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Vaccines to prevent severe acute respiratory syndrome coronavirus-induced disease

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Abstract

An important effort has been performed after the emergence of severe acute respiratory syndrome (SARS) epidemic in 2003 to diagnose and prevent virus spreading. Several types of vaccines have been developed including inactivated viruses, subunit vaccines, virus-like particles (VLPs), DNA vaccines, heterologous expression systems, and vaccines derived from SARS-CoV genome by reverse genetics. This review describes several aspects essential to develop SARS-CoV vaccines, such as the correlates of protection, virus serotypes, vaccination side effects, and bio-safeguards that can be engineered into recombinant vaccine approaches based on the SARS-CoV genome. The production of effective and safe vaccines to prevent SARS has led to the development of promising vaccine candidates, in contrast to the design of vaccines for other coronaviruses, that in general has been less successful. After preclinical trials in animal models, efficacy and safety evaluation of the most promising vaccine candidates described has to be performed in humans.

Keywords

RNA viruses; Coronaviruses; SARS coronavirus; Vaccines

1. Introduction

Severe acute respiratory syndrome (SARS) is an infectious disease caused by a coronavirus (SARS-CoV) (Marra et al., 2003; Rota et al., 2003). SARS was detected for the first time in the Guandong province of China in late 2002, spread rapidly around the World and resulted in more than 8000 cases, 10% of which resulted in death, in 33 countries, and areas of five continents (http://www.cdc.gov/mmwr/mguidesars.en.html). World Healh organization (WHO) declared the end of the SARS outbreak in July 5th 2003, nevertheless, several isolated outbreaks subsequently occurred because of accidental contaminations in laboratories of Taiwan, Singapore, and mainland China (http://www.who.int/csr/sars/en/). In late 2003 and early 2004, newly infected persons who had contact with animals infected with SARS-CoV strains significantly different from those predominating in the 2002–2003 outbreak were reported in Guandong, China (Peiris et al., 2004). These events indicate that a SARS epidemic may recur at any time in the future, although this event most likely would require the generation of new isolates evolving from SARS-CoV-like virus circulating in animals (Jiang et al., 2005). As a consequence of this possibility, and because SARS-CoV could be used as a

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biological weapon, it has been declared as a category C priority pathogen by the National Institute of Allergy and Infectious Diseases Biodefense (http://www2.niaid.nih.gov/Biodefense/bandcpriority.htm).

SARS-CoV infection results in severe acute respiratory disease, pneumonia, diarrhea, and sometimes death (Peiris et al., 2003). SARS-CoV is a zoonotic virus that crossed the species barrier, most likely originating from bats, and has been amplified in other species, preferentially civets (Lau et al., 2005; Li et al., 2005; Woo et al., 2006). The SARS-CoV like viruses that exist in animals do not cause typical SARS-like disease in the natural host and are not easily transmitted from animals to humans. Under certain conditions, the virus may have evolved into the early human SARS-CoV, with the ability to be transmitted from animals to humans and from humans to humans, resulting in localized outbreaks and mild human disease (Jiang et al., 2005). In fact, the virus most likely crossed the species barrier before the 2002 outbreak, as sera samples collected in 2001 were positive in 1.8% of the cases (Zheng et al., 2004). Furthermore, 40% of animal traders, whereas only a 5% of vegetable traders in Guandong markets were seropositive for SARS-CoV without showing signs of disease (Guan et al., 2003).

SARS-CoV vaccines are urgently needed to prevent potential SARS epidemics. As SARS-CoV disease severity has been linked to age, with higher mortalities for ages over 45, special attention should be paid to vaccine development to protect elderly people. Several types of vaccines are being developed, including inactivated viruses, subunit vaccines, virus-like particles (VLPs), DNA vaccines, heterologous expression systems, and vaccines derived from SARS-CoV genome by reverse genetics. This paper will review previously published SARS vaccination strategies and announce new accomplishments in SARS-CoV based vaccination approaches while focusing on the correlates of protection, detected serotypes, vaccination side effects, and bio-safeguards that can be engineered into recombinant vaccine approaches based on the SARS-CoV genome.

2. Complexity of SARS-CoV serotypes

Knowledge on the diversity of serotypes is essential information for vaccine design. Phylogenetic analysis of SARS-CoV isolates from animals and humans strongly suggest that the virus originated from animals, most likely bats (Lau et al., 2005; Li et al., 2005; Poon et al., 2005), was amplified in palm civets, and transmitted to human population via live animal markets (Kan et al., 2005).

The neutralization of a set of eight pseudoviruses expressing the spike glycoprotein of eight SARS-CoV strains selected from the three phases of the SARS epidemic (early, middle and late), plus another human isolate collected at the end of 2003 (GD03), and two civet cat isolates from 2003 (SZ16 and SZ3) has been studied (Chinese-Consortium, 2004; Yang et al., 2005). Human monoclonal antibodies against the S protein of the Frankfurt isolate (FRA-1), derived from Epstein-Barr virus transformed B lymphocytes were used. The virus tested in the neutralization assays included pseudotypes made with the S protein from members of the four main genetic clusters defined with the Bayesian analysis of the SARS-CoV glycoproteins (Fig. 1A) (Deming et al., 2006):

- **i.** Group 1, originating from animals isolated in 2003. A prototype of this group is the isolate SZ16 which primarily uses civet but not human angiotensin-converting enzyme 2 (ACE2) as a receptor.
- **ii.** Group 2, low pathogenic viruses originating from civets, raccoon dogs, or sporadic human cases, such as strain GD03 reported from a sporadic SARS case in December 22, 2003. This virus represented an independent introduction of a less pathogenic

virus, having an S glycoprotein sequence that is the most divergent of all human strains (Chinese-Consortium, 2004). In general, group 2 isolates appear to have a receptor binding domain (RBD) that is capable of recognizing the human ACE2 receptor, and have been successfully cultured. GD03 S glycoprotein contains 18 amino acid substitutions relative to group 3 Urbani S protein, many of which map within neutralizing epitopes between amino acids 130–150 and 318–510, corresponding to the RBD. Recombinant viruses encoding the GD03 S glycoprotein have been isolated using reverse genetics (Baric et al., 2006). Recombinant icGD03 virus replicates about 0.5–1.0 logs less efficiently in human airway epithelial cells (6.8×10^7 PFU/ml) as compared to *wt* Urbani (3.0×10^7 PFU/ml) at 42 h postinfection.

iii. Group 3, highly pathogenic viruses representing the 2002–2003 epidemic strains associated with the early, middle, or late phase. Prototypes of these viruses are the early isolate and middle isolates GZ02, and CUHK-W, respectively, and the late Urbani, FRA-1, or Toronto 2 (TOR-2) strains.

iv. Group 4, bat SARS-CoV strains have not been successfully cultured but were sequenced from samples taken from *Rhinolophus* spp. like the Chinese horseshoe bat. These viruses differ from Urbani by about 12–22% in amino acid sequence and generally have about 3–4 out of 13 contact interface residues with human ACE2 receptor. Using the S glycoprotein gene, an unrooted Bayesian analysis suggests that bat strains are most closely related to early phase human strains.

Basically, all virus pseudotyped with S proteins from different strains were neutralized to the same extent, except the human GD03 and the two civet cat isolates SZ16 and SZ3, indicating that there were at least two human SARS-CoV serotypes, most likely originated from two independent transmissions of the virus from civet cat to human (Baric et al., 2006; Yang et al., 2005). It will be important to determine if recombinant viruses bearing zoonotic S glycoproteins display similar neutralization kinetics as pseudotyped viruses bearing SARS spikes.

The SARS-CoV-like isolated from bats identified in *Rhinolophus* spp. has a nucleotide identity with TOR-2 strain higher than 92% (Lau et al., 2005; Li et al., 2005). In addition, six novel coronaviruses from six different bat species have been described (Woo et al., 2006). Four of these coronaviruses belong to group 1, and two of them to group 2. Based on sequence data these authors have proposed the classification of bat CoV in three subgroups (2a, 2b, and 2c). Subgroup 2b comprises both SARS-CoV and a bat-SARS-CoV (Rp3 isolate). The sequences of these SARS-CoV isolates differ in the S1 domain of the S protein, where sequence identity fell to 64%. This sequence divergence in the S1 domain corroborated the serum neutralization studies, which indicate that although bat sera have a high level of cross-reactive antibodies, they failed to neutralize human or civet cat SARS-CoV when tested in Vero E6 cells (Li et al., 2005). In contrast, other authors (Lau et al., 2005) have reported that 42% of the bat sera samples tested neutralized human SARS-CoV isolate (HKU-39849) using FRhK-4 cells. The discrepancy could be due to the presence of a different SARS-CoV-like virus serotype in the bats studied by the two different groups, or to the two test systems used in the evaluation. Consequently vaccine design should take into account this antigenic diversity. Interestingly, recent characterization of the antigenic structure of SARS-CoV S protein with a large panel of Mabs has shown that at least one epitope providing susceptibility to SARS-CoV that maps in the RBD for ACE-2 is highly conserved in most virus strains and therefore, may confer protection to most SARS-CoV strains (He et al., 2006).

3. Antigens involved in the protection against CoV induced infections

SARS-CoV has at least seven structural proteins (S, 3a, E, M, 7a, 7b, and N) (Fig. 2) (Huang et al., 2006;Schaecher and Pekosz, 2006;Weiss and Navas-Martin, 2005). SARS-CoV S, 3a, E, and M proteins are viral membrane proteins with domains exposed to the external face of

the virus (Ito et al., 2005;Shen et al., 2005) that, in principle, could be involved in protection by induction of neutralizing antibodies. It has been shown that proteins S and 3a induce *in vitro* SARS-CoV neutralizing antibodies, with S protein being the main component of protective immunity (Qiu et al., 2005;Saif, 2004). Although strong immune responses are elicited against both S and N proteins (Buchholz et al., 2004;Subbarao et al., 2004;Wang et al., 2005;Zhu et al., 2004), passive transfer studies illustrate that only S-specific antibodies confer protection from SARS-CoV replication in the mouse model (Bisht et al., 2004;Subbarao et al., 2004).

The relevance of S protein in protection against SARS-CoV has been reinforced by the identification of neutralizing antibodies from convalescent patients. The majority of the coronavirus neutralizing epitopes are located within the spike protein of the virus (Buchholz et al., 2004; Hogan et al., 2004). Two domains are defined in the spike protein, the amino-terminus (S1) and the carboxy-terminus (S2) halves. In some coronaviruses, but apparently not in the case of SARS-CoV, the S protein is processed in these two halves (Weiss and Navas-Martin, 2005; Wong et al., 2004). Recent evidence has determined that SARS-CoV neutralization is sensitive to deglycosylation of the spike protein, suggesting that conformational epitopes are important in antibody recognition (Song et al., 2004). The RBD located in the S1 subunit of S protein contains multiple conformational neutralizing epitopes. This suggests that recombinant proteins containing RBD, and vectors encoding the RBD sequence, can be used to develop safe and effective SARS vaccines (Jiang et al., 2005). The continuous viral epitopes targeted by antibodies in plasma samples from convalescent SARS patients have been identified by biopanning with a M13 phage display dodecapeptide library (Zhong et al., 2005). These epitopes converged to very short peptide fragments, one on each of the spike, nucleocapsid, 3a, 9b and nsp3 proteins. Immunoassays found that most of the patients (82%) that recovered from SARS developed antibodies to the epitope-rich region on the spike S2 domain, indicating that this domain also is an immunodominant site on the S protein. These S2-targeting antibodies were shown to effectively neutralize SARS-CoV. Moreover, it is possible that S2-specific antibodies provided protective immunity to help the patients recover from viral infection (Zhong et al., 2005). In fact, among the rabbit antibodies elicited by different fragments covering the entire S protein expressed in *E. coli*, some of them were specific for aa 1029–1192, which include the heptad-repeat sequence of the S2 domain that interacts to form S protein trimers, and had neutralizing activities, indicating that this region of the S protein also carries neutralizing epitopes.

SARS-CoV 3a protein consist of 274 amino acids, contains three putative transmembrane domains, and is expressed on the virus and cell surface (Ito et al., 2005; Tan et al., 2004b). The topology of 3a protein on the cell surface was experimentally determined; the first 34 aa, located before the first transmembrane domain, are facing the extracellular matrix (Akerstrom et al., 2006), and its C-terminal, after the third transmembrane domain (aa 134–274), is facing the cytoplasm (Tan et al., 2004b). Interestingly, in two separate cohorts of SARS patients, one from Taiwan (Liu et al., 2004) and the other from Hong Kong (Zhong et al., 2005), B cells recognizing the N-terminal region of 3a protein were isolated from patients. Moreover, significant proportion (40%) of the convalescent SARS patients examined in a dot blot assay using a synthetic peptide with a sequence corresponding to amino acids 12–27 of the N terminus of the protein were positive (Zhong et al., 2005). In addition, it was recently reported that the N-terminal domain of 3a protein elicits strong and potentially protective humoral responses in infected patients (Zhong et al., 2006). Accordingly, rabbit polyclonal antibodies raised against a synthetic peptide corresponding to aa 15–28 of 3a protein inhibit SARS-CoV propagation in Vero E6 cells, in contrast to antibodies specific for the C-terminal domain of the protein (Akerstrom et al., 2006).

SARS-CoV E, M, and 7a proteins have shown low immunogenicity (Tan et al., 2004a). Sera from three convalescent phase SARS patients do not recognize these proteins expressed in mammalian cells. Accordingly, SARS-CoV E protein peptides were not recognized by convalescent patient antisera using a protein microarray (Qiu et al., 2005). In a study using rabbit antibodies to 13 recombinant fragments associated with SARS-CoV S, E, M, N, 3a, 3b, 6, 7a, and 9b proteins, strong neutralizing antibodies were only elicited by the S1 fragment (aa 241–591) of S protein (Qiu et al., 2005). The incorrect folding of the proteins expressed in *E. coli* could be responsible for the lack of detection of antibodies to other viral proteins, such as the 3a protein that is known to induce neutralizing antibodies (see above).

The M protein of transmissible gastroenteritis coronavirus (TGEV) is required for virus assembly and budding, and M protein specific antibodies significantly, but weakly, neutralize TGEV and mediate complement-dependent lysis of TGEV infected cells (Delmas et al., 1986; Risco et al., 1995; Woods et al., 1987). Consistent with the TGEV data described above, it has been shown that SARS-CoV M protein also induced virus neutralizing antibodies in the absence of complement (Buchholz et al., 2004). In addition, a mixture of S and M proteins showed a synergistic effect in the *in vitro* synthesis of TGEV neutralizing antibodies by immune leukocytes (Anton et al., 1995). In the case of SARS-CoV, immunization of hamsters with a parainfluenza virus vector has shown a differential role of S, M, E, or N proteins in protection (Buchholz et al., 2004). Parainfluenza virus expressing S protein alone provided complete protection against SARS-CoV challenge in the lower respiratory tract and partial protection in the upper respiratory tract. This protection was slightly augmented by co-expression with M and E proteins (Buchholz et al., 2004). Nevertheless, expression of M, E, or N proteins in the absence of S protein did not confer detectable protection. These results identify S as a main SARS-CoV neutralization and protective antigen among the structural proteins, and confers a limited role to SARS-CoV M protein in protection.

SARS-CoV N protein specific antibodies do not neutralize the virus *in vitro* as it could be expected for an internal virus protein (Pang et al., 2004). However, SARS-CoV N protein induces T-cell responses (Gao et al., 2003). Accordingly, DNA immunization using SARS-CoV N gene induces potent Th1 polarized immune responses in mice, as well as specific antibodies in these animals. In fact, the highest levels of humoral response and T cell proliferation activity were induced by the N gene construct (Jin et al., 2005). Analysis of the immune response to another coronavirus (TGEV), using an *in vitro* antibody synthesis system, has shown that the optimum combination of viral proteins to stimulate the production of TGEV neutralizing antibodies *in vitro* was a mixture of S and N proteins, or a combination of S protein oligomers (rosettes) and the N or N protein-derived peptides (Anton et al., 1996; Anton et al., 1995). These data, in principle, suggest that N protein could be used in a vaccine to promote the synthesis of S-specific neutralizing antibodies.

9b protein (98 aa) elicits antibodies in SARS-CoV patients, indicating that it is expressed in natural disease and that it is immunogenic (Qiu et al., 2005; Zhong et al., 2005). In fact 100% of convalescent phase patients sera were positive for 9b protein. Based on this data, it has been speculated that 9b protein could be structural (Qiu et al., 2005). Nevertheless, the presence of 9b protein in SARS-CoV virions needs to be further proved.

In summary, with the available data, SARS-CoV proteins S and 3a elicit strong neutralizing antibody responses, whereas protein M only induces a reduced neutralizing humoral immune response. These antigens probably are relevant in the protection against SARS-CoV. In addition, other structural proteins (such as E, M, 7a, 7b, and N), and possibly protein 9b, could also play a role in protection.

4. Role of humoral and cellular compartments in protection against SARS

To study the role of the humoral immune response to SARS-CoV, spike specific monoclonal antibodies that neutralize the virus have been developed (Berry et al., 2004; Subbarao et al., 2004; Traggiai et al., 2004; Zhang et al., 2004). Passive transfer of these monoclonal antibodies or immune serum into naïve mice protected them from infection with SARS-CoV (Subbarao et al., 2004). Using Epstein-Barr virus transformed human B cells, the memory repertoire of a patient who recovered from SARS-CoV infection has been rescued (Traggiai et al., 2004). Some of the monoclonal antibodies exhibited neutralization activity *in vitro*, and some of them also conferred protection in a mouse model of SARS-CoV infection. In addition, human IgG monoclonal antibodies neutralizing SARS-CoV developed using phage display libraries protected ferret from lung disease and virus shedding in pharyngeal secretions (ter Meulen et al., 2004). In both mouse and ferret models, administration of human monoclonal antibodies with *in vitro* neutralization activity reduced SARS-CoV titers in the lungs 3 to 6-log10-unit, protecting from lung pathology in ferrets (ter Meulen et al., 2004). Overall, these data indicate that humoral immune responses alone can protect against SARS.

Immune responses to SARS-CoV, elicited by a DNA vaccine encoding a codon optimized SARS spike protein (Yang et al., 2004), or the S1 fragment, induced neutralizing antibodies (Zeng et al., 2004), as well as T-cell responses. Nevertheless, protection from SARS-CoV challenge was mediated by a humoral immune response but not by a T-cell-dependent mechanism (Yang et al., 2004).

Surprisingly, immunodeficient mice can clear a SARS-CoV infection, showing the role of innate immune responses in the defenses against SARS-CoV. C57BL/6 mice that lack NK-T cells ($CD1^{-/-}$), or NK cells, or those that lack T and B cells ($Ragl^{-/-}$) cleared the virus by day 9 after infection (Glass et al., 2004) and displayed high induction of proinflammatory cytokines. These data suggest that the NK cells and the adaptative immune response were not essential for virus clearance in mice. Possibly, interferon pathways were relevant in viral clearance. The importance of interferon response was reinforced by infecting Stat1-deficient mice with SARS-CoV (Hogan et al., 2004). Stat1 is important to the regulation of interferons, and Stat1-deficient mice produced one hundred-fold increase in viral titer over control mice. Additionally, the mutant mice developed interstitial pneumonia, not seen in control mice (Hogan et al., 2004) but not alveolar damage, as seen in lungs of human patients. It is unclear at this time if the observed pathological differences between human and Stat 1-deficient mouse lungs were due to time of sampling or to differences in host responses (Hogan et al., 2004).

5. Development of non-replicating SARS-CoV vaccines

5.1. Inactivated virus vaccines

Neutralizing antibodies were detected 2–3 weeks after the onset of disease in immunocompetent SARS patients, and 90% of patients recovered without hospitalization (Ksiazek et al., 2003). These data indicate that most patients successfully respond to SARS-CoV infection. Although many types of vaccines for SARS-CoV have been attempted such as expression of recombinant proteins, or the use of virus vectors, these vaccines require considerable research set-up time (Bradbury, 2003). Therefore, the classical approach using inactivated, cell-culture based SARS-CoV is likely to be the easiest way for SARS vaccine development, based on the experience with available vaccines including inactivated or live polio and rabies vaccines (Montagnon, 1989; Zhou et al., 2005). This is the case of companies that favored the development of an inactivated candidate whole virus vaccine, based on the well-established technologies for the development of such vaccines (Spruth et al., 2006).

The fast spreading of SARS initially prompted a Chinese company (Sinovac Biotech of Beijin) to develop a vaccine in collaboration with the Chinese Academy of Medical Sciences that started tests of the vaccine, an inactivated form of SARS-CoV as early as 2004, using 30 volunteers (Marshall and Enserink, 2004). No side effects were reported for the first patients that were injected with the inactivated virus.

SARS-CoV inactivation to produce the killed vaccines has been performed using different methods. For instance, SARS-CoV purified by ultracentrifugation has been inactivated with β-propiolactone and administered with or without Alhydrogel as adjuvant (See et al., 2006). The virus subcutaneously administered to mice was more efficient than recombinant adenoviruses expressing either the S or N proteins, provided by the intranasal or intramuscular routes. An alternative method was the inactivation of SARS-CoV by β-propiolactone before initiating the purification step. Immunization with this virus was compared with vaccination using DNA or adenovirus vectors. The humoral immune response was most effective using inactivated virus with adjuvants such as MF59 (Chiron Vaccines) or Alum (Pierce, Rockkford, IL), and was associated with stimulation of the CD4 but not the CD8 response, supporting the use of inactivated SARS-CoV as vaccine (Kong et al., 2005).

SARS-CoV has also been purified up to 98% homogeneity by ultrafiltration, gel filtration, and exchange chromatography, and inactivated with β-propiolactone. Cynomolgus macaques were immunized with different amounts of the purified virus in the absence or in the presence of adjuvant. Monkeys were challenged by the nasal route 30 days post-immunization. High levels of neutralizing antibodies that prevented the replication of SARS-CoV and interstitial pneumonia were induced (Qin et al., 2006).

Interestingly, no side effects were observed even in the presence of low titer neutralizing antibodies, indicating that the purified SARS-CoV vaccine is safe in monkeys.

In other approaches, SARS-CoV partially purified in sucrose cushions was completely inactivated with formaldehyde. This virus efficiently competed the binding of infectious virus to cells, indicating that the inactivated virus kept a functional RBS (Qu et al., 2005). Polyethyleneglycol precipitated virus alone or in the presence of cholera toxin B (CTB, Sigma) or CpG, administered to mice by the intranasal route elicited serum SARS-CoV-specific neutralizing antibodies, and IgA specific antibodies in the trachea and lungs (Qu et al., 2005).

In another approach, formaldehyde inactivated whole virus, prepared in Vero cells, was used in intramuscular immunization of 2–5 year-old rhesus monkeys (Zhou et al., 2005). After 3 weeks, monkeys were challenged with SARS-CoV. Doses of 0.5 or 5 μg/monkey protected most of the monkeys against challenge with 10^8 pfu of SARS-CoV, and higher doses (50 μg) conferred complete protection. In contrast, the control animals developed a typical SARS-CoV infection after challenge. The immunization preferentially induced Th1 responses, but also enhanced other cellular immune responses, including the production of IFN-γ that can increase the activity of natural killer cells and inhibit virus replication. No systemic side effects were observed in vaccinated animals post-immunization, even at the high dose (5000 μg/monkey) and after two injections.

One vaccine manufactured to large scale using fermenter cultures of Vero cells in serum free medium has been based in a double-inactivated, whole virus vaccine (Spruth et al., 2006). Virus infection at a moi of 0.001 resulted in generation of high viral titers (around 10^8 $TCID_{50}$/ml). Culture supernatants were harvested and inactivated by formalin treatment followed by UV inactivation. This two step inactivation procedure was utilized in order to ensure a high safety margin with respect to residual infectivity. Mice immunized twice with l μg of SARS-CoV vaccine using adjuvant (0.2% aluminium hydroxide) developed high antibody titers against SARS-CoV spike protein, as determined by an ELISA test. The use of the adjuvant $Al(OH)_3$

had only a minor effect on the immunogenicity of the vaccine. In addition, cell mediated immunity, as measured by the production of INF-γ and IL-4 stimulation, was elicited. The vaccine confers 100% protection that was correlated to antibody titer against the SARS-CoV S protein and to neutralizing antibody titer. Induction of neutralizing antibodies with titers ≥114 and an S-specific ELISA titer ≥25,600 resulted in 100% protection against intranasal challenge with 10^5 $TCID_{50}$ of infectious virus.

5.2. Subunit vaccines

A polypeptide containing amino acids 14–762 of the SARS-CoV spike protein has been expressed using the baculovirus system (Bisht et al., 2005). The affinity purified protein was administered to mice with either saponin or Ribi as adjuvants. Both regimens induced binding and neutralizing monoclonal antibodies, although the best results were obtained with saponin and polypeptide, which provided the highest antibody response. After challenge, protective immunity was shown by the reduction of SARS-CoV titers in the upper and lower respiratory tract. This subunit vaccine induced higher neutralizing antibody and more complete protection against an intranasal challenge than that achieved by inoculation of mice with live SARS-CoV (Subbarao et al., 2004), vaccinia virus Ankara (MVA) expressing the full length S protein (Bisht et al., 2004), or DNA expressing the full-length S or S protein lacking the transmembrane and cytoplasmic domains (Yang et al., 2004).

N protein by itself does not provide protection against SARS-CoV infections. Still, a large number of reports using N protein as an antigen have been published. The immune response of mice vaccinated with a purified N protein fused to glutathione S-transferase (GST) was analyzed and compared with the response of two DNA-based vaccination approaches (Gupta et al., 2006). The immunization with N-GST elicited a strong T-cell IL-4, and antibody responses but minimal IFN-γ response. This response differed markedly with the immune response shown by mice immunized with both DNA encoding unmodified cytoplasmic N protein, and DNA encoding N as a LAMP-1 chimera targeted to the lysosomal MHC II compartment. DNA immunizations elicited a strong T-cell IFN-γ and CTL responses. Nevertheless, the T-cell responses to the three immunogens were elicited by the same N peptides as shown by the ELISPOT analysis of antigen-activated T cells. *In vivo* protection experiments were not performed with these vaccines.

The N protein of SARS-CoV was expressed in *E. coli* and purified (Liu et al., 2006). Balb/c mice were vaccinated with N protein emulsified in Montanide ISA-51 containing the oligodeoxynucleotide CpG, or in PBS. In the first case, anti-N antibodies were found to be mainly IgG2a, suggesting a prevalence of Th1 immune response. In contrast, anti-N protein antibodies of mice immunized with N protein in PBS were found to be mainly IgG1. Reactivity of antisera raised against N protein formulated in ISA-51/CpG in mice and monkeys and that of sera from patients were tested with a panel of overlapping peptides. The region around residues 156–175 of N protein is immunogenic in the three models. In addition, peptides corresponding to residues 1–30, 86–100, 306–320 and 351–365 contained murine immunodominant T-cell epitopes. Using and IFN-γ secretion cell assay, peptides containing residues 81–95 were capable of stimulating $CD4^+$ and $CD8^+$ cell proliferation *in vitro*. Peptides corresponding to residues 336–350 were capable of stimulating INF-γ production in T-cell cultures derived from peripheral blood mononuclear cells (PBMCs) of macaques immunized with the N protein emulsified in ISA-51/CpG. No protection experiments were performed with this immunogen.

5.3. Virus like particles (VLPs)

The requirements for SARS-CoV VLP formation differs using different expression systems and cell types. Production of viral proteins in insect cells using baculovirus has shown

intracellular SARS-CoV VLPs assembly by expressing M and E proteins (Ho et al., 2004). Secretion of these VLPs to the extracellular media required the co-expression of S protein (Mortola and Roy, 2004). In these experiments secretion of VLPs was relatively efficient (200 μg per 1×10^9 infected cells). These results are at variance with those obtained by expressing SARS-CoV proteins in human 293 renal epithelial cells under the control of cytomegalovirus promoter, using DNA plasmids (Huang et al., 2004). In this system, any combination of genes that expressed M and N proteins, with or without S or E proteins, generated intracellular VLPs, and the pseudoparticles did not form in the absence of the M and N proteins. No single viral gene was able to support the formation of viral capsids within these cells. The additional expression of the S protein allowed the formation of budding particles with morphology typical of SARS and related coronaviruses. Different types of VLPs could be formed depending of the protein composition of these SARS-CoV VLPs. Protection by the administration of VLPs has not been reported.

6. Vaccines produced in plants

The development of plant-based vaccines against different coronaviruses [TGEV, infectious bronchitis virus (IBV), and porcine epidemic diarrhea virus (PEDV)] using the oral delivery of recombinant S protein that elicit protective immunity has been reported (Bae et al., 2003; Lamphear et al., 2004; Tuboly et al., 2000; Zhou et al., 2003). One of these studies include a S protein plant-based vaccine candidate against TGEV that has advanced into early phase farming trials (Tuboly et al., 2000). More recently, to develop a safe, effective, and an inexpensive vaccine candidate, the S1 domain of SARS-CoV S protein has been expressed in tomato and low-nicotine tobacco plants (Pogrebnyak et al., 2005). High expression levels of recombinant S1 protein (>0.1% total soluble protein) were observed in several transgenic lines by Western blot analysis using S protein specific antibodies. Plant-derived antigen induced systemic and mucosal immune responses in mice, which showed significantly increased levels of SARS-CoV specific IgA after oral ingestion of tomato fruits expressing S1 protein (Pogrebnyak et al., 2005). Sera of mice parenterally primed with tobacco-derived S1 protein revealed the presence of SARS-CoV-specific IgG.

7. DNA vaccines

Several approaches based on DNA vaccination have been described in order to elicit protection against SARS. Two of them used prime-boost strategies and showed that the combination of the DNA vaccine and the whole chemically inactivated vaccine can be used to enhance the magnitude of the immune response, and also to change the balance of humoral to cellular immune response (Zakhartchouk et al., 2005a). A combination of the DNA and inactivated virus induces Th1 immune responses while the whole killed virus vaccine induces Th2 immune responses.

Mice immunized intramuscularly with a DNA vaccine expressing S protein and intraperitoneally boosted with *E. coli* expressing S peptides showed high neutralization titers (>1:1280). This vaccine might have a practical value to immunize in farms growing civet cats due to its low cost (Woo et al., 2005).

Other DNA vaccines express N protein alone or linked to calreticulin. The first ones preferentially induce IgG responses of the IgG2a isotype, IFN-γ and IL-2, and $CD8^+$ CTL responses to N protein, but produce strong delayed-type hypersensitivity (DTH) that could have undesired side effects (Zhao et al., 2005). The expression of N protein linked to calreticulin increases major histocompatibility complex (MHC) class I presentation to $CD8^+$ T cells, in the absence of reported adverse effects in mice (Kim et al., 2004). These vaccines led to the generation of strong N-specific humoral and T-cell-mediated immune responses in mice, but no protection experiments were shown. N protein has also been expressed linked to

hLAMP (N-hLAMP), that target antigen to the MHC class II, leading to a stronger and higher memory cellular immune response associated to high IFN-γ production than immunization with N protein alone (Gupta et al., 2006).

Studies on DNA immunization to protect against SARS, using three forms of the spike protein: full-length S, and S proteins with the cytoplasmic or the transmembrane domains deleted have been reported (Yang et al., 2004). These vaccines induced neutralizing antibodies and T-cell responses, resulting in protective immunity in mice. Viral replication was reduced more than six orders of magnitude in the lungs of mice vaccinated with these S plasmid DNA expression vectors. Protection was mediated by a humoral but not a T-cell dependent immune mechanism, as shown by adoptive T-cell transfer in which donor T-cells were unable to reduce pulmonary viral replication in recipient animals. By contrast, passive transfer of purified IgG from immunized mice, but not control IgG provided immune protection against SARS-CoV (Yang et al., 2004). The vector expressing the S protein with the cytoplasmic domain partially deleted induced the most potent neutralizing antibody response.

8. Development of SARS-CoV vaccines based on viral expression vectors

8.1. Poxvirus expression vector based vaccines

Using the highly attenuated modified MVA, SARS-CoV S protein has been expressed by several groups (Bisht et al., 2004; Chen et al., 2005). The first one has shown that intranasal or intramuscular immunization of Balb/c mice elicited protective immunity as shown by the reduction of SARS-CoV titers in the upper and lower respiratory tract after challenge. Furthermore, passive transfer of serum from mice immunized with the recombinant MVA expressing S protein to naïve mice also reduced the replication of SARS-CoV in the respiratory tract after challenge, demonstrating a role for S protein specific antibodies in protection. The second group (Chen et al., 2005) showed the induction of neutralizing antibodies in mice, ferrets, and monkeys, although protection experiments were not performed. In an antibody absorption assay, the majority of the antibodies raised by the MVA recombinant expressing the full-length S protein were absorbed by an S protein fragment including aa 400–600, that includes the RBD, indicating that the major SARS-CoV neutralization mechanism likely occurs through blocking the interaction between the virus and the cellular receptor ACE2.

In contrast, other authors immunized ferrets using MVA expressing the SARS-CoV S protein, inducing a vigorous immune response that did not prevent virus infection and spreading (Czub et al., 2005; Weingartl et al., 2004). Liver inflammation was found in all MVA-spike vaccinated ferrets. These authors suggested that their results indicate the induction of antibody dependent enhancement (ADE) of disease similar to that caused by feline infectious peritonitis virus (FIPV).

8.2. Adenovirus vector based vaccines

SARS vaccines based on the use of adenovirus vectors have shown that expression of S protein alone or in combination with N protein led to the protection of mice against the challenge with SARS-CoV. The efficacy of immunization with adenovirus vectors was compared with that of chemically inactivated partially purified virus. Whole-killed virus vaccine was more effective in conferring protective immunity against live SARS-CoV (See et al., 2006). Other adenovirus vaccines tested in mice have expressed either the S or the N protein (Zakhartchouk et al., 2005b) and shown that the S2 domain and the N protein contain strong T-cell epitopes, but reported no challenge experiments. In the monkey model, adenovirus-based vaccines induce strong SARS-CoV-specific immune responses, indicating that these vectors are promising vaccine candidates but, again, no information on protection have been provided (Gao et al., 2003). The adenovirus Ad5 vector with a deletion in the E1 and E3 regions, have

also been used to express the S1 domain of the SARS-CoV S protein (490 aa) (Liu et al., 2005). Wistar rats immunized three times throughout consecutive weeks produce antiserum capable of protecting from SARS-CoV infection in cell culture. Histopathological examination found no evident side effects in the immunized animals. Nevertheless, *in vivo* protection experiments were not performed. Therefore, additional experiments are required with the adenovirus based SARS vaccines.

8.3. Venezuelan equine encephalitis (VEE) virus vector based vaccines

Severe disease and high death rates were noted in senescent human populations infected with SARS-CoV, while children under 12 years of age did not develop the severe disease that was seen in adults (Baric et al., 2006; Deming et al., 2006; Ng et al., 2004). These data suggest that the quality of the immune response may play a role in the outcome of virus infection. The ability of vaccines to induce robust immune responses in senescent populations has been evaluated to determine if protection can be elicited in elderly populations with senescent immune systems. To evaluate vaccine efficacy against homologous and heterologous strains, the Urbani S glycoprotein and nucleocapsid genes were inserted in VEE virus replicon particles (VRP-S or VRP-N) (Baric et al., 2006; Deming et al., 2006). In addition, expression of the influenza A HA glycoprotein (VRP-HA) was used as a control. Using reverse genetics, synthetically resurrected recombinant viruses bearing the GD03 S glycoprotein that replicated to high titers in Vero and human airway epithelial cells have been obtained (Baric et al., 2006; Deming et al., 2006). Importantly, human convalescent sera had plaque reduction neutralization titre of 50% ($PRNT_{50}$) values of about 1:1600 against late phase isolates like Urbani, yet were reduced about 10–15 fold against the heterologous icGD03 virus ($PRNT_{50}$ 1:150) (Fig. 3A). Young and senescent Balb/c mice with ages exceeding 1 year at the time of challenge, were vaccinated with VRP-HA, VRP-S, VRP-N, or a combination of VRP-S and VRP-N, and challenged with recombinant SARS-CoV expressing the Urbani S protein or the antigenically different GD03 S protein (Fig. 3B). In vaccinated animals, VRP-S vaccines provided complete short- and long-term protection against homologous challenge, protecting both young and senescent mice from the Urbani strain replication. After challenge, VRP-S and VRP-S + VRP-N vaccinated mice displayed little if any pathologic lesions in the lung, whereas VRP-HA vaccinated aged mice demonstrate pathological lesions in the lung similar to that reported in the literature (Roberts et al., 2005a). VRP-S vaccines also provided short-term protection in young mice challenged with the heterologous GD03 S strain, despite the significantly reduced ability of anti-Urbani S antibody to neutralize virus expressing GD03 S. In contrast, vaccination of senescent mice with VRP-S provided limited protection (~38%) and the combination of VRP-S + VRP-N vaccines provided little long-term protection against infection by the antigenically different SARS-CoV GD03, although virus titers were reduced about 10-fold compared with VRP-HA controls. The SARS-CoV GD03 challenge also produced pathological lesions in both the VRP-HA and SARS-vaccinated animals that were virtually indistinguishable from those produced by infection with the SARS-CoV-Urbani strain. Therefore, it is likely that declining immunity of senescent animals in combination with the reduced ability of antibody to neutralize heterologous challenge viruses resulted in vaccine failure in aged animals. It seems that vaccine approaches that induce less robust neutralization responses like DNA and killed vaccines, might completely fail in protecting senescent populations against SARS-CoV GD03 challenge.

SARS-CoV disease severity was linked to age and other co-morbidities, with mortality rates increasing with age and exceeding 50% in individuals over 65. It is also known that elderly respond poorly to new antigens as compared to younger populations, but overall this phenomena is poorly studied. Consequently, vaccine efficacy in the elderly is a key property of efficacious SARS-CoV vaccines. Immunosenescence is common in animal models and in clinical studies that occur during aging, and vaccine efficacy is often attenuated in the elderly

(Frasca et al., 2005; Song et al., 1997; Zheng et al., 1997). Immune complications include a generalized decrease in the function of B and T cell and innate immune function, diminished macrophage and granulocyte function, diminished cellular traffic, cell growth and differentiation and decreased natural killer cell numbers and activity. New and different vaccine regimens should be developed and tested with the ultimate goal of eliciting complete protection against antigenically heterologous forms of SARS-CoV, especially in the most vulnerable elderly populations, and that there is a need for further testing developing vaccines that induce an anti-N response in more animal models, similarly to what has been described in other viral systems (Frech et al., 2005; McElhaney, 2005).

8.4. Parainfluenza based vectors

A vector based in an existing live attenuated parainfluenza virus, that is being developed for intranasal pediatric immunization against human parainfluenza virus type 3 (HPIV3) was used to express SARS-CoV S protein (Bukreyev et al., 2004). Vector administration to monkeys resulted in the production of systemic immune response by mucosal immunization. After challenge with SARS-CoV, all monkeys in the control group shed SARS-CoV. In contrast, no viral shedding occurred in the group immunized with the parainfluenza vector expressing the S protein. Recombinant viruses expressing SARS-CoV structural S, M, and N proteins, individually or in combination, have been evaluated for immunogenicity and protection in hamsters that support the replication of both SARS-CoV and parainfluenza vector (Buchholz et al., 2004). A single intranasal administration of the vector expressing the S glycoprotein induced a high titer of SARS-CoV neutralizing antibodies, only two fold lower than that induced by SARS-CoV infection. This response provided complete protection against SARS-CoV challenge in the lower respiratory tract, and partial protection in the upper respiratory tract. In contrast, expression of M, N, or E proteins did not induce detectable serum SARS-CoV neutralizing antibodies.

8.5. Rhabdovirus based vector

A recombinant rabies virus vector has been used to express the S protein of SARS-CoV (Faber et al., 2005). Immunogenicity studies in mice showed the induction of SARS-CoV neutralizing antibodies after a single dose, but no protection studies have been shown. Similarly, an attenuated vesicular stomatitis virus (VSV) vector was used to express the S protein of SARS-CoV (Kapadia et al., 2005). Mice vaccinated with VSV-S developed SARS-CoV neutralizing antibodies that controlled challenge with SARS-CoV performed at 1 or 4 months after a single vaccination.

In summary, immunization to prevent SARS using different live vector systems has shown that protection is mainly mediated by humoral immune responses to the S protein. A warning signal was that, at least with some vectors such as VEE virus, the expression of N protein may lead to side effects.

9. SARS-CoV vaccine candidates engineered by reverse genetics

9.1. Vaccines based on the deletion of single or several accessory combinations of genes

The effect of the deletion of group-specific genes in different coronaviruses has been studied. Reports using mouse hepatitis virus (MHV) as a model have shown that deletion mutants removing ORFs 4, 5a, 7a, and HE are attenuating in the natural host (de Haan et al., 2002a). Similarly, studies deleting ORF 7 of TGEV (Ortego et al., 2003) and ORFs 3abc and 7ab of FIPV (Haijema et al., 2004) led to virus attenuation. However, SARS-CoV deletion mutants lacking ORFs 3a, 3b, 6, 7a, or 7b did not significantly influence *in vitro* and *in vivo* replication efficiency in the mouse model (Yount et al., 2005). All recombinant viruses replicated to wild-type levels in the murine model, suggesting that either the group-specific ORFs play little role

in *in vivo* replication efficiency or that the mouse model is not of sufficient quality for discerning the role of the group-specific ORFs in disease. In fact it has been surprising that ORFs like 3a, 7a, and 7b, that encode structural virus proteins, (Huang et al., 2006; Schaecher and Pekosz, 2006; Yount et al., 2005) have little influence on *in vivo* virus replication in the mouse model. Only deletion of ORF 3a has shown a minor decrease (about one log unit) in virus growth (Yount et al., 2005). Furthermore, deletion of more than one gene, such as deletion of ORFs 3a and 3b, and ORF6, showed a 1–1.5 log reduction in Vero cells but little effect on growth in the murine model at day 2 postinfection. Moreover, little difference in growth or pathogenesis were noted in the mice model between *wt* and deletion mutants lacking ORF3a and ORF7. Therefore, the effect of SARS-CoV gene deletions needs to be tested in more relevant animal models. Interestingly, the simultaneous deletion of larger combinations of group-specific genes such as 6, 7a, 7b, 8a, 8b, and 9b has lead to the production of an infectious SARS-CoV deletion-mutant that propagates in cell culture with a titer similar to that of the parental wild type virus. The potential of this deletion-mutant as a promising vaccine candidate is being studied (M.L. DeDiego, and L. Enjuanes, unpublished results).

9.2. Vaccines based on the deletion of structural proteins

A recombinant SARS-CoV (rSARS-CoV) that lacks the E gene generated from a bacterial artificial chromososme (BAC) was attenuated *in vitro* and in an animal model (DeDiego et al., 2007). The E gene was previously shown to be a non-essential gene for the group 2 MHV coronavirus (Kuo and Masters, 2003), although elimination of this gene from MHV genome reduced virus growth in cell culture more than one thousand-fold. In contrast, for group 1 TGEV coronavirus, expression of the E gene product was essential for virus release and spread. Propagation of E gene deleted TGEV (TGEV-ΔE) was restored by providing E protein *in trans* (Curtis et al., 2002; Ortego et al., 2002).

The role of E protein in SARS-CoV propagation was studied by constructing SARS-CoV with a deleted E gene (DeDiego et al., 2007). Interestingly, viable viruses were recovered in Vero E6 cells with a relatively high titer (around 10^6 pfu/ml) and also from Huh-7 and CaCo-2 cells with reduced titers (Fig. 4), indicating that SARS-CoV E protein is not essential for virus replication in cell culture. Electron microscopy observation of Vero E6 cells infected with the SARS-CoV *wt* or the ΔE deletion mutant showed much higher assembly efficiency for the *wt* virus (Fig. 5). In this respect, SARS-CoV-ΔE behaves as MHV, the other group 2 coronavirus studied, although SARS-CoV-ΔE grows to a considerably higher titer. The differential behavior of ΔE mutant viruses from different coronavirus groups may indicate basic differences in virion assembly or life cycles among the different groups.

The hamster model has been used to study SARS-CoV-ΔE virus pathogenicity, because it demonstrates elements present in human cases of SARS-CoV infections including interstitial pneumonitis and consolidation (DeDiego et al., 2007). An ideal animal model that completely reproduces human clinical disease and pathological findings has not been identified. Nevertheless, the hamster model reproducibly supports SARS-CoV replication in the respiratory tract to a higher titer and for a longer duration than in mice or non-human primates. Virus replication in this model is accompanied by histological evidence of pneumonitis, and the animals develop viremia and extrapulmonary spread of virus (Roberts et al., 2005b). Although overt clinical disease is absent, the hamster model is a useful model for the evaluation of SARS-CoV infection. Titers of recombinant SARS-CoV (rSARS-CoV) achieved in the respiratory tract of hamsters (Fig. 6) were similar to those previously reported (Roberts et al., 2005b) and were 100–1000-fold higher than titers of the rSARS-CoV-ΔE virus, suggesting that this mutant virus is attenuated. Histopathology examination of lungs from infected hamsters was performed at two and five days post-infection, because it has been shown that pulmonary disease was most notable at these time points. Detection of viral antigen was

reduced in lungs from rSARS-CoV-ΔE infected hamsters and pulmonary inflammation was less prominent in these animals than in rSARS-CoV-infected animals, indicating that rSARS-CoV-ΔE is attenuated *in vivo* (DeDiego et al., 2007). In fact, reduction of SARS-CoV titers in patients has been associated with a considerable reduction in pathogenicity and survival rates (Chu et al., 2004; Hung et al., 2004). In contrast, challenge of hamsters with recombinant viruses lacking single group specific ORFs3a and ORF7 did not result in significant reductions in virus titier or pathology (Baric et al., unpublished). Therefore, SARS-CoV-ΔE attenuated virus is a promising vaccine candidate that is being evaluated in different animal models (mice, ferrets and macaques).

9.3. Future trends on SARS-CoV reverse genetic vaccines

Live attenuated virus vaccines face a series of potential concerns including reversion to *wt* and recombination repair with circulating heterogeneous human coronaviruses or zoonotic SARS strains. Consequently, live virus vaccine formulations should include rational approaches for minimizing the potential for reversion to *wt* phenotype and simultaneously resist recombination repair. It is clear that modifications of SARS-CoV genome could lead to viruses with an attenuated phenotype that could be considered safe and effective vaccine candidates. The replicase as a target for attenuating coronaviruses is undiscovered territory, likely ripe with alleles that might influence replication efficiency and virulence. The SARS-CoV replicase represents a major target of future research endeavors.

Coronaviruses have a characteristic, strictly conserved genome organization with structural genes occurring in the order 5′-polymerase (pol)-S-E-M-N-3′. MHV virus mutants with the genes encoding the structural proteins located in different order were constructed (de Haan et al., 2002b). These recombinant viruses were tested for the ability to replicate in the natural host, the mouse. The results indicate that the canonical coronavirus genome organization is not essential for *in vivo* replication. Some of the mutants showed an attenuated phenotype, similarly to what has been observed for the VSV (Ball et al., 1999). Therefore, deliberate rearrangement of the viral genes may be useful in the generation of attenuated coronaviruses, which due to their reduced risk of generating viable viruses by recombination with circulating field viruses would make safer vaccines.

Vaccines based on modifications of the replicase gene could in principle be generated by mutagenesis, as modifications introduced in the MHV nsp-1coding regions have identified residues important for protein processing and viral RNA replication that may affect virus virulence and could be introduced in vaccine candidates (Brockway and Denison, 2005). SARS nsp1 blocks host macromolecular synthesis and abrogates IFN signaling (Kamitani et al., 2006), providing further evidence that nsp1 coding regions represent potential virulence determinants. Alternatively, Tyr6398His substitution in open reading frame (ORF) 1b-nsp14 has been demonstrated that attenuate MHV replication in mice (Sperry et al., 2005). Similarly, deletion of the nsp2 gene in MHV and SARS-CoV has been shown to yield viable attenuated mutant viruses that replicate about one log less efficiently than *wt* virus in cell culture and in animals, and may also provide a foundation for the design of live vaccines (Graham et al., 2005). As the nsp14 Tyr residue and nsp 2 are completely conserved, it may be possible to engineer common *Coronaviridae* attenuating alleles via recombinant DNA techniques. Alternatively, changes in gene order within the replicase or even relocation to the 3′-end of the genome, if tolerated, may led to attenuated virus phenotypes.

Other options to include safeguards into the genetically engineered vaccines, particularly those that can prevent the recovery of the original virulent phenotype by recombination between the vaccine strain and viruses circulating in the field (such as HCoV-229E, -OC43, or -NL63) have been developed. One of them is the construction of replication-competent, propagation-defective viruses (pseudovirions) that are defective in one gene conferring an attenuated

phenotype or even the ability for virus propagation (Enjuanes et al., 2005). These viruses could be grown in packaging cell lines providing *in trans* the missing protein. In the case of SARS-CoV, vaccine candidates without the E gene have been constructed. In order to prevent the rescue of the virulent phenotype by recombination with a circulating human coronavirus, the deletion of an essential gene, located in a position distant from gene E, and the relocation of the deleted gene to the position previously occupied by gene E has been proposed. A potential recombination leading to the rescue of gene E would lead to the loss of the essential gene (Enjuanes, 2005).

An alternative approach for developing safer, recombination resistant live coronavirus vaccines has been developed by modifying the transcriptional regulatory sequences (TRS) of a vaccine strain to a sequence incompatible with the TRS of any known circulating coronavirus. The idea being that recombinant events between *wt* coronaviruses and TRS remodeled SARS-CoV would result in genomes containing lethal mixed regulatory sequences that block expression of subgenomic mRNAs (Fig. 7A) (Yount et al., 2006). TRS sequences among coronaviruses are highly conserved and direct the expression of subgenomic mRNAs. Using a molecular clone, the SARS-CoV TRS network was remodeled from ACGAAC to CCGGAT (Fig. 7B). This rewiring of the genomic transcription network allows for efficient replication of the mutant virus, icSARS-CRG. The icSARS-CRG recombinant virus replicated to titers equivalent to *wt* virus and expressed the typical ratios of subgenomic mRNAs and proteins. Interestingly, some new transcripts were noted initiating from the replicase gene, most of which could encode N-terminal truncated ORF1a polyproteins. It is not clear if these novel transcripts might influence pathogenic outcomes, although in some instances nsp3 is truncated potentially allowing for the establishment of dominant negative phenotypes on replication in cell culture and animals. An attractive SARS-CoV vaccine could further be modified by building attenuating mutations on the genetic template of the recombination-resistant TRS rewired virus either for use as a safe, high titer seed stock for making killed vaccines or as a live virus vaccine. One interesting refinement of this approach would be to include secondary traps that are activated in recombinant genomes. In this instance, *wt* TRS sequences can be designed into intragenic sites in essential ORFs like the S and M glycoprotein genes. In recombinant viruses encoding *wt* leader TRS sequences, subgenomic transcription might initiate from within the essential structural genes and the resulting N-terminal deletions would likely be lethal or severely attenuating.

10. Potential side effects of SARS-CoV vaccines

Both humoral and T-cell-mediated responses to animal coronaviruses may exacerbate disease or cause new health problems (Zhong et al., 2005). T-cell responses have been implicated in the demyelinization of the brain and spinal cord following infection with neurotropic MHV (Castro and Perlman, 1995; Wu et al., 2001), a group 2 coronavirus like SARS-CoV. Adverse humoral responses to another group 2 coronavirus, bovine coronavirus (BCoV), have also been linked to the development of “shipping fever” in cattle (O’Connor et al., 2001). Moreover, previous exposure to FIPV, or passive or active immunization against this virus, a group 1 coronavirus, was found to cause the “early death syndrome” instead of providing immune protection (Pedersen et al., 1981; Weiss et al., 1980). This disease exacerbation was due to the virus-specific antibodies that facilitated and enhanced uptake and spread of the virus, causing ADE of infectivity (Porterfield, 1986; Vennema et al., 1990; Weiss and Scott, 1981), that is caused by spike protein specific antibodies (Corapi et al., 1995; Corapi et al., 1992; Olsen et al., 1993; Olsen et al., 1992).

With this scenario of side effects caused by some coronavirus vaccines, a safety concern is that SARS-CoV could induce similar antibody- or cell-mediated immune pathologies. This concern was increased mainly by three reports. One study utilizing lentivirus pseudotyped with various

SARS-CoV S proteins (Yang et al., 2005) indicated that within the S protein, the major target for vaccine and immunotherapy, there are minor differences among eight strains transmitted during human outbreaks in early 2003, whereas substantial functional changes were detected in S derived from a case in late 2003 from Guandong province (isolate GD03) and from two palm civets (SZ16 and SZ3). The GD03 spike pseudotyped virus is markedly resistant to antibody neutralization. Alternatively, antibodies that neutralized most human S glycoproteins, enhanced virus entry mediated by two civet cat virus S glycoproteins related to the GD03 isolate (Yang et al., 2005). In another report, it has been shown that the administration into ferrets of MVA-based SARS-CoV S vaccine, but not MVA alone, followed by live SARS-CoV challenge, resulted in enhanced hepatitis (Weingartl et al., 2004). These side effects have not been reported in other studies with SARS-CoV in ferrets, in which it has been shown that ferrets are a useful model for SARS-CoV (Martina et al., 2003). Similarly, these data were not reproduced by other groups (He et al., 2006). Furthermore, ADE of disease has not been observed with any human SARS-CoV strain, therefore it will be important to assess vaccines in relevant animal models as they become available. Antibodies directed against SARS-CoV were found to be protective and not to enhance viral infectivity in the mouse or hamster models (Bisht et al., 2004; Roberts et al., 2005b; Subbarao et al., 2004; Yang et al., 2004), using inactivated SARS-CoV or immunization with recombinant adenovirus vectors expressing the S and N proteins of SARS-CoV (See et al., 2006), although their effect in humans remains unknown (Zhong et al., 2005). Side effects have not been observed in other animal models such as African green monkeys that, 2 months after administration of SARS-CoV into the respiratory tract, were challenged with SARS-CoV and no evidence of enhanced disease upon re-challenge was shown (McAuliffe et al., 2004).

Consistent with these results, cynomolgus macaques immunized with different amounts of purified virus, in the absence or the presence of adjuvant, challenged by the nasal route 30 days post-immunization showed no side effects even in the presence of low titer neutralizing antibodies. Temperature, breathing, appetite, mental state, and all biochemical indexes were normal for immunized monkeys, and no abnormalities were observed in major organs such as lung, liver, kidney, etc. All control non-vaccinated animals showed interstitial pneumonia. These results indicate that the purified SARS-CoV vaccine is safe in monkeys (Qin et al., 2006). In summary, immunization of mice using either S protein or whole inactivated virus (Spruth et al., 2006), or of monkeys with whole inactivated SARS-CoV (Qin et al., 2006; Zhou et al., 2005), most frequently resulted in the absence of side effects after providing different types of SARS-CoV vaccines.

In contrast, VEE virus expressing N protein failed to induce protection in either young or senescent animals, and resulted in enhanced immunopathology following viral challenge between days 4 and 14 post-infection. Therefore, caution has to be taken before including N in vaccine formulations by expressing N protein using DNA immunization, or VEE vectors (Deming et al., 2006), as no protection was elicited in mice against homologous challenge, and no benefit to vaccination with a cocktail of both S and N proteins was observed. Rather, the co-expression of N protein in vaccine regimens which failed to simultaneously induce a strong neutralizing anti-S antibody response led to an increased number of lymphocytic and eosinophilic inflammatory infiltrates, which are also characteristic of the immune pathology observed with respiratory syncytial virus (RSV) infection following vaccination with formalin inactivated RSV (De Swart et al., 2002; Hancock et al., 1996). Therefore, the concern has been raised that expression of N protein may result in vaccine-enhanced pulmonary disease, as previously described for viruses like RSV (Kim et al., 1969). The data suggest that the presence of N protein in vaccines should be evaluated in each vaccine formulation (Deming et al., 2006). Although thus far, no human SARS-CoV S vaccine has been shown to be involved in ADE of disease, possible immunopathological complications of SARS vaccine candidates require rigorous clinical and immunological evaluation.

11. Concluding remarks

Whereas the production of effective and safe vaccines for animal coronavirus previously reported has not been satisfactory (Cavanagh, 2003; Enjuanes et al., 1995; Saif, 2004), the production of inactivated, subunit, or vaccines based on DNA, recombinant vectors, or by reverse genetics using SARS-CoV genomes seem more promising. An optimum animal model for SARS-CoV vaccine evaluation is still required. After preclinical trials in animal models efficacy and safety evaluation of the most promising vaccine candidates described has to be performed in humans.

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References

Akerstrom S, Tan YJ, Mirazimi A. Amino acids 15–28 in the ectodomain of SARS coronavirus 3a protein induces neutralizing antibodies. FEBS Lett 2006;580:3799–3803. [PubMed: 16781713]

Anton IM, Sune C, Meloen RH, Borras-Cuesta F, Enjuanes L. A transmissible gastroenteritis coronavirus nucleoprotein epitope elicits T helper cells that collaborate in the in vitro antibody synthesis to the three major structural viral proteins. Virology 1995;212:746–751. [PubMed: 7571447]

Anton IM, Gonzalez S, Bullido MJ, Corsin M, Risco C, Langeveld JP, Enjuanes L. Cooperation between transmissible gastroenteritis coronavirus (TGEV) structural proteins in the in vitro induction of virus-specific antibodies. Virus Res 1996;46:111–124. [PubMed: 9029784]

Bae JL, Lee JG, Kang TJ, Jang HS, Jang YS, Yang MS. Induction of antigen-specific systemic and mucosal immune responses by feeding animals transgenic plants expressing the antigen. Vaccine 2003;21:4052–4058. [PubMed: 12922142]

Ball LA, Pringle CR, Flanagan B, Perepelitsa VP, Wertz GW. Phenotypic consequences of rearranging the P, M, and, G genes of vesicular stomatitis virus. J Virol 1999;73:4705–4712. [PubMed: 10233930]

Baric, RS.; Sheahan, T.; Deming, D.; Donaldson, E.; Yount, B.; Sims, AC.; Roberts, RS.; Frieman, M.; Rockx, B. SARS coronavirus vaccine development. In: Perlman, S.; Holmes, K., editors. The Nidovirus: Towards Control of SARS and other Nidovirus Diseases. Vol. 581. Springer; New York: 2006. p. 553-560.

Berry JD, Jones S, Drebot MA, Andonov A, Sabara M, Yuan XY, Weingartl H, Fernando L, Marszal P, Gren J, Nicolas B, Andonova M, Ranada F, Gubbins MJ, Ball TB, Kitching P, Li Y, Kabani A, Plummer F. Development and characterization of neutralising monoclonal antibody to the SARS-coronavirus. J Virol Methods 2004;120:87–96. [PubMed: 15234813]

Bisht H, Roberts A, Vogel L, Bukreyev A, Collins PL, Murphy BR, Subbarao K, Moss B. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc Natl Acad Sci USA 2004;101:6641–6646. [PubMed: 15096611]

Bisht H, Roberts A, Vogel L, Subbarao K, Moss B. Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein. Virology 2005;334:160–165. [PubMed: 15780866]

Bradbury J. Custom-made vaccines at speed. Drug Discov Today 2003;8:518–519. [PubMed: 12821292]

Brockway SM, Denison MR. Mutagenesis of the murine hepatitis virus nsp1-coding region identifies residues important for protein processing, viral RNA synthesis, and viral replication. Virology 2005;340:209–223. [PubMed: 16051301]

Buchholz UJ, Bukreyev A, Yang L, Lamirande EW, Murphy BR, Subbarao K, Collins PL. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. Proc Natl Acad Sci USA 2004;101:9804–9809. [PubMed: 15210961]

Bukreyev A, Lamirande EW, Buchholz UJ, Vogel LN, Elkins WR, St Claire M, Murphy BR, Subbarao K, Collins PL. Mucosal immunization of African green monkeys (Cercopithecus aethiops) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet 2004;363:2122–2127. [PubMed: 15220033]

Castro RF, Perlman S. CD8+ T-cell epitopes within the surface glycoprotein of a neurotropic coronavirus and correlation with pathogenicity. J Virol 1995;69:8127–8131. [PubMed: 7494335]

Cavanagh D. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol 2003;32:567–582. [PubMed: 14676007]

Chen Z, Zhang L, Qin C, Ba L, Yi CE, Zhang E, Wei Q, He T, Yu W, Yu J, Gao H, Tu X, Gettie A, Farzan M, Yuen KY, Ho DD. Recombinant modified vaccinia virus ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. J Virol 2005;79:2678–2688. [PubMed: 15708987]

Chinese-Consortium SME. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science 2004;303:1666–1669. [PubMed: 14752165]

Chu CM, Poon LL, Cheng VC, Chan KS, Hung IF, Wong MM, Chan KH, Leung WS, Tang BS, Chan VL, Ng WL, Sim TC, Ng PW, Law KI, Tse DM, Peiris JS, Yuen KY. Initial viral load and the outcomes of SARS. Can Med Assoc J 2004;171:1349–1352. [PubMed: 15557587]

Corapi WV, Olsen CW, Scott FW. Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. J Virol 1992;66:6695–6705. [PubMed: 1383568]

Corapi WV, Darteil RJ, Audonnet JC, Chappuis GE. Localization of antigenic sites of the S glycoprotein of feline infectious peritonitis virus involved in neutralization and antibody-dependent enhancement. J Virol 1995;69:2858–2862. [PubMed: 7707508]

Curtis KM, Yount B, Baric RS. Heterologous gene expression from transmissible gastroenteritis virus replicon particles. J Virol 2002;76:1422–1434. [PubMed: 11773416]

Czub M, Weingartl H, Czub S, He R, Cao J. Evaluation of modified vaccinia virus Ankara based recombinant SARS vaccine in ferrets. Vaccine 2005;23:2273–2279. [PubMed: 15755610]

de Haan CAM, Masters PS, Shen S, Weiss S, Rottier PJM. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. Virology 2002a;296:177–189. [PubMed: 12036329]

de Haan CAM, Volders H, Koetzner CA, Masters PS, Rottier PJM. Coronavirus maintain viability despite dramatic rearrangements of the strictly conserved genome organization. J Virol 2002b;76:12491–12502. [PubMed: 12438575]

De Swart RL, Kuiken T, Timmerman HH, van Amerongen G, Van Den Hoogen BG, Vos HW, Neijens HJ, Andeweg AC, Osterhaus AD. Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. J Virol 2002;76:11561–11569. [PubMed: 12388717]

DeDiego ML, Alvarez E, Almazan F, Rejas MT, Lamirande E, Roberts A, Shieh WJ, Saki S, Subbarao K, Enjuanes L. A SARS coronavirus that lacks the E gene is attenuated in vitro and in vivo. J Virol 2007;81:1701–1713. [PubMed: 17108030]

Delmas B, Gelfi J, Laude H. Antigenic structure of transmissible gastroenteritis virus. II Domains in the peplomer glycoprotein. J Gen Virol 1986;67:1405–1418. [PubMed: 2425049]

Deming D, Sheahan T, Yount B, Heise M, Davis N, Sims A, Suthar M, Pickles R, Harkema J, Wihitmore A, West A, Donaldson E, Curtis K, Johnton R, Baric RS. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS med 2006;3:2359–2374.

Enjuanes, L., editor. Curr Top Microbiol Immunol. Vol. 287. Berlin: Springer; 2005. Coronavirus replication and reverse genetics.

Enjuanes L, Smerdou C, Castilla J, Anton IM, Torres JM, Sola I, Golvano J, Sanchez JM, Pintado B. Development of protection against coronavirus induced diseases. A review. Adv Exp Med Biol 1995;380:197–211. [PubMed: 8830481]

Enjuanes, L.; Sola, I.; Alonso, S.; Escors, D.; Zuniga, S. Coronavirus reverse genetics and development of vectors for gene expression. In: Enjuanes, L., editor. Coronavirus Replication and Reverse Genetics. Vol. 287. Springer; 2005. p. 161-197.

Faber M, Lamirande EW, Roberts A, Rice AB, Koprowski H, Dietzschold B, Schnell MJ. A single immunization with a rhabdovirus-based vector expressing severe acute respiratory syndrome coronavirus (SARS-CoV) S protein results in the production of high levels of SARS-CoV-neutralizing antibodies. J Gen Virol 2005;86:1435–1440. [PubMed: 15831955]

Frasca D, Riley RL, Blomberg BB. Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. Semin Immunol 2005;17:378–384. [PubMed: 15996480]

Frech SA, Kenney RT, Spyr CA, Lazar H, Viret JF, Herzog C, Gluck R, Glenn GM. Improved immune responses to influenza vaccination in the elderly using an immunostimulant patch. Vaccine 2005;23:946–950. [PubMed: 15603897]

Gao W, Tamin A, Soloff A, D'Aiuto L, Nwanegbo E, Robbins PD, Bellini WJ, Barratt-Boyes S, Gambotto A. Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet 2003;362:1895–1896. [PubMed: 14667748]

Glass WG, Subbarao K, Murphy B, Murphy PM. Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. J Immunol 2004;173:4030–4039. [PubMed: 15356152]

Graham RL, Sims AC, Brockway SM, Baric RS, Denison MR. The nsp2 replicase proteins of murine hepatitis virus and severe acute respiratory syndrome coronavirus are dispensable for viral replication. J Virol 2005;79:13399–13411. [PubMed: 16227261]

Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yuen KY, Peiris JS, Poon LL. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science 2003;302:276–278. [PubMed: 12958366]

Gupta V, Tabiin TM, Sun K, Chandrasekaran A, Anwar A, Yang K, Chikhlikar P, Salmon J, Brusic V, Marques ET, Kellathur SN, August TJ. SARS coronavirus nucleocapsid immunodominant T-cell epitope cluster is common to both exogenous recombinant and endogenous DNA-encoded immunogens. Virology 2006;347:127–139. [PubMed: 16387339]

Haijema BJ, Volders H, Rottier PJ. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J Virol 2004;78:3863–3871. [PubMed: 15047802]

Hancock GE, Speelman DJ, Heers K, Bortell E, Smith J, Cosco C. Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus. J Virol 1996;70:7783–7791. [PubMed: 8892899]

He Y, Li J, Li W, Lustigman S, Farzan M, Jiang S. Cross-neutralization of human and palm civet severe acute respiratory syndrome coronaviruses by antibodies targeting the receptor-binding domain of spike protein. J Immunol 2006;176:6085–6092. [PubMed: 16670317]

Ho Y, Lin PH, Liu CY, Lee SP, Chao YC. Assembly of human severe acute respiratory syndrome coronavirus-like particles. Biochem Biophys Res Commun 2004;318:833–838. [PubMed: 15147946]

Hogan RJ, Gao G, Rowe T, Bell P, Flieder D, Paragas J, Kobinger GP, Wivel NA, Crystal RG, Boyer J, Feldmann H, Voss TG, Wilson JM. Resolution of primary severe acute respiratory syndrome-associated coronavirus infection requires Stat1. J Virol 2004;78:11416–11421. [PubMed: 15452265]

Huang Y, Yang ZY, Kong WP, Nabel GJ. Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. J Virol 2004;78:12557–12565. [PubMed: 15507643]

Huang C, Ito N, Tseng CT, Makino S. Severe acute respiratory syndrome coronavirus 7a accessory protein is a viral structural protein. J Virol 2006;80:7287–7294. [PubMed: 16840309]

Hung IF, Cheng VC, Wu AK, Tang BS, Chan KH, Chu CM, Wong MM, Hui WT, Poon LL, Tse DM, Chan KS, Woo PC, Lau SK, Peiris JS, Yuen KY. Viral loads in clinical specimens and SARS manifestations. Emerg Infect Dis 2004;10:1550–1557. [PubMed: 15498155]

Ito N, Mossel EC, Narayanan K, Popov VL, Huang C, Inoue T, Peters CJ, Makino S. Severe acute respiratory syndrome coronavirus 3a protein is a viral structural protein. J Virol 2005;79:3182–3186. [PubMed: 15709039]

Jiang S, He Y, Liu S. SARS vaccine development. Emerg Infect Dis 2005;11:1016–1020. [PubMed: 16022774]

Jin H, Xiao C, Chen Z, Kang Y, Ma Y, Zhu K, Xie Q, Tu Y, Yu Y, Wang B. Induction of Th1 type response by DNA vaccinations with N, M, and E genes against SARS-CoV in mice. Biochem Biophys Res Commun 2005;328:979–986. [PubMed: 15707974]

Kamitani W, Narayanan K, Huang C, Lokugamage K, Ikegami T, Ito N, Kubo H, Makino S. Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. Proc Natl Acad Sci USA 2006;103:12885–12890. [PubMed: 16912115]

Kan B, Wang M, Jing H, Xu H, Jiang X, Yan M, Liang W, Zheng H, Wan K, Liu Q, Cui B, Xu Y, Zhang E, Wang H, Ye J, Li G, Li M, Cui Z, Qi X, Chen K, Du L, Gao K, Zhao YT, Zou XZ, Feng YJ, Gao YF, Hai R, Yu D, Guan Y, Xu J. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J Virol 2005;79:11892–11900. [PubMed: 16140765]

Kapadia SU, Rose JK, Lamirande E, Vogel L, Subbarao K, Roberts A. Long-term protection from SARS coronavirus infection conferred by a single immunization with an attenuated VSV-based vaccine. Virology 2005;340:174–182. [PubMed: 16043204]

Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89:422–434. [PubMed: 4305198]

Kim TW, Lee JH, Hung CF, Peng S, Roden R, Wang MC, Viscidi R, Tsai YC, He L, Chen PJ, Boyd DA, Wu TC. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. J Virol 2004;78:4638–4645. [PubMed: 15078946]

Kong WP, Xu L, Stadler K, Ulmer JB, Abrignani S, Rappuoli R, Nabel GJ. Modulation of the immune response to the severe acute respiratory syndrome spike glycoprotein by gene-based and inactivated virus immunization. J Virol 2005;79:13915–13923. [PubMed: 16254327]

Ksiazek TG, Erdman D, Goldsmith C, Zaki S, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell S, Ling AE, Humphrey C, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes J, LeDuc JW, Bellini WJ, Anderson LJ. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003;348:1953–1966. [PubMed: 12690092]

Kuo L, Masters PS. The small envelope protein E is not essential for murine coronavirus replication. J Virol 2003;77:4597–4608. [PubMed: 12663766]

Lamphear BJ, Jilka JM, Kesl L, Welter M, Howard JA, Streatfield SJ. A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine 2004;22:2420–2424. [PubMed: 15193404]

Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci USA 2005;102:14040–14045. [PubMed: 16169905]

Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF. Bats are natural reservoirs of SARS-like coronaviruses. Science 2005;310:676–679. [PubMed: 16195424]

Liu IJ, Hsueh PR, Lin CT, Chiu CY, Kao CL, Liao MY, Wu HC. Disease-specific B Cell epitopes for serum antibodies from patients with severe acute respiratory syndrome (SARS) and serologic detection of SARS antibodies by epitope-based peptide antigens. J Infect Dis 2004;190:797–809. [PubMed: 15272409]

Liu RY, Wu LZ, Huang BJ, Huang JL, Zhang YL, Ke ML, Wang JM, Tan WP, Zhang RH, Chen HK, Zeng YX, Huang W. Adenoviral expression of a truncated S1 subunit of SARS-CoV spike protein results in specific humoral immune responses against SARS-CoV in rats. Virus Res 2005;112:24–31. [PubMed: 16022898]

Liu SJ, Leng CH, Lien SP, Chi HY, Huang CY, Lin CL, Lian WC, Chen CJ, Hsieh SL, Chong P. Immunological characterizations of the nucleocapsid protein based SARS vaccine candidates. Vaccine 2006;24:3100–3108. [PubMed: 16494977]

Marra MA, Jones SJM, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YSN, Khattra J, Asano JK, Barber SA, Chan SY, Cloutier A, Coughlin SM, Freeman D, Girn N, Griffith OL, Leach SR, Mayo M, McDonald H, Montgomery SB, Pandoh PK, Petrescu AS, Robertson AG, Schein JE, Siddiqui A, Smailus DE, Stott JM, Yang GS, Plummer F, Andonov A, Artsob H, Bastien N, Bernard K, Booth TF, Bowness D, Czub M, Drebot M, Fernando L, Flick R, Garbutt M, Gray M, Grolla A, Jones S, Feldmann H, Meyers A, Kabani A, Li Y, Normand S, Stroher U, Tipples GA, Tyler S, Vogrig R, Ward D, Watson B, Brunham RC, Krajden M, Petric M, Skowronski DM, Upton C, Roper RL. The genome sequence of the SARS-associated coronavirus. Science 2003;300:1399–1404. [PubMed: 12730501]

Marshall E, Enserink M. Medicine. Caution urged on SARS vaccines. Science 2004;303:944–946. [PubMed: 14963300]

Martina BE, Haagmans BL, Kuiken T, Fouchier RA, Rimmelzwaan GF, Van Amerongen G, Peiris JS, Lim W, Osterhaus AD. SARS virus infection of cats and ferrets. Nature 2003;425:915. [PubMed: 14586458]

McAuliffe J, Vogel L, Roberts A, Fahle G, Fischer S, Shieh WJ, Butler E, Zaki S, St Claire M, Murphy B, Subbarao K. Replication of SARS coronavirus administered into the respiratory tract of African Green, rhesus and cynomolgus monkeys. Virology 2004;330:8–15. [PubMed: 15527829]

McElhaney JE. The unmet need in the elderly: designing new influenza vaccines for older adults. Vaccine 2005;23(Suppl 1):10–25.

Montagnon BJ. Polio and rabies vaccines produced in continuous cell lines: a reality for Vero cell line. Dev Biol Stand 1989;70:27–47. [PubMed: 2759353]

Mortola E, Roy P. Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. FEBS Lett 2004;576:174–178. [PubMed: 15474033]

Ng PC, Leung CW, Chiu WK, Wong SF, Hon EK. SARS in newborns and children. Biol Neonate 2004;85:293–298. [PubMed: 15218286]

O'Connor A, Martin SW, Nagy E, Menzies P, Harland R. The relationship between the occurrence of undifferentiated bovine respiratory disease and titer changes to bovine coronavirus and bovine viral diarrhea virus in 3 Ontario feedlots. Can J Vet Res 2001;65:137–142. [PubMed: 11480517]

Olsen CW, Corapi WV, Ngichabe CK, Baines JD, Scott FW. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J Virol 1992;66:956–965. [PubMed: 1309922]

Olsen CW, Corapi WV, Jacobson RH, Simkins RA, Saif LJ, Scott FW. Identification of antigenic sites mediating antibody-dependent enhancement of feline infectious peritonitis virus infectivity. J Gen Virol 1993;74:745–749. [PubMed: 7682252]

Ortego J, Escors D, Laude H, Enjuanes L. Generation of a replication-competent, propagation-deficient virus vector based on the transmissible gastroenteritis coronavirus genome. J Virol 2002;76:11518–11529. [PubMed: 12388713]

Ortego J, Sola I, Almazan F, Ceriani JE, Riquelme C, Balasch M, Plana-Durán J, Enjuanes L. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences *in vivo* virus replication and virulence. Virology 2003;308:13–22. [PubMed: 12706086]

Pang H, Liu Y, Han X, Xu Y, Jiang F, Wu D, Kong X, Bartlam M, Rao Z. Protective humoral responses to severe acute respiratory syndrome-associated coronavirus: implications for the design of an effective protein-based vaccine. J Gen Virol 2004;85:3109–3113. [PubMed: 15448374]

Pedersen NC, Boyle JF, Floyd K, Fudge A, Barker J. An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. Am J Vet Res 1981;42:368–377. [PubMed: 6267960]

Peiris JS, Yuen KY, Osterhaus AD, Stohr K. The severe acute respiratory syndrome. N Engl J Med 2003;349:2431–2441. [PubMed: 14681510]

Peiris JS, Guan Y, Yuen KY. Severe acute respiratory syndrome. Nat Med 2004;10:S88–S97. [PubMed: 15577937]

Pogrebnyak N, Golovkin M, Andrianov V, Spitsin S, Smirnov Y, Egolf R, Koprowski H. Severe acute respiratory syndrome (SARS) S protein production in plants: development of recombinant vaccine. Proc Natl Acad Sci USA 2005;102:9062–9067. [PubMed: 15956182]

Poon LL, Chu DK, Chan KH, Wong OK, Ellis TM, Leung YH, Lau SK, Woo PC, Suen KY, Yuen KY, Guan Y, Peiris JS. Identification of a novel coronavirus in bats. J Virol 2005;79:2001–2009. [PubMed: 15681402]

Porterfield JS. Antibody-dependent enhancement of viral infectivity. Adv Virus Res 1986;31:335–355. [PubMed: 2428214]

Qin E, Shi H, Tang L, Wang C, Chang G, Ding Z, Zhao K, Wang J, Chen Z, Yu M, Si B, Liu J, Wu D, Cheng X, Yang B, Peng W, Meng Q, Liu B, Han W, Yin X, Duan H, Zhan D, Tian L, Li S, Wu J, Tan G, Li Y, Liu Y, Liu H, Lv F, Zhang Y, Kong X, Fan B, Jiang T, Xu S, Wang X, Li C, Wu X, Deng Y, Zhao M, Zhu Q. Immunogenicity and protective efficacy in monkeys of purified inactivated Vero-cell SARS vaccine. Vaccine 2006;24:1028–1034. [PubMed: 16388880]

Qiu M, Shi Y, Guo Z, Chen Z, He R, Chen R, Zhou D, Dai E, Wang X, Si B, Song Y, Li J, Yang L, Wang J, Wang H, Pang X, Zhai J, Du Z, Liu Y, Zhang Y, Li L, Sun B, Yang R. Antibody responses to individual proteins of SARS coronavirus and their neutralization activities. Microbes Infect 2005;7:882–889. [PubMed: 15878679]

Qu D, Zheng B, Yao X, Guan Y, Yuan ZH, Zhong NS, Lu LW, Xie JP, Wen YM. Intranasal immunization with inactivated SARS-CoV (SARS-associated coronavirus) induced local and serum antibodies in mice. Vaccine 2005;23:924–931. [PubMed: 15603894]

Risco C, Antón IM, Suñé C, Pedregosa AM, Martín-Alonso JM, Parra F, Carrascosa JL, Enjuanes L. Membrane protein molecules of transmissible gastroenteritis coronavirus also expose the carboxy-terminal region on the external surface of the virion. J Virol 1995;69:5269–5277. [PubMed: 7636969]

Roberts A, Paddock C, Vogel L, Butler E, Zaki S, Subbarao K. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. J Virol 2005a;79:5833–5838. [PubMed: 15827197]

Roberts A, Vogel L, Guarner J, Hayes N, Murphy B, Zaki S, Subbarao K. Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. J Virol 2005b;79:503–511. [PubMed: 15596843]

Rota PA, Oberste MS, Monroe SS, Nix WA, Campganoli R, Icenogle JP, Peñaranda S, Bankamp B, Maher K, Chen MH, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman Dd, Peret TCT, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rassmussen M, Fouchier R, Gunther S, Osterhaus ADME, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 2003;300:1394–1399. [PubMed: 12730500]

Saif LJ. Animal coronavirus vaccines: lessons for SARS. Dev Biol (Basel) 2004;119:129–140. [PubMed: 15742624]

Schaecher, SR.; Pekosz, A. The SARS-CoV accessory protein ORF 7B is a Golgi localized transmenbrane protein, translated via a ribosomal leaky scanning mechanism, and is packaged into viral particles. The American Society for Virology 25th Annual Meeting; University of Wisconsin-Madison. 2006. p. 89

See RH, Zakhartchouk AN, Petric M, Lawrence DJ, Mok CP, Hogan RJ, Rowe T, Zitzow LA, Karunakaran KP, Hitt MM, Graham FL, Prevec L, Mahony JB, Sharon C, Auperin TC, Rini JM, Tingle AJ, Scheifele DW, Skowronski DM, Patrick DM, Voss TG, Babiuk LA, Gauldie J, Roper RL, Brunham RC, Finlay BB. Comparative evaluation of two severe acute respiratory syndrome (SARS) vaccine candidates in mice challenged with SARS coronavirus. J Gen Virol 2006;87:641–650. [PubMed: 16476986]

Shen S, Lin PS, Chao YC, Zhang A, Yang X, Lim SG, Hong W, Tan YJ. The severe acute respiratory syndrome coronavirus 3a is a novel structural protein. Biochem Biophys Res Commun 2005;330:286–292. [PubMed: 15781262]

Song H, Price PW, Cerny J. Age-related changes in antibody repertoire: contribution from T cells. Immunol Rev 1997;160:55–62. [PubMed: 9476665]

Song HC, Seo MY, Stadler K, Yoo BJ, Choo QL, Coates SR, Uematsu Y, Harada T, Greer CE, Polo JM, Pileri P, Eickmann M, Rappuoli R, Abrignani S, Houghton M, Han JH. Synthesis and characterization

of a native, oligomeric form of recombinant severe acute respiratory syndrome coronavirus spike glycoprotein. J Virol 2004;78:10328–10335. [PubMed: 15367599]

Sperry SM, Kazi L, Graham RL, Baric RS, Weiss SR, Denison MR. Single-amino-acid substitutions in open reading frame (ORF) 1b-nsp14 and ORF 2a proteins of the coronavirus mouse hepatitis virus are attenuating in mice. J Virol 2005;79:3391–3400. [PubMed: 15731233]

Spruth M, Kistner O, Savidis-Dacho H, Hitter E, Crowe B, Gerencer M, Bruhl P, Grillberger L, Reiter M, Tauer C, Mundt W, Barrett PN. A double-inactivated whole virus candidate SARS coronavirus vaccine stimulates neutralising and protective antibody responses. Vaccine 2006;24:652–661. [PubMed: 16214268]

Subbarao K, McAuliffe J, Vogel L, Fahle G, Fischer S, Tatti K, Packard M, Shieh WJ, Zaki S, Murphy B. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J Virol 2004;78:3572–3577. [PubMed: 15016880]

Tan YJ, Goh PY, Fielding BC, Shen S, Chou CF, Fu JL, Leong HN, Leo YS, Ooi EE, Ling AE, Lim SG, Hong W. Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers. Clin Diagn Lab Immunol 2004a; 11:362–371. [PubMed: 15013989]

Tan YJ, Teng E, Shen S, Tan THP, Goh PY, Fielding BC, Ooi EE, Tan HC, Lim SG, Hong W. A novel severe acute respiratory syndrome coronavirus protein, U274, is transported to the cell surface and undergoes endocytosis. J Virol 2004b;78:6723–6734. [PubMed: 15194747]

ter Meulen J, Bakker AB, van den Brink EN, Weverling GJ, Martina BE, Haagmans BL, Kuiken T, de Kruif J, Preiser W, Spaan W, Gelderblom HR, Goudsmit J, Osterhaus AD. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. Lancet 2004;363:2139–2141. [PubMed: 15220038]

Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat Med 2004;10:871–875. [PubMed: 15247913]

Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L, Nagy E. Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. Vaccine 2000;18:2023–2028. [PubMed: 10706964]

Vennema H, de Groot RJ, Harbour DA, Dalderup M, Gruffydd-Jones T, Horzinek MC, Spaan WJ. Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. J Virol 1990;64:1407–1409. [PubMed: 2154621]

Wang Z, Yuan Z, Matsumoto M, Hengge UR, Chang YF. Immune responses with DNA vaccines encoded different gene fragments of severe acute respiratory syndrome coronavirus in BALB/c mice. Biochem Biophys Res Commun 2005;327:130–135. [PubMed: 15629440]

Weingartl H, Czub M, Czub S, Neufeld J, Marszal P, Gren J, Smith G, Jones S, Proulx R, Deschambault Y, Grudeski E, Andonov A, He R, Li Y, Copps J, Grolla A, Dick D, Berry J, Ganske S, Manning L, Cao J. Immunization with modified vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory syndrome is associated with enhanced hepatitis in ferrets. J Virol 2004;78:12672–12676. [PubMed: 15507655]

Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol Mol Biol Rev 2005;69:635–664. [PubMed: 16339739]

Weiss RC, Scott FW. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. Comp Immunol Microbiol Infect Dis 1981;4:175–189. [PubMed: 6754243]

Weiss RC, Dodds WJ, Scott FW. Disseminated intravascular coagulation in experimentally induced feline infectious peritonitis. Am J Vet Res 1980;41:663–671. [PubMed: 6250426]

Wong SK, Li W, Moore MJ, Choe H, Farzan M. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. J Biol Chem 2004;279:3197–3201. [PubMed: 14670965]

Woo PC, Lau SK, Tsoi HW, Chen ZW, Wong BH, Zhang L, Chan JK, Wong LP, He W, Ma C, Chan KH, Ho DD, Yuen KY. SARS coronavirus spike polypeptide DNA vaccine priming with

recombinant spike polypeptide from Escherichia coli as booster induces high titer of neutralizing antibody against SARS coronavirus. Vaccine 2005;23:4959–4968. [PubMed: 15993989]

Woo PC, Lau SK, Li KS, Poon RW, Wong BH, Tsoi HW, Yip BC, Huang Y, Chan KH, Yuen KY. Molecular diversity of coronaviruses in bats. Virology 2006;351:180–187. [PubMed: 16647731]

Woods RD, Wesley RD, Kapke PA. Complement-dependent neutralization of transmissible gastroenteritis virus by monoclonal antibodies. Adv Exp Med Biol 1987;218:493–500. [PubMed: 2829565]

Wu GF, Dandekar AA, Pewe L, Perlman S. The role of CD4 and CD8 T cells in MHV-JHM-induced demyelination. Adv Exp Med Biol 2001;494:341–347. [PubMed: 11774490]

Yang ZY, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, Nabel GJ. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature 2004;428:561–564. [PubMed: 15024391]

Yang ZY, Werner HC, Kong WP, Leung K, Traggiai E, Lanzavecchia A, Nabel GJ. Evasion of antibody neutralization in emerging severe acute respiratory syndrome coronaviruses. Proc Natl Acad Sci USA 2005;102:797–801. [PubMed: 15642942]

Yount B, Roberts RS, Sims AC, Deming D, Frieman MB, Sparks J, Denison MR, Davis N, Baric RS. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. J Virol 2005;79:14909–14922. [PubMed: 16282490]

Yount B, Roberts RS, Lindesmith L, Baric RS. Rewiring the severe acute respiratory syndrome coronavirus (SARS-CoV) transcription circuit: engineering a recombination-resistant genome. Proc Natl Acad Sci USA 2006;103:12546–12551. [PubMed: 16891412]

Zakhartchouk AN, Liu Q, Petric M, Babiuk LA. Augmentation of immune responses to SARS coronavirus by a combination of DNA and whole killed virus vaccines. Vaccine 2005a;23:4385–4391. [PubMed: 16005746]

Zakhartchouk AN, Viswanathan S, Mahony JB, Gauldie J, Babiuk LA. Severe acute respiratory syndrome coronavirus nucleocapsid protein expressed by an adenovirus vector is phosphorylated and immunogenic in mice. J Gen Virol 2005b;86:211–215. [PubMed: 15604448]

Zeng R, Chow KY, Hon CC, Law KM, Yip CW, Chan KH, Peiris JS, Leung FC. Characterization of humoral responses in mice immunized with plasmid DNAs encoding SARS-CoV spike gene fragments. Biochem Biophys Res Commun 2004;315:1134–1139. [PubMed: 14985131]

Zhang H, Wang G, Li J, Nie Y, Shi X, Lian G, Wang W, Yin X, Zhao Y, Qu X, Ding M, Deng H. Identification of an antigenic determinant on the S2 domain of the severe acute respiratory syndrome coronavirus spike glycoprotein capable of inducing neutralizing antibodies. J Virol 2004;78:6938–6945. [PubMed: 15194770]

Zhao P, Cao J, Zhao LJ, Qin ZL, Ke JS, Pan W, Ren H, Yu JG, Qi ZT. Immune responses against SARS-coronavirus nucleocapsid protein induced by DNA vaccine. Virology 2005;331:128–135. [PubMed: 15582659]

Zheng B, Han S, Takahashi Y, Kelsoe G. Immunosenescence and germinal center reaction. Immunol Rev 1997;160:63–77. [PubMed: 9476666]

Zheng BJ, Wong KH, Zhou J, Wong KL, Young BW, Lu LW, Lee SS. SARS-related virus predating SARS outbreak, Hong Kong. Emerg Infect Dis 2004;10:176–178. [PubMed: 15030679]

Zhong X, Yang H, Guo ZF, Sin WY, Chen W, Xu J, Fu L, Wu J, Mak CK, Cheng CS, Yang Y, Cao S, Wong TY, Lai ST, Xie Y, Guo Z. B-cell responses in patients who have recovered from severe acute respiratory syndrome target a dominant site in the S2 domain of the surface spike glycoprotein. J Virol 2005;79:3401–3408. [PubMed: 15731234]

Zhong X, Guo Z, Yang H, Peng L, Xie Y, Wong TY, Lai ST. Amino terminus of the SARS coronavirus protein 3a elicits strong, potentially protective humoral responses in infected patients. J Gen Virol 2006;87:369–373. [PubMed: 16432024]

Zhou JY, Wu JX, Cheng LQ, Zheng XJ, Gong H, Shang SB, Zhou EM. Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. J Virol 2003;77:9090–9093. [PubMed: 12885926]

Zhou J, Wang W, Zhong Q, Hou W, Yang Z, Xiao SY, Zhu R, Tang Z, Wang Y, Xian Q, Tang H, Wen L. Immunogenicity, safety, and protective efficacy of an inactivated SARS-associated coronavirus vaccine in rhesus monkeys. Vaccine 2005;23:3202–3209. [PubMed: 15837221]

Zhu MS, Pan Y, Chen HQ, Shen Y, Wang XC, Sun YJ, Tao KH. Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine. Immunol Lett 2004;92:237–243. [PubMed: 15081618]

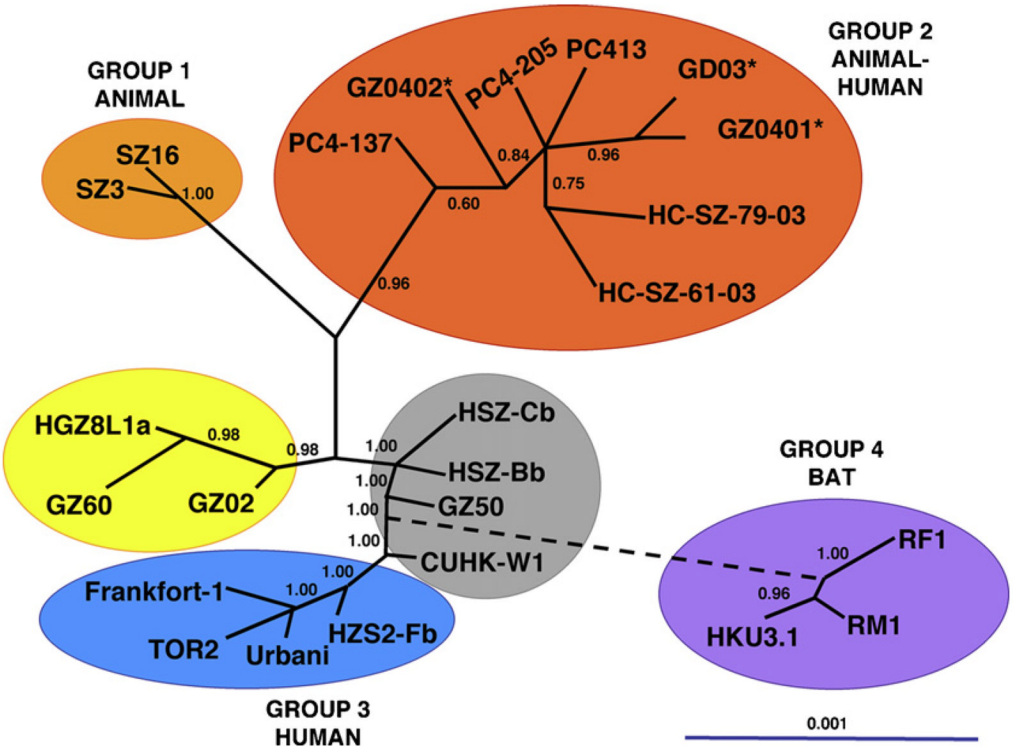

Fig. 1.

Phylogenetic analysis of human, bat, and civet cat-racoon dog virus spike sequences. An unrooted Bayesian phylogenetic gene tree of 24 SARS viruses divided into four groups. Group 1 includes viruses isolated from animals in southern China in 2003. Group 2 is a cluster of viruses isolated from animals and humans (*) in 2003. Group 3 includes viruses from all three phases of the human SARS epidemic of 2002–2003. Group 4 represents a cluster of viruses isolated from bats in 2005–06. A multiple sequence alignment of the spike gene of each virus was created using ClustalX 1.83 with default settings. Bayesian inference was conducted with Mr. Bayes, with Markov chain Monte Carlo sampling of four chains for 500,000 generations, and a consensus tree was generated using the 50% majority rule with a burn in of 1000. Branch confidence values are shown as posterior probabilities. The three human isolates that fall within the animal cluster (GZ0402, GD03, and GZ0401) may represent infections where a human acquired the virus from animals. The dashed line between Group 3 and Group 4 is used to represent a much longer line in the tree (~10 times longer), thus the distance of the line is not representative of the distance between bat and human SARS.

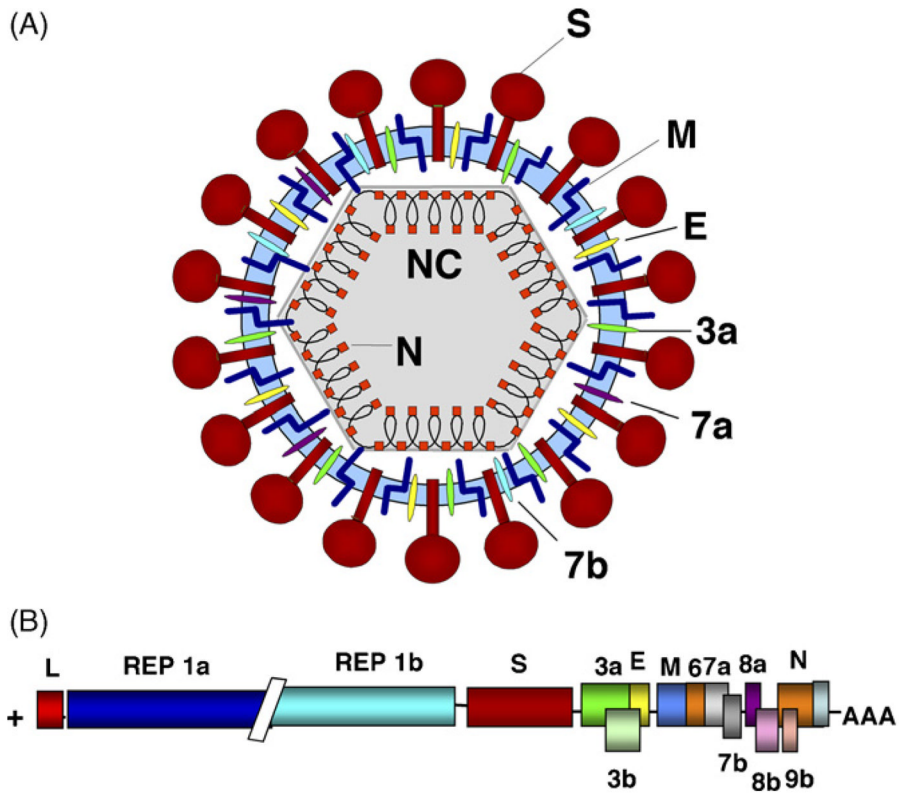

Fig. 2.

Structure and genome organization of SARS-CoV. (A). Schematic diagram of SARS-CoV structure. S, spike protein; M, membrane protein; E, envelope protein; N, nucleoprotein; 3a, 7a, and 7b, structural proteins of SARS-CoV. (B). Representation of a prototype SARS-CoV genome. Poly(A) tail is indicated by AAA. Numbers and letters indicate viral genes.

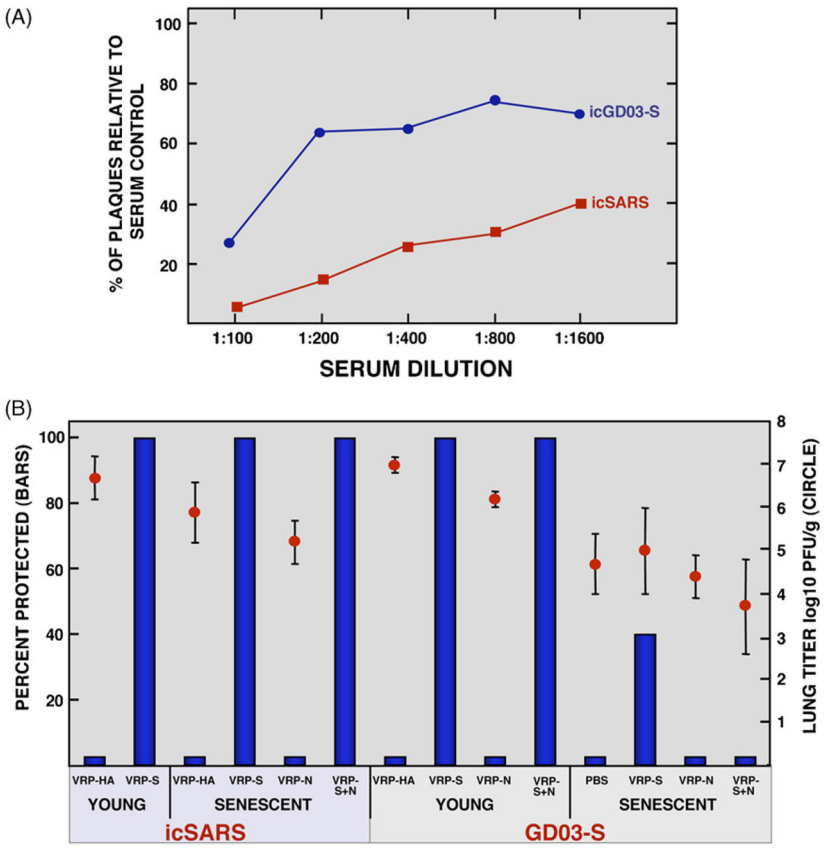

Fig. 3.

Neutralization of SARS-CoV pseudotypes and heterologous challenge studies. (A) Cross neutralization responses between *wt* Urbani (^) and icGD03 (▪). About 100 PFU of each virus was incubated for 30 min with varying concentrations of human antisera from a convalescent SARS patient or control serum and titered by plaque assay. (B) Compilation of vaccination results in mice inoculated with VRP-vectored vaccines and challenged with icSARS or icGDO3-S (Deming et al., 2006). The percent of mice without detectable replicating virus are shown as bars while the average titers of detectable virus are shown as red circles. Error bars represent the standard deviation of the measured samples. Mice were vaccinated with VRP-S, VRP-N, a cocktail of VRP-S and VRP-N (VRP-S+N), or mock vaccinated with either VRP-HA (VRP expressing influenza A HA protein) or PBS. Mice were intranasally challenged with either Urbani derived from the infectious clone (icSARS) or a chimeric virus expressing the GD03 spike glycoprotein (GD03-S). Mice challenged with icSARS were vaccinated when young (4–5 weeks), boosted 4 weeks later, and challenged either 8 weeks post boost (young) or 54 weeks post boost when old (Senescent). VRP-S and VRP-S+N provided protection in both groups against icSARS. Mice challenged with GD03-S were either vaccinated when 7 weeks old (young) or older than 26 weeks old (senescent), boosted approximately 4 weeks later, and challenged either 7 weeks post boost (young) or 32 weeks post boost (senescent).

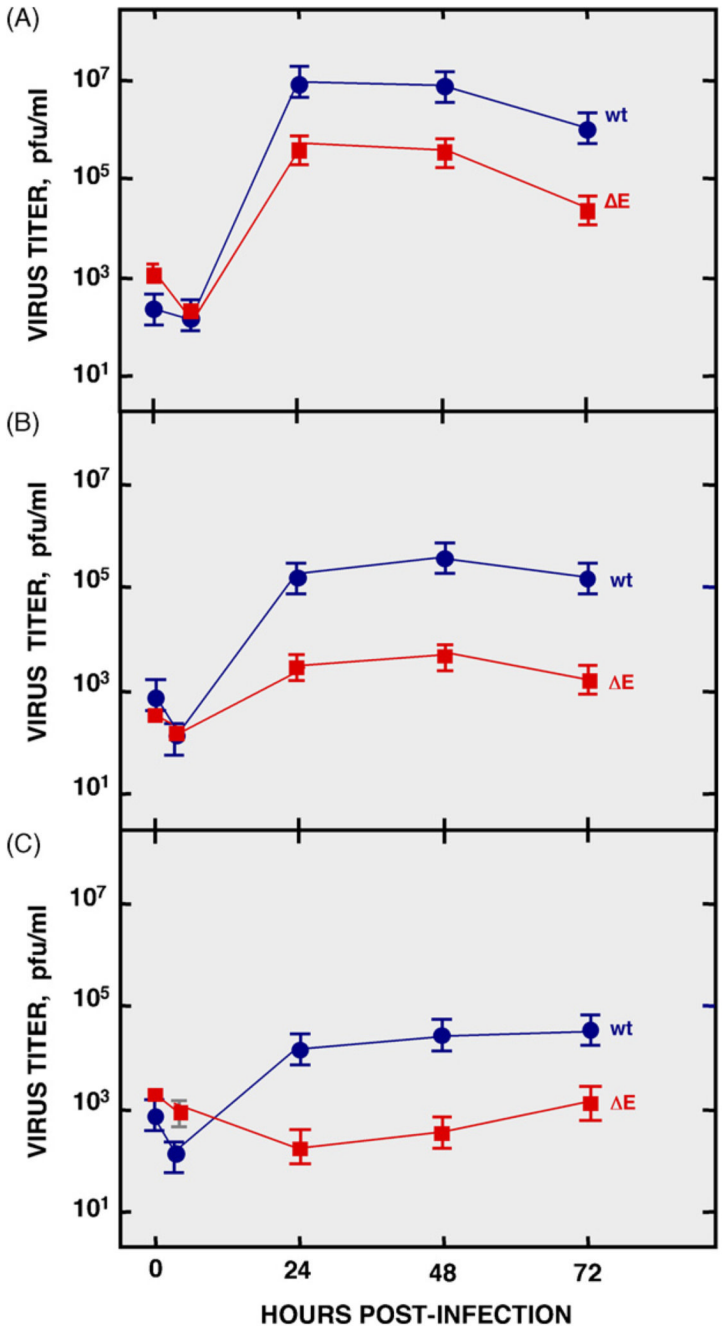

Fig. 4.

Propagation of SARS-CoV with a deleted E gene in different cell lines. Vero E6 (A), Huh-7 (B), and CaCo-2 (C) cells were infected at a moi of 0.5 with either the rSARS-CoV-ΔE or the recombinant wild-type virus. At different times post-infection, virus titers were determined by plaque assay on Vero E6 cells. Error bars represent standard deviations from the mean from three experiments.

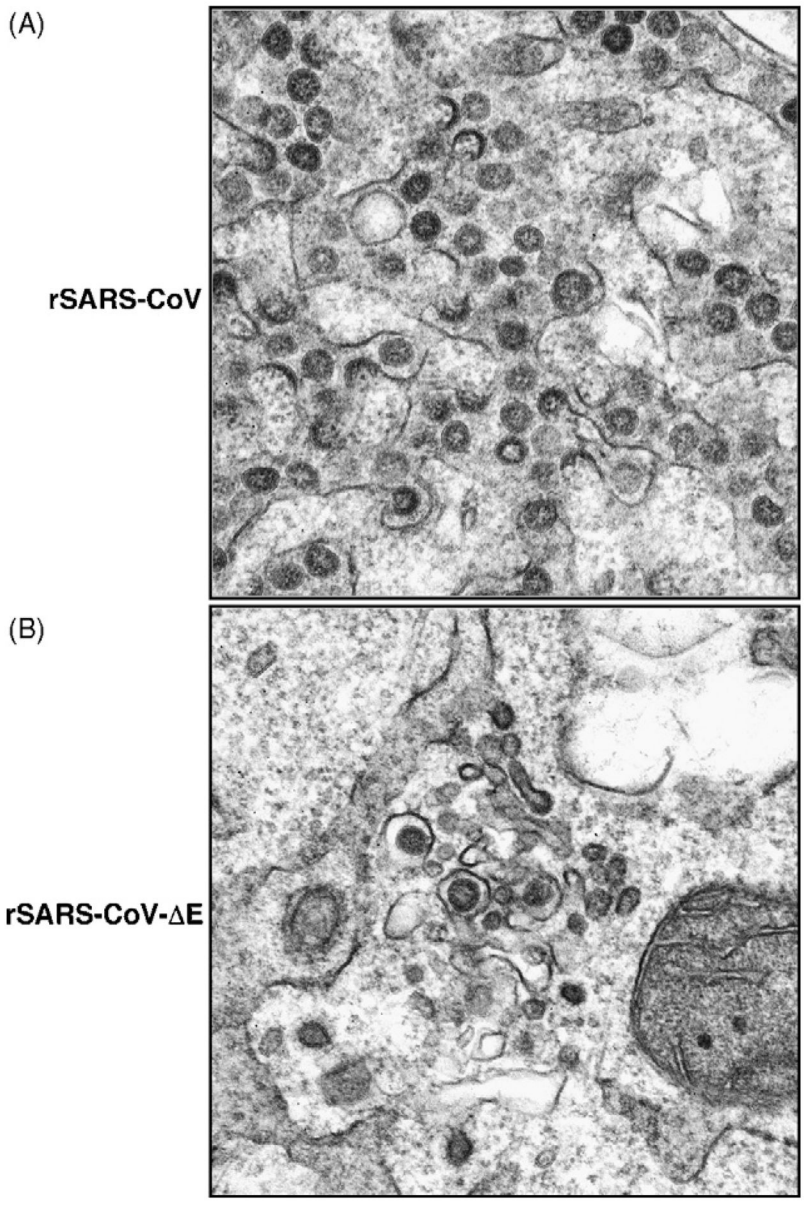

Fig. 5.
Assembly of SARS-CoV-ΔE deletion mutant in the ERGIG compartment. Electron micrographs of Vero E6 cells infected (moi 1.0) with SARS-CoV (A) and SARS-CoV-ΔE (B) at 24 h post-infection.

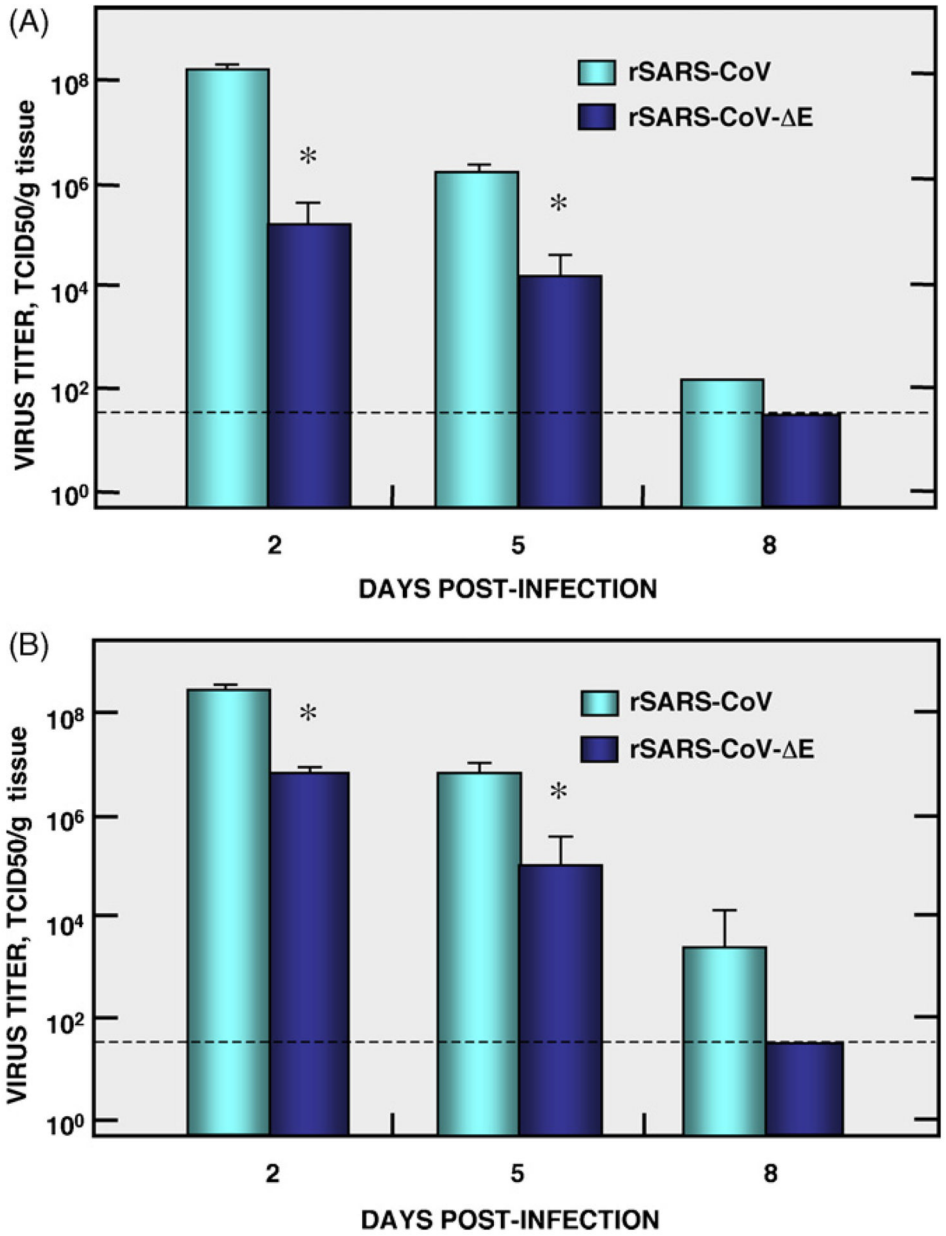

Fig. 6.

Growth of rSARS-CoV in the respiratory tract of hamsters. Hamsters were inoculated with 10^3TCID$_{50}$ of rSARS-CoV or rSARS-CoV-ΔE. Animals were sacrified and tissues were harvested at different times post-infection. Viral titers in lung (A) and nasal turbinates (B) were determined in Vero E6 cells monolayers. The non-parametric Mann–Whitney *U*-statistical method was used for ascertaining the significance of observed differences. Statistical significance was indicated by ($^*p$-value < 0.05). The dotted line indicates the lower limit of detection.

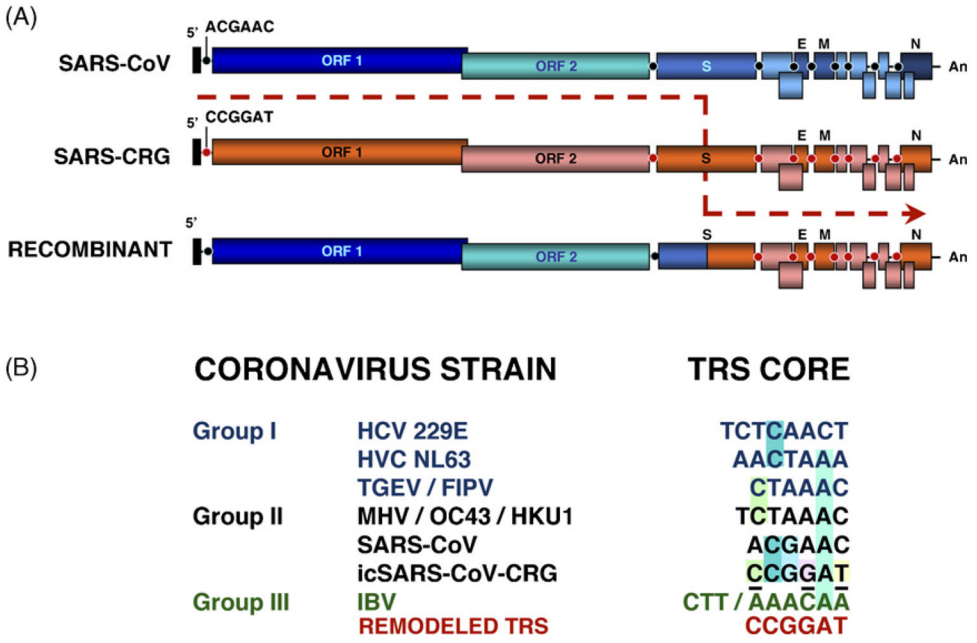

Fig. 7.

(A) Genome organization of SARS-CoV recombinant viruses to generate safe attenuated viruses. The wild-type SARS-CoV TRS, ACGAAC (blue circles), were changed to CCGGAT (red circles). Since the wild-type and mutant TRS signals are not compatible in regulating subgenomic transcription (Yount et al., 2006), a recombination event resulting in a viral genome with mixed TRS signals is not viable. (B) The icSARS-CoV TRS sequence is unique from that of other described coronaviruses. TRS sequences for select group 1, 2 and 3 coronaviruses are summarized. The TRS selected for the remodeled virus is shown at the bottom.