS-CoV and MERS-CoV in animal models\(^32–37\).

In addition to ADE, vaccine-induced enhancement of disease can also be caused by T helper 2 (TH2) cell-biased immunopathology, leading to ERD\(^35–37\). Although some studies of SARS-CoV in animal models do not show evidence of ADE or ERD\(^38–40\), safety should be considered when designing vaccines for SARS-CoV-2.

With continuing cases and deaths from the COVID-19 pandemic, researchers worldwide are racing to develop COVID-19 vaccines. According to the landscape document from the WHO, COVID-19 vaccine candidates generally fall into seven strategies (BOX 1), which can be divided into three broad categories: first, protein-based vaccines that generate target antigens in vitro such as inactivated virus vaccines, virus-like particles and protein subunit vaccines; second, gene-based vaccines that deliver genes encoding viral antigens to host cells for in vivo production such as virus-vecotected vaccines, DNA vaccines and mRNA vaccines; and, third, a combination of both protein-based and gene-based approaches to produce protein antigen or antigens both in vitro and in vivo, typically represented by live-attenuated virus vaccines. As of December 2020, the WHO has documented more than 214 COVID-19 vaccine candidates, with 51 of them in clinical evaluation, 13 in phase III trials and several vaccines now being authorized for use in some regions (WHO; COVID-19 Vaccine tracker). In this Progress article, we summarize and discuss the targets used...
**Box 1 | Vaccine strategies for SARS-CoV-2 vaccine candidates**

**Inactivated virus vaccines**
Viruses are physically or chemically inactivated but preserve the integrity of the virus particle, which serves as the immunogen.

**Virus-like particle or nanoparticle vaccines**
In this strategy, structural viral proteins are co-expressed to form non-infectious particles as the vaccine immunogen. They resemble real virions but they lack the virus genome.

**Protein subunit vaccines**
This strategy comprises only key viral proteins or peptides that can be manufactured in vitro in bacteria, yeast, insect or mammalian cells. The largest number of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine candidates in both clinical and preclinical stages are based on this strategy.

**Virus-vectored vaccines**
Gene(s) encoding pathogen antigen(s) are cloned into non-replicating or replicating virus vectors (such as adenovirus). The antigen(s) are produced by transduced host cells after immunization.

**DNA and mRNA vaccines**
DNA and mRNA vaccines have the advantage of rapid manufacturing against emerging pathogens. For DNA vaccines, viral antigen(s) encoded by a recombinant DNA plasmid are produced in host cells via a sequential transcription-to-translation process. By contrast, mRNA vaccines are synthesized by in vitro transcription and they produce viral antigen(s) in the cytoplasm through direct protein translation in vivo.

**Live-attenuated virus vaccines**
In this strategy, virus is attenuated by in vitro or in vivo passage or reverse-genetic mutagenesis. The resulting virus becomes non-pathogenic or weakly pathogenic but retains immunogenicity by mimicking live virus infection.

The figure shows the seven strategies being explored as vaccines for coronavirus disease 2019 (COVID-19).

in vaccine candidates, focusing on those candidates already advanced into clinical trials and with published data.

**SARS-CoV-2 proteins as targets**
SARS-CoV-2 contains four major structural proteins, namely spike (S), membrane (M) and envelope (E) proteins, all of which are embedded in the viral surface envelope, and nucleocapsid (N) protein, which is in the ribonucleoprotein core of the virus. S proteins are responsible for recognition of the host cellular receptor to initiate virus entry. M proteins are embedded in the envelope and shape the virion envelope. E proteins are small polypeptides that are crucial for CoV infectivity. N proteins make up the helical nucleocapsid and bind along the viral RNA genome. In addition to these structural proteins, SARS-CoV-2 encodes 16 non-structural proteins (nsp1–16) and 9 accessory proteins. Several of these viral proteins could potentially serve as targets of vaccine-induced immune responses.

**S protein.** S protein is the main protein used as a target in COVID-19 vaccines. S protein consists of a membrane-distal S1 subunit and a membrane-proximal S2 subunit and exists in the virus envelope as a homotrimer. The S1 subunit determines receptor recognition via its receptor-binding domain (RBD), whereas the S2 subunit is responsible for membrane fusion, which is required for virus entry (Fig. 1). In MERS-CoV, SARS-CoV or SARS-CoV-2, the RBD is located in the C-terminal domain of the S1 subunit. In some CoVs, the N-terminal domain (NTD) of the S1 subunit can be used for receptor binding (such as in mouse hepatitis virus) or might also be involved in virus attachment to host cells by recognizing specific sugar molecules (such as in TGEV, BCoV and IBV) or has an important role in the pre-fusion to post-fusion transition of the S protein. The S2 subunit contains the fusion peptide (FP), connecting region (CR), heptad repeat 1 (HR1) and HR2 around a central helix as a helix-turn-helix structure. Structural evidence has proposed a model for the rearrangement of SARS-CoV-2 S protein following recognition of the host cell receptor. S protein can be proteolytically cleaved at both the S1–S2 and S2′ cleavage sites, releasing the structural constraints on the FP (Fig. 1). The engagement of the RBD with its cellular receptor, human angiotensin-converting enzyme 2 (hACE2), leads to the dissociation of the S1 subunit and concomitantly initiates the refolding process of the spring-loaded S2 subunit, which protrudes the FP at its end for membrane fusion. The S2 subunit in its post-fusion conformation folds as a long helical bundle with the FP inserted into the host cell membrane (Fig. 1). Theoretically, nAbs can target the S protein to inhibit virus infection at multiple stages during the virus entry process. The RBD is the major target for nAbs that interfere with viral receptor binding. To date, most of the potent nAbs to SARS-CoV-2 target the RBD. In addition, nAbs targeting the NTD have been reported in SARS-CoV-2 and MERS-CoV infection, making it another potential target for inclusion in a vaccine. The S2 subunit is also a potential target for nAbs that interfere with the
structural rearrangement of the S protein and the insertion of FP required for virus–host membrane fusion67,68. The S protein is also a target for T cell responses; studies of SARS-CoV, MERS-CoV and SARS-CoV-2 have described both CD4+ and CD8+ T cell epitopes in the S protein1,4,29.

To date, three COVID-19 vaccine candidates based on adenovirus expressing the full-length S protein have entered phase III clinical trials (Table 1). One, which is being developed in China, is based on human adenovirus type 5 (Ad5)69,70. The second, being developed in the UK, uses recombinant chimpanzee adenovirus, ChAdOx1 (refs 71,72). The third vaccine candidate, from Russia, combines recombinant human Ad26 and Ad5 in a prime–boost vaccination regimen80.

The preclinical data showed that the recombinant ChAdOx1 vaccine induced nAb production and balanced T,1 and T,2 immune responses in NHPs28 (Table 1). The vaccine prevented COVID-19-associated pneumonia in NHPs and reduced viral loads in both bronchoalveolar lavage fluid and the respiratory tract19. Phase II trial studies showed that all of these vaccine candidates induced nAb production and T,1 cell-biased responses in humans53,54,75 (Table 1).

In addition to the adenovirus-vector strategy, a DNA vaccine candidate expressing the S protein has also completed phase II clinical trials. This vaccine elicited nAbs and S protein-specific T cell responses in both mice and guinea pigs41 (Table 1). Recently, a native-like trimeric S protein was reported for a COVID-19 subunit vaccine candidate being developed in China, in which the S protein is fused to a trimer-tag, the C-terminal region of human type Ia collagen, to form a disulfide-bonded homotrimer42. This strategy stabilizes the S protein antigen in its trimeric form and increases the antigen yield42. The recombinant trimeric protein structurally mimics the native S protein presented on virus particles and could serve as a target for nAbs, a strategy that is also being pursued in the development of an HIV vaccine43.

Vaccination of the S-trimer together with AS03 (a squalene-based adjuvant developed by GlaxoSmithKline) or CpG 1018 (a Toll-like receptor 9 agonist adjuvant developed by Dynavax) elicited high levels of nAbs and T,1 cell-biased responses in mice and NHPs. It could protect NHPs from challenge with SARS-CoV-2, with reduced viral loads and pathology in the lungs84. This candidate vaccine is currently under evaluation in phase I clinical trials (Table 1).

The S protein is metastable when produced as a recombinant protein and prone to transform from its pre-fusion to a post-fusion conformation, shedding the S1 subunit (Fig. 1). However, the S1 subunit

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**Fig. 1** | **Major targets used in COVID-19 vaccine candidates.** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) contains four major structure proteins: spike (S), membrane (M) and envelope (E) proteins, which are embedded on the virion surface, and nucleocapsid (N) protein, which binds viral RNA inside the virion. The S protein trimer in its pre-fusion conformation is shown. The S protein comprises the S1 subunit (which includes the N-terminal domain (NTD) and the receptor-binding domain (RBD)) (the receptor-binding motif (RBM) within the RBD is also labelled) and the S2 subunit (which includes fusion peptide (FP), connecting region (CR), heptad repeat 1 (HR1), heptad repeat (HR2) and central helix (CH)). The SARS-CoV-2 S protein binds to its host receptor, the dimeric human angiotensin-converting enzyme 2 (hACE2), via the RBD and dissociates the S1 subunits. Cleavage at both S1–S2 and S2′ sites allows structural rearrangement of the S2 subunit required for virus–host membrane fusion. The S2-trimer in its post-fusion arrangement is shown. The RBD is an attractive vaccine target. The generation of an RBD-dimer or RBD-trimer has been shown to enhance the immunogenicity of RBD-based vaccines. A stabilized S-trimer shown with a C-terminal trimer-tag is a vaccine target. The pre-fusion S protein is generally metastable during in vitro preparations and prone to transform into its post-fusion conformation. Mutation of two residues (K986 and V987) to proline stabilizes S protein (S-2P) and prevents the pre-fusion to post-fusion structural change.
| Strategy               | Construct          | Developer                                                                 | B and T cell responses*                                                                 | Clinical stage | Clinical trial identifier | First reported | Refs |
|------------------------|--------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|----------------|--------------------------|----------------|------|
| **Whole virus**        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Inactivated virus      | NA                 | Sinovac, with National Institute for Communicable Disease Control and Prevention, China | Induction of S-specific, RBD-specific and N-specific IgG, and nAbs in mice, rats and NHPs; no induction of either T<sub>H</sub>1 or T<sub>H</sub>2 cell responses in NHPs; induction of RBD-specific IgG and nAbs in humans; no obvious vaccine-induced T cell responses in humans | Phase III      | NCT04456595, 669/UN6.KEP/EC/2020, NCT04582344, NCT04617483 | 6 May 2020 | 5,145 |
|                        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Inactivated virus      | NA                 | Wuhan Institute of Biological Products, Sinopharm, with Wuhan Institute of Virology, Chinese Academy of Sciences, China | Induction of virus-specific IgG and nAbs in humans; no induction of either T<sub>H</sub>1 or T<sub>H</sub>2 cell responses in humans | Phase III      | ChiCTR2000034780, ChiCTR2000039000, NCT04612972 | 13 Aug 2020 | 146  |
|                        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Inactivated virus      | NA                 | Beijing Institute of Biological Products, Sinopharm, with Institute of Viral Disease Control and Prevention, China | Induction of nAbs in rabbits, guinea pigs, rats, mice, NHPs and humans; no induction of either T<sub>H</sub>1 or T<sub>H</sub>2 cell responses in NHPs | Phase III      | NCT04560881 | 6 Jun 2020 | 6,250 |
|                        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Inactivated virus      | NA                 | Institute of Medical Biology, Chinese Academy of Medical Sciences, China | Induction of S-specific and N-specific IgG and nAbs in humans | Phase I/II     | NCT04470609 | 9 Nov 2020 | 151  |
| **S protein**          |                    |                                                                           |                                                                                         |                |                          |                |      |
| Virus vector (Ad5)     | Full-length S      | CanSino Biological Inc. with Beijing Institute of Biotechnology, China | Induction of RBD-specific IgG and nAbs in humans; induction of T<sub>H</sub>1 cell responses | Phase III      | NCT04526990, NCT04540419 | 22 May 2020 | 76,77 |
|                        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Virus vector (ChAdOx1) | Full-length S      | University of Oxford, with AstraZeneca, UK | Induction of S-specific IgG and nAbs in mice and NHPs; induction of high T<sub>H</sub>1 cell responses but low T<sub>H</sub>2 cell responses in mice; induction of S-specific IgG and nAbs in humans, with nAb titres similar to convalescent plasma | Phase III      | ISRCTN89951424, NCT04516746, NCT04540393, CTRI/2020/08/027170 | 20 Jul 2020 | 78,74,152 |
|                        |                    |                                                                           |                                                                                         |                |                          |                |      |
| Virus vector (AdZ6 and Ad5) | Full-length S | Gamaleya Research Institute, Russia | Induction of RBD-specific IgG and nAbs in humans, with nAb titres similar to convalescent plasma; induction of IFNy-associated T cell responses | Phase III      | NCT04530396, NCT04564716, NCT04642339 | 4 Sep 2020 | 80   |
| **DNA**                |                    |                                                                           |                                                                                         | Phase II       | NCT04642638, ChiCTR2000040146 | 20 May 2020 | 81   |
| **Protein subunit**    |                    |                                                                           |                                                                                         | Phase I        | NCT04405908 | 21 Sep 2020 | 84   |
| LNP-mRNA               | Full-length S with two proline substitutions (K986P and V987P) | Moderna, with National Institute of Allergy and Infectious Diseases, USA | Induction of S-specific IgG and nAbs in mice, NHPs and humans, with nAb titres higher than convalescent plasma; induction of high T<sub>H</sub>1 cell responses but low T<sub>H</sub>2 cell responses in mice, NHPs and humans | Phase III      | NCT04470427 | 14 Jul 2020 | 88–89,153 |
is the immunodominant antigen during CoV infections due to its accessibility for immune recognition and it contains neutralizing epitopes mainly on its RBD. Strategies to stabilize the S protein in its pre-fusion conformation and enhance pre-fusion S protein expression are thought to increase the quality and quantity of vaccine-induced antibodies targeting the functionally relevant epitopes on the S1 subunit. Pallesen et al. reported two proline substitutions (2P) at the apex of the central helix and HR1 that can retain the S proteins of MERS-CoV, SARS-CoV and HKU1 in the antigenically optimal pre-fusion conformation \(^{89}\) [FIG. 1]. The resulting antigen, S-2P, induced much greater nAb titers than wild-type S protein in mice. Learning from the previous experience with these CoVs, the S-2P protein-based candidate (by Novavax) \(^{4,86–96}\) is now being used in several vaccine strategies against COVID-19. SARS-CoV-2 S-2P (comprising proline substitutions at residues K986 and V987) is used as the target antigen in three gene-based vaccine candidates (mRNA vaccines by Moderna/NIAID and BioNTech/Pfizer and a recombinant Ad26 vaccine by Janssen Pharmaceutical Companies) and a protein-based candidate (by Novavax) \(^{93–96}\) (TABLE 1). Moreover, mutation at the cleavage sites in the S protein is also believed to stabilize the pre-fusion conformation of the
S protein. S-2P in the Janssen Ad26-vectored vaccine (Ad26.COV2.S) and in the Novavax protein-based vaccine (NVX-CoV2373) contains additional mutations at the S1–S2 polybasic cleavage site from RRAR to SRAG and thus inhibits virus attachment.

The RBD binds to the host receptor via a receptor-binding motif (RBM) on its external subdomain in SARS-CoV, MERS-CoV or SARS-CoV-2 [refs. 47,63,101]. The surface of the S protein is extensively shielded from antibody recognition by glycans, with the notable exception of the RBD, which explains the immunodominance of RBD epitopes. Most SARS-CoV-2 nAbs bind to RBD and block the RBD–hACE2 interaction, thus inhibiting virus attachment.

RBD is an attractive vaccine target because it elicits high-quality, functionally relevant antibodies, while avoiding the potential risk of ADE, which is generally thought to be mediated by weak nAbs or non-nAbs. For example, antibodies targeting an epitope (S597–603) of the SARS-CoV RBD markedly enhanced SARS-CoV infection. The RBD also contains epitopes for T cell responses, as shown in studies of SARS-CoV, MERS-CoV and SARS-CoV-2 [refs. 94–96,109]. RBD-based antigens have been described in previous studies for SARS-CoV and MERS-CoV vaccine development.

To date, several RBD-based vaccines for COVID-19 have entered clinical trials. Yang et al. reported an RBD-based COVID-19 vaccine candidate generated using a protein subunit strategy [TABLE 1]. This vaccine induced nAbs in mice, rabbits and NHPs, and protected NHPs against challenge with SARS-CoV-2. They further showed that the immune sera, as opposed to splenic T cells, played the protective role in mice.

Indeed, stabilization of the S protein in its pre-fusion conformation represents an effective avenue to improve vaccine efficacy. Notably, although the data are yet to be published, recent press releases from BioNTech/Pfizer and Moderna/NIAD have announced that their mRNA vaccines have more than 90% efficacy in preventing COVID-19 in phase III clinical trials.

The UK has just approved the BioNTech/Pfizer vaccine.

Following their initial observations in MERS-CoV, SARS-CoV and SARS-CoV-2, Hsieh et al. generated a new variant form of the S protein ectodomain, known as HexaPro, which comprises six beneficial proline substitutions, including the two from S-2P [104]. These proline substitutions are located at flexible loops or N termini of helices in the FP, HR1 and CR, further constraining the structural rearrangement of the S2 subunit and stabilizing the pre-fusion S protein. HexaPro exhibits approximately 10-fold higher expression than S-2P, thus representing a promising antigen design.

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Consistent with this, a recent report of an RBD-based DNA vaccine also showed that the nAbs, and not the induced T cells, are the immune correlates of protection against COVID-19 in NHPs. An RBD-based mRNA vaccine is being developed in China and is currently in phase I trials. This candidate vaccine, ARCoV, which expresses SARS-CoV RBD delivered by lipid nanoparticles, induced both nAb production and Tₜₐ₁ cell-mediated responses in mouse and NHP models. Vaccination protected mice against challenge with a mouse-adapted SARS-CoV-2 strain.

However, the use of RBD in vaccines is compromised by its limited immunogenicity owing to its small molecular size and possible mixed forms of multiple complexes (as monomers, dimers or trimers). Strategies to overcome these drawbacks include increasing antigen size (for example, by fusing the RBD with an Fc domain [12,100]) or by RBD multimerization (for example, by displaying multiple copies of RBD on particles [117–119]). Recently, to address these limitations, our team described a generalizable strategy to design a dimeric form of the RBD of beta-CoV antigens suitable for use against SARS-CoV-2, MERS-CoV and SARS-CoV [fig. 1]. The RBDs from SARS-CoV, MERS-CoV or SARS-CoV-2 spontaneously form dimers in solution. Structural analyses showed, for MERS-CoV [10] and SARS-CoV [121], that both RBD protomers in a dimer stack on top of each other via the core subdomains and expose the RBM, the major site recognized by nAbs, indicating similar RBD-dimer structures for other CoVs. Structure-guided design yielded homogeneous RBD-dimers as a tandem-repeat single chain. The RBD-dimer antigen induced 10-fold to 100-fold higher nAb titres than the conventional RBD-monomer and was protective in a mouse model [122]. We developed a COVID-19 vaccine candidate, ZF2001, comprising the RBD-dimer as the target. This protein subunit vaccine is currently being evaluated in phase III clinical trials [TABLE 1]. In addition to the RBD-dimer, an mRNA vaccine, BNT162b1 (BioNTech/Pfizer), was reported to express an RBD-trimer stabilized by the foldon trimerization domain (fig. 1). The phase I/II studies of this vaccine encouragingly showed that two doses of the vaccine induced nAbs to levels higher than those in convalescent patients as well as inducing Tₜₐ₁ cell-mediated responses [123]. Indeed, it is assumed that multivalent antigens would allow the crosslinking of B cell receptors for better B cell activation.

Interestingly, a recent study described a vaccine candidate comprising multiple copies of the SARS-CoV-2 RBD displayed in arrays on nanoparticles that induced a markedly lower binding to nAb ratio than a 2-SP-based vaccine [124], indicating the humoral responses are focused on epitopes recognized by nAbs for the RBD-based vaccines, which are believed to have lower ADE potential.

S1-NDT and the S2 subunit. The S1-NDT also contains epitopes for CoV nAbs found in infected patients [90,92,107,126,127] and has been considered a potential target in CoV vaccines. NTD-targeting nAbs generally do not directly block receptor binding but rather interfere with receptor binding [90,126,127] or restrain the S protein conformational changes required for the pre-fusion to post-fusion transition [90,127]. It is notable that SARS-CoV-2 NTD-targeting nAbs generally exhibit lower neutralizing potency than RBD-specific nAbs [90]. We previously reported an NTD-based vaccine against MERS-CoV [128]. Vaccination with NTD protein elicited nAbs and NTD-specific T cell responses. Furthermore, it reduced lung abnormalities in a MERS-CoV challenge mouse model, although the immunogenicity and protective efficacy of the NTD protein were weaker than the RBD protein. The inclusion of NTD in a COVID-19 vaccine would broaden the neutralizing epitopes and reduce the potential of viral escape of host immunity. Yet, so far, NTD-based vaccines against COVID-19 have not been reported.
For the S2 subunit, peptides derived from HR1 or HR2 of the S2 subunit from SARS-CoV, MERS-CoV and SARS-CoV-2 have been described that inhibit viral fusion with target cells and thereby prevent virus infection. Moreover, nAbs have been reported to target the S2 subunit of CoVs, including SARS-CoV-2 (REFS[80,109-111]), suggesting the S2 subunit as a COVID-19 vaccine target. However, the membrane-proximal S2 subunit contains more extensive N-glycan shielding[32,13] and is less accessible for immune recognition than the S1 subunit and is therefore less immunogenic. Rabbits immunized with SARS-CoV-2 S2 protein showed much lower nAb titres than those immunized with the S1 subunit or RBD proteins[14]. S2 subunit-targeting antibodies isolated from convalescent patients showed weaker neutralizing activities against SARS-CoV-2 than RBD-targeting antibodies[8]. These studies suggest that the S2 subunit alone may not be an effective target for humoral responses. Nevertheless, because of the relative sequence conservation of the S2 subunit between virus species, the S2 subunit is targeted by cross-reactive antibodies and CD4+. T cell epitopes recognizing both SARS-CoV-2 and other human CoVs[24,114-116,117], suggesting a potential target in universal CoV vaccines.

M, E and N proteins. Unlike the S protein, CoV M and E proteins are poorly immunogenic for humoral responses, presumably owing to their small ectodomains for immune cell recognition and small molecular sizes[118] (FIG. 1). Adoptive transfer of sera from donors immunized with a virus vector expressing M or E protein did not protect mice against SARS-CoV-2 infection[14]. Therefore, M and E proteins have never been explored as vaccine targets alone against SARS-CoV-2 or other CoVs. Nonetheless, the sequence identity of M or E proteins among SARS-CoV, MERS-CoV and SARS-CoV-2 is much higher than for the S protein and RBD, suggesting the potential of M and E proteins as targets for cross-reactive T cells. Indeed, several T cell epitopes have been identified in M and E proteins in previous studies of SARS-CoV and MERS-CoV immunity[24]. In this regard, M and E proteins may help to broaden the T cell response and improve cross-protection if included in a SARS-CoV-2 vaccine.

The N protein is the most abundant viral protein and is highly immunogenic during CoV infections[119]. It is a major target for antibody responses and also contains T cell epitopes[14]. N-specific antibodies were reported to protect mice against mouse hepatitis virus, a mouse CoV, via Fc-mediated effector functions[14]. However, anti-N immune sera did not protect against SARS-CoV-2 infection in a mouse model[14]. Immunization with N protein can also elicit CD4+ and CD8+ T cell responses in mice[14]. N-specific CD8+ T cell epitopes are known to protect chickens against IBV infection[14]. Venezuelan equine encephalitis virus replicon particles expressing an N-specific CD4+ T cell epitope showed complete protection against SARS-CoV infection[14]. These virus replicon particles also conferred partial cross-protection against MERS-CoV owing to the protein sequence conservation between viruses, resulting in a reduced viral load[14]. However, its potential as a CoV vaccine target was largely undermined by early studies of SARS-CoV showing that vaccines expressing N protein did not provide protection and, on the contrary, enhanced infection-induced pneumonia via increased pulmonary eosinophil infiltration and T<sub>2</sub> cell-biased responses[14], causing ERD. Therefore, the inclusion of N protein in CoV vaccines is complicated by balancing viral clearance and immunopathogenesis and no N protein-based vaccine has been reported for COVID-19.

The whole virus as a target
Inactivated virus and live-attenuated virus vaccines use the whole virus as vaccine targets. They contain all structural proteins (S, N, M and E proteins) and live-attenuated virus vaccines can also generate non-structural and accessory proteins in vivo. Therefore, these vaccine candidates can induce broader antibody and T cell responses than the above-mentioned vaccines, which are based on a single protein or protein fragments.

Three vaccine candidates (all being developed in China) based on an inactivated virus strategy are currently in phase III clinical trials and another one is at the phase I/II stage (TABLE 1). In a preclinical study, mice immunized with an inactivated virus vaccine, PiCoVacc, elicited S protein-specific (including RBD-specific) and N-specific antibodies[24]. PiCoVacc and another inactivated virus vaccine, BBiBP-Cov, elicited substantial nAb production in NHPs but did not induce T cell responses[19]. Furthermore, they protected NHPs against challenge by SARS-CoV-2, suggesting an important role for humoral responses as the immune correlates of protection. Initial clinical trial studies of these two inactivated vaccine candidates showed that, similar to in NHPs, these vaccines elicited substantial nAbs in humans[14]. These inactivated vaccine candidates did not induce either T<sub>1</sub> or T<sub>2</sub> cell-associated cytokines in those vaccinated. As the uptake of inactivated vaccine components by host cells is very limited or negligible, antigen processing for peptide presentation to T cells is inefficient and may explain why these vaccines generally do not elicit T cell responses. Moreover, these inactivated vaccines all use Alum as the adjuvant, which is generally a poor stimulator of T cell responses (TABLE 1).

Concluding remarks
An ideal COVID-19 vaccine target would be expected to induce high titres of nAbs, reduce non-nAb production to minimize ADE potential, elicit robust T<sub>1</sub> cell-biased responses but low T<sub>2</sub> cell-biased responses to lower the ERD potential, maintain long-lasting immunological memory, and provide cross-protection between CoVs. No combination of different targets has yet been tested as a multiple-target vaccine but it might be worthwhile in the future to look at this possibility.

To date, several vaccine candidates have reached the final stages for vaccine safety and protection efficacy in large-scale clinical trials, with very recent announcements from BioNTech/Pfizer and Moderna/NIAID claiming safety and very high levels of protection efficacy for their leading mRNA vaccine candidates. It is worth noting that it is currently difficult to compare the various vaccines as there are no standardized assays for neutralization
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Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
L.D. and G.F.G. are listed as inventors on patent applications for a MERS-CoV RBD-dimer vaccine and on pending patent applications for RBD-dimer-based CoV vaccines. The pending patents for RBD-dimers as protein subunit CoV vaccines. The pending patents for RBD-dimers as protein subunit vaccines for MERS-CoV and SARS-CoV-2 have been licensed to Anhui Zhifei Longcom Biopharmaceutical Co. Ltd, China.

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