Chapter 14
Structure, Immunopathogenesis and Vaccines Against SARS Coronavirus

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Abstract A new disease, severe atypical respiratory syndrome (SARS), emerged in China in late 2002 and developed into the first epidemic of the 21st century. The disease was caused by an unknown animal coronavirus (CoV) that had crossed the species barrier through close contact of humans with infected animals, and was identified as the etiological agent for SARS. This new CoV not only became readily transmissible between humans but also was also more pathogenic. The disease spread across the world rapidly due to the air travel, and infected 8096 people and caused 774 deaths in 26 countries on 5 continents. The disease is characterized by flu-like symptoms, including high fever, malaise, cough, diarrhea, and infiltrates visible on chest radiography. The overall mortality was about 10%, but varied profoundly with age; the course of disease seemed to be milder in the pediatric age group and resulted rarely in a fatal outcome, but the mortality in the elderly was as high as 50%. Aggressive quarantine measures taken by the health authorities have successfully contained and terminated the disease transmission. As a result there are no SARS cases recorded recently. Nevertheless there is a possibility that the disease may emerge in the population with high vigor. Significant progress has been made in understanding the disease biology, pathogenesis, development of animal models, and design and evaluation of different vaccines, and these are the focus of this chapter.

14.1 Introduction

A new infectious disease, known as severe acute respiratory syndrome (SARS), appeared in the Guangdong province of southern China in 2002. It was characterized mainly by flu-like symptoms, including high fevers, dry nonproductive dyspnea, and infiltrates visible on chest radiography. In about a third of all cases, the resulting

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pneumonia led to acute breathing problems requiring artificial respirators [1]. The overall mortality for SARS was about 10%, but varied greatly with age with a mortality rate in the elderly as high as 50% [2, 3, 4]. A previously unidentified coronavirus was isolated from Vero and FRhK-4 cells that were inoculated with clinical specimens (nasopharyngeal, oropharyngeal and sputum) from SARS patients [5, 6, 7]. The association of the virus with the disease was confirmed when monkeys that were inoculated with the virus developed symptoms similar to those observed in human cases of SARS [8]. Although accurate information about the onset of the SARS epidemic is not available, the Chinese Ministry of Health reported an outbreak of unexplained pneumonia to the World Health Organization (WHO) in February 2003. The SARS associated coronavirus (SARS-CoV) is believed to have jumped from an animal host to humans in rural areas of the Guangdong province, and then spread rapidly throughout the world via air travel. During the period from November 2002 to July 2003, the epidemic of SARS spread to 29 countries, affected approximately 8,000 people, resulted in about 800 deaths and severely crippled the Asian economy. The overall cost of the outbreak was estimated to approach $100 billion, mostly as a result of cancelled travel and decreased investment in the affected region [9]. However, aggressive quarantine measures successfully controlled the emergence of SARS in 2003, yet in January 2004 two new confirmed cases of community acquired SARS has been reported in China. This suggests that this infectious disease has not been completely eliminated, and may dramatically re-emerge in the human population [10].

Based on the phylogenetic analysis of the replicase genes of Coronaviruses (CoVs), they are divided into three main sero groups: ***group I CoVs***, including transmissible gastroenteritis virus and human CoV 229E; ***group II CoVs***, including mouse hepatitis virus and bovine CoV; and ***group III***, including infectious bronchitis virus (Fig. 14.1) [11]. Phylogenetic analyses of the complete genome sequence of the SARS-CoV suggests that it is not closely related to any of the three previously identified coronavirus groups, nor does it seem to be a reassortant of known coronaviruses [12]. Its unique sequence suggests that the virus has evolved independently from the other members of the family for a long period of time. The search for a possible natural reservoir of the SARS-CoV is ongoing, since it could serve as the launch pad for another SARS outbreak. To date, these efforts have limited success because a virus with very close sequence homology was isolated from palm civets, raccoons, dogs, and the Chinese ferret badger, indicating that the virus may have jumped recently from these mammals to humans [13]. Cats may be infected with the virus and can spread it, but do not show clinical signs of infection [14]. Moreover, the virus has been detected on the body-surface and gut contents of cockroaches by PCR, but their organs were negative, so they might act as a mechanical vector of virus transmission [15]. The pandemic potential and pathogenicity of SARS-CoV, as well as the absence of effective licensed drugs, highlights the need for aggressive efforts directed toward the development of a safe and effective vaccine. The availability of a prophylactic vaccine would be a particularly desirable solution, since it would not only prevent disease in vaccinated people, but it would also reduce overall spread of the virus. While the development of coronavirus vaccines generally has

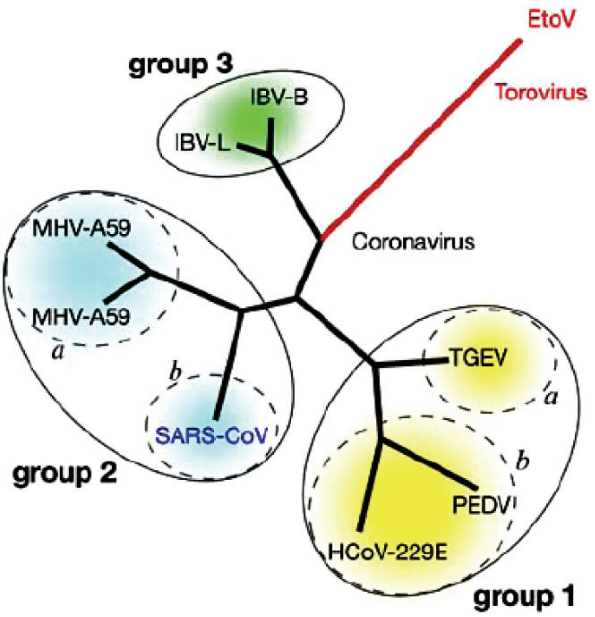

Fig. 14.1 Phylogenetic analysis of coronavirus replicase genes. SARS-CoV linage is derived from group 2 viruses. IBV, infectious bronchitis virus; TGEV, porcine transmissible gastroenteritis virus; EtoV, equine torovirus; MHV-A59, murine hepatitis virus A59; PEDV, porcine epidemic diarrhea virus; HCoV-229E, human coronavirus 229E (Figure is adapted from [11, 148])

been challenging, there are several encouraging factors which point towards the feasibility of developing a SARS-CoV vaccine: (1) the evidence that SARS-CoV is inducing an acute infection and disease (it is generally more complicated to develop a vaccine against a microorganism that induces a chronic infection); (2) the infection mounts a strong humoral response; (3) passive transfer of sera obtained from convalescent patients to SARS patients resulted in the reduction of the viral load that saved the lives of the patients; (4) the relative ease with which the virus can be propagated in vitro; and (5) as a proof of concept there are some effective licensed veterinary coronavirus vaccines based on inactivated or live attenuated virus, including those against a canine coronavirus and avian infectious bronchitis virus.

In this chapter, we will focus on: (a) Genes and proteins of the SARS-CoV; (b) correlates of protection; (c) animal models; (d) application of different technologies for developing SARS vaccine; (e) use of adjuvant and delivery systems for enhancing the potency of the vaccine, and (f) the potential issue of disease enhancement due to vaccines.

14.1.1 Gene Organization of SARS Coronavirus

Similar to other coronaviruses, SARS-CoV is an enveloped positive-strand RNA virus, featuring a large viral genome encoding for the three different types of proteins known as (i) structural, (ii) non-structural and (iii) accessory proteins. We will discuss these different proteins one by one.

14.1.2 Structural Proteins

This group of proteins includes the spike (S), envelope (E), matrix (M) glycoproteins, the nucleocapsid protein (N) [16, 17]. The S, M and E proteins are incorporated into the viral envelope, and S-protein dimers or trimers protrude from the viral membrane, providing CoVs with a characteristic corona-resembling shape (Fig. 14.2). For the structural proteins we will focus on Spike and nucleocapsid proteins, as they are the prime targets for developing an effective and efficacious vaccine.

14.1.3 SARS Spike Protein

The S protein of SARS-CoV is a large transmembrane glycoprotein of coronaviruses, and is responsible for virus binding, fusion and entry. Since the S protein is exposed on the surface of the virion, it is the major target for inducing neutralizing antibodies. Furthermore, the S protein plays critical roles in viral pathogenesis and virulence, and is also important for viral functions and antigenicity [18].

The S protein is a type I transmembrane glycoprotein with 1255 amino acids. All CoV S-proteins contain an N-terminal signal peptide, which facilitates transport into the endoplasmic reticulum, where the proteins are extensively glycosylated. Notably, SARS-CoV S has 23 consensus sites for N-linked glycosylation [16, 17, 19, 20, 21]. Comparison of SARS S protein with the S proteins of other coronaviruses, revealed that the sequence of the SARS-CoV S protein may have the following hypothetical features (i) a 13-amino-acid cleavable secretory signal [16], (ii) a putative S1 globular domain (residues 15–680) with a potential receptor binding

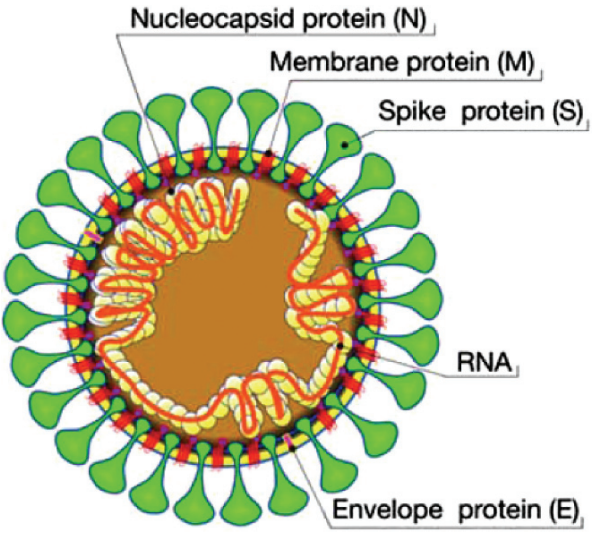

Fig. 14.2 Morphology of the SARS-CoV. A Schematic presentation of the virus. A lipid bilayer comprising the spike protein, the membrane protein and the envelope protein cloaks the helical nucleocapsid, which consist of the nucleocapsid protein that is associated with the viral RNA (adapted from [148])

site [7, 21, 22, 23], (iii) a putative S2 stalk domain (residues 681–1255) with a fusion peptide and heptad repeats, (iv) a hydrophobic transmembrane (TM) domain near the C terminus that could be responsible for anchoring the S protein to the virion lipid envelope [24], and (v) a cysteine-rich (Cy) domain immediately following the membrane anchor region, a feature common to all other coronaviruses which may be involved in stabilizing protein-lipid interactions.

The S1 domain is responsible for virus binding to the receptor on the target cells. It has been demonstrated that angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV [7, 22, 25, 26]. Investigators have mapped an approximately 200 amino acid region of S1 domain (318–510 amino acids) that is responsible for interacting with ACE2, its receptor. This 200 amino acid domain is known as the receptor-binding domain (RBD) [27, 28, 29]. The S2 domain contains a putative fusion peptide and two heptad repeat (HR1 and HR2) regions. Upon binding of RBD on the viral S protein to ACE2 on target cells, S2 changes conformation by interaction between the HR1 and HR2 regions to form fusogenic core and bring viral and target cell membrane into close proximity, resulting in virus fusion and entry [30]. The HR1 and HR2 regions can associate to form a six-helix bundle structure [30, 31], and a peptide derived from the HR2 region of SARS-CoV S protein had inhibitory activity on SARS-CoV infection [30]. This indicates that the fragments containing the functional domains on the S protein may be used as antigens for inducing antibodies to block virus binding or fusion. Notably, glycoproteins (GPs) of highly divergent viruses, including human immunodeficiency virus (HIV) [32], and mouse hepatitis coronavirus (MHV) [24, 33] exhibit a similar architecture. These GPs, referred to as class I fusion proteins, use similar mechanisms to promote membrane fusion, which has important implications for therapeutic intervention. However, despite the similarities in domain organization, the SARS-CoV S-protein does not exhibit significant sequence identity with S-protein of any other CoVs; the highest sequence conservation is found in heptad repeats (HRs) located within the S2 regions underlining their important function.

The S polypeptide is N-glycosylated co-translationally in the endoplasmic reticulum (ER) and further processed in the Golgi apparatus [24]. The S glycoproteins of human coronavirus 229E, transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus, feline infectious peritonitis virus, and canine coronavirus remain as single glycoproteins, those of mouse hepatitis virus, bovine coronaviruses, and human coronavirus OC43 are proteolytically cleaved into two subunits, S1 and S2, during the cellular transport process. Earlier studies of TGEV indicate that the S protein is an oligomer composed of three copies of the monomeric S glycoprotein [34]. Such a quaternary structure has been reported for other enveloped RNA viruses and has been demonstrated to be important for eliciting neutralizing antibodies against hemagglutinin A (HA) of influenza virus [35], the gp120-gp41 heterodimer of human immunodeficiency virus [36], and the G protein of vesicular stomatitis virus [37].

It is believed that S-protein is present as a trimer on the surface of SARS CoV. Li and colleagues [38] have characterized four sequential states of a purified recombinant S ectodomain (S-e) comprising S1 and the ectodomain of S2. They

are S-e monomers, uncleaved S-e trimers, cleaved S-e trimers, and dissociated S1 monomers and S2 trimer rosettes. Lowered pH induces an irreversible transition from flexible, L-shaped S-e monomers to clove-shaped trimers. Protease cleavage of the trimer occurs at the S1-S2 boundary; an ensuing S1 dissociation leads to a major rearrangement of the trimeric S2 and to formation of rosettes likely to represent clusters of elongated, post-fusion trimers of S2 associated through their fusion peptides. The states and transitions of S suggest conformational changes that mediate viral entry into cells. However, S-protein in different conformations is yet to be evaluated for its efficacy in inducing potent neutralizing antibody responses.

14.2 The Interaction of S-protein with Dendritic Cells

Binding of viral glycoproteins to cellular factors other than the receptor(s) does not enable entry but can enhance viral infection. Therefore, various pathogens, including HIV, are thought to interact with factors on dendritic cells (DCs) to promote their spread within infected individuals [39]. Binding of HIV to DCs facilitates infection of nearby susceptible cells through a mechanism that is not completely understood. The lectin DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) or related molecules might be instrumental to this process because DC-SIGN expressed on cell lines binds to the GP of HIV and catalyzes infection of adjacent receptor-positive cells. DC-SIGN also interacts with SARS-CoV S-protein and augments infection with retroviral particles bearing the S-protein on their envelope [40, 41]. This observation is reflected by efficient DC-mediated SARS-CoV transmission to target cells [40, 41]. Furthermore, additional factors other than DC-SIGN are clearly involved in viral transfer [40]. Notably, DCs are not permissive to virus infection, indicating that productive infection is not required for transmission [40, 41]. However, the interaction of SARS-CoV with DCs could contribute to SARS pathogenesis. Attachment of SARS-CoV to dermal DCs might facilitate viral spread in the skin, whereas DC-SIGN-positive alveolar macrophages could promote SARS-CoV replication in the lung. Moreover, internalization of SARS-CoV by DCs might provide the virus with means of immune escape.

14.3 Receptor for SARS-CoV

Efforts from several groups were focused on the identification of the cellular receptor for SARS-CoV. Li et al. [7] reported that the metallopeptidase angiotensin-converting enzyme 2 (ACE2) is a receptor for SARS-CoV. They had used a soluble S1-immunoglobulin (Ig) fusion protein for immunoprecipitation experiments with lysates from Vero E6 cells, the cell type used for the isolation of SARS-CoV. Subsequent proteomic analysis revealed ACE2 to be a high-affinity binding partner of S1. Inhibition of SARS-CoV infection of susceptible cells using antibodies against

ACE2, in conjunction with the observation that ACE2 expression in a resistant cell line for SARS CoV makes it highly susceptible for SARS infection, indicated that the interaction of S1 protein to ACE2 facilitated SARS-CoV infection [7]. Wong et al. also identified ACE2 as a SARS-CoV receptor by using a different approach [28]. Binding studies using soluble fragments of SARS-CoV S-protein revealed residues 318 to 510 to be the minimal receptor-binding domain [21, 27, 28]. An initial search for S-protein residues that are important for ACE2 binding pointed to E452 and D454, with the latter being crucial for association with ACE2 [28]. However, the exact regions of ACE2, which are in contact with the S-protein, remain to be identified. Recently, the structure of the ACE2 ectodomain has been resolved, revealing two ridges flanking the catalytic site [42]. Molecular modeling suggests that these ridges might interact with the S-protein [26]. Interestingly, binding of ACE2 to an inhibitor and probably also to substrate induces structural changes within these ridges [42] and might interfere with binding to the S-protein. Detailed analysis will be required to identify residues within the S-protein and ACE2 that are critical for their interaction; and therefore represent attractive targets for developing inhibitors. It is also conceivable that inducing potent antibodies against the receptor-binding domain could be an attractive strategy for developing a vaccine against the SARS.

These studies might also have important implications for the development of small animal models. Thus, ACE2 from African green monkeys enables efficient entry of SARS-CoV [43], and infection of some macaque species reproduces aspects of SARS in humans [8, 44], while viral replication in mice is less robust and does not induce disease [45]. It will, therefore, be important to determine if a potentially reduced interaction of SARS-CoV S-protein with murine ACE2 limits viral spread in these animals. If that were the case, then the generation of transgenic animals would be of greater significance [7]. ACE2, a carboxypeptidase that cleaves polypeptides from the renal-angiotensin system [46], is essential for cardiac function [47] and is expressed in various tissues and organs [48]. Importantly, major target cells of SARS-CoV, such as pneumocytes [1, 49, 50, 51], express ACE2 [48], and expression in cell lines correlates with permissiveness to SARS-CoV S-driven infection [43], indicating that ACE2 plays a central role in SARS-CoV replication.

14.4 Co-receptor or Alternative Receptors for SARS CoV

In contrast to HIV, the evidence for the requirement of a co-receptor or the existence of alternative receptors for SARS-CoV to entry into certain tissues has not yet been demonstrated. However, ACE2-dependent infection of organs other than the lung might contribute to SARS pathogenesis. For example, small intestinal enterocytes express ACE2 [48] and are permissive for SARS-CoV [49, 51, 52]. Moreover, the efficient infection of renal epithelial cells of different species [41] and isolation of the virus from kidney tissue of a SARS patient [6] suggest that SARS-CoV

infection of kidney cells might contribute to acute renal failure observed in some SARS patients [41, 53]. Infection of intestinal enterocytes and kidney cells could facilitate viral transmission via the fecal-oral route. Viral RNA has been detected in stool samples from SARS patients [1, 52], however, it is unclear if transmission via feces promoted viral spread during the 2003 outbreak. Hepatocytes from SARS patients were also infected [49], and some ACE2-expressing hepatoma cell lines are highly permissive to replication of SARS-CoV [41, 43, 54]. Infection of hepatocytes might therefore partially account for the altered levels of liver-specific enzymes commonly observed in SARS patients [1, 54]. Because liver tissue was found to be largely negative for ACE2 protein expression [48], it will be interesting to examine whether viral entry is facilitated by low levels of ACE2 expression or other factors.

14.5 SARS-CoV Spike Protein Triggering: Low pH Versus Receptor Engagement

Receptor engagement can activate the fusion machinery of viral glycoproteins in two ways. First, binding to receptor can directly activate the fusion process [25, 55], which is the case for HIV and murine leukemia virus (MLV) glycoproteins. Alternatively, receptor engagement can trigger the internalization of viral particles into endosomes where protonation activates glycoprotein-driven membrane fusion [25, 55]. Influenza hemagglutinin and the vesicular stomatitis virus G-protein (VSV-G) are activated by low pH. In the case of SARS CoV it has been demonstrated that inhibitors of vacuolar acidification also block infection by S-bearing pseudotypes, suggesting potential triggering of the fusion activity of SARS-CoV S-protein by low pH [41, 54, 56]. However, SARS-CoV S-driven cell-to-cell fusion can occur in the absence of low pH [21, 25]. Therefore, the S-protein of SARS-CoV might be able to mediate membrane fusion in a pH-dependent and independent fashion, and several parameters might control which stimulus is required under what conditions. One such parameter could be the association between the S1 and S2 subunits of the SARS-CoV S-protein. Many class I fusion proteins are cleaved into an outer and a transmembrane subunit by cellular proteases. In addition, the cleavage is essential for the functionality of the glycoprotein such as the case for HIV envelope. By contrast, S-proteins of group I CoVs are not cleaved at all, and cleavage of the S-protein of MHV, a group II CoV, appears to be cell-type dependent and not required for its functionality [24, 57]. Based on the limited set of data, it seems there are no obvious consensus sites for cellular proteases present in SARS-CoV S protein [16, 17], and efficient cleavage of the protein has not been reported [21, 24, 30, 58]. Notably, protease treatment of cells expressing S-protein resulted in an increased cell-to-cell fusion activity [56], indicating that cleavage of S protein might enable pH-independent, receptor-dependent triggering of the fusion activity. Further work is needed to clearly demonstrate the role of cellular proteases in triggering the S-protein driven membrane fusion of the SARS CoV.

14.6 Membrane Fusion

Two functional elements located in the transmembrane domain of CoV S-proteins are key to the membrane fusion: a putative fusion peptide and two heptads repeat (HR). The function of these elements has been elucidated in the context of prototype class I fusion proteins, such as HIV gp160 [59]. Cleavage of gp160 produces the fusion active form of the transmembrane subunit gp41, which is oriented perpendicular to the viral membrane and contains a fusion peptide (a stretch of hydrophobic amino acids) at its N-terminus. Two HRs (HR1 and HR2) are located between the fusion peptide and the transmembrane domain. During the fusion process, the fusion peptide inserts into the target cell membrane, HR2 folds back onto HR1, resulting in the formation of a six-helix bundle structure (trimer of dimers). In this conformation, the HRs are oriented in an anti-parallel fashion, thereby bringing the fusion peptide (inserted into the target cell membrane) and the transmembrane domain (inserted into the viral membrane) into close contact, which ultimately facilitates the membrane fusion [59].

Bosch et al. [24] demonstrated that fusion driven by the S-protein of MHV follows similar principles; however, a major difference compared to HIV was observed. The fact that the cleavage of many CoV S-proteins, including that of SARS-CoV, is not needed for exerting their function suggests that these proteins must have an internal fusion peptide similar to the G-protein of VSV. A computer-based analysis has predicted a potential fusion peptide at the N-terminus of HR1 in SARS-CoV S [59]. In light of these observations, the model for membrane fusion illustrated previously must be revised for the SARS-CoV S-protein. Thus, upon exposure to low pH it is possible that an internal fusion peptide, which is covalently associated with both the S1 and S2 subunits, inserts into the target cell membrane, and the membrane fusion is driven by the formation of the six-helix bundle between HR1 and HR2. In case of influenza HA, a low pH environment triggers irreversible conformational changes associated with membrane fusion, whereas exposure of VSV-G to low pH induces a reversible transition into the fusion active state [59]. The nature of pH-induced conformational changes in SARS-CoV S-protein remains to be determined. If the model proposed above accurately describes SARS-CoV S-driven membrane fusion, one would expect that peptides mimicking HR1 or HR2 should assemble into a six-helix bundle and that such peptides would inhibit SARS-CoV S-mediated membrane fusion. The latter speculation is based on evidence obtained with several viral class I fusion proteins, including MHV S-protein [24], for which HR-derived peptides were shown to inhibit fusion by preventing the formation of the six-helix bundle [59]. The peptide T20, which potently inhibits HIV gp160-driven membrane fusion when present in the low-nanomolar range, has been approved for use in patients, representing the first member of entry inhibitors, a new class of therapeutics [60]. Therefore, blocking the six-bundle formation either by chemical means such as T-20 is a proven therapeutic agent or whereas directing antibodies to the critical elements may represent an attractive target for developing a vaccine against SARS-CoV.

14.7 SARS Nucleocapsid Protein

The N protein, which binds to the genomic RNA via a leader sequence, recognizes a stretch of RNA that serves as a packaging signal and leads to the formation of the helical ribonucleoprotein (RNP) complex during assembly. The structure of the RNA-binding domain of the SARS-CoV N protein was determined by NMR spectroscopy in 2004 [61]. It consists of a five-stranded β sheet whose folding is unrelated to that of other RNA-binding proteins. The authors identified a binding site for single-stranded RNA (ssRNA), using NMR to determine the resonance of residues perturbed by the addition of RNA, and revealed a similar mode of interaction to RNA-binding proteins such as U1A RNP. They also identified small molecules from an NMR-based screen that bind to the RNA-binding domain and might impair its function. Antigenic peptides of the coronavirus N protein can be recognized by T cells on the surface of infected cells [62, 63]. The structure of the MHC-I molecule HLAA* 1101 in complex with such a peptide derived from the SARS-CoV N protein, a nonamer with a SARS-specific sequence, has recently been determined to 1.45 A° resolution [61]. It is similar to other MHC-I molecules and shows a similar peptide-binding mode, and thus this structure adds to the growing library of MHC-I structures and could be used as a template for peptide-based vaccine design.

14.8 Non-structural Proteins

The SARS-CoV replicase gene encodes for 16 non-structural proteins (nsp), with multiple enzymatic functions. These are known or predicted to include types of enzymes that are common components of the replication machinery of plus-strand RNA viruses: an RNA-dependent RNA polymerase activity (RdRp, nsp12); a 3C-like serine protease activity (Mpro or 3CLpro, nsp5); a papain-like protease 2 activity (PL2pro, nsp3); and a superfamily-1 helicase activity (HEL1, nsp13) [16, 64, 65]. In addition, the replicase gene encodes proteins that are indicative of 30–50 exoribonuclease activity (ExoN homologue, nsp14), endoribonuclease activity (XendoU homologue, nsp15), adenosine diphosphate-ribose 10-phosphatase activity (ADRP, nsp3) and ribose 20-O-methyltransferase activity (20-O-MT, nsp16). These enzymes are less common in plus-strand RNA viruses, and may therefore be related to the unique properties of coronavirus replication and transcription. Finally, the replicase gene encodes another nine proteins, of which little is known about their structure or function. The nsp 4, 10 and 16 have been implicated by genetic analysis in the assembly of a functional replicase–transcriptase complex.

14.9 Accessory Proteins

The genomic sequences of numerous SARS-CoV isolates have been determined. The ‘conserved’ open reading frames (ORFs) of the SARS-CoV genome occur in the same order as and are of similar size to those found in other coronaviruses. However, in addition to the conserved genes, the SARS-CoV genome contains eight novel

ORFs at the 3′ end (ORFs 3a, 3b, 6, 7a, 7b, 8a, 8b and 9b) [11]. To date, the functions of these genes remain largely unknown, although their absence from other genomes suggests unique functions that might be advantageous to SARS-CoV replication, assembly or virulence [66]. Only one of these so-called accessory proteins has a known structure and further studies are required to elucidate their precise functions.

14.10 Correlate of Protection for SARS

SARS CoV infection of humans results in the development of acute respiratory syndrome in about 10% of the patients, which usually results in mortality. Therefore, one of the major questions in SARS is how the immune system manages to control the infection in the majority of patients. The S glycoprotein is a major structural protein of SARS-CoV and a potential target for SARS-specific humoral immunity and/or cell-mediated immune responses. Recent studies in animal models have demonstrated that vaccines based on the S protein of SARS-CoV seem to induce a considerable neutralizing antibody response [58, 67, 68, 69] and provide protection via inhibition of viral replication after challenge with live SARS-CoV [68]. In addition, SARS-CoV specific IgG can be detected at week 3 after the onset of syndromes in SARS patients and persist for a long period of time [70, 71]. Polyclonal immune sera from convalescent SARS patients were passively transferred to treat SARS patients during the outbreak in 2003 [72]. These findings revealed that humoral immune responses, therefore, play an important role in controlling and clearing SARS-CoV infection in humans and mice. Moreover, the S protein in its native conformation might be a suitable candidate for vaccine approaches. Indeed, immunization of macaques with adenoviruses coding for S, M, and N triggered the production of neutralizing antibodies, providing at least some indirect proof that S-based vaccines hold promise. To clearly demonstrate that antibodies are really involved in the control of SARS CoV infection, investigators resorted to the use of passive transfer experiments, a practice of administering polyclonal immunoglobulin isolated from hyperimmune sera of animal or human origin, introduced by Von Behring and Kitasato. It has been used extensively in prophylactic as well as in therapeutic settings [73]. However, the risks related to the use of human blood products make them problematic as a standard therapy. Human monoclonal antibodies may be a solution to some of the problems. In addition the use of mAbs has the added advantage of using higher tittered and higher avidity antibody directed against the protective epitope(s). Furthermore, it may also alleviate the concern of disease enhancement, since generally low avidity antibodies are considered to cause disease enhancement.

14.11 Passive Transfer of Monoclonal Antibodies

The first human monoclonal antibody (mAb) resulted from a screen of a human non-immune single-chain variable region fragment (scFv) phage library constructed from B cells against the S1 domain of the SARS-CoV spike protein. The antibody

had a high neutralization activity in vitro and blocked syncytia formation of 293T cells expressing the S protein on their surface [74]. When the antibody was given prophylactically to mice at doses therapeutically achievable in humans, viral replication was reduced by more than 4 orders of magnitude and was no longer detectable [74]. Also employing phage display technology to screen a naïve antibody library for antibodies reactive against the S protein, ter Meulen and colleagues isolated a human mAb belonging to the IgG1 subclass [75]. Prophylactic administration of this antibody to ferrets, an animal model of SARS-CoV infection and disease, has reduced replication of SARS-CoV in the lungs of infected ferrets by 3.3 logs ($p < 0.001$), completely prevented the development of SARS-CoV induced lung pathology ($p < 0.013$), and abolished shedding of the virus in pharyngeal secretions. Traggiai and coworkers [76] chose B cells from a convalescent person to identify human mAbs recognizing SARS-CoV. Through a combination of magnetic and fluorescence-activated cell sorting they isolated memory B-lymphocytes, which were subsequently immortalized with Epstein-Barr virus (EBV). B lymphocyte clones were selected according to their ability to recognize the SARS-CoV proteins and/or to neutralize the virus. Surprisingly, only a small fraction of memory B cells specific for SARS-CoV antigens were directed against neutralizing epitopes present in the spike protein. One of the human mAbs was tested in a mouse model for its in vivo neutralizing activity [45]. It protected the lungs completely and the upper respiratory tract partially from virus replication when given two days prior to challenge with SARS-CoV. This approach has the advantage that it is fast, as it can be completed within 3 months, and efficient. It generates large numbers of antibodies that can immediately be screened for the most favorable affinity and epitope specificity. Passive immunization certainly has its merits especially when it comes to the prophylactic protection of high-risk groups like health care workers but might also be of therapeutic benefit when given early after onset of the disease.

14.12 The Role of Cell Mediated Immunity in SARS-CoV Infection

The role of cell-mediated immunity in the resolution of SARS-CoV infection in humans is still not well understood. In an study by Yang and colleagues, the investigators observed that memory T-cell responses against the S protein were persistent for more than 1 year after SARS-CoV infection by detecting the production of IFN-γ using ELISA and ELISpot assays [77]. Flow cytometric analysis demonstrated that both CD4+ and CD8+ T cells were involved in cellular responses against SARS-CoV infection. Interestingly, most of SARS-CoV S-specific memory CD4+ T cells were central memory cells with the CD45RO+ CCR7+ CD62L phenotype. However, the majority of memory CD8+ T cells revealed effector memory phenotype, CD45RO-CCR7-CD62L-. Thus, the study provides evidence that SARS-CoV infection in humans can induce cellular immune responses that are persistent for a long period of time. These data may argue for the design a vaccine that may be effective in

inducing not only potent neutralizing antibody responses but also cellular responses and T-helper cell responses as well. Nevertheless, further work would be needed to establish a direct correlation between strong cellular responses and reduced viral load.

14.13 Animal Models

An authentic animal model that represents infections of humans with SARS-CoV is critical for a better understanding of the disease. In addition, animal models are key for pre-clinical evaluations of the most effective vaccines leading to clinical evaluations. Efforts have been made by different groups to develop rhesus, mouse, and ferret challenge models for SARS-CoV infection. SARS-CoV can infect cynomolgus macaques (*Macaca fascicularis*) following intratracheal inoculation [8, 44, 78, 79]. The histopathologic pattern resembled that seen in humans dying of SARS when the animals were analyzed 4 or 6 days after infection with a Hong Kong SARS-CoV isolate [8, 44]. A different study using the Tor2 isolate found only mild, self limited, respiratory symptoms in some animals and low-level virus replication [78]. Experimental infection of the three species of Old World monkeys (African Green, rhesus, and cynomolgus monkeys) with SARS-CoV strain Urbani *via* the respiratory route revealed a quantitative difference in virus replication in the upper and lower respiratory tract of the three species; the virus replicated to higher titers and for a longer time in the respiratory tract of African Green monkeys (AGM) compared to cynomolgus or rhesus monkeys. The titer of serum neutralizing antibodies induced in these animals correlated with the level of viral replication in the respiratory tract. Histopathologic examination of African green monkey lungs were consistent with those reported by Kuiken et al. and Fouchier et al. although they found more evidence for pneumonitis at earlier time-points post infection (2 days vs. 4 or 6 days, respectively) [79]. Ferrets, cats, mice, and Golden Syrian hamsters have also been successfully infected with SARS-CoV. All of these animal models support viral replication in the upper and lower respiratory tract although no clinical signs were seen in SARS-CoV inoculated cats. Infected ferrets on the other hand became lethargic from day 2–4 post infection, and developed a lung pathology similar to but milder than those described for infected macaques. Both infected cats and ferrets were able to efficiently transmit the virus to other animals living in close proximity [14]. Following intranasal administration of SARS-CoV strain Urbani, the virus replicates in the respiratory tract of BALB/c mice [45] and Golden Syrian hamsters [80]. The kinetics of virus replication in both species resembles each other, peaking at day 2, post infection and the virus clears after 5–7 days. In hamsters, however, the virus reaches higher titers especially in the upper respiratory tract, and the animals are shedding the virus for a longer time. In contrast to mice, hamsters showed pathology in the upper and lower respiratory tract, as well as viremia and extrapulmonary spread of the virus to liver and spleen. Neither Golden Syrian hamster nor BALB/c mice showed any clinical signs of disease with the exception of aged mice. Twelve to fourteen months old BALB/c mice infected with SARS-CoV

demonstrated signs of clinical illness as characterized by weight loss, hunching, ruffled fur, and slight dehydration, which were resolved by day 7-post infection. Compared to young BALB/c mice, SARS-CoV replication was enhanced and prolonged in the aged mice; virus titers were higher in the lungs and accompanied by alveolar damage and interstitial pneumonitis [80]. All animal models described above differ from the human disease in two important aspects: (a) the period between infection and the peak in viral load is shorter; and (b) pathology is shortened and the disease rarely progresses to a fatal outcome. But despite these differences, these animal models are important and useful for vaccine evaluation.

14.14 Development of SARS Vaccine using Different Platform Technologies

Vaccine efficacy is generally measured by the ability of the antigen to raise a protective immunologic response from B and/or T cells after exposure to the pathogen. Ideally, by creating antigen-specific memory within the immune system, individuals will be protected from infection for decades. Several veterinary coronavirus vaccines are currently available, but their efficacy is variable. The vaccine for prevention of infectious bronchitis virus (IBV), which infects chickens, is effective [81], but the canine and porcine vaccines are only partially effective [82]. The feline infectious peritonitis (FIP) vaccine is actually deleterious to the health of the animal.

There are several independent and parallel vaccine approaches being evaluated against the SARS-CoV: (i) live attenuated or inactivated virus, (ii) DNA Vaccines; (iii) DNA prime and boost approach; iv) use of viral vectors for delivering the vaccine; (v) recombinant subunit vaccine, and (vi) use of virus like particles (VLP). All these approaches have potential advantages and disadvantages, and one needs to weigh the pros and cons for each technology, and select the technology, which is most potent in inducing protective immune responses.

14.14.1 Attenuated and Inactivated Whole Virus Based Vaccines

The choice of an inactivated vaccine is, without question, the most expeditious route that can be pursued to reach the clinical evaluation stage of a potential SARS-CoV vaccine. It has a safety record established by immunizing people with hundreds of million doses and they are generally easy to manufacture. Moreover, they are able to induce a broad immune response against all antigenic determinants of the virus. In fact, many groups from academic institutions and industry are working on the development of an inactivated vaccine against the SARS-CoV and in December 2004 Sinovac Biotech announced that all 36 subjects participating in a phase I human clinical trial testing an inactivated SARS-CoV vaccine had received their second and last vaccination (http://www.news-medical.net/?id=4560). So far, no adverse reactions have been reported and all participants are in good health. A recent

study by Zhou and colleagues [83] describes a formaldehyde inactivated whole virus vaccine, which was tested for its immunogenicity, safety, and protective efficacy in rhesus monkeys. The animals were immunized twice intramuscularly (i.m) at one-week interval with 0.5, 5, or 50 μg of non-adjuvanted vaccine and challenged 2 weeks after the second immunization. None of the vaccinated animals developed any clinical symptoms upon virus challenge. However, the control group showed only minor clinical signs. Two weeks after the second immunization SARS-CoV specific IgG and neutralization titers were highest in the 50 μg group and those animals were protected from virus challenge, as no replicating virus could be detected in the lungs 15 days post challenge. However, animals receiving lower dosages were only partially protected. The detection of increased IFN-γ concentrations and almost constant IL-4 concentrations in vaccinated animals indicated a Th-1 driven immune response. He and colleagues (2004) demonstrated that SARS-CoV inactivated by β-propiolactone (BPL) elicited high titers of antibodies in the immunized mice and rabbits that recognize the S protein, especially the receptor-binding domain (RBD) in the S1 region [84]. The antisera from the immunized animals efficiently bound to the RBD and blocked binding of RBD to angiotensin-converting enzyme 2, the functional receptor on the susceptible cells for SARS-CoV. With a sensitive and quantitative single-cycle infection assay, using pseudovirus bearing the SARS-CoV S protein, the investigators demonstrated that mouse and rabbit antisera significantly inhibited S protein-mediated virus entry with mean 50% inhibitory titers of 1:7393 and 1:2060, respectively. These data suggest that the RBD of S protein is a major neutralization determinant in the inactivated SARS vaccine, which can induce potent neutralizing antibodies to block SARS-CoV entry [84]. In another study, a BPL inactivated SARS-CoV vaccine was developed and tested for its immunogenicity and efficacy in BALB/c mice in collaboration with the NIH [85]. Animals were immunized at 0, 2, and 4 weeks with 5 μg of inactivated virus with or without the adjuvant MF59, an oil squalene-in-water emulsion, approved for human use in Europe for an influenza vaccine [86]. After three doses, the MF59 adjuvanted BPL-inactivated SARS virus vaccine induced a ten fold higher neutralizing titer (1:645) than the non-adjuvanted vaccine (1:64). IgG subclass determination indicated predominant Th2-type immune response to the adjuvanted vaccine. Two weeks after the last vaccine dose, mice were challenged intranasally and nasal turbinates and lung tissues were analyzed for infectious virus two days later. Complete protection from virus replication was observed in mice that received the MF59 adjuvanted vaccine; neither the nasal turbinates nor the lungs of these mice contained recoverable virus. Immunization with the non-adjuvanted vaccine resulted in complete protection of the upper respiratory tract and a significant ($p < 0.00001$) reduction of viral titers in the lower respiratory tract of 30,000-fold compared to the control groups. A number of other investigators have also evaluated whole virus vaccines using UV, BPL or formaldehyde to inactive the virus in mice and rabbits and combined them with different types of adjuvant [84, 87, 88, 89]. Regardless of the method or combination used, all vaccines elicited strong immune responses underscoring the potential of this approach. One major drawback inherent to all vaccines using inactivated viruses is the high risk of accidents during their production. High amounts of

infectious virus have to be cultivated and purified raising the probability of incidents. Using live attenuated virus strains instead of wild type virus could minimize this risk.

In summary, vaccines can be produced by inactivation of the virus, by using an attenuated or weak form of the virus, or by using recombinant forms of viral components. Inactivated virus vaccines are relatively safe because they cannot revert back to the live form. They are also relatively stable and may not even require refrigeration. This is important in developing countries and for ease in mobilization during outbreak or emergency situations. However, there are limitations to their use. Inactivated vaccines usually require several doses and some are weakly effective at stimulating an immune response. The vaccine to prevent hepatitis A is an example of an inactivated viral vaccine [90]. Furthermore, these vaccines are less characterized, may require special laboratory for the development therefore it may be even harder to get the FDA approval for clinical evaluation. An unfortunate example of this general lack of characterization is the inactivated respiratory syncytial virus, which caused two deaths and many hospitalizations due to disease enhancement in vaccinated infants.

14.14.2 DNA Vaccines

DNA vaccination is quite a powerful strategy, and receiving considerable attention due to its ability to induce both humoral and cellular immune responses. It induces immune responses to, and in some cases even leads to, the protection against various types of infections, such as influenza, malaria, and SARS [91, 92, 93]. A common feature of DNA vaccination is that the synthesis of the antigen occurs in intracellular compartments, allowing the processed antigen to enter the MHC class I pathway, in turn to generate CD8+ cytotoxic T-lymphocyte (CTL) activities. High CTL and antibody responses were observed after mice were injected three times with a recombinant plasmid vector expressing the N protein [94]. Mice immunized with a plasmid containing the S protein produced anti-SARS-CoV IgG and developed neutralizing antibodies and a T-cell mediated response resulting in a six-fold reduction in viral titers in the lungs. Plasmids encoding either the S1 or S2 regions of the spike protein elicited antibody production in mice. Neither the S1 or S2 antibodies alone were capable of neutralizing the virus; however, cooperatively they enabled neutralization of the virus, suggesting that both regions of the spike protein are important for host-cell viral entry.

Jin and colleagues have demonstrated that both humoral- and cellular-mediated specific responses could be induced by DNA vaccines for N, M, and E antigens [95]. All three DNA constructs induced SARS-CoV-specific antibodies in mice, however the highest antibodies were induced against N protein, followed by M and E proteins. In addition, T cell proliferation and DTH responses were also successfully induced in mice after vaccinations with these constructs. These results suggested that the DNA vaccine was effective to prime a specific anti-SARS-CoV response and

apparently generate a broad range of both T-helper and B-cell memory responses during the priming. This was also consistent with previous observations of the protective immunity [96, 97]. Furthermore, the DNA administrations generated a lower level of IL-10, suggesting that DNA vaccines may help polarizing Th-1 type of responses.

Recently, Zhu et al. [98] have demonstrated that a DNA construct based on the pcD3d vector could successfully induce SARS-CoV N protein-specific antibody titers and CTL responses. Kim [99] has demonstrated that vaccination with N DNA vaccine could successfully induce a SARS-CoV antigen-specific CD8+ T cell response and distinctly reduce the titer of recombinant vaccinia virus expressing SARS-CoV N protein after the challenge and that the co-expression of calreticulin (CRT), a 46 kDa Ca2+-binding protein, with N gene could enhance its ability to protect against viral challenges. Therefore, it is consistent that the N protein construct could induce the highest SARS-specific IgG, T cell proliferation, and in vivo CTL response were once again induced for N protein, followed by M and E. Furthermore, the highest Th-1 type responses based on IFN-γ and IL-2 production were also induced against the N protein. It is difficult to answer if N is truly more immunogenic compared to M and E, or the increased level of immune responses may be due to more epitopes in the N nucleocapsid protein since it contains 422 amino acid residues, while the M and E have 220 and 76 amino acids respectively. In addition, the differential concentrations of each antigen presented in the killed SARS-CoV preparation could have also contributed to the differences.

It has been demonstrated in several studies that there is an interaction between the N and M proteins. Shi and colleagues [100] have tried to answer two questions: (a) Can N or M membrane proteins be expressed in a DNA vaccine? and (b) Can the expression of a membrane protein (M) affect the immune responses induced by N protein in the context of a DNA vaccine? The animals were injected with 20 μg of the mixture of DNA vaccines encoding for M and N. The ELISA analysis using the N antigen or inactivated SARS-CoV particles as capture antigen showed that co-injection of SARS-M could enhance the antibody responses against N, especially of the IgG2a subclass. After lymphocytes were stimulated with 10 μg/ml purified N antigen, the CD4+ and CD8+ T cells of N and M plus N group were increased compared with those of control groups. Cytokine ELISA analysis revealed that co-injection of M could enhance the levels of IFN-γ, and IL-2 production induced by the N antigen. Virus challenge test was conducted in BSL3 bio safety laboratory with Brandt's vole SARS-CoV model, and the results indicated that co-immunization of M and N antigens could reduce the mortality and pathological changes in the lungs from virus infection in the mixed vaccine immunized group compared to the single vaccine groups.

In another study, Huang et al. [101] have also demonstrated that immunization of mice with SARS-CoV spike DNA vaccine induced antigen-specific cellular and humoral immune responses. The cellular immune responses were mediated by both CD4+ and CD8+ T cells [101].

14.14.3 DNA Prime Vector or Protein Boost Vaccines

Different forms of SARS coronavirus spike protein-based vaccines were evaluated for the generation of neutralizing antibody responses against SARS-CoV in a mouse model [102]. In this study, they compared six combinations: (a) intra-peritoneal (i.p.) immunization with recombinant spike polypeptide produced in *Escherichia coli* (S-peptide), (b) mice primed with tPA-optimized DNA vaccine (tPA-SDNA) and boosted with S-peptide i.p.; (c) mice primed with CTLA4HingeS ARS800 DNA vaccine (CTLA4-S-DNA) and boosted with S-peptide i.p.; (d) mice primed with oral live-attenuated *Salmonella typhimurium* (*Salmonella*-S-DNA-control) and boosted with. S-peptide i.p, (e) mice primed with oral live-attenuated *S. typhimurium* that contained tPA-optimize800 DNA vaccine (*Salmonella*-tPA-S-DNA) and boosted with. S-peptide i.p; and (f) mice primed with oral live-attenuated *S. typhimurium* that contained CTLA4 Hinge SARS800 DNA vaccine (*Salmonella* tPA-S-DNA) and boosted with. S-peptide i.p. There was no statistically significant difference among the Th-1/Th-2 profile among these six groups of mice with had high antigen-specific IgG levels. Sera of all six mice immunized i.p. with S-peptide, i.m. with DNA vaccine control and oral *Salmonella*-SDNA- control showed no neutralizing antibody against SARS-CoV. Sera of the mice immunized with i.m. tPA-S-DNA, i.m. CTLA4-S-DNA, oral *Salmonella*-S-DNA-control boosted with i.p. S-peptide, oral *Salmonella*-tPA-S-DNA, oral *Salmonella*-tPA-S-DNA boosted with i.p S peptide, oral *Salmonella*-CTLA4-S-DNA and oral *Salmonella*-CTLA4-S-DNA boosted with i.p. S-peptide showed neutralizing antibody titers of $<$1:20–1:160. Sera of all the mice immunized with i.m. tPA-S-DNA boosted with i.p. S-peptide and i.m. CTLA4-S-DNA boosted with i.p. S-peptide showed neutralizing antibody titers of $\geq$ 1:1280. The present observation may have major practical value, such as immunization of civet cats, since production of recombinant proteins from *E. coli* is far less expensive than production of recombinant proteins using eukaryotic systems.

Among all the combinations of vaccines examined in this study, mice primed with SARS-CoV human codon usage optimized spike polypeptide DNA vaccines and boosted with S-peptide produced by *E. coli* generated the highest titers of neutralizing antibody against SARS-CoV. It has been demonstrated that the S-peptide produced by *E. coli* did not induce neutralizing antibody against SARS-CoV infection. On the other hand, recombinant spike polypeptide generated by eukaryotic systems such as transfection of COS7 and BHK21 cells or DNA vaccine was able to elicit high neutralizing antibody titers against SARS-CoV infection [67, 103, 104]. This was probably because the S-peptide produced in *E. coli* did not have the same structure and conformation compared to the S-protein produced in the mammalian expression system. In this study, we documented that although recombinant S-peptide produced by *E. coli* itself was not able to generate neutralizing antibody against SARS-CoV infection, mice primed with spike polypeptide DNA vaccine and boosted with S-peptide from *E. coli* were able to generate high titers of neutralizing antibody against SARS-CoV. This indicates that the type of vaccine used for priming is crucial in determining the type of immune response developed after the boost.

Furthermore, it has also been demonstrated that the humoral immune response developed in mice primed with spike DNA vaccine and boosted with S-peptide from *E. coli* did not develop a Th1 type immune response. However, mice immunized with S-peptide from *E. coli* alone developed a Th1 type response. This indicates that a Th1 type immune response may not be essential for the generation of neutralizing antibodies against SARS-CoV. Although our results suggest that priming with DNA vaccines and boosting with S-peptide produced by *E. coli* was successful in the generation of neutralizing antibody against SARS-CoV, further experiments using infection models to evaluate its protective immunity are warranted, since anti-spike antibodies have been shown to enhance the infectivity of coronaviruses in some cell culture systems, as occurred with SARS-CoV and feline infectious peritonitis virus [105, 106].

Zakhartchouk and colleagues [107] evaluated the efficacy of DNA prime and whole killed SARS-CoV vaccines in combination vs. both the vaccines alone. They have clearly demonstrated that a combination of the vaccines is more immunogenic in mice than the DNA vaccine alone. Higher antibody responses (as compared to DNA vaccine and the whole killed virus vaccine alone) as well as higher cell-mediated responses (as compared to DNA vaccine alone) were elicited. Their finding also suggests that the S protein is expressed in 293 transfected cells as a single, uncleaved polypeptide, but in two differentially glycosylated forms. A combination of the vaccines and the DNA vaccine induced Th1-dominated immune response, while two injections of the whole killed vaccine induced Th2-biased response. It has been shown previously, that aluminum adjuvants skewed the immune response towards a Th2 response and a DNA vaccine enhanced T-cell immune responses [108, 109]. Immunity associated with a Th1-type immune response is thought to be essential for the control intracellular pathogens; therefore, changing the bias of the immune response may be an attractive feature of a vaccine combination strategy.

14.14.4 Use of Viral Vectors for Delivering the Vaccine

Compared to the DNA vaccines, whole killed virus, and live attenuated virus, delivering the vaccines using viral vectors is more effective for the induction of functional immune responses. Various viral vectors such as recombinant adeno associated virus (rAAV), rhabdovirus, adenovirus, and MVA have been used for the delivering the gene or genes of interest for developing SARS vaccines. In this section, we will briefly review various viral vectors.

14.14.4.1 rAAV for Vaccine Delivery

Du and colleagues have used rAAV for delivering the RBD in mice [110]. The investigators have demonstrated: (1) a single dose of RBD-rAAV vaccination could induce sufficient neutralizing antibody against SARS-CoV infection; (2) two more repeated doses of the vaccination boosted the neutralizing antibody to about 5 times

of the level achieved by a single dose of the immunization; and (3) the level of the antibody continued to increase for the entire duration of the experiment (5.5 months). It was very interesting to see that neutralizing activity of these antibodies continued to increase with the number of immunizations. These data suggest that the RBD-rAAV vaccination can deliver a prolonged immune response. This may be due to the fact that the gene expression of the recombinant AAV goes through a slow onset initially, taking a course of a few days or weeks, followed by persistent gene expression for many months, which is supported by reports that the AAV may express foreign genes long-term in vivo in different organisms without resulting in significant toxicity [111, 112, 113]. However, the limitation of this study is that it did not provide the information if the antibody response has reached its highest level at the time the study was ended, and also the longevity of the antibody responses. Nevertheless, it is quite clear that the rAAV has the potential to be used as a delivery vector. In addition, it has been generally observed that some of the viral delivery systems, i.e., adenovirus and vaccinia virus may not be used for repeated immunizations either due to the pre-existing antibodies against the vector or the induction of vector specific antibodies during the primary immunization. The vector specific antibodies limit the efficacy of subsequent immunizations for enhancing the immune response for the target gene by repeated immunizations [114, 115]. However, these results clearly demonstrate that this is not the case at least for rAAV. This may be due to the lower antigenicity of the AAV delivery vector used in this study.

14.14.4.2 Use of Rhabdovirus for Vaccine Delivery

Rhabdovirus (RV) has been introduced as a vaccine vector [116, 117, 118, 119, 120, 121, 122, 123] that could also be used for the expression of relevant SARS virus antigens. There are several advantages of RV that suggest its suitability as an expression vector for SARS virus proteins: (i) the modular genome of RV is organized with short transcription stop/start sequences flanking the genes making it readily amenable to manipulation [116]; (ii) the RV genome is RNA and the life cycle of RV is exclusively cytoplasmic so no DNA recombination, reversion or integration is observed [119, 124]; (iii) stable incorporation of large and multiple foreign genes of up to 6.5 kb offers advantages over plus stranded RNA virus vectors [119]; (iv) RV is non-cytopathic in infected cells and expresses high levels of foreign proteins over extended periods of time [118, 119]; (v) RV can induce a protective immune response in a variety of animals (e.g. dog and mongoose) following immunization by the oral route and attenuated RV can target cells in the tonsils and buccal mucosa [125]; (vi) multiple mutations introduced into the RV genome that completely abolish the pathogenicity of RV render the RV vector extremely safe [119] and replication-defective RVs can be produced that are safe for even completely immunocompromised individuals [126, 127]; and (vii) since RV contains a nucleocapsid protein that has the properties of a superantigen [128], the RV vector is a unique vaccine delivery vehicle. They have evaluated the ability of RV for delivering nucleocapsid protein or envelope spike protein genes in mice. A single inoculation with the RV-based vaccine expressing SARS-CoV S protein

induced a strong SARS-CoV-neutralizing antibody response. The ability of the RV-SARS-CoV S vector to induce strong immune responses after a single inoculation makes this a promising candidate for further evaluation in larger animals as well as in challenge studies to determine the protective efficacy of the immune responses.

14.14.4.3 Use of Adenovirus for Vaccine Delivery

Replication-deficient human adenovirus type 5 (AdH5) is yet another promising vector that can induce strong transgene product-specific cellular and humoral responses. However, one of the major limitations associated with this vector is the presence of neutralizing antibodies (NAbs) against AdH5. Therefore, Zhi and colleagues [129] developed a chimpanzee adenovirus C7 (AdC7) vector to circumvent interference by pre-existing immunity to AdH5, and to evaluate the impact of pre-existing immunity to human adenovirus on the efficacy of adenovirus-based vaccines against SARS CoV. Efficacy was assessed after intramuscular injection of the vector into mice and was measured as the frequency of SARS-CoV-specific T cells and neutralizing antibodies (Nab) against SARS-CoV. Immunogenicity of the AdH5-based vaccine was significantly attenuated or completely abolished when the pre-existing anti-AdH5 NAb titer was higher than 40. In contrast, preexisting anti-AdH5 NAbs have a minimal effect on the potency of the AdC7-based genetic vaccine. Taken together, these results warrant further development of AdC7 as a vaccine vector for human trials.

14.14.4.4 Use of MVA for Vaccine Delivery

As early as in 2004, Bisht and colleagues have determined the immunogenicity of S protein delivered by modified vaccinia Ankara (MVA) in a BALB/c mouse challenge model following the intranasal or intramuscular route of immunization [58]. Irrespective of the route of immunization, immunized mice induced serum antibodies that recognized the SARS S-protein in ELISA, and also neutralized SARS-CoV in vitro. Moreover, MVA_S administered by either route elicited protective immunity, as shown by reduced titers of SARS-CoV in the upper and lower respiratory tracts of mice after challenge. Passive transfer of serum from mice immunized with MVA_S to naive mice also reduced the replication of SARS-CoV in the respiratory tract after challenge, demonstrating a role for anti-S antibodies in protection. The attenuated nature of MVA and the ability of MVA_S to induce neutralizing antibody that protects mice support further development of a candidate vaccine.

More recently, Ba and colleagues have performed a head to head comparison of different delivery technologies such as DNA, MVA, and Ad5 for the full-length SARS-CoV S gene either alone or in combination [130]. They have reported that Ad5-S elicited the highest level of Nabs against SARS-pseudovirus, MVA-S induces about tenfold (IC50) lower levels of Nabs than Ad5-S, with DNA-S being the lowest. Therefore, the live vector Ad5 may offer some advantages for inducing the highest level of NAb response after one immunization. After the boost, DNA primed/DNA boosted animals induced the lowest level of NAb activity. On the other

hand, priming with MVA and boosting with Ad-5 induced the highest levels of antibody responses against the S protein. It was interesting to observe that mice primed with MVA-S or DNA-S and boosted with MVA-S induced relatively lower levels of antibodies compared to animal that were primed with MVA and boosted with Ad-5. Surprisingly, mice primed with Ad5-S and boosted with either Ad5-S or MVA-S (AA, AM) induced antibodies that were higher than what was observed following the DNA primed and DNA boosted regimen, but lower than the other combinations. This finding indicates the importance of heterologous MVA-S prime and Ad5-S boost regimen for inducing the substantial level of NAb response. The use of this regimen may offer an alternative approach to overcome the problems associated with the limitations of using live viral vectors for multiple immunizations due to the pre-existing immunity against the vectors.

Ishii et al. [131] constructed a series of recombinant Dis (rDIs), a highly attenuated vaccinia strain (highly restricted host range mutant of vaccinia virus isolated by successive 1-day egg passage of the DIE vaccinia strain), expressing a gene encoding four structural proteins (E, M, N and S) of SARS-CoV [131, 132]. These rDIs elicited SARS-CoV-specific serum IgG antibody and T-cell responses in vaccinated mice following intranasal or subcutaneous administration. Mice that were subcutaneously vaccinated with rDIs expressing S protein with or without other structural proteins induced a high level of serum neutralizing IgG antibodies and demonstrated marked protective immunity against SARS-CoV challenge in the absence of a mucosal IgA response. These results indicate that the potent immune response elicited by subcutaneous injection of rDIs containing S is able to control mucosal infection by SARS-CoV. Thus, replication-deficient DIs constructs hold promise for the development of a safe and potent SARS vaccine.

14.14.4.5 Recombinant Subunit Vaccines

To sidestep the problems linked to the risks of inactivated SARS-CoV vaccine production, a **recombinant subunit vaccine** based on the spike protein but also different SARS-CoV proteins could be designed. The S protein already has been shown to be the major antigenic site in the virus and antibodies directed against this protein efficiently block SARS-CoV infection in vitro and in vivo [76, 133, 134]. The protein can be expressed and purified in its full-length form as a cell membrane anchored trimer, or as a truncated protein lacking its transmembrane region, from the supernatant of transfected cells [104]. Using the baculovirus expressed S protein, He and colleagues characterized the antigenic structure of the S protein against a panel of 38 monoclonal antibodies (MAbs) isolated from the immunized mice [135]. The epitopes of most anti-S MAbs (32 of 38) were localized within the S1 domain, and those of the remaining 6 MAbs were mapped to the S2 domain. Among the anti-S1 MAbs, 17 MAbs targeted the N-terminal region (amino acids [aa] 12 to 327), 9 MAbs recognized the receptor-binding domain (RBD; aa 318 to 510), and 6 MAbs reacted with the C-terminal region of S1 domain that contains the major immunodominant site (aa 528 to 635). Strikingly, all of the RBD-specific MAbs had potent neutralizing activity, 6 of which efficiently blocked the receptor

binding, confirming that the RBD contains the main neutralizing epitopes and that blockage of the receptor association is the major mechanism of SARS-CoV neutralization. Five MAbs specific for the S1 N-terminal region exhibited moderate neutralizing activity, but none of the MAbs reacting with the S2 domain and the major immunodominant site in S1 showed neutralizing activity. All of the neutralizing MAbs recognized conformational epitopes. This panel of anti-S MAbs can be used as tools for studying the structure and function of the SARS-CoV S protein.

Bisht and colleagues [136] expressed a truncated version of the S protein (amino acid residue 14-762) in the baculovirus system, and purified the S protein to homogeneity, and evaluated in mice its ability to induce protective antibody responses. The truncated form of the S-protein elicited higher levels of neutralizing antibodies in mice and protected them against a viral challenge. In another study, He and colleagues have expressed full-length S protein (FL-S) or its extracellular domain (EC-S) in baculoviruses, and compared their immunogenicity in mice [137]. The immunized mice developed high titers of anti-S antibodies with potent neutralizing activities against SARS pseudoviruses constructed with the S proteins of diverse heterologous isolates of Tor2, GD03T13, and nSZ3, the representative strains of 2002 to 2003 and 2003 to 2004 human SARS-CoV and palm civet SARS-CoV, respectively. These data suggest that the recombinant baculovirus-expressed S protein vaccines possess important conformational neutralizing epitopes, which are important for protection against the challenge infection. They have further demonstrated that a recombinant S-protein containing193-amino acid receptor-binding domain (residues 318–510) fused in frame to human IgG1 Fc fragment induced highly potent antibody responses in the immunized rabbits. The antibodies recognized RBD on S1 domain and completely inhibited SARS-CoV infection at a serum dilution of 1:10,240. Rabbit anti-sera effectively blocked binding of S1, which contains RBD, to ACE2. This suggests that RBD can induce highly potent neutralizing antibody responses and has potential to be developed as an effective and safe subunit vaccine for prevention of SARS.

To map the precise epitope in the RBD He and colleagues have immunized mice with the RBD-IgG1 FC recombinant protein. The RBD-IgG1 FC induced high titer of RBD-specific Abs in the immunized mice, and these sera effectively neutralized infection by both SARS-CoV and SARS pseudoviruses [137]. They have developed a series of mAbs (27) and to characterize the neutralization determinants on the RBD of S protein. Six groups of conformation-dependent epitopes, designated as Conf I–VI, and two adjacent linear epitopes were identified by ELISA and binding competition assays. The Conf IV and Conf V mAbs significantly blocked RBD-Fc binding to angiotensin-converting enzyme 2, suggesting that their epitopes overlaps with the receptor-binding sites in the S protein. Most of the mAbs (23 of 25) that recognized the conformational epitopes possessed potent neutralizing activities against SARS pseudovirus with 50% neutralizing activity in doses ranging from 0.005 to 6.569 ug/ml. Therefore, the RBD of SARS S protein contains multiple conformational epitopes capable of inducing potent NAb responses, and is an important target site for developing vaccines and immunotherapeutics.

It was very interesting to note that S protein produced in baculo virus induced potent binding and neutralizing antibodies in mice and rabbits [137]. However, it has been shown by Bai and colleagues that the S protein expressed in E. coli failed to induce NAb in mice [138]. It is not clear what the reason could be. One potential difference between the S protein produced in E. coli and baculovirus is that the baculovirus produced protein is glycosylated, and the E. coli produced protein is non-glycosylated. However, further work is needed to define that it is the sugars that are directly involved in creating immunodominant epitopes, or sugars are involved in producing a correctly folded S-protein. In either situations, perhaps the expression of S-protein in mammalian expression system will be more desirable. In a proof of concept study, Chang et al. studied the effect of intron and exon splicing enhancers to improve the expression of STR2 (88 kDa), carrying three S fragments (S74–253, S294–739, and S1129–1255) in mammalian cells [139]. The investigators demonstrated that the addition of an 138 base-pair intron increased the expression of STR2 protein by 1.9, 2.5, and 4.1-fold in Vero E6, 293A cells, and CHO cells respectively. Furthermore, exon-splicing enhancers also increased the STR2 expression 1.7-2.8 fold. However, the combination of intron and exon splicing enhancer resulted in the suppression of STR2 expression. These results can provide an optimal strategy to enhance SARS-CoV S protein expression in mammalian cells and may contribute to the development of SARS-CoV subunit vaccine.

Based on the data reviewed here, it is quite evident that that full length, truncated, and also the receptor binding domain of S protein induced strong binding and neutralizing antibody responses against the S-protein in mice and rabbits. Furthermore, upon challenge with pathogenic SARS virus, these animals were protected. However, it is not clear if the S protein alone will be able to induce a long lasting immunity against the virus, or other proteins such as M and N will need to be included in an effective vaccine against SARS.

14.14.4.6 Use of VLPs for Developing a Vaccine

Subunit vaccines based on recombinant proteins can suffer from poor immunogenicity owing to incorrect folding of the target protein or poor presentation to the immune system. Virus-like particles (VLPs) represent a specific class of recombinant vaccines that mimic the outer structure of authentic virus particles but do not cause a productive infection since they lack the viral genome. Yet, they are recognized readily by the immune system and present viral antigens in their authentic or near-authentic conformation. Co-expression of mouse hepatitis virus (MHV) M and E proteins resulted in the assembly of particles. The S protein was dispensable but was incorporated when present. The resulting secreted VLPs are indistinguishable from authentic virions in size and shape. The N protein was neither required nor packaged into the particles when present [140]. Similar observations were made when SARS-CoV M, E, and S protein were co-expressed in insect cells using a baculovirus expression system [141, 142]. Whether those VLPs are able to elicit a protective immune response remains to be determined. In contrast, formation of SARS-Co VLPs in mammalian cells seems not to be dependent on the expression of the E but rather

the N protein. Therefore, SARS-Co VLP assembly in human 293 renal epithelial cells relies on the expression of the S, M, and N protein [143]. Whether this discrepancy is caused by the different expression systems needs to be studied further.

14.15 Use of Adjuvant for Enhancing Functional Immune Responses

The vaccination modality may play a crucial role in the type of antibodies or T cell responses induced [144]. Therefore, it is possible that different vaccination modalities such as inactivated virus, live virus vector delivery, DNA vaccine, protein subunit vaccines and VLP will elicit antibodies that are qualitatively different even if the immunogen is similar. Additionally, the formulation of immunogens with different adjuvants may affect the maturation of antibody responses differently. This is suggested by the qualitative differences recorded in antibody responses generated by a given antigen formulated in different adjuvants in case of HIV vaccines [145]. This observation suggests that the structure of specific epitopes within the immunogen may be presented differently in combinations with different adjuvants. Therefore, even if the overall structure of the immunogen remains stable, the exposure of specific epitopes may be modified. In a proof of concept study Hu and colleagues have demonstrated that co-inoculation of DNA vaccine and IL2 had induced a significantly higher immune response compared to the spike DNA vaccine alone [146]. In the same study, they evaluated the route of immunization upon the type and magnitude of immune responses and found that oral vaccination evoked a vigorous T-cell response and a weak IgG2a antibody responses; intramuscular immunization evoked a vigorous antibody response and a weak T-cell response, and vaccination by electroporation evoked a vigorous response with a predominant subclass IgG1 antibody response and a moderate T-cell response.

14.16 Potential of Disease Enhancement Due to Candidate SARS Vaccines

Enhanced disease in previously immunized individuals is a concern for the development of any vaccine. This may be particularly true for SARS-CoV vaccines since adverse effects have already been reported for one coronavirus vaccine, feline infectious peritonitis virus [147]. In fact, it has been demonstrated that some S variants were not only resistant to antibody neutralization [133], but also showed enhanced entry in the presence of certain antibodies in vitro [106]. The S protein from different SARS-CoV strains isolated during the outbreak in 2002/2003 and 2003/2004 but also S protein of virus isolated from civet cats was used to generate pseudo typed viruses. Each pseudovirus was incubated independently with immune IgG purified from mice vaccinated with S protein from strain Urbani. Inhibition of entry was demonstrated for the prototype strain (Urbani) but also for pseudoviruses

from 2002/2003 human isolates. However, one S (GD03T0013) pseudovirus was markedly resistant to antibody inhibition by the polyclonal IgG and, unexpectedly, entry of two pseudoviruses from palm civet S glycoproteins was markedly enhanced. A similar effect could be detected when human mAbs derived from a recovered SARS patient [76] were used instead of the polyclonal IgG. Antibodies with insufficient cross-neutralization capacity may enhance rather than protect from virus infection. Epitope mapping of human mAbs using recombinant S protein fragments demonstrated a significant reduction in reactivity with an S fragment (residues 318–510) containing a N479S substitution [134]. Sui et al. obtained similar results [133] when they tested the neutralization ability of another human mAb. The antibody could protect mice from a SARS-CoV (strain Urbani) challenge when given 1 day before inoculation. However, when tested in vitro on its ability to neutralize various S protein-pseudo typed viruses, this antibody did not bind to pseudo typed virus containing a D480G substitution in the spike. D480G is a naturally occurring variation in the S protein found in a SARS-CoV isolate (GD03T0013). Although this virus, which was isolated in December 2003 from a SARS patient and viruses isolated from civet cats seem to be only weakly pathogenic in humans, other mutations in the viral genome might occur that impact viral tropism and virulence. Variations in the viral genome will likely continue to occur in the animal reservoirs due to the high mutation rate of RNA viruses and especially coronaviruses. Additionally, antibodies mediating virus entry, could function as a facilitating portal for viruses to gain entrance into the human population. SARS-CoV-like strains which normally do not infect or hardly infect humans could get the chance to replicate in and thus also to adapt to the new host. Therefore, any antiviral strategy based on neutralizing antibodies, whether passive immunotherapy or active immunization, has to be carefully evaluated in appropriate animal models that closely resemble the human disease.

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