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CHAPTER EIGHT

Molecular Basis of Coronavirus Virulence and Vaccine Development

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Abstract

Virus vaccines have to be immunogenic, sufficiently stable, safe, and suitable to induce long-lasting immunity. To meet these requirements, vaccine studies need to provide a comprehensive understanding of (i) the protective roles of antiviral B and T-cell-mediated immune responses, (ii) the complexity and plasticity of major viral antigens, and (iii) virus molecular biology and pathogenesis. There are many types of vaccines including subunit vaccines, whole-inactivated virus, vectored, and live-attenuated virus vaccines, each of which featuring specific advantages and limitations. While nonliving virus vaccines have clear advantages in being safe and stable, they may cause side effects and be less efficacious compared to live-attenuated virus vaccines. In most cases, the latter induce long-lasting immunity but they may require special safety measures to prevent reversion to highly virulent viruses following vaccination. The chapter summarizes the recent progress in the development of coronavirus (CoV) vaccines, focusing on two zoonotic CoVs, the severe acute respiratory syndrome CoV (SARS-CoV), and the Middle East respiratory syndrome CoV, both of which cause deadly disease and epidemics in humans. The development of attenuated virus vaccines to combat infections caused by highly pathogenic CoVs was largely based on the identification and characterization of viral virulence proteins that, for example, interfere with the innate and adaptive immune response or are involved in interactions with specific cell types, such as macrophages, dendritic and epithelial cells, and T lymphocytes, thereby modulating antiviral host responses and viral pathogenesis and potentially resulting in deleterious side effects following vaccination.

1. INTRODUCTION

1.1 Focus of the Review

There are four "common" human coronaviruses (CoVs) that are endemic in the human population: HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1. The first two CoVs have been known since the 1960s, while the emergence of severe acute respiratory syndrome CoV (SARS-CoV) in 2002 led to an active search for novel CoVs and the identification of HCoV-NL63 and HCoV-HKU1 in 2004 and 2005, respectively (van der Hoek et al., 2004; Woo et al., 2005a). The common human CoVs are generally associated with relatively mild clinical symptoms and cause a self-limiting upper respiratory tract disease (common cold) (Walsh et al., 2013). In some cases, common CoVs may also be associated with more severe pathogenesis in the lower respiratory tract, such as bronchiolitis or pneumonia (Pene et al., 2003; van der Hoek et al., 2005; Woo et al., 2005b). Human CoVs cause more serious disease in young, elderly, or immunocompromised individuals, and they may lead to exacerbation of preexisting conditions, such as asthma or chronic obstructive pulmonary disease, frequently requiring hospitalization (Mayer et al., 2016; Varkey and Varkey, 2008). Considering that

the prevalence of these viruses ranges between 3% and 16% and that around 70% of the population is infected during childhood, with recurrent infections occurring throughout life (van der Hoek, 2007; Zhou et al., 2013b), common human CoVs represent a significant burden to public health.

More recently, two previously unknown animal CoVs emerged that were shown to cause deadly disease in humans. The first, SARS-CoV, emerged in Southern China and spread around the globe in late 2002 and early 2003, infecting at least 8000 people and killing nearly 10% of the infected individuals (Lee et al., 2003). The second, Middle East respiratory syndrome CoV (MERS-CoV), was first reported in 2012 in the Middle East and there are still ongoing reports of sporadic cases, particularly in Saudi Arabia and the United Arab Emirates. This virus has caused close to 1782 laboratory-confirmed cases, resulting in 634 deaths, and is the cause of an important outbreak in Korea that started in May 2015, leading to more than 186 confirmed cases with a death toll of 36, according to the World Health Organization (WHO) (http://www.who.int/emergencies/mers-cov/en/).

Current treatment strategies for SARS and MERS, and discussion of the discovery and development of new virus-based and host-based therapeutic options for CoV infection have been reviewed recently (Zumla et al., 2016). In this chapter, we will mainly focus on the development of vaccines suitable to prevent infections caused by highly pathogenic CoVs, particularly SARS-CoV and MERS-CoV, in humans. To date, a wide range of vaccine candidates have been developed for these viruses, including subunit, whole-inactivated virus, DNA, and vectored vaccines (see reviews by Du and Jiang, 2015; Enjuanes et al., 2008; Zhang et al., 2014a). However, in many cases, these vaccines were found to induce antibody-dependent enhancement of infectivity (ADEI) and eosinophilia. In contrast, live-attenuated vaccines have a long history of success and are the most frequently used vaccines in humans. This chapter will be focusing on a recently developed next generation of live-attenuated vaccines based on recombinant viruses. Attenuation of viruses generally relies on the previous identification of genes involved in viral virulence in specific hosts. Often, these genes encode proteins that antagonize the innate immune response, and their deletion leads to recombinant viruses that are attenuated in their virulence and, therefore, may be developed into candidate vaccines. The timeline from bench research to approved vaccine use is generally 10 years or more (Papaneri et al., 2015). The use of genetically engineered viruses may significantly reduce both the time and costs required for vaccine development and

production. In this chapter, specific features of promising vaccine candidates in meeting the earlier criteria will be reviewed.

1.2 CoV Genome Structure and Protein Composition

CoVs contain the largest genome known among RNA viruses, consisting of a single-stranded positive-sense RNA molecule of around 30 kb in length (Fig. 1) (de Groot et al., 2012). It is similar to cellular mRNAs, as it contains 5′-capped and 3′ polyadenylated ends. The 5′-terminal two-thirds of the genome contain two overlapping open reading frames (ORFs): ORF1a and ORF1b (Fig. 1). Translation of ORF1a yields polyprotein 1a (pp1a), and -1 ribosomal frameshifting allows translation of ORF1b to yield pp1ab (Ziebuhr, 2005). Together, these polyproteins are co- and post-translationally processed into 16 nonstructural proteins (nsps), most of them being involved in viral genome replication and subgenomic mRNA synthesis. The 3′-third of the genome encodes a series of structural proteins in the

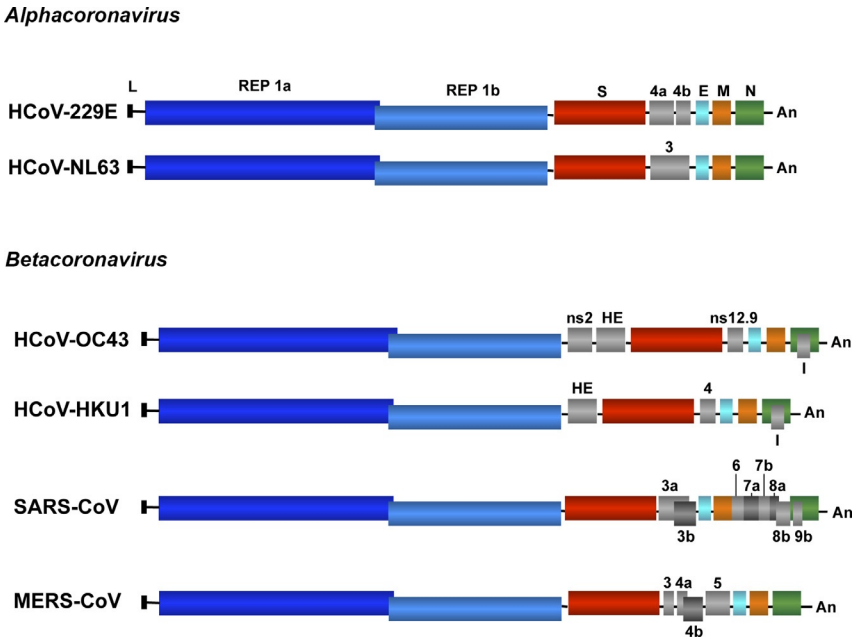

Fig. 1 Genome structure of human CoVs. Each *bar* represents the genomic organization of a human CoV. The tags above the bars indicate the name of each gene. Genus-specific genes are represented in *light* and *dark gray* colors. *An*, poly(A) tail; *I*, internal ORF; *L*, leader sequence; *REP* 1a and *REP* 1b, replicase gene (comprised of ORFs 1a and 1b).

order 5′-S-E-M-N-3′ and genus-specific proteins that vary in number among the different CoV members (Fig. 1) (de Groot et al., 2012; Sola et al., 2015).

Human HCoV-229E and HCoV-NL63 belong to the genus *Alphacoronavirus*, while HCoV-OC43, HCoV-HKU1, SARS-CoV, and MERS-CoV belong to genus *Betacoronavirus*. Although the overall genomic organization is conserved among all coronavirus genera, the members of this virus family encode a unique set of genus-specific proteins (Fig. 1) that, in general, are involved in the modulation of pathogenesis.

1.3 Requirement of B- and T-Cell Responses for Protection

Neutralizing monoclonal antibodies (mAbs) represent a promising therapeutic strategy against emerging CoV infections. Fully human neutralizing antibodies may be developed using different technologies and applied as therapeutic or prophylactic agents (Jiang et al., 2014; Tang et al., 2014; ter Meulen et al., 2004; Ying et al., 2014; Zhang et al., 2005; Zhu et al., 2007). Of special interest are the potent human cross-reactive neutralizing antibodies specific for SARS-CoV (Pascal et al., 2015). Since most of these antibodies target the receptor-binding domain of the spike (S) glycoprotein, antigenic variability within the S gene among human and animal strains must be considered in the design of broad-spectrum neutralizing molecules (Ying et al., 2015b). A combination of mAbs targeting different epitopes may be used to prevent the emergence of escape mutants and, potentially, generate a synergistic neutralizing effect (Ying et al., 2015a). In line with this, passive immunotherapy with mouse or dromedary immune serum was shown to be protective in animal models against SARS-CoV (Subbarao et al., 2004) and MERS-CoV (Zhao et al., 2015b), respectively, suggesting that this approach may be used prophylactically or therapeutically in infected patients.

Neutralizing antibodies induced by the S glycoprotein provide complete protection from lethal CoV infections (Sui et al., 2005). Moreover, an inverse correlation was observed between IgA secretion and MERS-CoV infectivity in patients, suggesting that virus-specific IgA production may be a suitable tool to evaluate the potency of candidate vaccines against MERS-CoV (Muth et al., 2015). However, the IgA response is short lived in patients. In contrast, virus-specific memory $CD8^+$ T cells persisted for up to 6 years after SARS-CoV infection, at which time memory B cells and virus-specific antibodies were undetectable (Yang et al., 2006). It has been shown that memory $CD8^+$ T cells specific for an immunodominant epitope

substantially protected aged mice from lethal SARS-CoV infection (Channappanavar et al., 2014). After challenge, memory $CD8^+$ T cells produced effector cytokines (interferon gamma, IFN-γ; tumor necrosis factor alpha, TNF-α; and interleukin 2, IL-2) and cytolytic molecules, reducing viral loads in the lung. However, dysregulation of some of these inflammatory mediators, including type I IFN and inflammatory monocyte–macrophage responses, caused lethal pneumonia in SARS-CoV-infected mice and, therefore, should be considered during vaccine design to minimize immunopathology (Channappanavar et al., 2016). In addition to the protective effect mediated by memory $CD8^+$ T cells, SARS-CoV-specific $CD4^+$ T cell and antibody responses are likely necessary for complete protection. The requirement of T-cell responses in MERS-CoV protection was also suggested by immunization experiments in macaques (Muthumani et al., 2015). Therefore, for effective protection, CoV vaccines should elicit not only antibody responses but also specific memory $CD4^+$ and $CD8^+$ T cells.

1.4 Antigenic Complexity of SARS- and MERS-CoV

Information on the complexity of CoV serotypes is crucial for predictions on whether antibodies against a previous CoV infection or a specific vaccine may protect from reinfection, which has important implications for vaccine design and neutralizing antibody therapy. Antigenic variability in the S protein, the major target of neutralizing antibodies, is extremely low between different MERS-CoV strains (Drosten et al., 2015). A recent serological study using infectious MERS-CoV isolates collected from patients in Saudi Arabia in 2014 showed no significant differences in serum neutralization, indicating that all these isolates belong to the same serotype (Muth et al., 2015). Based on these data, it seems likely that the S genes of all currently circulating MERS-CoVs are interchangeable in candidate vaccine formulations. The potential relevance of neutralizing antibodies directed against other envelope proteins remains to be studied.

MERS-CoV and all the closely related viruses isolated from camels and bats belong to the same viral species, with bat viruses being at the root of the phylogenetic tree. Most likely, the virus circulating in camels was acquired from bats and represents the origin of viruses identified in humans over the past few years. Recombination events within the spike gene of viral ancestors were likely involved in the emergence of MERS-CoV (Corman et al., 2014). Therefore, camels may serve as reservoirs for the maintenance and diversification of the MERS-CoVs responsible for human infection (Sabir

et al., 2016). The resultant genetic variability may lead to additional antigenic diversity, with obvious consequences for vaccine design.

Because SARS-CoV differs immunologically from other betacoronaviruses with little cross-reactivity of antiviral antibodies (Hou et al., 2010), the development of vaccine candidates suitable to provide broad protection should address the diversity of the main immunogenic determinants of the S protein (Zhou et al., 2013a). For example, SARS-CoV-specific domain in the S protein was found to contain an epitope (80R) that critically determines the sensitivity of a given virus to neutralizing antibodies specific for this epitope. Variants of this epitope have been found in SARS-like-CoVs from civet cats and in human SARS-CoVs that evolved during the epidemics. While the majority of SARS-CoVs from the first outbreak were sensitive to the 80R-specific antibody, the GD03 strain isolated from the index patient of the second outbreak was resistant, confirming the importance of the S protein's natural antigenic variability in eliciting neutralizing antibody responses (Sui et al., 2005).

The SARS-CoV S glycoprotein is a major target of protective immunity in vivo. Two specific human mAbs recognizing the S protein exhibited potent cross-reactivity against isolates from the two SARS outbreaks and palm civets, but not bat strains (Zhu et al., 2007). A combination of two neutralizing mAbs could prevent the emergence of neutralization escape mutants or at least attenuate viral virulence in vivo. However, although neutralizing mAbs directed against epitopes located at the interface between the viral S protein and its cellular receptor, angiotensin-converting enzyme 2 (ACE2), proved to have great potency and breadth in neutralizing multiple viral strains (Sui et al., 2014), both the single and combined use of one or two mAbs, respectively, failed to prevent the emergence of antibody escape variants. Therefore, the use of one or two neutralizing mAbs that target a structurally flexible SARS-CoV epitope may be of limited value for in vivo immunotherapies and should be combined with neutralizing mAbs that bind a second conserved epitope with low structural plasticity.

1.5 Animal Models for CoV Vaccine and Antivirals Studies

Suitable animal models that reproduce the pathology caused by human CoVs are required for studies of pathogenesis and vaccine testing. Unfortunately, no appropriate animal models have been developed to date for any of the four common human CoVs. A transgenic (Tg) mouse model expressing

human aminopeptidase N was generated for HCoV-229E but was not suitable for pathogenesis studies as it was based on immunodeficient Stat1$^{-/-}$ mice (Lassnig et al., 2005). A mouse model has been extensively used for studies of HCoV-OC43, as this human CoV causes lethal infections in mice (Jacomy and Talbot, 2003). However, following inoculation of mice with respiratory isolates of HCoV-OC43, the virus was found to adapt rapidly to grow in brain tissue, while viral RNA remained nearly undetectable in the lung (Butler et al., 2006; St-Jean et al., 2004), suggesting that the model does not reproduce the respiratory pathology seen in humans, limiting its value for studies of virus-induced pathology.

The use of animal models for SARS-CoV and MERS-CoV for studying pathology in humans has been recently reviewed, including clinical symptoms, viral replication, and pathology in humans, nonhuman primates (NHPs), rabbits, ferrets, marmosets, hamsters, and mice (Gretebeck and Subbarao, 2015; van Doremalen and Munster, 2015). Additional models for MERS-CoV based on dromedary camels and other animal species have also been reported (Falzarano et al., 2014; Haagmans et al., 2016; van Doremalen and Munster, 2015). Their large size and the lack of clear clinical signs of disease make camels a less suitable model for studying MERS-CoV pathology. Marmosets show clinical signs following infection with MERS-CoV virus but, in this case, research animals and appropriate reagents suitable to characterize the immune response are scarce or not available, limiting the use of this model system (van Doremalen and Munster, 2015). Hamsters cannot be naturally infected by MERS-CoV, largely preventing their use as an animal model. MERS-CoV S protein-mediated binding to its receptor, human dipeptidyl peptidase-4 (DPP4), involves interactions with 14 amino acid residues. Appropriate replacements of five residues that differ between hamster and human DPP4 render the hamster DPP4 a functional receptor for MERS-CoV (van Doremalen et al., 2014). Thus far, Tg hamsters have not been used due to the lack of specific gene targeting tools. With the availability of the CRISPR-Cas9 system, the situation may now change and hamsters susceptible to MERS-CoV might be developed but their suitability as animal models of MERS-CoV-induced disease remains unclear at present.

MERS-CoV is able to infect rabbits but does not cause histopathology or clinical symptoms although the virus can be detected in lungs. The virus is shed from the upper respiratory tract, providing a possible route of MERS-CoV transmission in this animal species (Haagmans et al., 2015). Clearly, the large size of rabbits also limits their use in BSL-3 containment laboratories.

Mice are an ideal model for pathogenesis studies of many viruses because of their small size and the availability of suitable genomic and immunological reagents. A key difference between SARS-CoV and MERS-CoV is that SARS-CoV infects several strains of mice, whereas MERS-CoV does not (Coleman et al., 2014b; Gretebeck and Subbarao, 2015). A standard procedure was the adaptation of SARS-CoV and MERS-CoV to grow in mice and reproduce the disease caused in humans. This strategy was directly applied in the case of SARS-CoV using conventional mouse strains without the need for Tg mice expressing the human ACE2 receptor (Day et al., 2009; Frieman et al., 2012; Roberts et al., 2007). Mouse-adapted SARS-CoV obtained by passing the virus 15 times in mice (SARS-CoV-MA15) has been an excellent model as it reproduces very well the pathology caused by SARS-CoV in humans, including mortality (DeDiego et al., 2011, 2014b). In contrast, in the case of MERS-CoV, the virus was grown in Tg or knockin humanized mice susceptible to MERS-CoV (K. Li, P. McCray, and S. Perlman, 2016, personal communication).

The first type of MERS-CoV-susceptible mice includes Tg mice that express hDPP4, the virus receptor, using promoters such as those from surfactant protein C, cytokeratin 18 (Li et al., 2016), or cytomegalovirus (Tao et al., 2015). In these mice, the LD50 has been estimated to be <10 $TCID_{50}$ of MERS-CoV. Although MERS-CoV grows almost equally well in the lungs and the brain in these Tg mice, these animals proved to be very useful for protection studies (Agrawal et al., 2015; Zhao et al., 2015a). In some Tg mouse strains, in which the hDPP4 was expressed under control of the surfactant protein C promoter, the virus was found to grow primarily in the lung, which might extend their use to viral pathogenesis studies (C. Tseng, 2016, personal communication).

The other type of MERS-CoV-susceptible mice available to date includes knockin strains in which 3 or 13 exons of mouse DPP4 have been replaced with the homologous sequences from hDPP4 (Agrawal et al., 2015; Li et al., 2016; Pascal et al., 2015). A major difference to the Tg mice described earlier is that the mouse-adapted MERS-CoV only produced disease in knockin mice after the virus has been passed 30 times in the knockin mouse (K. Li, P. McCray, and S. Perlman, 2016, personal communication), whereas the LD50 of MERS-CoV in these mice was around 10^4 pfu/mouse. Another mouse lineage was generated using CRISPR-Cas9 technology by altering mDPP4 amino acid residues 288 and 330 that are known to interact with the MERS-CoV S protein, leading to mice that closely reproduced the disease observed in humans, including mortality. In this case,

the LD50 was around 10^6 pfu/mouse (A. Crockrell and R. Baric, 2016, personal communication), significantly higher than that observed in the knockin mice (Cockrell et al., 2014; Peck et al., 2015; van Doremalen and Munster, 2015) and (K. Li, P. McCray, and S. Perlman, 2016, personal communication). Interestingly, all knockin mice and those generated with the CRISPR technology supported MERS-CoV replication in the lungs, making them extremely useful models for pathogenesis studies.

An alternative approach for the rapid generation of a mouse model for MERS-CoV has been the transduction of mice with adenoviral vectors expressing the human host-cell receptor DPP4 (Zhao et al., 2014a). These mice developed a pneumonia characterized by extensive inflammatory cell infiltration, with virus clearance occurring 6–8 days after infection. Using these mice the efficacy of a therapeutic intervention (poly I:C) and a potential vaccine based on Venezuelan equine encephalitis (VEE) virus has been demonstrated (Zhao et al., 2014a). An important advantage of this approach is that it may be rapidly adapted to other viruses that may emerge in the future, especially in cases in which a suitable mouse model is not available.

2. SUBUNIT, INACTIVATED, AND VECTORED VACCINES

2.1 Subunit Vaccines

Vaccines based on recombinant MERS-CoV S protein, in particular its RBD, have demonstrated partial efficacy in protecting immunized macaques from MERS-CoV infection, reducing pneumonia, and viral titers (Lan et al., 2015). Several fragments of the MERS-CoV S protein were found to induce MERS-CoV neutralizing antibody responses in mice and rabbits (Du et al., 2013; Jiang et al., 2014; Ma et al., 2014b; Mou et al., 2013), similar to what has been shown previously for SARS-CoV (Du et al., 2008; Wang et al., 2012). The fragment-containing residues 377–588 of MERS-CoV proved to be sufficient to protect Ad5/hDPP4-transduced and hDPP4-Tg mice against MERS-CoV. The immunogenicity of this fragment was further improved, resulting in strong humoral and cellular immune responses, by linking the fragment to human Fc and using an adjuvant (Tang et al., 2015; Zhang et al., 2016). These reports confirm that the MERS-CoV S protein is very well suited for the development of MERS subunit vaccines. The full-length S protein contains several non-neutralizing immunodominant domains that may compromise the

immunogenicity of major neutralizing domains or induce harmful immune responses as demonstrated for the S protein of SARS-CoV (Weingartl et al., 2004). Intranasal vaccination with the RBD domain of the MERS-CoV S protein induced much stronger local mucosal immune responses in the lung than subcutaneous immunization (Ma et al., 2014a). Other studies showed that full-length monomeric or trimeric recombinant SARS-CoV and MERS-CoV S proteins are able to induce protective responses in mice (Honda-Okubo et al., 2015; Li et al., 2013) and the presentation of MERS-CoV full-length S protein as nanoparticles in combination with appropriate adjuvants elicits neutralizing antibodies in immunized mice (Coleman et al., 2014a).

Alternatively, DNA vaccines expressing full-length S protein or smaller fragments are effective against MERS-CoV infection. An optimized DNA vaccine encoding the full-length S protein of MERS-CoV elicited antigen-specific neutralizing antibodies in mice, camels, and rhesus macaques, with six of eight vaccinated macaques showing no radiographic evidence of infiltration after MERS-CoV challenge (Muthumani et al., 2015). Potent antigen-specific cellular immune responses were induced in the immunized macaques, suggesting that T-cell responses may also play a role in MERS-CoV protection. Two companies actively working to develop MERS-CoV DNA vaccines (Inovio Pharmaceuticals Inc., Philadelphia, USA and GeneOne Life Science, Seoul, Korea) will soon perform a Phase I clinical trial for this DNA-based vaccine (Inovio News Release, http://ir.inovio.com/news/news-releases/news-releases-details/2015/Inovio-Pharmaceuticals-Partners-with-GeneOne-Life-Science-for-MERS-Immunotherapy-Clinical-Development/default.aspx).

In addition to the DNA-only strategy, DNA priming and protein boosting could be an alternative vaccination approach for MERS-CoV. It was shown that priming of mice and NHPs with DNA encoding the full-length S gene and boosting with the S1 subunit protein-induced robust neutralizing antibody responses against several MERS-CoV strains and protected NHPs from MERS-CoV challenge (Wang et al., 2015).

Chimeric virus-like particles (VLPs) containing SARS-CoV S protein and influenza matrix protein 1 protected mice against challenge with SARS-CoV (Liu et al., 2011) and may induce strong immune responses due to its polymeric nature. To increase their efficacy, subunit vaccines or VLPs have to be administered together with adjuvants (Bolles et al., 2011; Tseng et al., 2012). Subunit vaccines only include subviral components that do not represent the full antigenic complexity of the virus, resulting in limited protective

efficacy due to limited Th1-mediated immune responses and, in some cases, unbalanced immune responses that may lead to immunopathology.

2.2 Vaccines Based on Inactivated Whole Virus

Many vaccines based on chemically inactivated SARS-CoV virions have been evaluated in animal models such as hamsters, mice, ferrets, and NHPs (Bolles et al., 2011; Iwata-Yoshikawa et al., 2014; Roberts et al., 2010; Tseng et al., 2012). In all cases, production of neutralizing antibodies with different levels of protection was observed. Challenge of mice with SARS-CoV was reported to cause Th2-type immunopathology, suggesting that SARS-CoV components induced hypersensitivity. Further studies suggested that the immunopathology leading to eosinophilia was linked, at least in part, to the viral nucleoprotein (Bolles et al., 2011). Also, immunization with oligomers of the SARS-CoV S protein were shown to promote eosinophilia following viral challenge in different animal model systems (Tseng et al., 2012), suggesting that potential side effects of vaccines based on inactivated SARS-CoV or MERS-CoV should be carefully evaluated prior to use in humans. Moreover, inactivated whole-virus vaccines raise biosafety concerns due to the risk of vaccine preparations containing infectious virus. To minimize this risk, genetically attenuated viruses may be used as the starting point for the production of killed virus inactivation.

2.3 Vectored Vaccines

Vectored vaccines are generally based on vectors with a proven safety record. These vaccines allow production and release of immunogenic antigens from infected cells for a limited period of time. Vectors based on viruses from different families (poxvirus, adenovirus, measles, and togavirus (VEE)) have been used in the development of vaccines for CoVs. In the case of MERS-CoV, the most advanced and promising candidate is the modified vaccinia virus Ankara (MVA), a viral vector that does not replicate in mammalian cells and, therefore, holds great promise as a vaccine platform (Altenburg et al., 2014; Haagmans et al., 2016; Song et al., 2013; Volz et al., 2015). Using this vector, S protein fragments of different length were expressed: full-length, extracellular S1 domain, or the RBD. In all cases, neutralizing antibodies and T-cell responses for MERS-CoV were induced. One of these vectors induced mucosal immunity and reduced the shedding of MERS-CoV by a factor of one thousand after challenge with the virulent virus in dromedary camels, thus preventing spread from the animal reservoir

(Haagmans et al., 2016). An MVA-vectored vaccine will be entering clinical trials in 2016 supported by the German Center for Infection Research (DZIF) (Paddock, 2015).

A second, more advanced vectored vaccine is based on recombinant adenovirus expressing S protein fragments of different size (Guo et al., 2015; Kim et al., 2014; Shim et al., 2012). Sublingual immunization with a recombinant adenovirus encoding SARS-CoV S protein induced systemic and mucosal immunity in a mouse model system (Shim et al., 2012). Systemic and mucosal immunity were also elicited in mice by single immunization with human adenovirus type 5 or 41 vector-based vaccines carrying the S protein of MERS-CoV (Guo et al., 2015). Whether or not, these immune responses confer protection against viral infection remains to be evaluated.

Immunization with measles virus vectors expressing the SARS-CoV S protein induced neutralizing antibodies and strong Th1-biased responses, a hallmark of live-attenuated viruses and a highly desirable feature for an antiviral vaccine (Escriou et al., 2014), though eradication of measles virus may represent an obstacle for the application of this type of vaccines. VEE replicon particles expressing the SARS-CoV S protein provided protection against lethal homologous and heterologous challenge in an aged mice model (Sheahan et al., 2011).

Therefore, in principle, several well-known vectors offer the possibility of protection against CoV infection. Although these vectors are based on live viruses with a reasonable record of safety, they are limited to presenting one or a reduced number of CoV antigens to the immune system, in contrast to live vaccines based on the whole, attenuated CoV.

3. LIVE-ATTENUATED VACCINES

We consider it likely that the main strategy for producing more efficacious vaccines in the near future will be based on live-attenuated vaccines that lack specific virulence markers. The most rapid laboratory responses involved in vaccine generation is usually achieved by using subunit vaccines, whole-inactivated virus, or by the construction of vectored vaccines. Successful protection is frequently associated with the generation of a strong neutralizing immune response. Nevertheless, control of virus emergence with this type of vaccines frequently fails due to the relatively low titers and short-lived duration of neutralizing antibody responses and, at times, due to induction of immune pathogenesis including eosinophilia reactions. In contrast, live-attenuated vaccines have a long history of success and are

generally more immunogenic than nonreplicating vaccines, because they produce a comprehensive spectrum of native viral antigens over a prolonged time span, presenting antigens to the immune system as in natural infections. For virus attenuation, we favor modern approaches that may overcome reversion to virulence, as described later.

3.1 Strategies to Engineer Attenuated CoVs as Vaccine Candidates

An alternative approach to the design of attenuated viruses as vaccine candidates that several laboratories have followed is the identification of virulence-associated viral genes (DeDiego et al., 2014a; Stobart and Moore, 2015; Totura and Baric, 2012) that, in many cases, encode nonessential immunomodulatory proteins. Modification or deletion of these genes leads to the generation of attenuated viruses that may be useful as vaccine candidates. Our laboratory has used this strategy extensively to generate CoV vaccines. Also, other groups have used this approach successfully to produce vaccine candidates for influenza, respiratory syncytial virus, measles, dengue, and mumps (Kirkpatrick et al., 2016; Stobart and Moore, 2015).

Engineering attenuated CoVs as vaccine candidates requires the availability of reverse-genetics systems suitable to introduce deletions of (or mutations in) virulence genes as well as appropriate animal models for the evaluation of efficacy and safety of these candidate vaccines. To date, no vaccines have been developed for common human CoV infections. Nevertheless, infectious cDNA clones have been engineered for the three human CoVs that can be efficiently propagated in tissue culture: HCoV-229E, HCoV-OC43, and HCoV-NL63 (Donaldson et al., 2008; St-Jean et al., 2006; Thiel et al., 2001). Similarly, infectious cDNA clones have been generated for SARS-CoV (Almazan et al., 2000; Yount et al., 2003) and MERS-CoV (Almazan et al., 2013; Scobey et al., 2013), providing an excellent basis for the rational development of live-attenuated vaccines based on recombinant viruses.

3.2 Coronavirus Virulence

Virulence of CoVs is generally associated with specific virulence genes that, in most cases, antagonize cellular innate immune responses but are nor required for efficient virus replication. CoV infection affects many host-cell pathways that modulate pathogenesis. Innate immune response is the first

line of host-cell defense against viruses, and any viral factor modulating this pathway may have a strong impact on pathogenesis. CoV proteins nsp3, 4, and 6 are actively involved in the formation of host membrane vesicles and structures that affect CoV replication and evasion of the immune system by hiding the double-stranded RNA (dsRNA) generated during virus replication (Angelini et al., 2013). Pathogen-associated molecular patterns (PAMPs), such as ssRNA, dsRNA, or viral proteins, trigger the activation of transcription factors leading to proinflammatory cytokines and type I IFN induction. PAMPs activate protein kinase RNA-activated, which leads to translation initiation factor eIF2 phosphorylation and inhibition of host translation, and 2′-5′ oligoadenylate synthase (OAS), which triggers RNase L and RNA degradation (Fig. 2). The activation of cytoplasmic sensors RIG-I and MDA-5 triggers the upregulation of IFN regulatory factors (IRF)-3 and IRF-7, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) through mitochondrial antiviral-signaling protein (Fig. 2). In addition, Toll-like receptors (TLRs) activate the MyD88 response protein and adaptor molecule TRIF-dependent pathways, which also upregulate transcription factors IRF-3, IRF-7, NF-kB, and activator protein 1 (AP-1) (Fig. 2) (DeDiego et al., 2014a). Some CoV proteins act as IFN antagonists by inhibiting its production or signaling (Fig. 2) (DeDiego et al., 2014b; Totura and Baric, 2012; Zhong et al., 2012). Potent cytokines involved in controlling viral infections and priming adaptive immune responses are generated in response to CoV infection (Totura and Baric, 2012; Zhou et al., 2015). However, uncontrolled induction of these proinflammatory cytokines can also increase pathogenesis and disease severity as described for SARS-CoV and MERS-CoV (DeDiego et al., 2011, 2014a,b; Selinger et al., 2014). The cellular pathways mediated by IRF-3, IRF-7, activating transcription factor (ATF)-2/jun, AP-1, NF-kB, and nuclear factor of activated T cells, are the main drivers of the inflammatory response triggered after SARS-CoV infection, with the NF-kB pathway most strongly activated (DeDiego et al., 2011). CoV proteins that elicit this type of innate immune response may be modified to generate attenuated viruses suitable for vaccine development.

3.3 IFN Sensitivity of Human CoVs

Human CoVs are able to suppress IFN induction to different extents and, in general, are sensitive to the addition of exogenous IFN (Kindler et al., 2013). SARS-CoV in particular is highly resistant to the antiviral state induced by

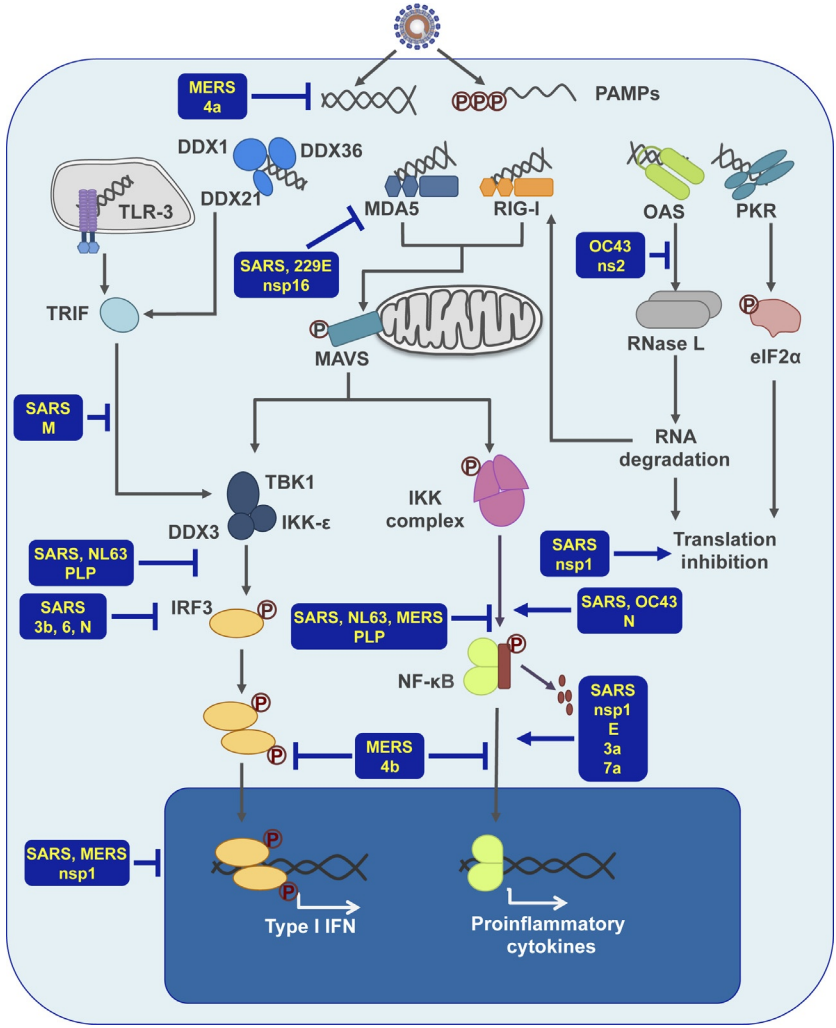

Fig. 2 Innate immunity signaling pathways affected by human CoV proteins. The main pathways leading to IFN and proinflammatory cytokine production are represented in the figure. These signaling routes are activated by dsRNA, which acts as a pathogen-associated molecular pattern (PAMP). The viral proteins affecting these pathways are indicated in the *dark blue boxes*. *SARS*, SARS-CoV; *MERS*, MERS-CoV; *229E*, HCoV-229E; *OC43*, HCoV-OC43; *NL63*, HCoV-NL63.

IFN treatment, suggesting that it possesses many mechanisms to counteract IFN-induced antiviral responses (Zielecki et al., 2013).

The betacoronavirus HCoV-OC43 is an exception for the sensitivity of human CoVs to IFN, as infection of certain cell types by this virus is favored

in the presence of exogenous IFN. A possible explanation for this observation may be that HCoV-OC43 uses IFN-inducible transmembrane (IFITM) proteins 2 and 3 for cell entry (Zhao et al., 2014b). Interestingly, IFITM3-mediated antiviral activity was observed for the alphacoronaviruses HCoV-229E and HCoV-NL63, but not for the betacoronaviruses SARS-CoV and MERS-CoV (Wrensch et al., 2014), suggesting that IFITM proteins may have a positive effect on all betacoronavirus infections.

3.4 Innate Immunity Modulators Encoded by Common Human CoVs

A number of nsps have been implicated in human CoV pathogenesis, such as nsp1, nsp3, and nsp16, and, possibly, might be used to produce highly attenuated strains of common human CoVs as vaccine candidates.

Nsp1 protein is only encoded by members of the *Alphacoronavirus* and *Betacoronavirus* genera, with similar functions in all cases despite low-sequence conservation. The nsp1 proteins of HCoV-229E and HCoV-NL63 inhibit protein expression, most likely via their association with 40S ribosomal subunits, and they also act as IFN antagonists (Narayanan et al., 2015; Zust et al., 2007).

The nsp3 transmembrane replicase protein contains several functional domains (Fig. 3) that are conserved among all common human CoVs: two papain-like proteases (PLPs), two ubiquitin-like domains, and an ADP-ribose-1″-phosphatase (ADRP) domain. A role for ADRP in pathogenesis of human CoVs has been proposed, although it is not required for

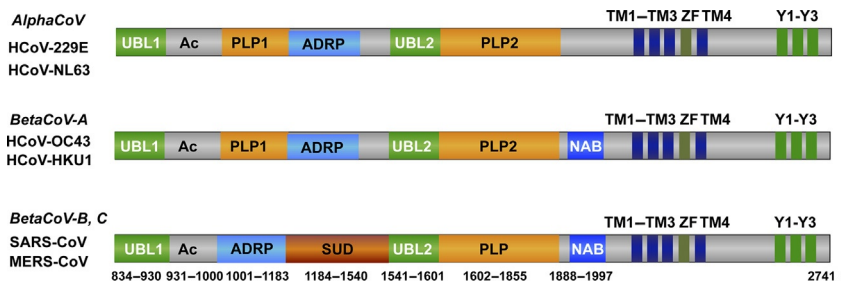

Fig. 3 Multidomain structure of CoV nonstructural protein nsp3. The approximate boundaries of each domain in SARS-CoV protein are indicated underneath by the amino acid numbers in the replicase polyprotein. *Ac*, Glu-rich acidic domain; *ADPR*, ADP-ribose-1″-phosphatase (also called macrodomain or X domain); *NAB*, nucleic acid-binding domain; *SUD*, SARS-unique domain; *TM1–TM4*, transmembrane domains forming an additional domain containing a metal-binding region (*ZF*); *UBL*, ubiquitin-like domain; *Y1–Y3*, Y domains preceding the C-terminal PLP cleavage sequence at nsp3/4.

viral growth in cell culture. HCoV-229E and SARS-CoV mutants lacking ADRP activity grow to similar titers compared to wild-type viruses in MRC-5 and Vero cells, respectively (Kuri et al., 2011; Putics et al., 2005). ADRP-deficient mutants of mouse hepatitis virus (MHV) were attenuated in vivo, most probably due to deficient induction of proinflammatory responses (Eriksson et al., 2008). ADRP mutants of HCoV-229E and SARS-CoV were reported to be more sensitive to IFN compared to wild-type viruses, suggesting that ADRP activity is involved in counteracting IFN activity (Kuri et al., 2011).

The PLP domains of nsp3 have also been linked to the modulation of innate immune response. The HCoV-NL63 PLP2 domain has been extensively characterized and acts as an IFN antagonist (Clementz et al., 2010), and apart from its PLP activity, it has deubiquitinating (DUB) and deISGylating activity (Chen et al., 2007; Clementz et al., 2010). HCoV-NL63 PLP2 DUB activity seems to be involved in modulation of NF-kB activation (Clementz et al., 2010) and p53-mediated modulation of IFN production (Takaoka et al., 2003). PLP2 deubiquitinates the cellular protein MDM2, leading to p53 proteasomal degradation and, as a consequence, decreased IFN production (Yuan et al., 2015).

Nsp16 has ribose 2′-*O*-methyltransferase (2′-*O*-MTase) activity that is required to produce the 5′ cap1 structures on viral RNAs. The methylation proved to be important to avoid viral RNA to be recognized as being "nonself" by the host-cell sensor MDA5 and subsequent activation of cellular innate immune responses. HCoV-229E mutants that lack 2′-*O*-MTase activity replicated to 10^2-fold lower titers than the wild-type virus in MRC-5 cells, confirming the relevance of nsp16 activity. Moreover, the 2′-*O*-MTase mutant was more sensitive to treatment with type I IFN, and macrophages infected with the mutant virus produced more type I IFN than those infected with the parental virus (Zust et al., 2011). The role of nsp16 in modulation of innate immunity seems to be conserved among all CoVs, as similar results were obtained for MHV and SARS-CoV, and a SARS-CoV mutant lacking 2′-*O*-MTase activity was attenuated in vivo (Menachery et al., 2014).

CoVs contain a variable number of genus-specific genes that have been implicated in modulation of pathogenesis. In fact, all common human CoVs contain a genus-specific gene located between the S and envelope (E) genes, named ORF4 (HCoV-229E and HCoV-HKU1), ORF3 (HCoV-NL63), or ns12.9 (HCoV-OC43) (Fig. 1). These genes encode transmembrane proteins that differ significantly in both their sequences and predicted structures

(Muller et al., 2010; Zhang et al., 2014b, 2015), but may have related functions, such as ion channel activity and morphogenesis (Donaldson et al., 2008; Zhang et al., 2014b, 2015). In fact, the SARS-CoV 3a protein, HCoV-NL63 protein 3, and the HCoV-229E 4a protein are each able to complement *in trans* the absence of HCoV-OC43 ns12.9 protein (Zhang et al., 2015). The proteins are not essential for viral growth in cell culture but virus yield is increased between 6- and 25-fold if the protein is expressed (Zhang et al., 2014b, 2015). Also, there is evidence that these protein homologs may play an important role in vivo. For example, all clinical isolates of HCoV-229E contain a full-length ORF4, while in cell culture-adapted strains, this gene is spontaneously divided into two ORFs, 4a and 4b, or replaced by a truncated ORF encoding only the first transmembrane domain (Dijkman et al., 2006). Recombinant HCoV-NL63-Δ3 virus grows to a 10-fold lower titer than the parental virus in primary human airway epithelium (HAE) culture (Donaldson et al., 2008). Finally, a recombinant HCoV-OC43-Δns12.9 virus was attenuated in vivo (Zhang et al., 2015). These results suggest that ORF3 genes can be modified for the generation of attenuated viruses that may be used as vaccine candidates.

The HCoV-OC43 ns2 gene is predicted to encode a 2′-5′-phosphodiesterase (PDE) based on its homology to the MHV ns2 gene (Roth-Cross et al., 2009). The MHV ns2 protein-mediated PDE activity interferes with the OAS-RNase L pathway, affecting pathogenesis by counteracting cellular innate immune responses (Zhao et al., 2012). Moreover, the absence of ns2 PDE activity can be complemented by other viral or cellular PDEs (Zhang et al., 2013).

Common human betacoronaviruses encode a hemagglutinin-esterase (HE) gene (Fig. 1). The HE protein is a transmembrane protein that is incorporated in the viral envelope, acting as a receptor-destroying enzyme with a role in viral entry (Huang et al., 2015). Interestingly, HCoV-OC43 mutants that lack the HE gene or encode an inactive HE protein produced replication-competent propagation-defective viruses (Desforges et al., 2013). These viruses, in principle, may be useful as vaccine candidates.

3.5 SARS-CoV Genes as Modulators of the Innate Immune Response

SARS-CoV encodes many known immunomodulatory proteins, such as nsp1, nsp3, nsp14, membrane (M), E, and nucleocapsid (N) (Fig. 2) (DeDiego et al., 2011, 2014a; Totura and Baric, 2012; Zhong et al., 2012). Modification or deletion of some of them may lead to attenuated

viruses that could be used as live vaccine candidates. Many of these proteins also specifically inhibit cellular signaling pathways associated with the innate immune response.

It is generally thought that deletion or genetic modification of SARS-CoV genes encoding proteins that have IFN antagonist activity or other viral defense mechanisms, such as nsp1, nsp3, nsp16, M, 3b, 6, and N, will result in attenuated viruses, as has been shown for SARS-CoV nsp1 (Jimenez-Guardeno et al., 2015) and nsp16 mutants, respectively (Menachery et al., 2014).

As an alternative approach to produce attenuated CoVs to be used as vaccine candidates, the modification of viral replication fidelity has been proposed (Graham et al., 2012; Smith et al., 2013). Live-attenuated RNA virus vaccines are efficacious but subject to reversion to virulence. Among RNA viruses, replication fidelity is recognized as a key determinant of virulence and escape from antiviral therapy, though reduced fidelity is detrimental for some viruses. The replication fidelity of CoVs has been estimated to be approximately 20-fold higher than that of other RNA viruses and is mediated by a $3' \rightarrow 5'$ exonuclease (ExoN) activity (Minskaia et al., 2006) that probably functions in RNA proofreading (Eckerle et al., 2010). It has been demonstrated that engineered abrogation of SARS-CoV ExoN activity results in a stable mutator phenotype with profoundly decreased fidelity both in cell culture and in vivo, and attenuation of pathogenesis in young, aged, and immunocompromised mice (Graham et al., 2012). The ExoN null genotype and mutator phenotype are stable and do not revert to virulence, even after serial passage or long-term persistent infection in vivo. ExoN inactivation thus has potential for broad applications in the stable attenuation of CoVs.

Our laboratory has focused on the role of virulence of the SARS-CoV E and 3a proteins, and of the MERS-CoV E and 5 proteins, and on studying effects of their partial or complete deletion on virus attenuation and possible implications for vaccine development (DeDiego et al., 2007, 2011; Regla-Nava et al., 2015). The safety of these vaccine candidates has been further enhanced by genetic modifications of the nsp1-coding sequence (Jimenez-Guardeno et al., 2015). The CoV E protein is 76–109 aa in length, depending on the virus, and has diverse functions in CoV morphogenesis and the secretory pathway (Westerbeck and Machamer, 2015). The importance of E in different CoV species and genera varies greatly, ranging from being absolutely essential in some members of the genera *Alphacoronavirus* (TGEV) and *Betacoronavirus* (lineage C, MERS-CoV) to having nonessential

functions in other betacoronaviruses (MHV (lineage A), SARS-CoV (lineage B)). In SARS-CoV, two strategies have been used to abrogate E function: complete deletion of E protein, and the introduction of small deletions of 8–12 aa at the E protein carboxyl-terminus (Jimenez-Guardeno et al., 2015). A similar strategy is currently being applied to MERS-CoV (J. Gutierrez, I. Sola, and L. Enjuanes, unpublished results). The SARS-CoV E protein is nonessential for virus replication and dissemination, and its deletion has led to attenuated forms of SARS-CoV that are promising vaccine candidates (see later).

Viral infections often induce endoplasmic reticulum (ER) stress, specifically the unfolded protein response (UPR). UPR induces three main signaling pathways to avoid the accumulation of proteins with altered folding in the ER. Stress in the ER is also interconnected with the innate immune response and other host-cell responses (Hetz, 2012). Both HCoV-OC43 and HCoV-HKU1 induce ER stress by a mechanism that is associated with S protein expression leading to virulent CoVs in a mouse model (Favreau et al., 2009; Siu et al., 2014a). Similarly, in the case of SARS-CoV, the E protein has been linked to virulence and the induction of UPR (DeDiego et al., 2011).

E protein has a major role in inflammasome activation and the associated exacerbated inflammation elicited by SARS-CoV in the lung parenchyma (Nieto-Torres et al., 2014). This exacerbated inflammation causes edema leading to acute respiratory distress syndrome and, frequently, to the death of experimentally infected animals or human patients. The E protein is a viroporin that conducts Ca^{2+} ions. Changes in intracellular Ca^{2+} concentration, mediated by E protein ion channel activity, are responsible for inflammasome activation (Nieto-Torres et al., 2015). Elimination of ion channel activity by the introduction of aa substitutions within the transmembrane domain of E protein led to virus attenuation (Nieto-Torres et al., 2014).

At its carboxyl-terminus, the E protein contains another virulence factor, a PDZ domain-binding motif (PBM) that, during SARS-CoV infection, could potentially target more than 400 cellular proteins containing PDZ domains with possible effects on viral pathogenicity. Interestingly, deletion or modification of the E protein PBM resulted in attenuated SARS-CoVs that are good vaccine candidates (Jimenez-Guardeño et al., 2014; Regla-Nava et al., 2015).

Deletion of full-length E protein led to the attenuation of SARS-CoV in several animal models (Lamirande et al., 2008; Netland et al., 2010).

The immunogenicity and protective efficacy of a live-attenuated vaccine based on a recombinant SARS-CoV lacking the E gene (rSARS-CoV-ΔE) was first studied using hamsters. After immunization with rSARS-CoV-ΔE, hamsters developed high serum neutralizing antibody titers and were protected from challenge with homologous (SARS-CoV Urbani) and heterologous (GD03) SARS-CoV in the upper and lower respiratory tract. Deletion of the E protein modestly diminished viral growth in cell culture but abrogated virulence in mice (Netland et al., 2010). We have shown that immunization with rSARS-CoV-ΔE almost completely protected BALB/c mice from fatal respiratory disease caused by mouse-adapted SARS-CoV, and partly protected hACE2 Tg mice from lethal disease, although hACE2 Tg mice, which express the human SARS-CoV receptor, were extremely susceptible to infection. Furthermore, we also showed that rSARS-CoV-ΔE induces antiviral T-cell and antibody responses. To improve vaccine efficacy, we engineered and adapted rSARS-CoV to efficiently grow in mice (rSARS-CoV-MA15). To this end, we incorporated six nucleotide substitutions into the SARS-CoV genome (Frieman et al., 2012; Roberts et al., 2007) using our reverse-genetics system based on bacterial artificial chromosomes. Using the rSARS-CoV-MA as a backbone genome, a second set of E-deletion vaccine candidates was generated (Fett et al., 2013). rSARS-CoV-MA15-ΔE was safe, causing no disease in 6-week-, 12-month-, or 18-month-old BALB/c mice. Immunization with this virus completely protected mice at three ages from lethal disease and induced a more rapid virus clearance. Compared to rSARS-CoV-ΔE, rSARS-CoV-MA-ΔE immunization elicited significantly greater neutralizing antibody titers and virus-specific $CD4^+$ and $CD8^+$ T-cell responses. After challenge, inflammatory cell infiltration, edema, and cell destruction were decreased in the lungs of rSARS-CoV-MA15-ΔE-immunized mice, compared to those from rSARS-CoV-ΔE-immunized 12-month-old mice, suggesting that this mouse-adapted virus is a safe candidate vaccine.

To identify E protein domains that contribute to SARS-CoV-MA15-ΔE attenuation, several rSARS-CoVs with mutations or deletions in the E protein were generated. Substitutions in the amino terminus or deletion of regions in the internal and carboxy-terminal regions of the E protein led to virus attenuation (Regla-Nava et al., 2015). Attenuated viruses induced minimal lung injury, diminished edema, limited neutrophil influx, and increased $CD4^+$ and $CD8^+$ T-cell counts in the lungs of BALB/c mice, compared to animals infected with the wild-type virus. The attenuated viruses completely protected mice against challenge with the lethal parental

virus, considerably increasing the survival of infected animals and indicating that these viruses are promising vaccine candidates.

Ideally, a vaccine should provide full protection, and this was the case for the SARS-CoV vaccine candidate that was engineered by deleting the full-length E protein. Nevertheless, a good vaccine must also be genetically stable, and the initial rSARS-CoV-ΔE vaccine was unstable both in cell culture and in vivo. In fact, this mutant virus regained fitness after serial passage in cell culture, resulting in the partial duplication of the membrane gene, and while the chimeric protein increased viral fitness in vitro, the virus remained attenuated in mice. When the full-length E gene was deleted or its PBM coding sequence mutated, revertant viruses either evolved a novel chimeric gene including PBM or restored the sequence of the PBM in the E protein, respectively (Jimenez-Guardeno et al., 2015). During SARS-CoV-ΔE passage in mice, the virus incorporated a mutant variant of the 8a protein including a PBM, resulting in reversion to a virulent phenotype. These data, and additional evidence, led us to conclude that the virus requires a PBM on a transmembrane protein to compensate the removal of this motif from the E protein (Jimenez-Guardeno et al., 2015). Therefore, to increase the genetic stability of the vaccine candidate, we introduced small attenuating deletions in the E gene that did not affect the endogenous PBM, preventing the selection of revertants incorporating novel PBMs into the virus genome and leading to a genetically and functionally stable vaccine candidate. To further increase vaccine safety, we introduced additional attenuating amino acid substitutions in the nsp1 protein. Recombinant viruses including attenuating mutations in the E and nsp1-coding sequences maintained their attenuation after passage in vitro and in vivo. Furthermore, these viruses fully protected mice against challenge with the lethal parental virus, and are therefore safe and stable vaccine candidates for protection against SARS-CoV (Jimenez-Guardeno et al., 2015).

3.6 MERS-CoV Genes as Modulators of the Innate Immune Response

The relevance of MERS-CoV proteins in modulating cellular innate immune responses varies depending on the experimental approach used. Two main strategies have been applied, either the expression of individual virus proteins or the deletion of appropriate protein-coding sequences from the full-length virus genome. Using the first approach, overexpressing MERS-CoV proteins in human 293T transfected cells, the structural, and accessory proteins M, 4a, 4b, and 5 were identified as potential IFN

antagonists (Matthews et al., 2014; Yang et al., 2013). In contrast, when the role of these virus proteins was analyzed in cells infected with MERS-CoV deletion mutants in which individual genes (3, 4a, 4b, and 5, respectively) were deleted, it was observed that, among these proteins, 4b was the major antagonist of the innate immune response induction (J. Canton, S. Perlman, L. Enjuanes, and I. Sola, 2016, unpublished results). Deletion of ORF4a resulted in a MERS-CoV mutant with increased INF sensitivity. The effect may be associated with the dsRNA-binding domain of the 4a protein that was shown to interact with PACT in an RNA-dependent manner; thereby, reducing the activation of RIG-I and MDA5 in overexpression assays (Siu et al., 2014b). Also, the combined deletion of the accessory genes 3, 4a, 4b, and 5 in a recombinant MERS-CoV (rMERS-CoV-ΔORF3-5) showed a 1–1.5 log reduction in viral titer compared with the full-length MERS-CoV (Scobey et al., 2013). Therefore, engineering MERS-CoV with deletions in one or more genes could lead to promising vaccine candidates.

The complete deletion of the E gene in MERS-CoV led to the generation of replication-competent propagation-defective viruses that are promising vaccine candidates (Almazan et al., 2013). Because E protein induces cell apoptosis (An et al., 1999; DeDiego et al., 2011), the establishment of stable cell lines constitutively expressing this protein to complement viruses lacking E protein has not been possible. To overcome this limitation, we have generated packaging cell lines that transiently express E protein in an inducible manner and used them to grow replication-competent propagation-defective MERS-CoV-ΔE (J. Gutierrez-Alvarez, I. Sola, and L. Enjuanes, 2016, unpublished results). Additionally, fully stable transformed mammalian cells were generated in which E protein expression was under the control of an optimized Tet-On system for doxycycline-inducible gene expression, drastically reducing leaky expression (Das et al., 2016; Markusic et al., 2005) and (J. Gutierrez, I. Sola, and L. Enjuanes, 2016, unpublished results).

In an alternative strategy, replication- and propagation-efficient rMERS-CoVs were generated that expressed a slightly shortened E protein lacking 8–12 aa at the carboxyl-terminus, as previously reported for SARS-CoV vaccine candidates (J. Gutierrez-Alvarez, S. Perlman, I. Sola, and L. Enjuanes, 2016, unpublished results). Thus, the lessons learned from previous SARS-CoV vaccine biosafety studies, such as the use of attenuated viruses expressing a shortened E protein (see earlier), may also be applicable to engineering MERS-CoV vaccines.

MERS-CoV replicase proteins nsp1, nsp3, and, possibly, nsp14, may also interfere with the signaling pathways associated with the innate immune response through different mechanisms (Lokugamage et al., 2015; Yang et al., 2014). Therefore, modification or deletion of the replicase gene sequences encoding these proteins may also lead to the generation of attenuated viruses. Similar to nsp1 of SARS-CoV, which inhibits host gene expression at the translational level, it has been reported that MERS-CoV nsp1 exhibits a conserved function, inhibiting host mRNA translation and inducing host mRNA degradation. This information could be exploited to produce MERS-CoV vaccine candidates (Lokugamage et al., 2015).

As in the common human CoVs, SARS-CoV and MERS-CoV PLPs have deISGylating and DUB activities and act as interferon antagonists (Barretto et al., 2005; Chen et al., 2007; Clementz et al., 2010; Frieman et al., 2009; Ratia et al., 2006; Zheng et al., 2008). Mutations introduced into the MERS-CoV PLP coding sequence to specifically disrupt ubiquitin binding without affecting viral polyprotein cleavage led to PLP variants without DUB activity that lost their wild-type ability to inhibit IFN-β promoter activation in reporter assays. These findings directly implicate DUB and deISGylating functions of PLP in the inhibition of IFN-β promoter activity, and such modification may lead to attenuated MERS-CoV. In addition, PLP catalytic activity was required by both MERS-CoV and SARS-CoV to reduce induction of endogenous proinflammatory cytokines in infected cells (Mielech et al., 2014), consistent with the important functions of ubiquitination and modification of cellular proteins by interferon-stimulated gene 15 (ISG15) in regulating cellular innate immune pathways. On the other hand, it has been shown that the SARS-CoV PLP interferes with the formation of the signaling complexes including STING (stimulator of interferon genes), TRAF3, TBK1, and IKKε; thus, preventing downstream phosphorylation, dimerization, and nuclear translocation of IRF3 mediated by STING and TBK1 (Fig. 2) (Chen et al., 2014; Yang et al., 2014). This inhibition is not dependent on the PLP catalytic activity (Chen et al., 2014).

These studies provide valuable information on how MERS-CoV PLP-mediated antagonism of the host innate immune response is orchestrated and offers additional attractive options for designing attenuated viruses as vaccine candidates.

4. VACCINE BIOSAFETY

4.1 ADEI and Eosinophilia Induction

In general, subunit vaccines against SARS-CoV and MERS-CoV administered with and without adjuvants provide different degrees of protection (Haagmans et al., 2016; Jaume et al., 2012; Wang et al., 2014). However, as mentioned earlier, these vaccines should be used with caution in humans because of possible ADEI mechanisms, especially when antibody levels are low. Highly concentrated antisera against SARS-CoV were shown to neutralize virus infectivity, whereas diluted mono- and polyclonal anti-S protein antibodies both caused ADEI in human promonocyte cell cultures, leading to cytopathic effects and increased levels of TNF-α, IL-4, and IL-6 (Haagmans et al., 2016; Jaume et al., 2012; Wang et al., 2014).

In addition, it has been documented that immunization of mice with VLPs or inactivated virus, both in the presence or absence of adjuvant, induced eosinophilic immunopathology in young and aged mice (Bolles et al., 2011; Tseng et al., 2012). Using a double-inactivated SARS-CoV vaccine, protection was observed after homologous and heterologous challenge. Protection against a nonlethal heterologous challenge was poor, and enhanced immune pathology was comparable with that seen in SARS-CoV N protein-immunized mice. Importantly, aged mice displayed increased eosinophilic immune pathology in the lungs, and mice were not protected significantly against virus replication.

The induction of immunopathology was also observed during challenge in ferrets and NHPs immunized with candidate vaccines based on VLPs, the whole-inactivated SARS-CoV virus, and rDNA-produced S protein (Tseng et al., 2012). The pulmonary damage after challenge with SARS-CoV was associated with a Th2-type immunopathology with prominent eosinophil infiltration, and upregulation of genes associated with the induction of eosinophilia was observed. Interestingly, this immunopathology in the lungs upon SARS-CoV infection could be avoided by administration of TLR agonist adjuvants (Iwata-Yoshikawa et al., 2014).

To increase the duration of immune responses elicited by vaccines and to prevent the CoV-induced lung immunopathology observed after challenge or natural infection, the effects of adjuvants have been studied. In immunizations either with recombinant CoV S protein or with inactivated whole-virus vaccines, the effects of different adjuvants including alum, CpG, and Adva, a new delta-inulin-based polysaccharide adjuvant, were analyzed

(Honda-Okubo et al., 2015). While all vaccines protected against lethal infection, addition of an adjuvant significantly increased serum neutralizing antibody titers and reduced lung virus titers on day 3 postchallenge. Whereas adjuvant-free or alum-formulated vaccines were associated with a significant increase in lung eosinophilic immunopathology on day 6 postchallenge, this was not seen in mice immunized with vaccines formulated with the delta-inulin adjuvant. The absence of eosinophilic immunopathology in vaccines containing delta-inulin adjuvants was found to correspond to enhanced T-cell IFN-γ-recall responses rather than reduced IL-4 responses, suggesting that immunopathology is primarily caused by an inadequate vaccine-induced Th1 response and illustrating the need to induce durable IFN-γ responses using appropriate adjuvants.

4.2 Interaction of CoV Vaccine Candidates with Cells of the Immune System

The possibility of productive infection in macrophages (MØ) and dendritic cells (DCs) by MERS-CoV is under debate. Whereas some reports provided evidence for the infection of human MØs and DCs (Table 1) (Chu et al., 2014, 2016; Scheuplein et al., 2015; Ying et al., 2016), others have not been able to productively grow virus in MØs from Tg mice-expressing hDPP4 (C. Tseng, personal communication). The different origin of the cells might explain these divergent results but more work is needed to clarify the susceptibility of MØs and DCs to MERS-CoV.

Common human CoVs, such as HCoV-NL63, HCoV-OC43, and HCoV-HKU1, as well as the highly pathogenic SARS-CoV and MERS-CoV infect ciliated epithelial cells in HAE culture, whereas HCoV-229E infects nonciliated cells (Dijkman et al., 2013; Kindler et al., 2013). Human CoVs do not elicit the production of proinflammatory cytokines in human primary respiratory epithelial cells or ex vivo human lung tissue culture, and the production of type I and III IFN was consistently found to be low (Chan et al., 2013; Kindler et al., 2013; Zielecki et al., 2013), suggesting that all human CoVs have evolved mechanisms to antagonize the host innate immune response, mediated by a variety of viral proteins.

Epithelial cells, MØs, and DCs play a role during virus replication in the lung, and may have an impact on pathogenesis. Both HCoV-229E and HCoV-OC43 can infect MØs, although only the former does it efficiently (Collins, 2002; Patterson and Macnaughton, 1982). DCs serve as sentinels in the respiratory tract, and are the connection between innate and adaptive immunity in the lung. HCoV-229E efficiently infects and kills DCs, which

Table 1 Infection of Immune Cells by SARS-CoV and MERS-CoV

	Cells							
Virus	**MØ**		**DC**		**Epithelial**		**T**	
MERS-CoV	**PROD**		**PROD**		**PROD**		**ABOR**	
	CXCL-10	↑	CXCL-10	↑	CXCL-10	↓	Apoptosis	
	CCL-2, -36, -5	↑	CCL-5	↑	CCL-5	↓	Lymphopenia	
	IL-8, -12	↑	IL-12	↑	IL-1β	↓		
			IFN-γ	↑	IL-6, -8	↓		
	IFN-α/β	↓	IFN-α/β	↓	IFN-α/β	↓		
					IFN-III	↓		
SARS-CoV	**ABOR**		**ABOR**		**PROD**		**ABOR**	
	CXCL-10	↑	CXCL-10	↑	CXCL-10	↓	Apoptosis	
	CCL-2	↑	CCL-5	↑	CCL-5	↓	Lymphopenia	
	IL-8, -12	↑	CCL-2, -3		IL-1β	↓		
			IL-12	↑	IL-6, -8	↓		
	IFN-α/β	↓	IFN-γ	↑	IFN-α/β	↓		
			IFN-α/β	↓	IFN-III	↓		

has been proposed as a potential mechanism to delay host adaptive immunity, providing time to replicate in the host (Mesel-Lemoine et al., 2012).

The interaction of MERS-CoV with human MØs and DCs (Chu et al., 2014, 2016; Scheuplein et al., 2015; Ying et al., 2016) induces CXCL-10, CCL-2, CCL-3, CCL-5, IL-8, and IL-12 (Zhou et al., 2014). MERS-CoV also infects mouse monocyte-derived dendritic cells, inducing CXCL-10, CCL-5, IL-12, and IFN-γ, although no IFN-β and only marginal IFN-α levels were detected (Chu et al., 2014; Zhou et al., 2015). In contrast, in human plasmacytoid dendritic cells, MERS-CoV induced large amounts of type I and III IFNs, especially IFN-α (Scheuplein et al., 2015), although the infection was abortive.

SARS-CoV also infects human MØs and DCs, but viral replication is abortive and no infectious virus particles are produced (Cheung et al., 2005; Law et al., 2005; Tseng et al., 2005; Yilla et al., 2005; Ziegler et al., 2005). Despite the lack of productive infection in human MØs, SARS-CoV induced the expression of proinflammatory chemokines, including CXCL-10 and CCL-2 but, in contrast, antiviral cytokines such as IFN-α and INF-β were basically absent (Cheung et al., 2005; Law et al., 2005, 2009; Tseng et al., 2005). In unproductive infections of DCs, SARS-CoV induced CXCL-10, CCL-2, CCL-3, CCL-5, and

TNF-α (Law et al., 2005; Tseng et al., 2005). Dysregulated type I IFN and inflammatory monocyte-MØ responses in SARS-CoV-infected mice resulted in high levels of cytokines and chemokines and impaired virus-specific T-cell responses leading to lethal pneumonia (Channappanavar et al., 2016).

Both MERS-CoV and SARS-CoV infect human primary T cells and induce massive apoptosis and lymphopenia, although they do not produce infectious virus in these cells (Chu et al., 2016; Gu et al., 2005; Zhou et al., 2015). Infection of T cells may play a role in controlling the pathogenesis elicited by both MERS-CoV and SARS-CoV (Chen et al., 2010).

Similarly to the field viruses, live SARS-CoV and MERS-CoV vaccines may interact with host MØs and DCs, promoting the synthesis of cytokines and chemokines that could lead to undesired side effects including imbalanced proinflammatory immune responses. The analysis of the type of interleukins and cytokines produced during vaccine administration should be included in safety studies of candidate vaccines and could involve murine leukocytes derived from susceptible Tg mice and human cells collected from healthy donors.

5. CORONAVIRUS ANTIVIRAL SELECTION

Despite extensive efforts commencing with the SARS epidemic in 2003, no antiviral drugs suitable to treat CoV infections have been approved by the FDA (Barnard and Kumaki, 2011; Kilianski and Baker, 2014). Nevertheless, there are several antiviral compounds in preclinical development that may be useful to control human CoV infections (Adedeji and Sarafianos, 2014; Zumla et al., 2016). A number of these compounds target highly conserved replicase proteins, and therefore should be effective against a broad range of CoVs, including common human CoVs. Key enzymatic activities, such as the viral main protease, PLP, and helicase have been employed as targets for CoV-specific antiviral drugs (Kilianski and Baker, 2014). These conserved antiviral targets are generally thought to tolerate fewer mutations compared to structural protein genes due to higher fitness pressure; thus, possibly reducing the risk of resistant variants emerging rapidly during antiviral treatment.

Other compounds inhibiting virus entry or morphogenesis have also been tested though, with few exceptions, these viral life cycle steps are poor antiviral targets, as escape mutants are easily recovered, especially when viral

structural proteins are targeted (Kilianski and Baker, 2014). The cell attachment of HCoV-229E and HCoV-NL63 has been inhibited with different antibody combinations (Pyrc et al., 2006, 2007), and subsequent steps of cell entry have been inhibited by S protein-derived heptad-repeat 2 (HR2) peptides in the case of HCoV-NL63 (Pyrc et al., 2006). Similarly, inhibitors of vacuolar acidification were effective against HCoV-229E in cell culture and against HCoV-OC43 both in cells and in vivo (Keyaerts et al., 2009; Pyrc et al., 2007).

The identification of signaling pathways involved in SARS-CoV-mediated pathogenesis has provided selection systems for drugs that significantly increase the survival of infected mice. For example, the identification of NF-kB as the main signaling pathway leading to an exacerbated inflammatory response during SARS-CoV infection enabled the selection of an antiviral suitable to control this infection (DeDiego et al., 2014b). Similarly, the identification of the increased phosphorylation of p38 MAPK by SARS-CoV E protein-activated syntenin also led to a dramatic increase (>80%) in the survival of infected mice treated with p38 MAPK inhibitors (Jimenez-Guardeno et al., 2015) (Fig. 4).

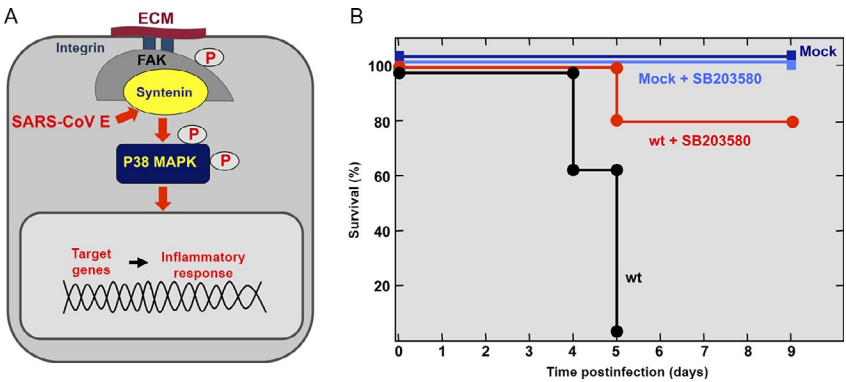

Fig. 4 Inhibitors of p38 MAPK activation protects mice infected with recombinant SARS-CoV. (A) Syntenin initiates a signaling cascade resulting in the phosphorylation (and activation) of p38 MAPK, a protein involved in the expression of proinflammatory cytokines. (B) Inhibition of p38 MAPK phosphorylation led to the survival of 80% of the mice infected with recombinant SARS-CoV, confirming the antiviral potency of this drug (Jimenez-Guardeno et al., 2015). *E*, SARS-CoV envelope protein; *ECM*, extracellular matrix; *FAK*, signaling adhesion kinase protein; *Mock*, noninfected mice; *P*, phosphorylated residue; *P38 MAPK*, p38 MAP kinase; *SB203580*, inhibitor of p38 MAPK; *Wt*, mice infected with virulent SARS-CoV.

6. CONCLUSIONS

New animal and human CoVs are constantly emerging or reemerging, as there are animal reservoirs that maintain them, including bats and birds present in high numbers and with high mobility. As a consequence, the development of technologies suitable to respond swiftly to newly emerging CoVs by producing vaccines is highly desirable. This goal could be achieved with the production of subunit or inactivated vaccines, but optimal combinations of antigen and adjuvant need to be established to minimize the risk of ADEI or eosinophilia that frequently occurred with CoV vaccines developed in the past. We believe that the vaccines of the future will be mainly based on live-attenuated viruses because of their superior potential to induce balanced Th1/Th2 immune responses, the potent and long-lasting immunity, and the comprehensive B and T-cell repertoire induced by this type of vaccines. However, the development of safe live-attenuated virus vaccines requires strong experimental support to confirm sufficient attenuation in the target species, possibly resulting in longer development and production times.

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