EXPERT REVIEWS

SARS vaccines: where are we?

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In this review, the current state of vaccine development against human severe acute respiratory syndrome (SARS) coronavirus, focusing on recently published data is assessed. We discuss which strategies have been assessed immunologically and which have been evaluated in SARS coronavirus challenge models. We discuss inactivated vaccines, virally and bacterially vectored vaccines, recombinant protein and DNA vaccines, as well as the use of attenuated vaccines. Data regarding the correlates of protection, animal models and the available evidence regarding potential vaccine enhancement of SARS disease are discussed. While there is much evidence that various vaccine strategies against SARS are safe and immunogenic, vaccinated animals still display significant disease upon challenge. Current data suggest that intranasal vaccination may be crucial and that new or combination strategies may be required for good protective efficacy against SARS in humans.

Keywords: animal model • antibody • efficacy • SARS • T lymphocyte • vaccine

Severe acute respiratory syndrome (SARS) caused 8098 reported human infections and 774 deaths in 32 countries in a single fall-to-spring period (2002–2003), and also led to travel restrictions and significant effects on the global economy [201]. The etiologic agent of SARS was identified as a new human coronavirus (CoV), order *Nidovirales*, family *Coronaviridae,* by the sequencing of its genome [1,2] and by experimental infection of macaques to fulfill Koch's postulates [3]. Serologic evidence suggests zoonotic transmission of SARS-associated CoV (SARS-CoV) into the human population for several years before the recognized outbreak [4], and transmission to humans has continued, resulting in at least four independent nonlaboratory-associated cases in 2004 [5–7,202]. The movement of SARS-CoV into the human population over several years suggests a need to prepare vaccines for protection from this potentially emerging agent. SARS-CoV is of particular concern as a zoonosis because it can replicate in a large number of animals including dogs, cats, pigs, mice, ferrets, foxes, monkeys and rats [8–10], in addition to Chinese palm civets, raccoon-dogs and bats, which appear to be the natural host [11,12].

SARS is primarily a respiratory disease, with the highest concentration of SARS-CoV found in the respiratory tract [13–15], although this virus is also detectable in other organs and tissues, as well as in stool [16–18]. The incubation period for the disease ranges from 2 to 10 days, and infectivity is maximal during the second week of disease [19,20]. The disease is characterized by fever, chills, malaise, dyspnea, cough, diarrhea and pneumonia [13–15]. Diffuse alveolar damage along with inflammatory cell infiltrate consisting particularly of macrophages are hallmarks found in SARS patients [21]. The fever of most patients abates within 2 weeks and is accompanied by resolution of chest symptoms and radiologic changes [3,13–15,22]. The major mode of transmission of SARS-CoV is believed to be through droplet spread [2,23], although SARS-CoV can remain viable when dried on surfaces for up to 6 days [24]. The majority of SARS patients are adults with only a few cases in children aged 15 years or younger [19,20,25]. The overall case–fatality rate is approximately 10% [19,203].

Currently, there are no approved antiviral drugs that effectively target SARS-CoV, hence vaccination is the most likely mode of preventing SARS in people, especially for those at highest risk (e.g., healthcare workers). A successful SARS vaccine could be used prophylactically to protect healthcare workers, laboratory personnel and other at-risk individuals. No vaccines are currently licensed for any of the human CoVs, but vaccines have been produced for a number of CoVs for use in chickens, cattle, dogs, cats and swine [26–28].

The positive-stranded RNA genome of SARS-CoV is 29.7 kb in length and contains approximately 14 open reading frames (ORFs), described in Table 1, with identification of each ORF by the four nomenclature systems [1,2].

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Table 1. Severe acute respiratory virus-associated coronavirus open reading frames.

Protein Tor2 (GenBank)	aa number (size)	Gene nomenclature systems		
		Qiu [139]	*Marra* [1]	*Rota* [2]
ORF 1a/b				
S	1255	S	S	S
3a	**274**	**PUP1**	**ORF3**	**X1**
3b	**154**	**PUP2**	**ORF4**	**X2**
E	**75**	**E**	**E**	**E**
M	221	M	M	M
6	**63**	**PUP3**	**ORF7**	**X3**
7a	**122**	**PUP4**	**ORF8**	**X4**
7b	**44**		**ORF9**	
8a	**39**		**ORF10**	
8b	**84**		**ORF11**	**X5**
N	422	N	N	N
9b	**98**	**PUP5**	**ORF13**	
Not designated	70		ORF14	

Bold ORFs have been experimentally deleted [103,106].
aa: Amino acid; E: Envelope; M: Matrix; ORF: Open reading frame.

These ORFs encode proteins that provide targets for vaccine and drug development. CoV enters target cells via receptor-mediated endocytosis driven by the spike (S) glycoprotein, which protrudes from the surface of the virion. The S protein serves as the major viral attachment protein, critical to virus binding and fusion of the viral envelope [29], and thus has been a major target antigen for vaccine development. The receptor–S protein interaction is a major determinant of species specificity and tissue tropism for CoV [30]. Angiotensin-converting enzyme 2 (ACE2) and CD209L were identified as functional receptors for SARS-CoV; however, entry through ACE2 is more efficient [31,32]. The receptor-binding domain of the S protein is a critical neutralization determinant.

Several strategies may be considered for vaccination against SARS-CoV, including an inactivated or whole-killed virus (WKV) vaccine, a live-attenuated SARS-CoV vaccine, a viral vector such as adenovirus (Ad) or vaccinia virus expressing SARS-CoV genes, bacterial vectors, recombinant SARS-CoV proteins or DNA vaccines. Live-attenuated CoV, killed CoV, DNA vaccines and viral vectored vaccines have all been used to successfully vaccinate against animal CoVs [28,33,34].

Inactivated virus vaccines

Inactivated, or WKV, vaccines are attractive because they are easily prepared (at least conceptually) and present an antigenic moiety similar to what the immune system will encounter in invading virus particles. In addition, these vaccines present multiple proteins on their surface for immune recognition (Figure 1). Antibodies were detected in patient sera to at least eight different proteins that may be in the viral particle membrane [35]. In addition to S, matrix (M) and envelope (E), four other ORFs (3a, 6, 7a and 7b) have been confirmed to encode additional structural proteins [36–40]. These data indicate that there are multiple epitopes and proteins that may be targets of protective antibodies. Mice vaccinated with inactivated SARS-CoV generated antibodies to a number of proteins including S, nucleocapsid (N), M and 3CL [41]. The main difficulties encountered with the production of inactivated vaccines are the biosafety level 3 growth of large amounts of pathogen and the difficulty ensuring that all virus has been successfully inactivated. For SARS-CoV, the large-scale production of UV-inactivated virus has been successfully described [42].

A number of laboratories have pursued the development of inactivated whole SARS-CoV virus vaccines and demonstrated that they induce SARS-CoV neutralizing antibody [41,43–47]. However, demonstrations of efficacy against live SARS-CoV challenge are rare. In one study, WKV vaccine was shown to protect against pulmonary SARS-CoV replication in BALB/c mice, although characterization of the immune response was not reported [48]. Our consortium (The SARS Accelerated Vaccine Initiative) prepared a β-propiolactone-inactivated WKV SARS-CoV (Tor-2 strain) vaccine and compared its immunogenicity and efficacy to a combination of attenuated Ads expressing either S or N proteins for the ability to protect against live SARS-CoV challenge in a permissive mouse model [49]. Our results showed that the WKV vaccine, in the presence or absence of alum adjuvant, provided protection against live SARS-CoV challenge by the induction of high levels of neutralizing antibodies and the reduction of SARS-CoV load in the respiratory tract compared with mock-vaccinated mice [49]. CoV-like particles have also been developed by coexpression of SARS-CoV S protein with E, M and N proteins of mouse hepatitis virus, thus mimicking WKV. This preparation was shown to induce neutralizing antibodies and to protect mice against SARS-CoV replication in lungs [50], but there was no direct experimental comparison made with the WKV vaccine.

The lack of significant clinical disease in many mouse models, however, leads one to question whether efficacy would be maintained in a host where SARS is more virulent. WKV has also been tested in ferrets, a model that shows clinical signs and significant lung pathology [51,52]. Formalin-inactivated whole-virus Urbani strain of SARS-CoV without adjuvant induced some neutralizing antibodies, and led to earlier clearance of virus after challenge, but provided only mild protection in ferrets [53]. Lung tissues were analyzed 23 days postchallenge and did not show significant changes between mock and vaccinated animals, but this time point may have been too late to reveal vaccination-induced differences in disease. The authors commented, "the vaccine was not immunologically robust". Our consortium also

tested β-propiolactone-inactivated WKV and compared it with Ad-vectored S and N vaccines in ferrets [52], and found that both vaccines induced neutralizing antibody responses and reduced viral replication and shedding in the upper respiratory tract, and progression of virus to the lower respiratory tract. The vaccines also diminished hemorrhage in the thymus, and reduced the severity and extent of pneumonia and damage to lung epithelia. However, despite high neutralizing antibody titers, protection was incomplete for all vaccine preparations and administration routes tested.

Formaldehyde-inactivated SARS-CoV was used to vaccinate rhesus monkeys [54], and the vaccine was shown to be safe and immunogenic (it induced neutralizing antibodies). However, evidence of protection was lacking, as only one of the two PBS-vaccinated monkeys showed any clinical signs after challenge, and they were mild. Inactivated SARS-CoV vaccine has been administered to 36 human subjects and was found to be safe, well tolerated and able to elicit SARS-CoV-specific neutralizing antibodies; however, lacking a natural challenge, there is no data on efficacy [55]. While it is widely accepted that endogenous antigen production (via recombinant or attenuated bacterial or viral delivery) yields superior T-lymphocyte responses, we found that SARS-CoV-specific IFN-γ-secreting T-cell responses were similar in WKV and Ad vector S/N-vaccinated mice [49], suggesting that WKV may induce a T-cell response equal to vectored vaccines. Thus, the accumulated data indicate that WKV vaccines are safe and they induce SARS-CoV neutralizing antibodies and can even activate T lymphocytes; however, compelling evidence of protective efficacy is scant or absent.

Recombinant vector vaccines

Recombinant virus vaccines have several features that make them efficient in inducing B- and T-cell-mediated immune responses, including their ability to infect cells and persist in the body, their ability to infect antigen-presenting cells directly, and the fact that viral proteins and the infection itself can have strong adjuvant activity [56]. Recombinant viruses express the foreign target protein in the cytoplasm of the host cell, much like an intracellular pathogen. Thus, the endogenous antigen is available for processing by the cellular antigen-processing machinery for expression with MHC class I for presentation to $CD8^+$ T lymphocytes and development of cytotoxic T cells. As a result, recombinant viruses result in activation of cellular immunity often necessary for elimination of infected cells. For SARS-CoV vaccines, several viruses have been used to express SARS-CoV proteins with the goal of inducing both strong cellular immunity and neutralizing antibodies.

Adenovirus vectored SARS-CoV vaccines

Some of the advantages of Ad vaccines include their lack of pathogenicity in humans, especially for replication-deficient mutants [57], oral or nasal administration, which promotes mucosal immunity, and the well-characterized genome of Ads [58]. Disadvantages of Ads compared with other viral delivery systems include their limited cloning capacity, the fact that human Ads have a restricted host range, often making animal testing difficult [59], and that a large percentage of the human population

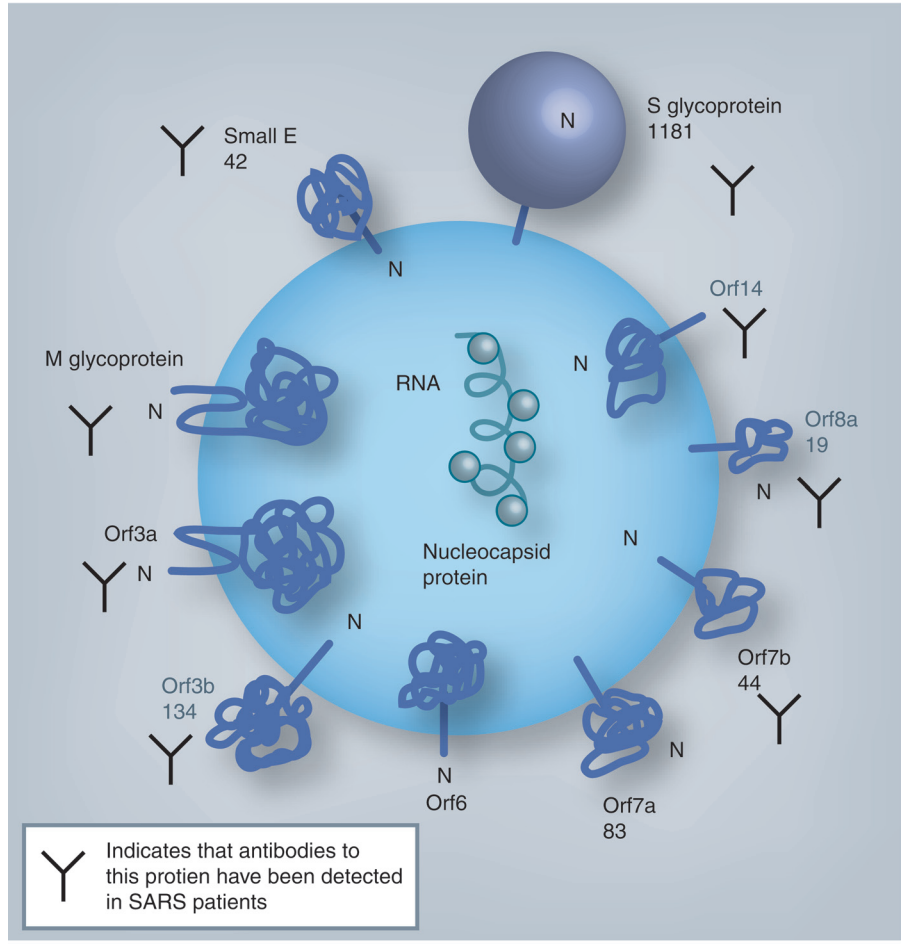

Figure 1. Severe acute respiratory syndrome-associated coronavirus virion. Known structural surface proteins including the viral attachment protein S, small E and M protein are shown in black text, as well as potential (gray text) predicted membrane proteins that may or may not be in the virion. Predicted orientations of the protein are also shown relative to their N, as well as the topology regarding the size of the protein mass relative to the membrane of the virus particle [1]. Nomenclature is shown as in Genbank, except for ORF14, which is designated as in the original severe acute respiratory syndrome (SARS) genome sequence manuscript [1]. For proteins that are primarily external, the number of predicted amino acids displayed on the surface of the virion is shown. Y indicates that antibodies to thiS protein have been detected in SARS patient sera [35]. E: Envelope; M: Membrane/matrix; N: Amino terminal; S: Spike.

has pre-existing immunity against the vector due to natural infections [56]. The last difficulty may be circumvented by the use of prime–boost protocols where a different vector (e.g., DNA vaccine) is used to prime the immune response followed by a boost with a recombinant Ad [56].

First, it was demonstrated that Ad vectors encoding SARS proteins were immunogenic. Rhesus macaques immunized intramuscularly with a combination of three Ad5–SARS-CoV Ad-based vectors (N, S and M protein) all generated antibody responses against spike S1 fragment, SARS-CoV-neutralizing antibodies and T-cell responses against the N protein [60]. Vaccination of C57BL/6 mice with recombinant SARS N protein–Ad was able to induce SARS-CoV-specific IFN-γ secretion and T-cell proliferation, but not neutralizing antibodies [57].

Protection against SARS-CoV challenge by Ad-vectored vaccines was first tested in mice [49]. We developed recombinant Ad constructs expressing genes for either SARS-CoV S or N proteins, and immunized SARS-CoV-susceptible 129S6/SvEv mice [61] with both vectors combined, either intranasally or intramuscularly. The vaccine induced high levels of neutralizing antibody, anti-SARS-CoV N protein IFN-γ secretion, and significantly reduced viral titers and RNA in the lungs of challenged mice [49]. Interestingly, although the intramuscular route was more effective in inducing neutralizing SARS serum antibodies, the intranasal route of administration induced IgA and was more effective in blocking SARS-CoV replication in nose and lung tissue (1000-fold in nasal secretions). This finding suggests that the intranasal administration of recombinant Ad N and S proteins may induce crucial protective mucosal immunity. In the same study, we compared adeno-vectored S and N vaccines with a preparation of inactivated SARS-CoV. The combined adeno-S and -N vaccine induced significantly lower neutralizing antibodies and similar anti-N IFN-γ-secreting cells compared with inactivated SARS-CoV vaccine, but the inactivated vaccine provided superior protection measured as SARS-CoV lung titers and RNA [49]. We also compared these vaccines in a ferret model in which ferrets show clinical signs, including fever and lung damage [52]. Both the whole-killed SARS-CoV vaccine and the combination of Ads encoding N and S proteins induced neutralizing antibody responses, reduced viral replication in the respiratory tract, and decreased tissue damage in the thymus and lungs [52]. The adeno-S and -N vaccine delivered intranasally elicited a poor serum-neutralizing antibody response but provided the best protection from lung replication and lung damage [52], indicating that serum-neutralizing antibodies are not a sufficient measure of protective efficacy of a vaccine. In addition, despite high neutralizing antibody titers in some vaccines, protection was incomplete for all vaccine preparations (with one homologous boost) and administration routes, suggesting that combinations of vaccines may be necessary to provide adequate protection against SARS in susceptible animals and humans.

Adenovirus constructs expressing SARS-CoV S protein have also been evaluated in a ferret model using a heterologous prime–boost with human and chimpanzee Ads in order to avoid interference from the immune response to the first Ad vaccination during the boost. This vaccine regimen reduced viral load and the severity of pneumonia in ferrets, and it was also shown to be immunogenic in rhesus macaques [51].

Poxvirus vectors

Poxvirus recombinants are attractive as vaccine vectors owing to their ease of production, stability, capacity for encoding large genes, cytoplasmic gene expression, and ability to induce long-lasting cellular and humoral immune responses [56]. The replication-deficient poxvirus vector, modified vaccinia Ankara (MVA) strain, encoding SARS-CoV S protein delivered either intranasally or intramuscularly, induced neutralizing antibodies and reduced viral replication in the respiratory tract of challenged mice [62]. An MVA–S recombinant vaccine was also employed in one ferret study with apparent increased liver pathology in vaccinated groups after SARS-CoV challenge [63]. While these data suggest that liver pathology be evaluated in SARS-CoV vaccine studies, no other report has shown liver damage linked to vaccination [62].

Recombinant platform vaccines

Several other viral and bacterial vaccine platform technologies have been employed to encode S protein for SARS-CoV vaccine development. Monkeys vaccinated intranasally with parainfluenza encoding the SARS-CoV S protein produced neutralizing antibodies and had significantly reduced viral titers in the respiratory tract after challenge [64]. Parainfluenza virus encoding the S protein was also protective from SARS-CoV challenge in hamsters, and the inclusion of M and E proteins enhanced efficacy [65]. Recombinant adeno-associated virus encoding SARS-CoV S protein vaccine induced SARS-CoV neutralizing antibodies, T-cell responses, and decreased viral titers and lung damage in mice [66]. As with the Ad studies [49,52], intranasal administration led to IgA production and improved protection from SARS-CoV challenge. Newcastle disease virus, an avian-tropic virus that exhibits limited replication in primate respiratory tissues, was also used for SARS-CoV vaccination. Monkeys vaccinated with Newcastle disease virus expressing S protein had up to 1000-fold less virus in the lung tissue after SARS-CoV challenge [67]. A replication-defective vesicular stomatitis virus recombinant expressing the SARS-CoV S protein induced neutralizing antibodies and T-cell responses, and provided protection of immunized mice from SARS-CoV [68].

Several other recombinant strategies have been tested for immunogenicity but not yet for efficacy. For example, live-attenuated recombinant measles viruses expressing SARS-CoV S and N proteins both induced high antibody titers against their cognate antigen. Anti-S antibodies were SARS-CoV neutralizing and N protein induced specific cellular immune responses [69]. Rabies vector has been used to express S proteins and elicit neutralizing antibodies in mice [70]. Recombinant baculoviruses expressing N or S proteins induced both humoral and cellular immune responses in vaccinated mice [71]. Attenuated *Salmonella* expressing SARS-CoV N protein elicited cytotoxic T-lymphocyte activities and induced IFN-γ-producing T cells

in mice. Interestingly, intranasal vaccination also showed advantages in this bacterial system, inducing the highest IgG and IgA levels [72].

Subunit vaccines: antigen targets & trials

Subunit vaccines comprised of purified antigen are advantageous owing to their safety and simplicity; however, protective efficacy is sometimes lacking. In particular, exogenously produced proteins are typically presented with MHC class II and thus often do not generate robust cytotoxic T-cell responses. CoV S proteins are the favorite targets in CoV subunit vaccine development since this viral protein contains determinants known to elicit protective immune responses [73,74]. Consequently, the SARS-CoV S glycoprotein, shown to be responsible for receptor binding to cellular ACE2, is an attractive target for the development of both vaccine and therapeutics [75,76]. This approach is strongly supported by the finding that a human monoclonal antibody that binds to the N-terminal of S protein potently neutralizes SARS-CoV infection and inhibits syncytia formation through blocking of receptor binding [77]. Moreover, the S protein has been shown to induce serum-neutralizing antibodies and confer protective immunity against SARS-CoV challenge in mice and African green monkeys [62,64,78]. Since several other proteins are also expressed on the surface of the virion (Figure 1) and elicit antibodies detectable in sera of convalescent SARS patients [35], other proteins may also be useful to augment protective immunity. For example, antibodies to M proteins have also been shown to have neutralizing activity [79]. In addition, it has also been shown that SARS-CoV S protein can generate CD4 and CD8 T-cell responses [37].

Studies from other animal CoV vaccines have also shown that the CoV N protein may represent another antigen candidate for vaccine development [27,80]. Although antibodies to CoV N proteins have no virus-neutralizing activity *in vitro* [79], there is evidence that the protein may provide *in vivo* protection by induction of cell-mediated immunity [73,81,82], as it has been shown to generate CoV-specific $CD8^+$ T cells [82–85] and provide protection in animals following infection [84,86]. The expressed SARS-CoV N protein has been shown to be a vaccine candidate by inducing antigen-specific T-cell responses, but no *in vivo* protection experiments were performed with these vaccines [87,88]. A review was published recently addressing the progress in subunit vaccines [89].

DNA vaccines

For many pathogens, both antibody and T-cell-mediated immunity is a desirable outcome of vaccination, and generally only live-recombinant or -attenuated organism vaccines efficiently induce cellular immunity [90]. DNA vaccines, comprised of plasmid DNA encoding proteins from pathogens, have been demonstrated to induce both humoral and cellular immune responses, the latter due to the mimicking of the effects of live viruses, in that antigenic proteins are endogenously produced and efficiently presented by MHC class I, thus inducing $CD8^+$ T-cell responses [90]. Furthermore, the stability, simplicity, safety and ease of manufacture make DNA vaccines an attractive alternative to the use of live vaccines [90]. Several DNA vaccine candidates have been reported for SARS-CoV proteins, including those for S [12,78,91–94], M [95] and N proteins [87,88], all of which can generate antibody and cellular immune responses [94].

A DNA vaccine expressing S protein induced both T-cell and neutralizing antibody responses, and reduced SARS-CoV replication in the lungs [78]. Furthermore, this study showed that protection was mediated by antibodies to the S gene, and was not T-cell dependent in mice [78]. Careful construction of the S plasmid (with splice sites and viral RNA export sequences) has now been shown to markedly increase efficacy of S-DNA vaccine in the mouse model [93], but these vaccines have not been tested in other animal models. A multiple-epitope DNA vaccine strategy elicited induction of antibody responses in mice to two epitopes, S (437–459) and M (1–20), which were able to neutralize SARS-CoV infectivity *in vitro* [95], but protection was not assessed.

Mice vaccinated with the N-DNA vaccine produced N-specific antibody and cytotoxic T-lymphocyte activity [87,96], although in one study this was also reported to induce a delayed-type hypersensitivity reaction, which might be problematic in a vaccine [96]. N-DNA vaccine, in which the N protein is expressed and linked to LAMP in order to enhance MHC class II presentation, increased memory responses [97]. DNA vaccination with SARS-CoV N protein linked to calreticulin to increase MHC class I presentation not only generated potent N-specific humoral and T-cell-mediated immune responses against N protein-expressing cells but also significantly reduced the titer of challenging vaccinia virus expressing the N protein [88]. These data suggest that such a response might also successfully target SARS-CoV-infected cells. A N-DNA vaccine candidate was also investigated in HLA-transgenic mice and elicited a specific $CD8^+$ T-cell response in this model [98]. DNA vaccines expressing the M protein have also been shown to induce neutralizing antibody and cytotoxic T-lymphocyte activity in mice [99]. Interestingly, in a study comparing S-, M- and N-DNA vaccines, M gave the strongest T-cell response [94].

Although DNA vaccines show great promise in preclinical models, their efficacy in clinical studies has often been disappointing. Thus, various prime–boost strategies have been developed that increase efficacy. DNA vaccination may be performed in conjunction with a heterologous prime or boost with proteins, inactivated viral vaccine candidates or viral vectors [12,37,95,100,101]. These strategies often provide superior immune responses and can also determine the magnitude and type of immune response (e.g., Th1/Th2). While DNA vaccines for SARS may hold promise, evidence of protection in a good SARS animal model is needed.

Attenuated vaccines

The most long-lasting and protective vaccines are those comprised of an attenuated pathogen or a closely related but avirulent live virus, such as the use of the naturally occurring vaccinia virus, a low-virulence member of the same genus, to vaccinate against smallpox. These vaccines are more efficacious due to their persistence in the host, possession of pathogen-encoded immune-activating moieties, and their appropriate location both in the body and in the cell, yielding endogenouS protein production and

efficient MHC class I presentation, generating a robust cytotoxic T-cell response. The difficulty with attenuated vaccines is that attenuating point mutations may revert causing virulence, and deletion-attenuated mutants may recombine with naturally occurring environmental wild viruses to regain virulence, as has been seen with the attenuated oral poliovirus. Given safety concerns, it is often difficult to gain regulatory approval of attenuated vaccines without strong proof that the threat of disease is sufficient to warrant the use of such a vaccine. For SARS, this threshold has not yet been met, but some interesting attenuated mutants have been developed. The immunogenicity and protective efficacy of a live-attenuated vaccine consisting of a recombinant SARS-CoV lacking the small E gene were studied. Deletion of E causes reduced viral morphogenesis and virus titers *in vitro* and *in vivo*, and thus attenuates the virus [102,103]. Hamsters immunized with this deletion mutant developed high levels of serum-neutralizing antibodies and were protected from clinical signs (decreased activity) and replication of homologous (SARS-CoV, Urbani) and heterologous (GD03) SARS-CoV in the upper and lower respiratory tract [104]. Thus, deletion of the structural E gene may be a first step toward development of a live-attenuated SARS-CoV vaccine. The deletion of the *nsp-1* gene in the related CoV mouse hepatitis virus (MHV) has been shown to create a highly efficacious attenuated vaccine, suggesting that this approach may also be attempted for the development of a SARS-attenuated vaccine [105]. Two comprehensive studies on gene deletion and attenuation effects showed that deletion of ORFs 3a, 3b, 6, 7a, 7b, 8a, 8b or 9b (highlighted in Table 1) had little or no effect on viral replication both *in vitro* and *in vivo* [103,106]. It is not known if deletion of these genes might have an attenuating effect in higher mammals. However, given the disappointing protection afforded by most SARS-CoV vaccine strategies explored to date, it seems that further exploration of attenuated SARS-CoV vaccines is justified. In that vein, exploration has been undertaken on the effects of rearrangement of the SARS-CoV genome, which has been shown to be attenuating in MHV [107]. This system has the additional advantage of making recombination of the vaccine with wild CoVs (thus restoring virulence) less likely. Other strategies to protect from virulence-restoring recombination events include multiple attenuating gene knockouts, with or without growth of the virus in *trans*-complementing cells lines, and replacement of transcriptional regulatory sequences (analogous to promoters in most systems) with sequences incompatible for wild-type gene expression. These strategies have been fully and elegantly described in a recent review [108].

Animal models

Animal models developed for SARS include macaques [3,109], African green monkey [64,110], ferrets [9], mice [111,112] and hamsters [113], and the Chinese masked palm civet [114]. An excellent review of animal models was recently released [115], and we will focus here on some of the newer developments, especially in ferrets.

Mouse models are of questionable use for efficacy studies as they do not reproduce the clinical signs or severe disease of SARS in humans [111,112], unless immunodeficient or aged mice are used [116]. However, the model has been improved by the use of mouse-adapted SARS and human ACE2 transgenic mice, although both models still have significant caveats [115,117–119]. Hamsters also do not exhibit clinical signs of SARS-CoV infection. Ferrets have been used widely for the study of influenza and are susceptible to SARS-CoV infection, with lung pathology and virus shedding [9,120]. One ferret study indicated that upon intranasal administration of SARS-CoV Toronto 2 strain, no clinical signs were observed, although viral RNA could be detected in pharyngeal swabs [63,121]. However, several other studies showed the ferret to be one of the better models for the display of clinical signs, viral replication and lung pathology [51], reflecting SARS pathogenesis in humans [122].

In the most recently reported ferret study[52], SARS-CoV challenge resulted in ferrets with clinical signs of infection (elevated temperature, nasal discharge and sneezing). No other animal, including cynomolgus macaques, has been reported to regularly experience fever, which is the most common sign in human SARS-CoV infection, (>99%) [123,124]. Thus, ferrets are a good model for SARS-CoV because they support replication in the upper and lower respiratory tracts, develop clinical disease, shed virus from the upper airway and develop severe lung pathology. Ferrets are also outbred, allowing the assessment of a range of individual responses that are documented in human SARS. Finally, the ferret model is a nonrodent model and is significantly less expensive and difficult to study than nonhuman primates. The main disadvantages of this model are that the ferret immune system is not well defined, there is a dearth of reagents and, as they are outbred, larger numbers are needed to assess statistical significance.

Vaccine enhancement of disease

The greatest fear among vaccinologists is the creation of a vaccine that is not only ineffective, but which exacerbates disease. Unfortunately, CoV vaccines have a history of enhancing disease, notably with feline CoVs [80,125]. While several mechanisms may exist, the best understood is antibody-mediated entry of virus into cells via immunoglobulin Fc receptors. This has been demonstrated to occur for the SARS-CoV S protein in human B-cell lines [126], however, the same group showed that SARS-CoV S protein-vaccinated animals showed no signs of enhanced lung pathology or hepatitis, and indeed that the viral load was reduced following challenge with SARS-CoV; although hamsters may not respond in a way similar to humans immunologically [126]. Other groups have also shown that administration of anti-S antibody does not enhance disease upon SARS-CoV challenge in mice or ferrets [78], again suggesting that antibody is not enhancing disease in SARS-CoV infection.

There have only been two reports of possible vaccine-induced pathology in SARS vaccine trials to date. In one study, ferrets vaccinated with the poxvirus vector MVA expressing SARS-CoV S protein displayed increased liver pathology after challenge [63] compared to other groups, but liver pathology has not been increased with any other SARS-CoV vaccines. In our ferret vaccine trials using S and N proteins, we noted a delayed histopathology in vaccinated groups, but no increase in pathology compared

to unvaccinated groups [52]. The lack of vaccine enhancement of disease is further supported by a recent study in WKV-vaccinated and challenged ferrets that were followed for 3 weeks [53]; however, the exact details and combination of vaccine vector and antigen may control this phenomenon. In the other study that raised vaccine safety concerns, vaccination with the N protein expressed in Venezuelan equine encephalitis virus replicon particles was reported to increase eosinophilic infiltration and damage in lungs of mice challenged with SARS-CoV [127]. This has not been reported in other studies, and in our studies with combination S and N protein expressed in Ad, no eosinophillic infiltration was noted in mice or ferrets [49,52]. While each vaccine and antigenic combination must be thoroughly evaluated for safety and efficacy, the overall picture for SARS-CoV vaccines shows no particular reason for concern with vaccine enhancement of disease. In the cases where it has been reported, it appears to be confined to a particular expression system rather than specifically related to any antigen. In the vast majority of studies, immunogenicity has been elicited without any negative impact on health after challenge with the virulent pathogen.

What mediates protection

Individuals convalescing from SARS develop high titers of neutralizing antibodies [128], and the appearance of antibodies coincides with the onset of resolution of SARS pneumonia [129,130]. In addition, antibodies to SARS S glycoprotein or whole SARS-CoV administered in several animal models have been shown to prevent or reduce SARS-CoV replication and disease [48,62,68,78,94,111,113,120,131–133]. However, we have shown that, while inactivated SARS with alum vaccine induced 15-fold higher serum-neutralizing antibody titers than the other vaccines (Ad-vectored), this vaccine did not universally protect ferrets better from SARS-CoV challenge [52]. These data provide a cautionary note about SARS rodent models, and indicate that the induction of strong neutralizing antibodies does not equate with protective efficacy in a relevant animal model where clinical signs are apparent and significant lung damage is seen. The ability of an antibody to neutralize virus infection is the easiest activity to measure, but it is not the only important function of antibody in antiviral defense. An important lesson may be drawn from the field of poxvirology where one of the most protective antigens (A33R) generates protective antibodies that are not neutralizing [134–136].

Several reports have indicated that intranasal vaccination may provide superior protection compared to other routes. A protollin-formulated SARS S protein delivered intranasally protected mice from SARS-CoV replication in the lung better than the same vaccine delivered intramuscularly, despite comparable serum levels of neutralizing anti-SARS IgG [137]. Presumably, this is due to the induction of IgA, which was detected only in the intranasally vaccinated animals. We found similar results in both mice and ferrets; the same vaccine given intranasally and intramuscularly gave stronger protection when delivered by the intranasal route, particularly in terms of viral load in the lung and shedding in nasal secretions [49,52]. Intranasal administration increases protection, despite greatly reduced serum antibody responses. These data underscore the importance of mucosal immunity.

Evaluation of T-lymphocyte responses in SARS protection has been problematic. Rodents may not provide an adequate disease model, and other models are difficult to evaluate because animals are outbread and there are inadequate reagents for measuring T-cell responses. Further studies are needed to determine the relative contributions of humoral and cell-mediated immunity in protection from SARS disease. Interestingly, in a study comparing S-, M- and N-DNA vaccines, M generated the strongest T-cell responses [94], and recovered SARS patients have long-lasting CD4 and CD8 memory to the M antigen [138]. These data suggest that further research should be directed toward evaluating the potential efficacy of the M antigen, as well as other viral proteins.

Conclusion

Much research to elucidate potential antigens, routes of vaccination, and methods for the design of SARS vaccines has been completed. Immunogenicity has been widely demonstrated, but identification of correlates of protection, and generation of immune responses that protect from clinical signs and lung damage remain elusive. Results suggest that a protective SARS vaccine should be possible; however, protection in mammals that are susceptible to severe disease (e.g., ferrets and humans) may be more difficult than the mouse models suggest. To date, the data indicate that the most efficacious vaccine strategy might be a heterologous combination of intranasal and systemic vaccination, since each delivers different aspects of protection. Given the incomplete protection of current vaccines, it seems unwise to discount T-cell responses, which have not been adequately evaluated, or the protection that might be afforded by the inclusion of additional viral proteins (especially those displayed on the virion and on the surface of infected cells) in SARS-CoV vaccine development.

Expert commentary & five-year view

Since protection from disease has not been demonstrated for any vaccine tested in an animal model that mimics human SARS disease, further vaccine development to improve vaccine efficacy is needed. While it is clear that antibodies to S protein offer some protection against SARS, it is equally apparent that high neutralizing antibody titers are not sufficient to protect animals from serious tissue damage after SARS-CoV challenge. Thus, it will likely be necessary to generate protective T-lymphocyte responses or antibodies to other SARS proteins, or to improve protection. Data collected thus far suggest that strategies including mucosal immunizations coupled with a heterologous systemic route of vaccination may improve efficacy. Alternatively, the development of safe attenuated SARS vaccines may be able to offer both the quality and quantity of immune response required to stop serious SARS-CoV-induced tissue damage. It will be necessary to conduct more trials with direct comparison of vaccines (and combinations of prime–boost) in appropriate animal models that more closely mimic human disease course. It will also be important to pursue an understanding of the role of T-cell immunity against SARS, since little is known at this time. Development of improved T-cell analysis reagents for ferrets will aid in this endeavor. The rate of SARS vaccine progress in the next 5 years likely depends on the perceived disease threat

from SARS. If another epidemic or pandemic occurs, funding and research for vaccine development will be a priority. Likewise, efficacy in humans can only be demonstrated if there is another SARS outbreak among a population of vaccinated and control subjects.

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Key issues

- Severe acute respiratory syndrome (SARS) caused thousands of human infections worldwide and hundreds of deaths in just a few months, and severely impacted the global economy. Evidence indicates that SARS-coronavirus (CoV) has continued to jump from animals to humans over several years, suggesting that another pandemic may occur.
- While SARS patients generated antibodies to multiple SARS proteins and antiviral T-lymphocyte responses, correlates of protective immunity are unknown.
- Inactivated virus, viral vector, bacterial vector, subunit and DNA vaccines encoding several antigens have been developed and shown to be immunogenic.
- Vaccine trials in animal models that mimic human disease indicate that no vaccine strategy tested to date would satisfactorily protect from disease, arguing for further vaccine development research, especially focusing on:
  – Mucosal immunization, which appears to be important for protection
  – Understanding T-cell immunity, which has been difficult to analyze
  – Combinations of heterologous vaccines in prime–boost regimens or attenuated SARS-CoV vaccines.

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