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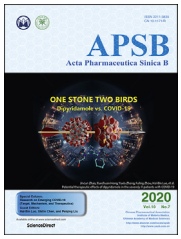

REVIEW

Highly pathogenic coronaviruses: thrusting vaccine development in the spotlight

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Abstract Coronaviruses (CoVs) are a large family of viruses that cause illness ranging from the common cold to more severe diseases such as Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) disease (COVID-19) has caused major public health crises. There have been more than 4,400,000 reported cases of COVID-2019 and more than 300,000 reported deaths to date (16/05/2020). SARS-CoV, MERS-CoV and SARS-CoV-2 have attracted widespread global attention due to their high infectivity and pathogenicity. To date, there is no specific treatment proven effective against these viral infectious diseases. Vaccination is considered one of the most effective strategies to prevent viral infections. Therefore, the development of effective vaccines against highly pathogenic coronaviruses is essential. In this review, we will briefly describe coronavirus vaccine design targets, summarize recent advances in the development of coronavirus vaccines, and highlight current adjuvants for improving the efficacy of coronavirus vaccines.

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1. Introduction

In 2019, a novel strain of coronavirus was found in humans[1]. On February 11, 2020, World Health Organization (WHO) announced a new name for the epidemic disease: Corona Virus Disease (COVID-19). Meanwhile, the International Committee on Taxonomy of Viruses named the novel coronavirus as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). As of May 16th, 2020, the epidemic of COVID-19 has caused more than 4,400,000 laboratory-confirmed cases and more than 300,000 reported deaths[2].

COVID-19 is the third known zoonotic coronavirus disease[3] after Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). SARS is a zoonosis caused by SARS-CoV, which has infected 8096 humans, including 774 deaths (mortality rate 9.6%), in at least 29 countries[4]. Another highly pathogenic coronavirus, MERS-CoV, has been reported in 27 countries with reported viral infection and 858 associated deaths (mortality rate 34.4%) [5]. Research indicates that SARS-CoV was transmitted from civet cats to humans and MERS-CoV was transmitted from dromedary camels to humans. However, the intermediate host of SARS-CoV-2 has not been identified[6–8].

SARS-CoV-2, together with SARS-CoV and MERS-CoV, has posed significant threats to international health due to theirs high pathogenicity and infectivity. Vaccination is an important strategy to provide protection from infectious diseases. However, to date, no vaccine has been approved to prevent coronavirus infection, indicating the need for further development of novel and effective vaccines against coronavirus infection.

In this review, we will illustrate vaccine design targets, review current advances and potential strategies for vaccine development based on the spike (S) protein of SARS-CoV and MERS-CoV, and focus on how to improve the efficacy of vaccines through adjuvant formulations. Overall, these strategies may provide useful guidance for vaccine development of SARS-CoV-2.

2. Spike protein: a key target for coronaviruses vaccine

Coronaviruses are widespread in nature. It can cause respiratory and intestinal infections in animals and humans. According to the phylogenetic relationships, coronavirus can be divided into four genera: Alpha, Beta, Gamma and Delta. Alpha and beta genera can infect mammals, while gamma and delta genera are mostly avian coronaviruses[9]. There are seven known coronavirus that can infect humans: 229E, OC43, NL63, HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2. The first four viruses cause only mild minor respiratory illness. The other three strains—SARS-CoV, MERS-CoV and SARS-CoV-2—are zoonotic and lead to severe respiratory syndrome[10–12].

Coronaviruses are the largest single positive-strand RNA viruses with a genome of 27−32 kb[13]. It is named for its corona-like appearance (Fig. 1A). The virus genome mainly encodes four structural proteins: spike (S), nucleocapsid (N), membrane (M), envelope (E) proteins. S protein forms the spikes on the surface of coronaviruses and mediates adsorption and fusion of the virus and host cells. N protein forms a helical capsid, which locates inside the viral membrane to protect viral RNA. M and E proteins are important components of the viral envelope, and together mediate the assembly process of the virus[14]. Among the four structural proteins, S protein is the leading mediator of virus entry and is the main factor that determines the virulence and host range of the virus.

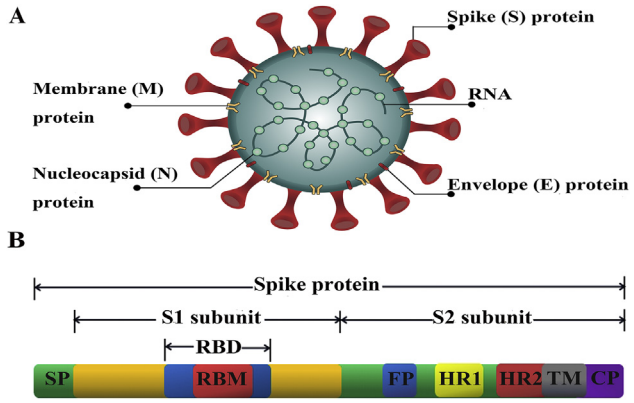

Figure 1 Coronavirus and spike protein (S) structures. (A) Schematic structure of coronavirus and its key structural proteins, including spike (S), nucleocapsid (N), membrane (M), envelope (E) proteins. (B) Schematic structure of coronavirus S protein and its functional regions. S protein is composed of S1 and S2 subunits. SP, signal peptide. RBD, receptor-binding domain. RBM, receptor-binding motif. FP, fusion peptide. HR1 and HR2, heptad repeat one and two regions. TM, transmembrane. CP, cytoplasmic tail.

SARS-CoV, MERS-CoV, and SARS-CoV-2 have strong human-to-human characteristics, which is attributed to the interaction between S protein and host cell surface receptors. SARS-CoV and SARS-CoV-2 use the angiotensin-converting enzyme 2 (ACE2) as a receptor[15,16], whereas MERS-CoV uses dipeptidyl peptidase 4 (DPP4; also known as CD26) as a receptor[17]. The distribution of receptors in humans and their affinity with S proteins determine the extent of tissue tropism and the intensity of transmission of coronavirus. The epidemiology and biological characteristics of SARS-CoV, MERS-CoV and SARS-CoV-2 are summarized in Table 1[2–8,12,15–17].

S protein is a large type I transmembrane glycoprotein whose trimers constitute the spike structure on the surface of the virus. The S protein (Fig. 1B) can be divided into two functional subunits: an N-terminal S1 domain contains signal peptide and receptor binding domain (RBD), and a C-terminal S2 domain contains fusion peptide and two heptapeptide repeats (HR1 and HR2) to facilitate viral fusion. RBD mediates the binding of virus and cell receptor, which then triggers a conformational change of the S protein, exposing HR1 and HR2 to form a 6-helix bundle fusion core structure, further leading to membrane fusion and viral RNA release[18–21]. Furthermore, S protein carries B-cell epitopes, which induces the body to produce neutralizing antibody and provides immune protection[22]. Because the S protein is involved in viral infection and is responsible for inducing host immune response and virus-neutralizing antibodies, it has been considered a key target for vaccine design.

Antigen-specific targets of S protein include full-length S protein, S1 subunits, RBD and S2 subunits. Viral vector vaccines encoding full-length S protein or S1 subunits have been demonstrated to induce high levels of neutralizing antibodies in various animal models[23,24]. However, some non-neutralizing epitopes on full-length S protein or S1 subunits may compete with neutralizing epitopes, leading to several safety concerns, including

Table 1 Epidemiology and biological characteristics of SARS-CoV, MERS-CoV and SARS-CoV-2, as of 16 May 2020.

Characteristic		SARS-CoV	MERS-CoV	SARS-CoV-2
Clinical epidemiology	Total global number	8096	2494	4,434,653
	Number of deaths	774	858	302,169
	Mortality	9.6%	34.4%	6.8%
	Affected countries	29	27	216
	Transmission region	Globally	Regionally	Globally
The predominant cell receptor		Human angiotensin-converting enzyme 2 (ACE2)	Human dipeptidyl peptidase 4 (DPP4 or CD26)	Human angiotensin-converting enzyme 2 (ACE2)
Receptor binding affinity		High	High	Higher than SARS-CoV
Pathogenic mechanism		Primarily infects ciliated bronchial epithelial cells and type II pneumocytes, resulting in massive viral replication and cell damage	Primarily infects unciliated bronchial epithelial cells and type II pneumocytes, resulting in massive viral replication and cell damage	Primarily infects ciliated bronchial epithelial cells and type II pneumocytes, resulting in massive viral replication and cell damage

inflammatory and immunopathological effects such as pulmonary eosinophilic infiltration and antibody-dependent enhancement (ADE) following subsequent viral challenge of vaccinated animals[22,25,26]. ADE is a phenomenon in which non-neutralizing antibodies are produced following an infection or a vaccination leads to enhanced infection[27]. One approach to mitigate the adverse effects of ADE is to narrow the immune response to target only critical or beneficial epitopes[25]. Vaccines based on RBD elicited a robust protective immune response and neutralizing antibodies. At the same time, RBD does not contain non-neutralizing epitopes that may cause harmful immune responses, which is a hot spot for CoV vaccine development. It is worth mentioning that RBD has relatively low immunogenicity and often requires repeated doses and adjuvants[28–30]. Because the S2 subunit is highly conserved and not prone to mutation, S2 region has become an important target for the development of protective vaccines. However, reports regarding the presence of neutralizing epitopes in S2 and a protective role for antibodies to S2 have been inconsistent. Several studies demonstrated that S2 domain could induce specific cellular immune response and a high level of total IgG but little neutralizing antibodies against coronavirus infection[31,32]. On the contrary, there are also reports that showed that S2 domain contains neutralizing epitopes and could induce neutralizing antibodies[33,34].

N protein serves multiple functions in viral replication, transcription, and assembly of the viral genome complex, which is more conservative than other proteins, such as S and M. Therefore, N protein has been also widely reported as a target antigen. N proteins have been shown to be highly immunogenic and capable of triggering T cell responses[35]. Remarkably, many studies indicated that the serum containing anti-N protein does not contain neutralizing antibodies against coronavirus infection[36,37]. In addition, vaccines based on N protein not only failed to protect from homologous or heterologous challenge, but resulted in enhanced immunopathology with eosinophilic infiltrates within the lungs of SARS-CoV-challenged mice[38].

3. Advances in the development of SARS-CoV vaccines and MERS-CoV vaccines

It is critical for CoV vaccines to induce robust humoral and cellular immunities. Previous studies indicated that the level of serum neutralizing antibody is correlated inversely with virus titers in the lungs, which effectively increased the survival rate of the vaccine host[39–41]. Production of high titer neutralizing antibodies can block MERS-CoV replication in the lungs of vaccinated mice (Fig. 2), confirming the importance of neutralizing antibody in fighting virus infection[41]. Similarly, the importance of T-cell responses against CoV infections was also highlighted in many studies[42–45]. For example, depletion of $CD4^+$ and $CD8^+$ T cells in mice prior to challenge by SARS-CoV resulted in decreased survival rates to 35% and 45%, respectively (Fig. 3)[44]. Interestingly, virus-specific memory T cells but not neutralizing antibodies could be detected 6 years after infection in SARS survivors, suggesting memory T cell responses may provide broad and long-term protection against SARS-CoV infection[45]. In general, both neutralizing antibody levels and T cell responses should be considered in current CoV vaccination strategies.

No CoV vaccine is currently approved for use in humans. Most of the currently developed CoV vaccines are in the preclinical stage. In addition to the traditional inactivated and attenuated live vaccines, other development of candidate CoV vaccines was mainly focused on the S protein of coronaviruses. These types of S protein-based vaccines include nucleic acid-, viral vector-, virus-like particle (VLPs) and subunit vaccines. Because the research of SARS-CoV-2 vaccine is still in the early stages, the following review will discuss strategies for the development of SARS-CoV and MERS-CoV vaccines in the hope of providing useful guidance for the development of SARS-CoV-2 vaccines.

3.1. Inactivated and live-attenuated virus vaccines

SARS or MERS inactivated by physical (ultraviolet or radiation) or chemical (methanol, β-propiolactone and formalin) methods has been shown to cause high levels of neutralizing antibodies and protective immunity in several animal models including mice, rabbit and ferret[46–48]. However, eosinophil infiltration in the lungs of animals vaccinated with inactivated SARS-CoV vaccine or MERS-CoV vaccine has raised concerns about the safety and ultimate protective efficacy of inactivated vaccine[49,50]. In addition, a double-inactivated SARS-CoV (doubly inactivated by formalin and UV irradiation) vaccine provides poor protection against lethal disease in aged-animal models following heterologous challenge[51]. This result may be attributed to respiratory dendritic cells (rDCs) migration to draining lymph nodes (DLNs) progressively decreases as mice age, decreases in virus-specific $CD8^+$ T cell

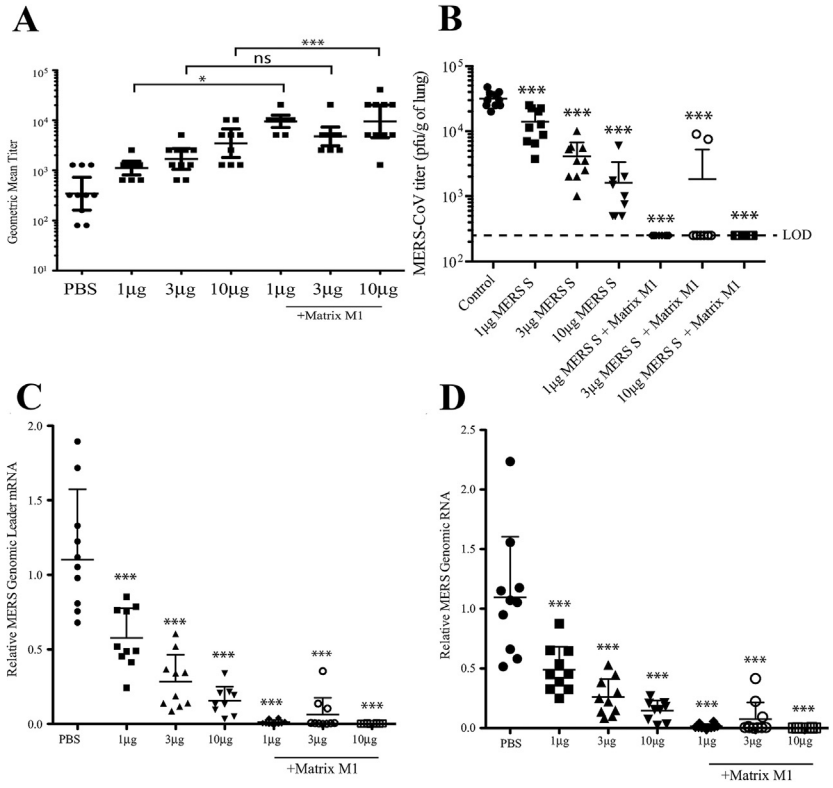

Figure 2 Vaccination of MERS S nanoparticle plus Matrix M1 protects mice from MERS-CoV challenge. (A) Neutralizing antibody levels against infections of live MERS-CoV. GMT ± standard deviation is graphed for each group of 10 mice. Dots represent individual mice. $^{*}P < 0.05$, $^{***}P < 0.001$, ns means not significant. (B) Lung MERS-CoV replication was determined by plaque assay. (C) MERS-CoV specific Leader mRNA expression (D) MERS-CoV genomic RNA expression. Mean ± standard deviation are graphed for each group of 10 mice. Dots represent individual mice. LOD means limit of detection. $^{***}P < 0.001$. The figure was adapted with permission from Ref. 42. Copyright ©2017 Elsevier.

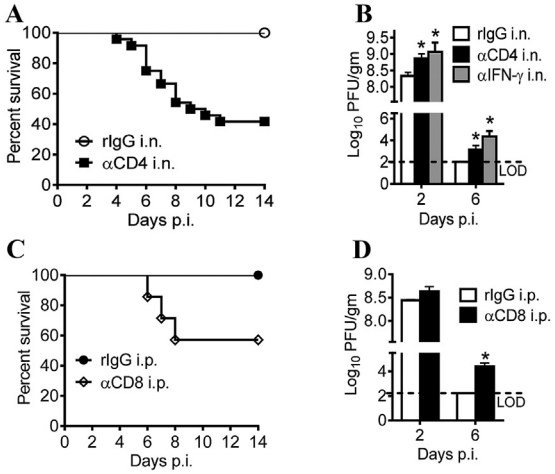

Figure 3 Airway T cells are protective against SARS-CoV challenge. (A) Survival rate of SARS-CoV infected mice after depletion of airway $CD4^{+}$ T cells. $n = 5$, rIgG i.n.; $n = 24$, aCD4 i.n. (B) Virus titers of SARS-CoV infected mice after depletion of airway $CD4^{+}$ T cells. Titers are expressed as PFU/g tissue. $n = 3$ mice/group/time point. $^{*}P < 0.05$. Data are representative of two independent experiments. (C) Survival rate of SARS-CoV infected mice after depletion of airway $CD8^{+}$ T cells. $n = 5$, rIgG i.p.; $n = 7$, aCD8 i.p. (D) Virus titers of SARS-CoV infected mice after depletion of airway $CD8^{+}$ T cells. $n = 3$ mice/group/time point. $^{*}P < 0.05$. Data are representative of two independent experiments. The figure was adapted with permission from Ref. 45. Copyright © 2016 Elsevier.

responses in lungs and more severe disease in older mice infected with SARS-CoV[52,53]. Based on the above results, candidate vaccines against emerging coronaviruses should emphasize the efficacy in older animal with virus infection.

The live-attenuated virus vaccine, composed of recombinant SARS-CoV lacking the E gene (rSARS-CoV-E), produces significant neutralizing antibodies and virus-specific T cell responses[54,55]. More recently, a live attenuated SARS-CoV was generated through mutation of transcription regulatory networks (TRNs), where the attenuated virus effectively limits virulence reversal and protects mice against challenge[56]. Another study reported that rMERS-CoV-ΔE, a mutant of the MERS-CoV prepared using a new DNA cloning vector system, only replicates in a small number of cells, but can produce enough antigen to stimulate protective immunity in the host[57]. Although vaccine candidates based on the live-attenuated coronaviruses have the potential to induce a highly effective immune response and protection, it may present biosafety problems associated with virulence recovery[54].

3.2. *Nucleic acid-based vaccines*

Nucleic acid vaccines, including DNA and RNA vaccines, are based on plasmids or messenger RNA that encode vaccine antigens, and they are introduced into the host to produce immunological response to protect organisms against diseases[58]. The efficacy of DNA-based vaccines against SARS-CoV and MERS-CoV

infections has been widely evaluated. In a phase I clinical trial, a SARS DNA vaccine produces cellular immune responses and neutralizing antibody in healthy adults[59]. Additionally, a DNA vaccine encoding the MERS-CoV S protein induces strong $CD8^+$ and $CD4^+$ T cell immunity and antigen-specific neutralizing antibodies in mice, camels and nonhuman primates (NHPs), and -protects vaccinated rhesus macaques from infection by MERS-CoV[60,61]. GLS-5300, a DNA vaccine expressing MERS-CoV S-protein antigen, is the first MERS-CoV vaccine to advance into human trials. The vaccine induced durable immune responses, as most participants maintained detectable S1 binding antibodies and had cellular immune responses at almost 1 year after the last vaccination[62]. Nevertheless, CoV DNA vaccines based on full-length S protein may cause a Th2-related harmful immune response, leading to liver damage in vaccinated animals. One study comparing the immunogenicity of MERS-CoV DNA vaccines expressing S or S1 in mice showed that plasmids expressing the S1 (pS1) subunit triggered a balanced Th1/Th2 response, thereby avoiding the risk of immunopathological risk associated with Th2 response[63]. Moreover, immunization of mice with pS1 vaccine induced significantly higher levels of IFN-γ compared to pS vaccine[63].

Messenger RNA (mRNA) vaccines carry transcripts encoding antigens, and use the host cell translational machinery to produce the antigens, which then stimulates an immune response[64]. Because of high yields of *in vitro* transcription reactions, mRNA has the potential for rapid, inexpensive and scalable manufacturing, which greatly shortens the development time and can respond quickly to epidemics. Compared to DNA vaccines, mRNA vaccines do not need to pass an additional membrane barrier (nuclear membrane), so it does not have safety concerns about integration into the host genome[65]. Due to the above advantages, mRNA vaccines are becoming a powerful tool against coronavirus infection.

However, their application has been restricted by the instability and inefficient *in vivo* delivery of nucleic acid (DNA or mRNA)[66,67]. To provide protection from degradation and facilitate their entry into targeted cells, efficient delivery systems for nucleic acid vaccines, particularly the nanocarriers, have been explored extensively.

3.3. Viral-vector vaccines

Viral vectors have a molecular mechanism that assists the target gene to enter cells and infect them, which is an important vector platform for CoV candidate vaccines. Viral vector-based vaccines encoding S protein of MERS-CoV and SARS-CoV have been widely studied. To date, adenovirus (Ad), modified vaccinia ankara (MVA), attenuated parainfluenza virus (BHPIV3) and rabies virus (RV) have been used as vaccine vector[68–72]. A previous report has indicated SARS-CoV S-specific neutralizing antibodies and mucosal responses are elicited in African green monkeys immunized with BHPIV3/SARS-S vector vaccines, protecting African green monkeys against SARS-CoV infection[68]. Another study reported that a single inoculation with the RV-based vaccine expressing SARS-CoV S protein can induce a strong SARS-CoV-neutralizing antibody response[69]. In addition, MERS-CoV S-specific neutralizing antibodies and antigen specific T cell response, are induced in mice after immunizing them with human adenovirus or MVA-based MERS-CoV S-expressing vaccines[70,71]. Furthermore, compared with MERS-CoV S-encoding Ad5 vaccines, MERS-CoV S1-encoding Ad5 vaccines might induce higher levels of neutralizing antibodies[72]. In a recent study, rAd5 constructs expressing CD40-targeted S1 fusion protein (rAd5-S1/F/CD40L) exhibited full protection against lethal MERS-CoV challenge, and prevented severe perivascular hemorrhage within the lungs as compared to non CD40-targeted vaccine (rAd5-S1)[74]. Currently, MERS-CoV S protein expressed by chimpanzee adenovirus (ChAdOx1) or modified vaccinia Ankara (MVA) vectors are at phase I clinical trial[74,75]. Indeed, viral vectors expressing S protein can induce viral neutralizing antibodies *in vivo*, providing an effective platform for the development of SARS-CoV-2 vaccine. However, some viral-vectors, such as certain serotypes of adenoviruses, may fail to induce effective immune responses owing to the high prevalence of virus-neutralizing antibodies in the human population resulting in elimination of viral vectors[76]. Thus, caution should be taken when developing CoV vaccines using viral vectors.

3.4. Virus-like particle (VLP) vaccines

Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of native viruses but devoid of infectious genetic materials. VLP is a potential candidate for the development of safe and effective CoV vaccines, which can efficiently stimulate innate and adaptive immune response functions. Bacterial, insect, yeast and mammalian cells expression systems have been widely used in the production of VLPs. One study indicated a chimeric VLPs that coexpression of SARS-CoV S protein and E, M and N proteins of mouse hepatitis virus resulted in the efficient production of neutralizing antibodies, thus inhibiting SARS-CoV replication in the lung[77]. The other chimeric VLPs, expressing SARS-CoV S protein and influenza M1 protein, can induce neutralizing antibodies and protect mice against deadly challenges[78]. Similar as SARS-CoV VLP vaccines that induce high titers of neutralizing antibodies against CoV infection, MERS-CoV VLP vaccines also elicit antigen specific cellular immune response against infections of MERS-CoV[79]. The VLP vaccine is a potential tool to provide protection against novel pandemic pathogens. However, VLPs as a preventive vaccine still have many problems to be considered. For example, viral mutations might allow the virus to evade antibody-mediated neutralization.

3.5. Subunit vaccines

Subunit vaccines are composed of highly purified antigens which require only a part of the pathogen to generate a protective immune response. Subunit vaccines are characterized by high security, controllable performance and easy production on a large scale, thereby gradually becoming the focus of more and more researchers. Compared to the full-length S protein, RBD contains several critical neutralizing epitopes and lacks non-neutralizing epitopes that may cause harmful pathological responses. Therefore, RBD-based subunit vaccines not only can induce effective neutralizing antibodies, but also avoid adverse immune responses. From the safety and effectiveness perspectives, the RBD-based CoV vaccines are more attractive candidates in the development of CoV vaccines.

Since the SARS and MERS outbreaks, subunit vaccines based on SARS-CoV and MERS-CoV RBD have been extensively studied and tested, showing sufficient effectiveness and strong

Table 2 Vaccine strategies of SARS-CoV and MERS-CoV.

Vaccine strategy	Process of production	Result and reference	
		SARS-CoV	MERS-CoV
Inactivated virus vaccines	Virus particles are inactivated by physical or chemical methods	1 Induces S-specific antibody responses and neutralizing antibodies in mice (1:7393) and rabbit (1:2060), neutralizes pseudotyped SARS-CoV[46]. 2 Induces neutralizing antibodies in mice in ferret (1: 128−256), neutralizes SARS-CoV; reduces the virus titer in the respiratory tract, and provides protective immunity[47].	Induces S-specific antibody responses and neutralizing antibodies in mice ($>1:10^3$); neutralizes pseudotyped MERS-CoV[48].
Live-attenuated virus vaccines	Genomes are mutated by mutagenesis or targeted deletions	1 Induces SARS-CoV-specific antibody responses and neutralizing antibodies in 6-week-old mice ($1:10^2$–10^3) and 12-month-old mice ($1:10^2$–10^3), neutralizes SARS-CoV Urbani strain; elicits T-cell responses and protects all mice (6-week-old/12-month-old) against challenge with virulent virus[54]. 2 Induces SARS-CoV-specific antibody responses and T cell responses in BALB/c and hACE2 Tg mice; protects 60%−70% of mice against challenge with virulent virus[55]. 3 The TRN-rewired SARS-CoV is attenuated and protect against lethal SARS-CoV challenge[56].	rMERS-CoV-E generated by reverse genetics system is a replication-competent, propagation-defective virus[57].
Nucleic acid-based vaccines	Genetically engineered DNA/mRNA encode antigenic compounds	Induces S-specific antibody responses and neutralizing antibodies in 80% subjects, neutralizes pseudotyped SARS-CoV; elicits T-cell responses in all subjects[59].	1 Induces S-specific antibody responses and neutralizing antibodies in mice ($>1:10^2$), camels (1:600−700) and rhesus macaques ($>1:10^2$), neutralizes MERS-CoV strain (EMC/2012); elicits T-cell responses in rhesus macaques and protects 100% of rhesus macaques from viral challenge[60]. 2 Induces S1-specific antibody responses and neutralizing antibodies in 77% subjects, neutralizes MERS-CoV strain (EMC/2012); elicits T-cell responses in 64% subjects[62].
Viral-vector vaccines	Inserting foreign gene units into the viral genome by homologous recombination	1 Induces neutralizing antibodies in African green monkeys (≈1:16) immunized with BHPIV3-SARS-S vector vaccine, neutralizes SARS-CoV; protects all african green monkeys against challenge with virulent virus[68]. 2 Induces neutralizing antibodies in mice (1:160) immunized with RV-SARS-S vector vaccine; neutralizes SARS-CoV[69].	1 Induces neutralizing antibodies in mice (1: 64−128) immunized with MVA-MERS-S vector vaccine, neutralizes MERS-CoV; elicit T-cell responses and reduces virus titers in the lung [71]. 2 Induces S-specific antibody responses and neutralizing antibodies in mice ($>1:10^3$) immunized with Ad5/Ad41-MERS-S vector vaccine, neutralizes pseudotyped MERS-CoV; elicits T-cell responses[71]. 3 Induces S-specific IgG subtype antibody (IgG1 and IgG2a) and neutralizing antibodies in mice ($>1:10^3$) immunized with Ad5-MERS-S1 vector vaccine, neutralizes MERS-CoV strain (EMC/2012)[72].

Table 2 (*continued*)

Vaccine strategy	Process of production	Result and reference	
		SARS-CoV	MERS-CoV
			4 Induces S1-specific IgG subtype antibody (IgG1 and IgG2a) and neutralizing antibodies in mice ($1:10^2$–10^3/$1:10^3$–10^4) immunized with rAd5-S1/F/CD40 vaccine, neutralizes pseudotyped and live MERS-CoV[73].
Virus-like particle (VLPs) vaccines	Genes clone viral structural proteins into expression system	1 Induce neutralizing antibodies in mice (1: 200 ± 97.7), neutralizes SARS-CoV; reduces virus titers in the lung[77]. 2 Induce neutralizing antibodies in mice (1:875−1525), neutralizes SARS-CoV Urbani strain; reduces virus titers in the lung and protects all mice against challenge with virulent virus[78].	Induced RBD-specific antibody responses and neutralizing antibodies in mice (1: 320), neutralizes pseudotyped MERS-CoV; elicit T-cell responses[79].
Subunit vaccines	Antigenic components including immunogenic pathogen fragment without nucleic acid	1 Induce S-specific antibody responses and neutralizing antibodies in mice ($1 : 4.0\times10^3\pm3.5\times10^2$), neutralizes SARS-CoV BJ01 strain; protects 80% of the mice from the virus challenge[80]. 2 Induces RBD-specific antibody responses and neutralizing antibodies in mouse ($1 : 5.8\times10^4\pm4.9\times10^3$/ $1 : 1.0\times10^3\pm2.4\times10^2$), neutralizes pseudotyped and live SARS-CoV; elicit T-cell responses and protects all mice against challenge with virulent virus[81].	Induced RBD-specific antibody responses and neutralizing antibodies in rhesus monkey (1:1600), neutralizes pseudotyped MERS-CoV; elicits T-cell responses and reduces virus titers[84].

protection against CoV infection in various animal models[80−84]. For example, RBD-Fc (RBD fused with human IgG1 Fc) elicits long-term humoral immune response, and produces neutralizing antibodies that protects the vaccinated mice from the SARS-CoV challenge without causing immunopathological damage[80]. Also, a newly designed RBD without the Fc tag induces robust humoral and T cell responses, particularly neutralizing antibodies in immunized mice, protecting mice against SARS-CoV infection[81]. It has been demonstrated that S377-588-Fc, which is a fusion protein of RBD fragment S377-588 (spike residues 377−588) and human IgG1 Fc, induces the higher-titer IgG antibodies and neutralizing antibodies among all RBD fragments in mice[82]. A study has also shown that i.n. vaccination of MERS-CoV RBD-Fc induces humoral IgG antibody response comparable to those induced by s.c. vaccination, including neutralizing antibodies, but more robust systemic cellular immune responses and higher local mucosal immune responses in mouse lungs[83]. In the rhesus macaque, a recombinant receptor-binding domain (rRBD) protein vaccine can also induce sustained and robust immunological responses[84]. These studies suggest that RBD-based CoV vaccines have potential for preventing respiratory infections caused by CoV, further enhancing beneficial strategies for emerging coronavirus infection. However, it is worth noting that highly purified proteins are generally low immunogenicity and often require the addition of vaccine adjuvants.

To summarize, an effective vaccine against coronavirus infection often needs to induce the body to produce strong humoral immune response and cellular immune response. The current advancements and vaccine strategies in the development of in the development of SARS-CoV vaccines and MERS-CoV vaccines are listed in Table 2 [46−48,54−57,59−62,68−73,77−81,84]. Apart from inactivated and live-attenuated virus vaccines, nucleic acid-, viral vector- and VLPs-based vaccines, particularly subunit vaccines containing the RBD of CoV S protein, are critically important.

4. Adjuvant systems for improving the immunogenicity of coronavirus subunit vaccines

Highly purified proteins in subunit vaccines are usually not inherently immunogenic, as they generally do not directly stimulate the innate immune system. However, the development of effective CoV vaccines requires the activation of powerful humoral and cellular immunity to induce protective immunity and virus clearance in the body. Therefore, adjuvants are needed to be incorporated in subunit vaccines to enhance the immunogenicity of these weaker antigens and evoke the required antigen-specific immune response phenotype, thus improving the overall potency of poorly immunogenic subunit vaccines. The following review will discuss adjuvants commonly used in subunit vaccines against coronavirus infection.

4.1. Aluminum-based adjuvants

Aluminum (Alum) adjuvant is the longest and most frequently used adjuvant in licensed vaccines, with an extensive safety

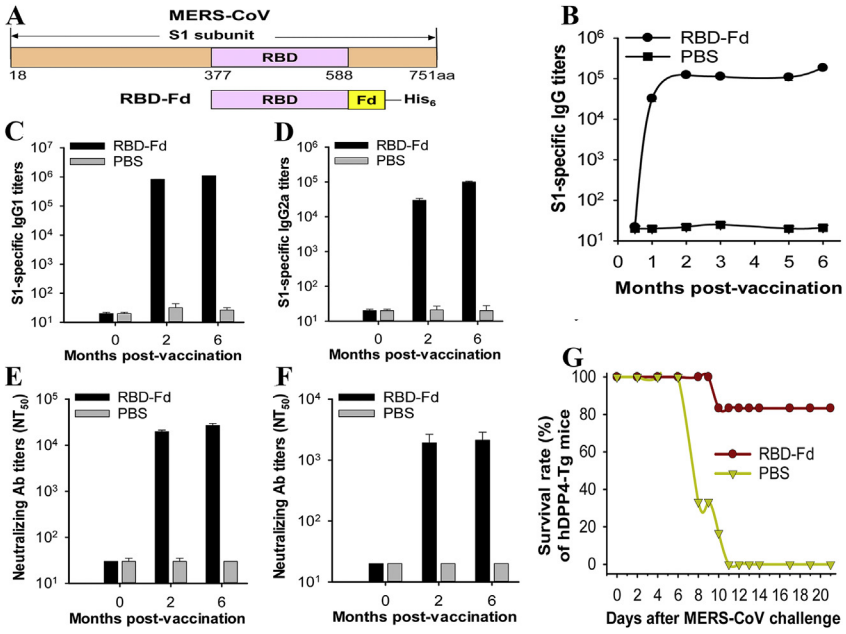

Figure 4 MERS-CoV RBD in trimeric form with MF59 protects human dipeptidyl peptidase 4 (hDPP4) transgenic mice from MERS-CoV infection. (A) Schematic structure of MERS-CoV S1 subunit and construction of RBD-Fd. A His6 tag was added at the C-terminus of RBD-Fd (B) MERS-CoV S1-specific IgG antibody titers. (C) and (D) MERS-CoV S1-specific IgG1 and IgG2a antibody titers. (E) and (F) neutralizing antibody levels against infections of pseudotyped and live MERS-CoV of EMC2012 strain. (G) Survival rate of MERS-CoV infected mice after vaccination. Fd: foldon. The figure was adapted with permission from Ref. 104. Copyright © 2017 Elsevier.

record. Alum is a Th2-type adjuvant that induces strong humoral immune response, including the production of neutralizing antibodies[85]. Therefore, Alum is incorporated into a range of vaccines against viral infection where neutralizing antibodies to viral antigens are required for protection, including human papillomavirus, rabies and hepatitis B[86].

Aluminum adjuvant has been widely used in the development of CoV vaccine due to a variety of advantages noted above. Several studies have indicated that RBD-based subunit vaccines in the presence of alum induce powerful serum-specific and neutralizing antibodies, providing a degree of protection against viral challenges[84,87]. It is noteworthy to mention that eliciting powerful cellular and humoral immunity is critical for a potential CoV vaccine. Virus-specific T cells can secrete IFN-γ and promote virus clearance. Meanwhile, effector T cells can further differentiate into memory T cells, which is expected to respond quickly and effectively to subsequent CoV infection[88,89]. Although alum successfully induces antibody-mediated protective

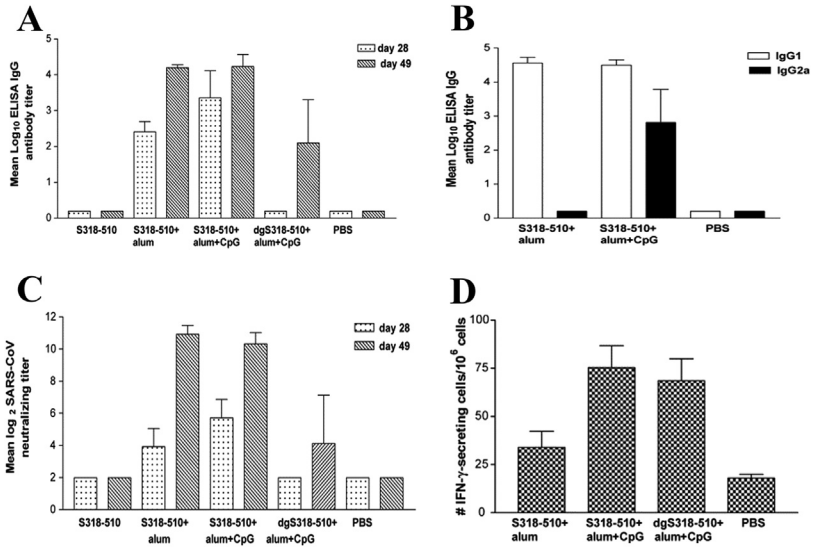

Figure 5 Mice immunized with SARS-CoV spike protein amino acids 318−510 (S318−510) with alum plus CpG elicited strong antibody and cellular immune responses. (A) SARS-CoV-specific IgG antibody titers. (B) SARS-CoV-specific IgG1 and IgG2a antibody titers. (C) Neutralizing antibody levels against infections of SARS-CoV of Tor-2 strain. The figure was adapted with permission from Ref. 124. Copyright © 2007 Elsevier.

Table 3 Application of adjuvants in subunit vaccines.

Adjuvant	Composition	Mechanism	Antibody responses and neutralizing antibody	Cellular immune response
Alum	Aluminum hydroxide/ Aluminum phosphate	1 Promotes a strong Th2-biased response (humoral immune response). 2 Depot effect 3 Inflammatory response[86]	1 Induces MERS-CoV RBD-specific antibody responses and neutralizing antibodies (1:1600) in rhesus macaque; neutralizes pseudotyped MERS-CoV[84]. 2 Induces MERS-CoV RBD-specific antibody responses[87].	No-report
Emulsions				
Freund's adjuvant	IFA: an water-in-oil emulsion formed by mixing mineral oil with an emulsifier CFA: killed bacteria *M. tuberculosis* added to IFA	1 Continuous release of immunogenic substances in oil droplets. 2 Inflammatory response. 3 Stimulates the production of antibodies[90–92]	Induces SARS-CoV RBD-specific antibody responses and neutralizing antibodies ($>1{:}10^4/1{:}10^2{-}10^3$) in mice; neutralizes pseudotyped and live SARS-CoV[93].	Elicits SARS-CoV RBD-specific T cell responses in mice[93].
Montanide ISA51	IFA		1 Induces MERS-CoV RBD-specific antibody responses and neutralizing antibodies ($>1{:}10^3$) in mice; neutralizes live MERS-CoV[82]. 2 Induces neutralizing antibodies ($1{:}240 \pm 139$) in mouse; neutralizes live MERS-CoV[97].	No-report
Sigma adjuvant system (SAS)	Oil-in-water emulsion containing monophosphoryl lipid A	1 Enhances antigen uptake at the injection site. 2 Induces the production of cytokines and chemokines. 3 Recruits immune cells to the injection site[98–100].	1 Induces SARS-CoV RBD-specific antibody responses and neutralizing antibodies ($1 : 6.9\times10^5$/ $1 : 1.6\times10^3$) in mice; neutralizes pseudotyped and live SARS-CoV[101]. 2 Induces neutralizing antibodies ($1{:}10^2$–	No-report

(*continued on next page*)

Table 3 (*continued*)

Adjuvant	Composition	Mechanism	Antibody responses and neutralizing antibody	Cellular immune response
			10^3) in mice; neutralizes eight MERS-CoV strain[102].	
MF59	Squalene-based oil-in-water emulsion		1 Induces MERS-CoV RBD-specific IgG subtype antibody (IgG1 and IgG2a) and neutralizing antibodies (1:100 −673) in mice; neutralizes live MERS-CoV[103]. 2 Induces MERS-CoV S1-specific IgG subtype antibody (IgG1 and IgG2a) and neutralizing antibodies (1:10^3–10^4) in mice; neutralizes pseudotyped and live MERS-CoV[104].	No significant increase in T-cell response[28].
Toll-like receptors (TLRs) agonists				
TLR3 agonist	Double-stranded RNA (dsRNA) analogue	1 The recognition of receptor stimulates innate immune responses such as antiviral and inflammatory responses. 2 Induce adaptive immune responses. 3 Activate immune cells and induce the production of Cytokines[106,107].	No-report	1 Induces the expression of IFN-associated molecule, elicits T cell responses[110]. 2 Induces the production of type-I IFN, elicits T cell responses[111].
TLR4 agonist	LPS/MPLA	Induces SARS-CoV S-specific antibody and virus-specific antibody (>1:10^4)[116].	Induces the production of Th1 cytokines, elicits T cell responses[116].	

TLR9 agonist	CpG DNA	No-report		1 Induces the production of IFN-a and IFN-γ, enhance NK cell cytotoxicity[120]. 2 Induces cytotoxicity T cells response and memory T cells response[121].
Stimulator of interferon genes (STING) agonists				
STING agonist	cdGMP	1 Activates the production of host defense molecules and cytokines. 2 Induces adaptive immune responses[123,124].	Induces MERS-CoV RBD-specific IgG subtype antibody (IgG1 and IgG2a) and neutralizing antibodies (1:40−320) in mice; neutralizes live MERS-CoV[126].	Induces the production of IFN-γ and memory T cells response[126].

immunity, its ability to induce cellular immune responses is limited. One approach to overcome the limitations of alum is to use it in combination with other adjuvants to enhance cellular immune responses.

4.2. *Emulsions*

Another approach that has an extensive history of use as CoV vaccine adjuvants are emulsions. Freund's adjuvant is a water-in-oil emulsion, divided into complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA). As a powerful agonist for Th1 cells, CFA can induce Th1 cytokines and enhance cellular and humoral immune responses. While IFA generally induce Th2 cytokines[90–92]. Mice immunized with SARS-CoV rRBD antigen together with Freund's adjuvant induce not just high titer of neutralizing antibodies, but relatively high levels of CTL and Th responses[93]. Freund's adjuvant induces a more balanced Th1 and Th2 immune response, providing more comprehensive protection against coronavirus infection. Freund's adjuvant is not approved for use in human vaccines due to its toxicity[94]. Despite this, Montanide ISA-51, also known as incomplete Freund's adjuvant (IFA), has been approved for human use in 2012[95,96]. Similar as classic Freund's water emulsion, the addition of Montanide ISA-51 in RBD-based vaccines can produce strong antigen-specific neutralizing antibodies response against CoV infection[82,97].

The toxicity of Freund's adjuvant mainly comes from the non-degradable oil in the ingredients. To avoid the toxicity problem of Freund's adjuvant, oil-in-water emulsions prepared from biocompatible oils such as squalene (*e.g.*, SAS and MF59) were developed[98–100]. The Sigma adjuvant system (SAS) is a stable oil-in-water emulsion containing monophosphoryl lipid A, which has been used in animal experiments. One study indicated that the SARS-CoV rRBD protein with SAS protect mouse against lethal SARS-CoV challenge, where the protection correlated well with the high titer of neutralizing antibodies[101]. In addition, MERS-CoV S1 protein together with SAS was identified to elicit robust serum neutralizing activity against several MERS-CoV strains in immunized mice[102].

MF59, another squalene-based oil-in-water emulsion, has been licensed in Europe for adjuvanted influenza vaccines. It is revealed that MERS-CoV RBD in trimeric form with MF59 elicits highly efficacious Th2-based IgG1 and Th1-based IgG2 antibody responses, as well as neutralizing antibodies against pseudotyped and live MERS-CoV, protecting 83% of hDPP4-Tg mice from lethal MERS-CoV infection (Fig. 4)[103]. Similarly, the activation of IgG subtype antibody response and production of neutralizing antibodies following immunization with RBD-Fc and MF59, resulting in the fully protection against MERS-CoV infection[104]. Additionally, MF59 induces stronger and broader IgG subtype antibody response than several other commercial adjuvants, including Freund's adjuvant, aluminum, Monophosphoryl lipid A and Montanide ISA51[28]. Based on the security and effectiveness of MF59, it becomes a promising candidate adjuvant for the development of coronavirus subunit vaccine.

4.3. *Toll-like receptors (TLRs) agonist*

The innate immune system recognizes pathogen-associated molecular patterns (PAMPS) mainly through pattern recognition receptors (PRRs). As one of the best characterized PRRs, Toll-like receptors (TLRs), which are widely distributed in antigen

presenting cells and play an important role in triggering innate immunity and priming the adaptive immune response[105]. Therefore, several TLR agonists have been investigating as virus-specific vaccine adjuvants to induce strong and sustained immune responses[106,107].

The Toll-like receptor (TLR) families have been characterized as key players in RNA virus detection and antiviral immunity. Polyinosinic acid–polycytidylic acid (PolyI:C) is a synthetic double-stranded RNA (dsRNA) analogue that acts as a TLR3 receptor agonist, inducing the production of type I IFN and inflammatory cytokines through a TRIF-dependent pathway[108]. Previous reports have indicated mice deficient in the TLR3 signaling way are extremely susceptible to SARS-CoV infection, showing increased lung pathology and higher viral titers[109]. Additionally, intranasal treatment with PolyI:C induces both innate and T cell immune responses against viral infections, protecting aged animals from infection by IAV or SARS-CoV, as well as providing more rapid virus clearance[110]. Furthermore, MERS-CoV S protein together with poly I:C effectively trigger CD8 T-cells response to accelerate MERS-CoV clearance without immunopathological effects[111]. These results suggest that PolyI:C, a potent type I IFN inducer, should be evaluated as a promising adjuvant in CoV subunit vaccines.

TLR4 mainly recognizes lipopolysaccharide (LPS) derived from the cell wall of gram-negative bacteria. Unlike other TLRs, TLR4 agonists mediate the production of inflammatory cytokines and type I IFN *via* MyD88 as well as TRIF signaling pathway[112]. It has been demonstrated that pretreatment with TLR4 ligands provides protective immunity against infections by SARS-CoV[110]. Monophosphoryl lipid A (MPLA)—a TLR4 agonist—is an attenuated version of lipopolysaccharide (LPS). MPLA has proven its safety and effectiveness in licensed vaccines, including human papillomavirus and hepatitis B vaccines[113,114]. Previous work has shown that inclusion of MPLA as an adjuvant in influenza vaccine promotes mucosal and systemic (Th1-skewed) immune responses after pulmonary vaccination[115]. In addition, SARS-CoV S protein with adjuvant TLR3 and TLR4 agonists successfully induce high expression of antigen-specific IgG and neutralizing antibodies without eosinophilic infiltrations and elicit Th1/17 cytokine responses in the lungs after the SARS-CoV challenge infection in the mouse model[116]. The above results indicated that TLR4 agonist, as a powerful Th1-type adjuvant, can effectively improve the immunogenicity of the CoV subunit vaccines and reduce the Th2-related eosinophilic pathological response.

TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) motifs that are commonly found in bacterial and viral DNA, and then mediates the antiviral response of type I IFN *via* the TLR9-MyD88 pathway[117]. CpG-containing oligodeoxynucleotides (CpG ODN) sequence 1018, as an adjuvant for immunization against hepatitis B virus (HBV), has been proven to significantly increase neutralizing antibody titers in clinical trials[118]. Recently it has been approved for adults in the United States[119]. A novel CpG ODN (BW001) has been shown to effectively activate B and NK cells and stimulate the body to secrete high level of IFN-a and IFN-γ, thereby producing strong anti-SARS-CoV activity[120]. Furthermore, CpG have been identified to be superior to other TLR agonists (TLR3 agonists: PolyI:C; TLR7/8 agonists: R848) in the secretion of inflammatory cytokines and activation of SARS-CoV S peptide specific $CD8^+$ T cell response[121]. Due to the strong ability of CpG to induce cellular immunity, it is often used in combination with alum to supplement the defects of alum in inducing cellular immunity. For instance, mice immunized with SARS-CoV S protein amino acids 318−510 (S318−510) together with alum and CpG ODN show higher humoral and cellular immune responses than those immunized with S318−510 antigen and alum alone (Fig. 5)[29,122]. The above findings suggest that CpG can be used as a potent adjuvant for coronavirus vaccines.

4.4. *Stimulator of interferon genes (STING) agonists*

STING (stimulator of interferon genes), a central component in the innate immune response, plays an important role in defense against viral and intracellular bacterial infections. STING is a transmembrane protein localized to the endoplasmic reticulum. Following stimulation by cytosolic cyclic dinucleotides (CDNs), STING undergoes a conformational change, resulting in a downstream signaling cascade involving the activation of NF-κB signaling pathway and the production of type I interferon[123,125]. STING agonists are potent adjuvants capable of eliciting robust humoral and $CD8^+$ T cell immune responses in mice by simulating the early phase of viral infection without concomitant excess inflammation[126]. Meanwhile, recombinant MERS-CoV RBD antigens with cyclic diguanylate monophosphate (cdGMP), a canonical STING agonist, have shown to effectively elicit neutralization antibody and antigen-specific T cell responses[126].

To summarize, the application of vaccine adjuvant requires a thorough understanding of the effect of adjuvants on immune response and mechanisms of action. The application of adjuvants in subunit vaccines are listed in Table 3. In addition to safety considerations, the design of adjuvants must also pay attention to the ability to selectively induce and regulate the types of immune responses in the body, so as to effectively promote the humoral and cellular immunity to combat coronavirus infection. It is also noteworthy to mention that an existing well-established adjuvant could be combined with new immunostimulants (*e.g.*, TLRs agonist) to improve the breadth and intensity of the immune responses, which has become a potential strategy for exploring efficient adjuvant systems.

5. Conclusions and perspectives

SARS-CoV-2 has spread rapidly since its outbreak and has now posed a risk to countries worldwide, making it urgent to develop a safe and effective vaccine against the infection. This review introduces the structure and functions of coronavirus S protein and summarizes the advancements and potential strategies of SARS-CoV and MERS-CoV vaccines based on CoV S protein. The CoV S protein-based vaccines are further classified into different types, including viral vector, nucleic acid (DNA and RNA), VLP and protein-based vaccines. Subunit vaccines have become the focus of current research due to their numerous advantages, but it often requires appropriate adjuvants to enhance their immunogenicity. In the USA, aluminum, MF59, and CpG are adjuvants included in licensed vaccines. These three adjuvants have shown induction of serum neutralizing antibodies and protection against infection in mice challenged with an infectious virus, which might be used for CoV subunit vaccine administration[28,84,87,103,120,121]. However, alum alone cannot induce a potent Th1 response unless combined with another adjuvant, such as CpG. This adjuvant combination will improve the effectiveness of the CoV subunit vaccines[122]. Similarly, in order to effectively activate the complex and orderly

natural immune system and produce the expected acquired immune response, it is sometimes necessary to use an adjuvant combination[29]. Here, we believe that adjuvant formulations that induce a balanced Th1 and Th2 immune response can more effectively improve the immunogenicity of the antigen[72,83,103], becoming a new trend in the development of coronavirus adjuvants. Ideally, an effective CoV vaccine is required to induce both robust humoral and cell-mediated immunities. Even though many promising vaccine candidates have been reported, there are still no commercial vaccines available against SARS-CoV and MERS-CoV. The comprehensive lessons and experiences brought by the outbreak of SARS and MERS provide valuable insights and progress on how to respond to COVID-19.

With the spread of COVID-19, scientific institutions and pharmaceutical companies around the world are racing against time to develop SARS-CoV-2 vaccine. According to WHO, there are at least 100 research projects on SARS-CoV-2 vaccines in the world[127]. In addition to traditional inactivated virus and live-attenuated virus vaccines, the developments of most SARS-CoV-2 vaccines are also based on some new technological routes, such as mRNA vaccines, subunit vaccines, viral vector vaccines, and DNA vaccines. Up to now, eight candidate vaccines have entered clinical trials, including three inactivated vaccines from Wuhan Institute of Biological Products[128], Beijing Institute of Biological Products[129] and Sinovac[130], two adenovirus vector vaccines (Ad5-nCoV from CanSino Biologicals[131] and COV001 from Inovio[132]), two mRNA vaccines (mRNA-1273 from Moderna[133] and BNT162 from BioNTech[134]) and one DNA vaccine (INO-4800 from Inovio[135]). Although some progress has been made in the development of SARS-CoV-2 vaccines, it is important to realize that vaccine development is a rigorous scientific exploration process. Due to many challenges of verifying the effectiveness of vaccines, it is still very likely that no SARS-CoV-2 vaccine will be available in the market for human in near future. Therefore, SARS-CoV-2 vaccine development still requires the unremitting efforts of researchers in the world.

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Author contributions

Chunting He and Ming Qin collected and reorganized the literature material. Chunting He wrote the manuscript. Xun Sun revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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