The spike protein of SARS-CoV — a target for vaccine and therapeutic development

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Abstract | Severe acute respiratory syndrome (SARS) is a newly emerging infectious disease caused by a novel coronavirus, SARS-coronavirus (SARS-CoV). The SARS-CoV spike (S) protein is composed of two subunits; the S1 subunit contains a receptor-binding domain that engages with the host cell receptor angiotensin-converting enzyme 2 and the S2 subunit mediates fusion between the viral and host cell membranes. The S protein plays key parts in the induction of neutralizing-antibody and T-cell responses, as well as protective immunity, during infection with SARS-CoV. In this Review, we highlight recent advances in the development of vaccines and therapeutics based on the S protein.

Zoonotic virus
A virus that normally exists in vertebrate animals, but can also be transmitted to humans and can cause disease in both animals and humans.

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Severe acute respiratory syndrome (SARS) was the first new infectious disease identified in the twenty-first century. This acute, and often severe, respiratory illness originated in the Guangdong province of China in November 2002 (REF. 1). A global effort coordinated by WHO led to the identification, in April 2003, of a new coronavirus, SARS-coronavirus (SARS-CoV), as the agent that caused the outbreak[2].

SARS-CoV is an enveloped, single and positive-stranded RNA virus[2]. Its genome RNA encodes a non-structural replicase polyprotein and structural proteins, including spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins[3–5]. SARS-CoV, a zoonotic virus, resides in hosts that form its natural reservoir, such as bats, but can also infect intermediate hosts, such as small animals (for example, palm civets), before being transmitted to humans[6–8]. SARS-CoV can infect and replicate in several cell types in the human body and causes serious pathological changes (BOX 1, FIG 1). A further understanding of the life cycle and pathogenesis of SARS-CoV will help us to develop vaccines and therapeutics to prevent and treat SARS-CoV and SARS-like coronavirus (SL-CoV) infections in the future.

After its first occurrence, SARS rapidly spread around the world along international air-travel routes, reaching all five continents and 29 countries, resulting in 8,098 cases and 774 deaths by 23 September 2003 (REF. 9). The overall fatality of SARS is about 10% in the general population, but >50% in patients aged 65 years and older (WHO update 49; see Further information). The global outbreak of SARS was brought under control in July 2003 by effective quarantine, patient-isolation and travel restrictions. Four sporadic SARS cases caused by different SARS-CoV isolates than those that predominated in the 2002–2003 outbreak were reported in late 2003 and early 2004 (REFS 10–12). The most recent epidemic of SARS occurred in Beijing and Anhui in China in April 2004 and originated from laboratory contamination (WHO update 7; see Further information). Since then, no new case of SARS has been reported, possibly because of continued global vigilance and surveillance and laboratory bio-safety practices, as well as the euthanizing or quarantining of animals that may have been exposed to SARS-CoV[13,14]. Although the outbreaks of SARS seem to be over, SARS is still a safety concern because of the possible reintroduction of a SL-CoV into humans and the risk of an escape of SARS-CoV from laboratories[15,16].

Infection with SARS-CoV can trigger a series of humoral and cellular immune responses. Specific antibodies against SARS-CoV (immunoglobulin G (IgG) and IgM) were detectable approximately 2 weeks post-infection, reaching a peak 60 days post-infection and remaining at high levels until 180 days post-infection (REF. 17). High titres of neutralizing antibodies and SARS-CoV-specific cytotoxic T lymphocyte responses were detected in patients who had recovered from SARS[18,19], and the levels of the responses correlated well with the disease outcome[20]. This suggests that both humoral and cellular immune responses are crucial for the clearance of infection by SARS-CoV.

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Box 1 | **Pathology of SARS and the life cycle of SARS-CoV infection**

Severe acute respiratory syndrome-coronavirus (SARS-CoV) spreads primarily through droplets (respiratory secretions) and close person-to-person contact. After the virus enters into the body, it binds to primary target cells that express abundant virus receptor, the angiotensin-converting enzyme 2 (ACE2), including pneumocytes and enterocytes in the respiratory system. The virus enters and replicates in these cells. The matured virions are then released to infect new target cells[121] (FIG. 1). SARS-CoV can also infect mucosal cells of intestines, tubular epithelial cells of kidneys, epithelial cells of renal tubules, cerebral neurons and immune cells[122,123]. Infectious viral particles in patients with SARS can be excreted through respiratory secretions, stool, urine and sweat. SARS-CoV infection damages lung tissues owing to elevated levels of production and activation of proinflammatory chemokines and cytokines[124], resulting in atypical pneumonia with rapid respiratory deterioration and failure.

Neutralizing antibodies and/or T-cell immune responses can be raised directly against several SARS-CoV proteins[21–23], but mainly target the S protein[20,24–26], suggesting that S protein-induced specific immune responses play important parts in the fight against SARS-CoV infection[18]. SARS-CoV S protein also has a key role in the ability of SARS-CoV to overcome the species barrier, as adaptive evolution of S protein can contribute to the animal-to-human transmission of SARS-CoV[27]. Because the S protein of SARS-CoV is involved in receptor recognition, as well as virus attachment and entry, it represents one of the most important targets for the development of SARS vaccines and therapeutics.

Structure of the SARS-CoV S protein

The spikes of SARS-CoV are composed of trimers of S protein, which belongs to a group of class I viral fusion glycoproteins that also includes HIV glycoprotein 160 (Env), influenza haemagglutinin (HA), paramyxovirus F and Ebola virus glycoprotein[28]. The SARS-CoV S protein encodes a surface glycoprotein precursor that is predicted to be 1,255 amino acids in length, and the amino terminus and most of the protein is predicted to be on the outside of the cell surface or the virus particles[3]. The predicted S protein consists of a signal peptide (amino acids 1–12) located at the N terminus, an extracellular domain (amino acids 13–1,195), a transmembrane domain (amino acids 1,196–1,215) and an intracellular domain (amino acids 1,216–1,255)[29–32] (FIG. 2a). Similarly to other coronaviruses, the S protein of SARS-CoV can be cleaved into the S1 and S2 subunits by proteases, such as trypsin[33], factor Xa[34] and cathepsin L[35]. The trypsin cleavage site occurs at R667–S668 (REF. 36), whereas cathepsin L cleavage is mapped to T678–M679 in the S protein[35]. Cathepsin L cleaves the S protein of SARS-CoV upstream of, rather than adjacent to, the fusion peptide, and the cleavage is required for activation of the membrane fusion domain of the S protein following entry into target cells[35].

Angiotensin-converting enzyme 2 (ACE2) has been identified as the receptor of SARS-CoV[37]. A fragment that is located in the S1 subunit and spans amino acids 318–510 is the minimal receptor-binding domain (RBD)[30,38,39]. Crystallographic studies have shown the structure of RBD complexed with its receptor ACE2 (REFS 29,40). During the interaction of RBD with the receptor, RBD presents a concave surface for the N terminus of the receptor peptidase, on which amino acids 445–460 anchor the entire receptor-binding loop of the RBD core (FIG. 2b). This loop (amino acids 424–494 of the RBD), which makes complete contact with the receptor ACE2, was referred to as receptor-binding motif (RBM) (FIG. 2a). The RBM region is tyrosine rich. Among the 14 residues of RBM that are in direct contact with ACE2, six are tyrosine, representing both the hydroxyl group and hydrophobic ring. The RBD region also contains multiple cysteine residues that are linked by disulphide bonds[29] (FIG. 2c). Two residues in particular, those at positions 479 and 487, determine SARS disease progression and SARS-CoV tropism (host range)[41,42]. Any residue changes in these two positions might therefore enhance animal-to-human or human-to-human transmission[29].

Human and animal SARS-CoVs depend on ACE2 for cell entry. Animal SARS-CoV could evolve to infect humans by a series of transmission events between animals and humans. For example, a chimeric recombinant SARS-CoV that bears the S protein of civet SARS-CoV (icSZ16-S) can adapt to human airway epithelial cells and displays enhanced affinity for human ACE2 (REF. 43). Changes of only a few residues in the RBD of the civet SARS-CoV S protein, which is responsible for binding with the peptidase domain of ACE2, result in enhanced human ACE2-binding affinity of SARS-CoVs from animals, including civets, mice and rats, facilitating efficient cross-species infections[29]. However, the SL-CoV from bats does not infect ACE2-expressing cells[7,8], suggesting that, unlike SARS-CoVs from human and civets, the SL-CoV from bats does not use ACE2 as a cellular receptor. Thus, the SL-CoV from bats might be the precursor of animal SARS-CoVs, which may act as the intermediates for animal-to-human transmission.

Functions of the SARS-CoV S protein

SARS-CoV S protein has pivotal roles in viral infection and pathogenesis[44,45]. S1 recognizes and binds to host receptors, and subsequent conformational changes in S2 facilitate fusion between the viral envelope and the host cell membrane[30,33].

Receptor binding. The RBD in S1 is responsible for virus binding to host cell receptors[30,37,39]. ACE2 from SARS-CoV-permissive Vero E6 cells efficiently binds S1, and its soluble form blocks S1 from associating with Vero E6 cells. In addition, SARS-CoV replicates efficiently in ACE2-transfected cells, and anti-ACE2 antibodies block virus entry and replication in Vero E6 cells. This shows that ACE2 is a functional receptor for SARS-CoV[37,46,47]. A total of 18 residues of ACE2 keep contact with 14 amino acids in the RBD of SARS-CoV S protein[29]. K341 of ACE2 and R453 of the RBD are important for the complex formation[48]. N479 and T487 of the RBD are important for the high-affinity association of S protein with ACE2 (REF. 42). A point mutation at R441 or D454 of the RBD disrupts the antigenic structure and binding activity of RBD to ACE2 (REFS 30,49).

Adaptive evolution
A process that enables living organisms to cope with environmental stresses and pressures for survival in a new host. For example, under positive selective pressure, civet SARS-CoV can evolve and subsequently adapt to the human host.

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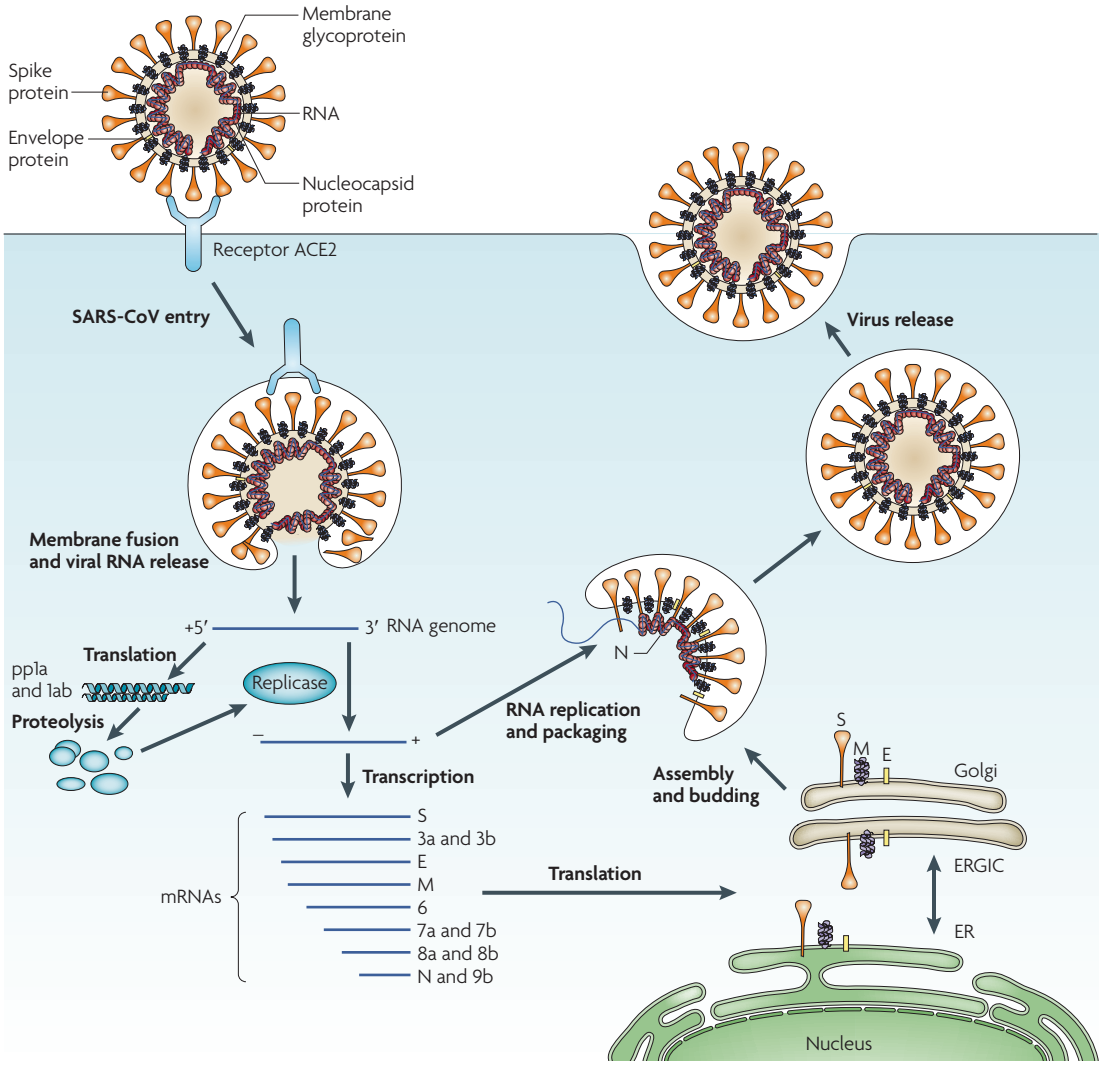

Figure 1 | **The life cycle of SARS-CoV in host cells.** Severe acute respiratory syndrome-coronavirus (SARS-CoV) enters target cells through an endosomal pathway[113,121,125–127]. S protein first binds to the cellular receptor angiotensin-converting enzyme 2 (ACE2)[129], and the ACE2–virus complex is then translocated to endosomes, where S protein is cleaved by the endosomal acid proteases (cathepsin L)[105] to activate its fusion activity. The viral genome is released and translated into viral replicase polyproteins pp1a and 1ab, which are then cleaved into small products by viral proteinases. Subgenomic negative-strand templates are synthesized from discontinuous transcription on the plus-strand genome and serve as templates for mRNA synthesis. The full-length negative-strand template is made as a template for genomic RNA. Viral nucleocapsids are assembled from genomic RNA and N protein in the cytoplasm, followed by budding into the lumen of the ERGIC (endoplasmic reticulum (ER)–Golgi intermediate compartment)[128]. Virions are then released from the cell through exocytosis.

SARS-CoV can also bind to host cells through alternative receptors, such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) and/or L-SIGN (liver/lymph node-SIGN)[50,51]. Seven asparagine-linked glycosylation sites in the S protein, including residues at positions 109, 118, 119, 158, 227, 589 and 699, are crucial for DC-SIGN- or L-SIGN-mediated virus entry. These residues differ from those of the ACE2-binding domain located at amino acids 318–510 (REF. 52). This would suggest that S protein can also use DC-SIGN or L-SIGN as a receptor, independently of ACE2. However, the actual function of DC-SIGN and L-SIGN needs to be further verified.

Viral fusion. The fusion process that is mediated by S protein of SARS-CoV is similar to that mediated by class I viral fusion proteins of other viruses, such as HIV-1 and murine hepatitis virus (MHV)[53,54], but may occur in the acidic environment of the endosomes, rather than on the cell surface. S2 contains heptad repeat 1 (HR1) and HR2 domains, which play an important part in SARS-CoV fusion with target cells. Binding of the RBD of S1 to the receptor ACE2 triggers a conformational change of the S2 from a pre-fusion form to a post-fusion form, resulting in insertion of the putative fusion peptide (amino acids 770–788)[31] into the target cell membrane and association of HR1

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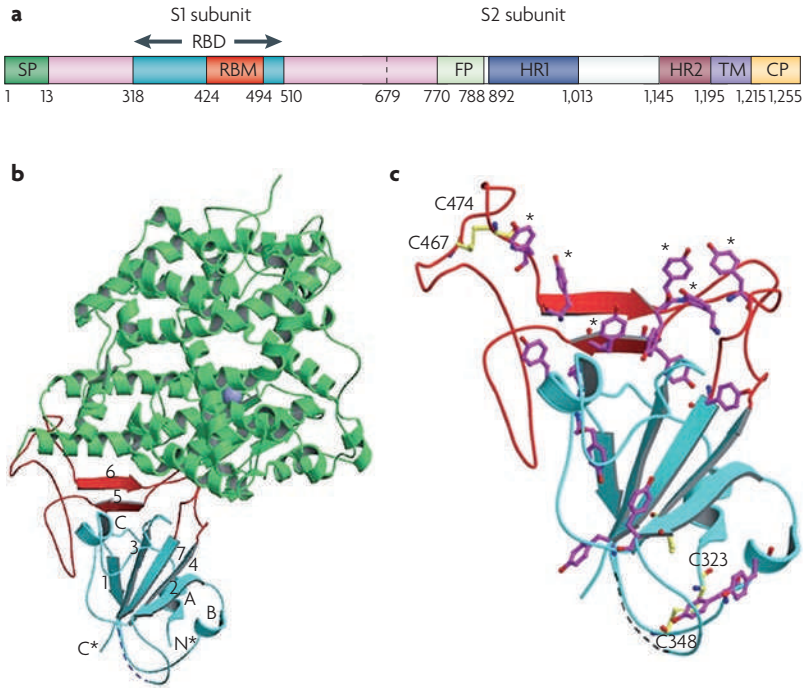

Figure 2 | **SARS-CoV S protein structure and its complex with the receptor ACE2.** **a** | Schematic of the S protein[29–32]. The residue numbers of each region represent their positions in the S protein of severe acute respiratory syndrome-coronavirus (SARS-CoV). **b** | Crystal structures of the RBD complexed with the receptor. RBD (the core structure is cyan and the loop RBM is red) interacts with the receptor angiotensin-converting enzyme 2 (ACE2; green). A five-stranded anti-parallel β-sheet (β1–β4 and β7) that connects with three short α-helices (αA–αC) constitutes the core, whereas a two-stranded β-sheet (β5 and β6) forms the loop. N* and C* represent the amino and carboxyl termini of the RBD, respectively. **c** | The RBD tyrosine (magenta) and cysteine (yellow) residue distribution[29]. The asterisks represent six ACE2-contacting tyrosines on the RBD, and two disulphide bonds are shown to link C323 to C348 and C467 to C474. CP, cytoplasm domain; FP, fusion peptide; HR, heptad repeat; RBD, receptor-binding domain; RBM, receptor-binding motif; SP, signal peptide; TM, transmembrane domain. Parts **b** and **c** are adapted, with permission, from REF. 29 © (2005) American Association for the Advancement of Science.

and HR2 domains to form a six-helix bundle fusion core structure. This brings the viral envelope and target cell membrane into close proximity for fusion. The crystal structure of the SARS-CoV fusion core is described in detail in REF. 55 (FIG. 3). Similarly to the S protein of MHV, but not gp41 of HIV-1, SARS-CoV S protein has a longer HR1 region than HR2 region. The six-helix bundle fusion core has a rod-shaped structure with a length of ~70 Å and a diameter of ~28 Å. Three HR1 helices form a parallel trimeric coiled-coil that is surrounded by three HR2 helices in an oblique, antiparallel manner[55] (FIG. 3). A synthetic peptide derived from the HR2 region could interact with an HR1 peptide to form a stable six-helix bundle and inhibit SARS-CoV infection in a dose-dependent manner[53]. Consequently, both the HR1 and HR2 regions in the S2 domains are expected to participate in the viral fusion and entry processes and will serve as attractive targets for the development of anti-SARS-CoV therapeutics and vaccines.

SARS pseudovirus
A synthetic virus that bears the SARS-CoV S protein and contains an Env-defective, luciferase-expressing genome of a retrovirus (for example, HIV), and can infect but does not replicate in cells that express receptors for SARS-CoV.

Vaccines based on the SARS-CoV S protein

The roles of S protein in receptor binding and membrane fusion indicate that vaccines based on the S protein could induce antibodies to block virus binding and fusion or neutralize virus infection. Among all structural proteins of SARS-CoV, S protein is the main antigenic component that is responsible for inducing host immune responses, neutralizing antibodies and/or protective immunity against virus infection. S protein has therefore been selected as an important target for vaccine and antiviral development. A comparison of these approaches is provided in TABLE 1.

It has been reported that antibodies raised to amino acids 485–625 in S1 or 1,029–1,192 in S2 neutralize infection by SARS-CoV strains (for example, Tor2 and Sin2774) in Vero E6 cells[56,57]. Vaccination of African green monkeys with an attenuated parainfluenza virus that encodes the full-length S protein of SARS-CoV Urbani strain resulted in the production of S protein-specific neutralizing antibodies, which protected vaccinated monkeys from subsequent homologous SARS-CoV challenge[58], suggesting that immunization with the S protein of SARS-CoV is highly effective in the prevention of SARS.

Vaccines based on the full-length S protein. Several vaccines that are based on the full-length S protein of SARS-CoV have been reported. Yang *et al.*[59] showed that a DNA vaccine encoding the full-length S protein SARS-CoV Urbani strain could induce both T-cell and neutralizing-antibody responses, as well as protective immunity, in a mouse model. Other groups have also shown that vaccination of mice or monkeys with highly attenuated modified vaccinia virus Ankara (MVA), which encodes the full-length S protein of the SARS-CoV Urbani strain or HKU39849 strain, elicited S-specific neutralizing antibodies and protective immunity, as evidenced by decreased virus titres in the respiratory tracts of animals after homologous SARS-CoV challenge[60,61]. Passive transfer of murine serum to naive mice also protected these mice from the challenge of homologous SARS-CoV[60,61]. In addition, vaccination of mice or hamsters with a full-length S protein trimer protected these animals from infection by homologous SARS-CoV (HKU39849 strain)[62]. Furthermore, a recombinant baculovirus-expressed full-length S protein of the Urbani strain and its trimer could induce sufficient neutralizing antibodies against human and palm civet SARS pseudoviruses that bore S proteins of homologous and heterologous SARS-CoV variants (for example, Tor2, GD03T13 and SZ3 strains) in vaccinated mice[63]. These reports suggest that the full-length S protein is highly immunogenic and induces protection against SARS-CoV challenge and that neutralizing antibodies alone may be able to suppress virus proliferation, further justifying the rationale that vaccines can be developed based on the S protein.

Although full-length S protein-based SARS vaccines can induce neutralizing antibody responses against SARS-CoV infection, they may also induce harmful immune responses that cause liver damage of the vaccinated animals or enhanced infection after challenge with

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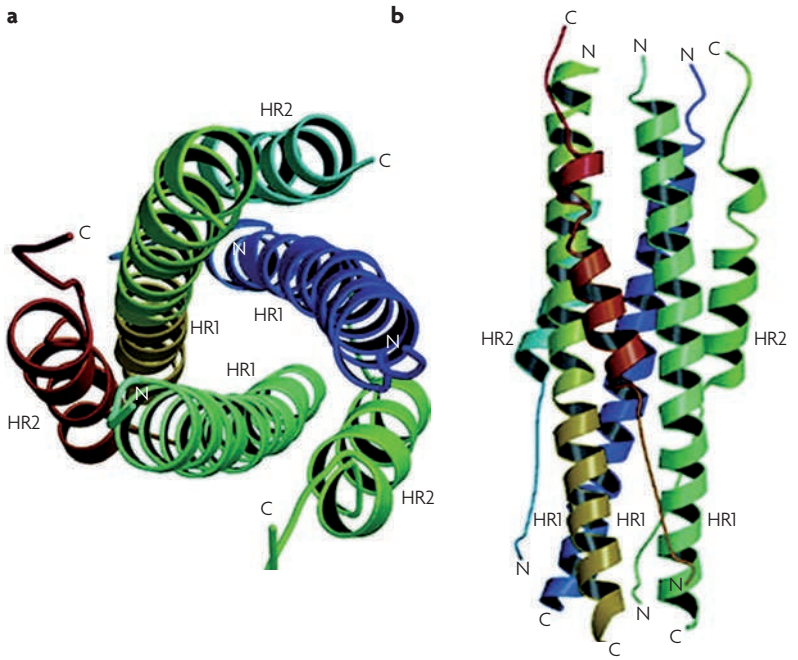

Figure 3 | **The fusion core structure.** The fusion core is a six-helix bundle with three HR2 α-helices packed in an oblique antiparallel manner against the hydrophobic grooves on the surface of the central HR1 trimer[55,130]. A top (**a**) and side (**b**) view is shown of the severe acute respiratory syndrome-coronavirus (SARS-CoV) S protein six-helix bundle fusion core structure formed by the HR1 and HR2 domains in the S2 subunit. C, carboxyl; N, amino. Figure adapted, with permission, from REF. 55 © (2004) American Society for Biochemistry and Molecular Biology.

homologous SARS-CoV[64,65], raising concerns about the safety and ultimate protective efficacy of vaccines that contain the full-length SARS-CoV S protein.

Vaccines based on the RBD. Previous studies have shown that the RBDs of the S proteins of the coronaviruses MHV and HCoV-229E contain major antigenic determinants that can induce neutralizing antibodies[66,67]. We have discovered that the recombinant RBD (rRBD) antigen of SARS-CoV is highly reactive with the neutralizing antibodies against SARS pseudoviruses that bear S proteins of SARS-CoV (Tor2 strain) in the antisera of mice and rabbits immunized with inactivated SARS-CoV[68]. The RBD strongly reacts with the antisera from patients with SARS in the convalescent phase, and depletion of the RBD-specific antibodies from patients with SARS results in significant elimination of the neu ralizing activity[69]. Chen *et al.*[61] have also shown that most neutralizing antibodies of antisera of mice, rabbits and monkeys induced by a live-attenuated MVA virus that expressed the full-length S protein could be absorbed and removed by rRBD. Using a fusion protein that contained the RBD linked to human IgG1 Fc fragment (designated RBD-Fc) as an immunogen, we have successfully induced highly potent neutralizing antibodies against SARS-CoV BJ01 strain in immunized rabbits with neutralizing titres greater than 1:10,000 (REF. 70). The antibodies effectively cross-neutralize infection by SARS pseudoviruses that bear S proteins of both homologous and heterologous SARS-CoV isolates, including the representative strains of human 2002–2003 and 2003–2004 SARS-CoV (Tor2 and GD03, respectively) and palm civet SARS-CoV (SZ3)[71]. Immunization of mice with RBD-Fc induces long-term protective immunity against challenge with homologous SARS-CoV BJ01 strain[70,72]. Administration of an adeno-associated virus (AAV)-based vaccine that contains RBD (RBD-rAAV) by intramuscular and mucosal pathways elicits sufficient neutralizing antibodies to inhibit homologous SARS-CoV (GZ50) challenge in the established mouse model, and the immune responses can be enhanced by priming with RBD-rAAV and boosting with RBD-specific peptides[73–75].

The SARS-CoV S protein can also induce CD8$^+$ T-cell responses. One H-2(b)- and one H-2(d)-restricted T-cell epitope are mapped to RBD (S436–S443 and S366–S374, respectively)[24]. Immunization of mice with a RBD-based subunit vaccine (S318–S510) elicits both antibody and cellular immune responses against SARS-CoV[26]. The RBD of S protein contains multiple conformation-dependent epitopes and is the main domain that induces neutralizing antibody and T-cell immune responses against SARS-CoV infection[76,77], making it an important target for vaccine development. The approaches for developing RBD-based vaccines against SARS-CoV have provided useful information for designing vaccines against other viruses with class I fusion proteins, as these proteins also contain RBDs in their S proteins.

It should be noted that the efficacy of these vaccine candidates is mainly tested in young-mouse and primate animal models. These models are usually less robust, providing virus replication but lacking clinical symptoms and diseases. It is necessary, therefore, to develop more-robust animal models of human diseases for evaluation of vaccine efficacy. Baric and colleagues[78,79] have recently reported several lethal SARS-CoV challenge models in BALB/c mice that recapitulated the age-related SARS disease by using recombinant SARS-CoV that bore the S protein of early human and zoonotic strains (GZ02 and HC/SZ/61/03, respectively). They also developed another pathogenic model for young mice after 15 passages of the Urbani isolate in BALB/c mice, which resulted in a lethal virus, MA15, that replicates to high titres in the lungs of mice, causing clinical disease of SARS[78,79]. Other reports[80,81] list examples for the use of senescent mouse models for vaccine evaluation. One candidate vaccine, Venezuelan equine encephalitis virus replicon particles, that expressed the Urbani SARS-CoV S protein partially protected the aged mice from challenge with a recombinant heterologous SARS-CoV that bore epidemic and zoonotic S proteins (icGDO3-S), providing a model to mimic the age-related susceptibility observed in the elder population[80]. The animal models discussed above can be used as valuable tools to evaluate the efficacy of SARS vaccines.

S protein-based therapeutics

Peptides that interrupt the RBD–ACE2 interaction. It has been shown that rRBD blocks S protein-mediated entry of lentivirus pseudotypes into ACE2-expressing

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Table 1 | **S protein-based vaccines and antiviral therapies against SARS-CoV**

Category	Advantages	Disadvantages	Refs
*Vaccines**			
Full-length S protein	Induces effective neutralizing-antibody and T-cell responses, as well as protective immunity	Might induce harmful immune responses that cause liver damage or enhanced infection	64,65
DNA-based	Easier to design; induces immunoglobulin G, neutralizing-antibody and T -cell responses and/or protective immunity	Might have low efficacy in humans; repeated doses may cause toxicity	59,131
Viral vector-based	Induces neutralizing-antibody responses, protective immunity and/or T-cell responses	Might induce ADE effect; possibly present pre-existing immunity	60,61,65
Recombinant S protein-based	Induces high neutralizing-antibody responses and protective immunity	Mainly humoral responses; need repeated doses and adjuvants	62
RBD	Induces highly potent neutralizing-antibody and T-cell responses and protective immunity	Not identified	70–73
DNA-based	Induces neutralizing-antibody and T-cell responses and/or protective immunity	Induces low responses; might not neutralize mutants	132–134
Viral vector-based	Induces neutralizing-antibody responses, protective immunity and/or T-cell responses	Possible genomic integration of foreign DNA; viral vector instability	75,135
Recombinant RBD protein-based	Safer and more effective than other RBD vaccines; induces neutralizing-antibody and T-cell responses, protective immunity and cross protection	Needs repeated doses and adjuvants	26,70–72
*Therapeutics**			
Peptides	Inhibits virus infection by preventing S protein-mediated receptor binding and blocking viral fusion and entry	Low antiviral potency	53,82–84, 136–138
RBD–ACE2 blockers	Blocks RBD–ACE2 binding and S protein-mediated infection	Not identified	82,83
S cleavage inhibitors	Might interfere with S cleavage	Not identified	84,136,137
Fusion core blockers	Easy to design; inhibits virus infection with high specificity	Not identified	53,89,90,138
Neutralizing antibodies	Highly potent virus inhibition and/or neutralization activity against homologous and heterologous SARS-CoV isolates	Might enhance SARS-CoV entry; further studies needed	139
Neutralizing mouse antibodies	Easier to generate than human neutralizing antibodies; neutralizes SARS-CoV *in vitro* and prevents virus replication	Repeated use can cause HAMA response; might not recognize mutants with key substitutions in S protein	65,94, 140,141
Neutralizing human antibodies	Inhibits virus entry, neutralizes virus infection, induces cross protection and reduces disease severity and viral burden; more suitable to development as human immunotherapeutics	Not identified	97,142,143
Small compounds	Oral bioavailability	Low antiviral potency	103–105, 107–109
Protease inhibitors	Blocks virus entry and/or inhibits protease (cathepsin L) proteolysis	Not identified	103–105
S protein inhibitors	Specifically inhibits S protein-mediated SARS-CoV fusion and entry into the host cell	Not identified	107–109
Small interfering RNAs	Reduces virus replication and/or silences S gene expression	Low antiviral potency; limited usefulness	113–117

*All candidates are at the preclinical study stage. ACE2, angiotensin-converting enzyme 2; ADE, antibody-dependent enhancement; HAMA, human–anti-mouse antibody, RBD, receptor-binding domain, SARS-CoV; severe acute respiratory syndrome-coronavirus.

293T cells with a half maximal inhibitory concentration (IC_{50}) of less than 10 nM[30]. Similarly, a peptide that overlaps the RBD sequence (amino acids 471–503) blocks the RBD–ACE2 interaction, inhibiting SARS-CoV entry into Vero cells with an IC_{50} of approximately 40 μM[82]. A polypeptide that contains two RBD-binding motifs of ACE2 (amino acids 22–44 and 351–357) linked by a glycine exhibits high potent inhibitory activity on SARS pseudovirus infection in ACE2-expressing HeLa cells with an IC_{50} of 100 nM[83]. These findings suggest that peptides derived from both RBD and ACE2 that block RBD–ACE2 binding could be developed as novel therapeutics against SARS-CoV infection. However, the *in vivo* inhibitory activity of these peptides should be evaluated in animal models before considering further development.

Peptides that interfere with the cleavage of S protein. Cleavage of the S protein trimer is an important event in infection, making the potential cleavage site between S1 and S2 domains another target for development of anti-SARS-CoV agents. Synthetic peptides, including P6 (amino acids 598–617) and P8 (amino acids 737–756), both of which are close to the S1–S2 connection and cleavage site, exhibit potent inhibitory activity against the GZ50 strain of SARS-CoV infection in fetal rhesus kidney

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(FRhK4) cells, and have IC_{90} values of approximately 100 and 25 μM[84]. This suggests that binding of the peptides to the S protein interferes with the cleavage of S1 and S2, inhibiting the production of functional S1 and S2 subunits and subsequent fusion of the viral envelope and the host cell membrane. Again, the *in vivo* antiviral efficacy of these peptides should be tested in animal models.

Peptides that block the HR1–HR2 interaction from forming a fusion-active core. In the early 1990s, Jiang *et al.*[85] and Wild *et al.*[86] discovered the highly potent anti-HIV peptides derived from the HIV-1 gp41 HR2 region. One of the HR2 peptides, T20 (enfuvirtide), was approved by the US Food and Drug Administration for the treatment of patients with HIV or AIDS, especially those who have failed to respond to the current antiretroviral drugs. These HR2 peptides could interact with the viral gp41 HR1 region at fusion-intermediate conformation and block six-helix bundle formation, resulting in the inhibition of HIV fusion at the nanomolar level[87,88]. Because the SARS-CoV S protein S2 domain also contains HR1 and HR2 sequences, we anticipated that peptides derived from the HR2 region of the SARS-CoV S protein S2 domain would also have antiviral activity against SARS-CoV. We designed and synthesized several peptides that overlapped the HR2 sequence and found that one of these, designated CP-1, could interact with an HR1 peptide to form a stable six-helix bundle and inhibited infection by SARS-CoV WHU strain in Vero E6 cells with an IC_{50} of approximately 20 μM[53]. Later, several other research groups also identified anti-SARS-CoV peptides from the S2 domain HR2 region that had viral fusion inhibitory activity at the micromolar level[84,89,90]. An NMR study has shown that in the pre-fusion intermediate state, the HR2 region forms a symmetric coiled-coil trimer, which has not been observed for other class I viral fusion proteins. The poor antiviral activity of anti-SARS-CoV peptides, compared with the anti-HIV peptides, could be attributed to the tendency of the SARS-CoV S protein HR2 region to form the trimeric coiled-coil. Replacement of the key residues in the HR2 peptide to reduce its ability to form the trimer, but increase its affinity of binding with the HR1 region, to form the six-helix bundle could lead to improvement of its antiviral efficacy. The peptidic antiviral drugs for SARS and other emerging infectious diseases with short incubation periods could have more advantages than the anti-HIV drug enfuvirtide, as enfuvirtide must be injected twice per day for the patient's lifetime. This results in an intolerable injection-site reaction and a high cost to patients, whereas a few injections of the peptidic drugs against SARS-CoV in the early stage of the acute phase could be enough to save patients' lives. One of the disadvantages of using HR2-based peptide inhibitors is the potential selection of escape mutants with altered host-range phenotypes[91].

mAbs that target the S protein

Neutralizing mouse mAbs. Using rRBD and inactivated SARS-CoV as immunogens, we have successfully generated a panel of highly potent neutralizing mouse monoclonal antibodies (mAbs) that could block receptor binding and cross-neutralize infection by pseudoviruses that bore S proteins of the representative human SARS-CoV strains that caused the 2002–2003 and 2003–2004 outbreaks (Tor2 and GD03T13) and palm civet SARS-CoV (SZ3)[63,69,71,92]. Mouse mAbs that target other fragments of the SARS-CoV S protein (for example, amino acids 1,143–1,157) could also effectively inhibit SARS-CoV infection[56,93]. These neutralizing mouse mAbs can be administered to patients with SARS for early and urgent treatment of SARS-CoV infection[9], but cannot be repeatedly used owing to the risk of a human–anti-mouse antibody response. Such a response could rapidly clear the murine antibody from the blood, thus preventing the mouse antibodies from producing the desired therapeutic effect and causing the patient to have an allergic reaction[94]. Some antibodies against trimeric S protein have the potential to mediate FcγRII-dependent entry into B cells *in vitro* and thereby cause antibody-dependent enhancement[62].

Neutralizing human mAbs. A range of neutralizing human mAbs have been generated from B cells of patients infected with SARS-CoV[95,96] or from human immunoglobulin transgenic mice immunized with full-length SARS-CoV S protein[97–99]. These S-specific mAbs, such as 80R and CR3014, could block SARS-CoV S protein binding with the ACE2 receptor and neutralize infection by human SARS-CoV strains Tor2 and HKU39849 and/or palm civet SARS-CoV strain SZ3 (REFS 32,100,101). mAbs m396 and S230.15 neutralize human SARS-CoV and/or pseudoviruses that bear S proteins of human SARS-CoV strains (Urbani, Tor2 and GD03) and palm civet SARS-CoV strains (SZ3 and SZ16)[97]. Human anti-S mAbs S109.8, S215.17, S227.14 and S230.15 cross-neutralize infection by a panel of recombinant SARS-CoV strains bearing variant S proteins that are representative of human strains (GZ02, CUHK-W1 and Urbani) and zoonotic strains found in palm civet (HC/SZ/61/03) and raccoon dog (A031G)[99]. Some human mAbs, such as 80R[32], m396 (REF. 97), 201 and 68 (REF. 102), exhibit potent antiviral effects against homologous SARS-CoV challenge in young-mouse replication models. However, others, such as S109.8, S227.14 and S230.15 (REF. 99), could induce broad protection against lethal homologous and heterologous SARS-CoV challenge in both young- and aged-mouse models, providing a strategy to minimize the emergence of mAb escape mutants.

Antiviral compounds and small molecules

Inhibitors of cathepsin L. Cathepsin L activates S protein-mediated membrane fusion by facilitating receptor-dependent and acid-dependent conformational changes in the S2 domain. This occurs in endosomes in which a low pH allows for optimal proteolytic activity[35,103,104]. Thus, cathepsin L inhibitors, such as E63c, E64d and MDL28170, can block viral entry or inhibit *in vitro* infection of SARS-CoV or SARS pseudoviruses[103,105,106]. These findings suggest that compounds which inhibit

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the activity of cathepsin L protease could be developed as therapeutics for the inhibition of SARS-CoV infection, but their *in vivo* antiviral activity should be further tested in animal models.

Other compounds and small molecules that target the S protein. Several other compounds and small molecules that target the S protein have been reported. For example, amiodarone blocks the *in vitro* spread of SARS-CoV by inhibiting virus infection at a post-endosomal level[107]. Yi *et al.*[108] identified two small molecules, tetra-O-galloyl-beta-D-glucose (TGG) and luteolin, which have inhibitory activity, that blocked SARS-CoV or SARS pseudovirus entry into Vero E6 cells. Kao *et al.*[109] identified 18 small molecules that targeted S protein–ACE2-mediated viral entry. One of these, VE607, exhibits potent inhibitory activity on SARS pseudovirus entry into ACE2-expressing 293T cells. These reports suggest that the small molecules discussed above can function as effective antiviral inhibitors against S protein-mediated viral entry. However, further studies are needed to determine the *in vivo* efficacy of these antiviral agents in animal models and select optimal formulations to deliver effective concentrations of the drugs to the target tissues.

Gene targeting with small interfering RNA. RNA interference induced by a small interfering RNA (siRNA) has been successfully used recently as a specific and efficient method for silencing specific viral genes, interrupting protein synthesis and suppressing virus replication[110,111]. It has been demonstrated that siRNAs directed against S sequences of SARS-CoV inhibited SARS-CoV replication in virus-infected Vero E6 cells[112]. Several research groups[113–117] reported that S-specific siRNAs could reduce S protein expression by blocking S mRNA accumulation or reducing the number of copies of the viral genome in FRhK4 cells, indicating that S gene expression in SARS-CoV-infected cells can be effectively silenced by S-specific siRNAs. The *in vivo* study used a rhesus macaque model to indicate that siRNA duplexes (siSC2–5) that targeted the S protein and ORF1b of SARS-CoV could suppress SARS-like symptoms, inhibit virus replication in the monkey respiratory tract and protect lungs from acute damage[118]. The findings discussed above reveal the function of siRNA in the inhibition of SARS-CoV infection, replication and/or interruption of S gene expression, raising hopes for the development of effective, novel antiviral agents against SARS-CoV.

Conclusions and prospects

In summary, the S protein of SARS-CoV possesses some unique features that are different from other type I glycoproteins. Many class I fusion proteins, such as HIV Env, influenza HA and MHV S, are post-translationally cleaved at the N-proximal region of the fusion peptide by specific proteases into the surface and transmembrane subunits. By contrast, cleavage of the SARS-CoV S protein may occur far upstream of the predicted fusion peptide (FIG. 2a). Unlike the S proteins of coronaviruses cleaved by furin-like proteases, the S protein of SARS-CoV can be cleaved by cathepsin L at position T678 or by trypsin at R667. In contrast to the entrance mechanism of HIV, SARS-CoV can enter cells from an acidic environment of the endosome[119]. Nevertheless, SARS-CoV can also enter the target cell surface, which is mediated by proteases on the cell surface through a non-endosomal-dependent pathway[120].

The interaction between the SARS-CoV S protein and ACE2 is essential for SARS-CoV entry. The natural evolution of the epidemic SARS-CoV strains probably occurred over a long period, through the repeated transmission of viruses from animals to humans and from humans to animals, resulting in mutations in both the SARS-CoV S protein and ACE2, so that human and animal SARS-CoVs could enter cells that bore human or animal ACE2. Further understanding of the tropism of the virus and the mechanism of the SARS-CoV S protein in receptor binding and entry is therefore important for the development of anti-SARS-CoV therapeutics and vaccines.

As the major component for the development of vaccines against SARS, S protein, and especially the RBD, has been shown to induce highly potent neutralizing antibodies to block virus binding and membrane fusion and/or protective immunity against virus infection. Owing to the absence of human SARS cases in recent years, future SARS epidemics will probably originate from zoonotic transmission. SARS vaccines should therefore protect against not only human SARS-CoV strains, including those from early, middle and late phases of the epidemic, but also those of zoonotic origin. Although current vaccine candidates effectively neutralize SARS-CoV in young-animal replication models without clinical symptoms, they may not protect an elderly population against SARS-CoV infection. Thus, it is essential to test the vaccine candidates in robust lethal-challenge models using aged animals. Future vaccines should effectively protect both the young and the elderly populations from infection by either human or animal SARS-CoV strains that may cause future SARS epidemics.

Peptides and non-peptidic small molecules that target the functional domain of the SARS-CoV S protein, particularly the RBD in the S1 subunit and the HR2 region in the S2 subunit, are mainly virus entry inhibitors and can be further developed as anti-SARS-CoV therapeutics. To develop these molecules as effective and safe antiviral drugs for the treatment of SARS, the urgent task is to improve their potency. Mouse and human mAbs that target the S protein of SARS-CoV have shown potent inhibition and/or neutralization to homologous and heterologous SARS-CoV isolates and can be further developed as immunotherapeutics or passive immunization agents for therapy and prophylaxis of SARS-CoV infection. Future studies are needed to test the *in vivo* efficacy of these antiviral agents in animal models.

Amiodarone
A medication commonly used to treat patients with irregular heart beats or cardiac arrhythmias, including ventricular tachycardia and ventricular fibrillation.

Luteolin
A flavonoid extracted from Chinese herbs, including *Prunella vulgaris* and *Saussurea lappa* Clarks.

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Overall, the feasibility of using peptides and small molecules as anti-SARS therapeutics is partially limited by their low antiviral potency. Furthermore, the possibility of enhancing viral entry might restrict mAbs as immunotherapeutics for long-term use. It is likely, however, that S protein-based vaccines will bear fruit in the near future, as they have been proven to induce long-term and potent neutralizing antibodies and/or protective immunity against SARS-CoV. But the *in vivo* efficacy of these vaccine candidates in elderly and lethal-challenge models, and their protection against zoonotic virus infection, should be determined before a clinical study is initiated. To take these factors into full consideration, a combination of different strategies with multiple vaccines and antiviral therapeutics may be needed to induce broad and cross protection against various virus strains, especially isolates that have mutated quickly. Early clinical studies that were based on such strategies have been carried out, but it is difficult to push the clinical trials of these candidate vaccines and therapeutics forwards owing to a lack of SARS-CoV-infected subjects and insufficient financial support. Thus, most big pharmaceutical companies have no interest in developing SARS vaccines and therapeutics because of the concern of profitability. However, studies on SARS will provide important information for designing novel strategies for prophylaxis and therapies of other newly emerging infections caused by enveloped viruses with class I fusion proteins.

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