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Potential adjuvants for the development of a SARS-CoV-2 vaccine based on experimental results from similar coronaviruses

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ABSTRACT

The extensive efforts around the globe are being made to develop a suitable vaccine against COVID-19 (Coronavirus Disease-19) caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2). An effective vaccine should be able to induce high titers of neutralizing antibodies to prevent the virus from attaching to the host cell receptors. However, to elicit the protective levels of antibodies, a vaccine may require multiple doses or assistance from other immunostimulatory molecules. Further, the vaccine should be able to induce protective levels of antibodies rapidly with the least amount of antigen used. This decreases the cost of a vaccine and makes it affordable. As the pandemic has hit most countries across the globe, there will be an overwhelming demand for the vaccine in a quick time. Incorporating a suitable adjuvant in a SARS-CoV-2 vaccine may address these requirements. This review paper will discuss the experimental results of the adjuvanted vaccine studies with similar coronaviruses (CoVs) which might be useful to select an appropriate adjuvant for a vaccine against rapidly emerging SARS-CoV-2. We also discuss the current progress in the development of adjuvanted vaccines against the disease.

1. Introduction

The SARS-CoV-2, a member of family Coronaviridae and the causative agent of COVID-19 disease, has spread rapidly around the globe since the first outbreak in Wuhan, China in December 2019. The World Health Organization (WHO) declared the outbreak as the sixth public health emergency of international concern on 30th January 2020 [1-4]. Despite the tremendous efforts to contain the virus, its spread is ongoing. Though the majority of cases resolve spontaneously, some develop various fatal complications, including organ failure, septic shock, pulmonary edema, severe pneumonia, and Acute Respiratory Distress Syndrome (ARDS) [5-7]. Before the current pandemic, highly pathogenic CoVs have hit the world as severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and Middle East Respiratory Syndrome (MERS) coronavirus in 2012. The SARS-CoV-2 has been identified as a β-coronavirus, and like SARS-CoV, it binds to angiotensin-converting enzyme 2 (ACE2) receptors [8,9]. Recent data on genome sequencing of SARS-CoV-2 revealed that it shares approximately 79.6% similarity with SARS-CoV at the nucleotide level [9], which varies between the different genes. SARS-CoV-2 contains a linear single-stranded positive-sense RNA as genetic material that encodes for the spike (S), envelop (E), membrane (M), and nucleocapsid (N) proteins [10]. The spike protein that binds to host cell receptors shares about 72% nucleotide similarity between these two [8,11-13]. The S protein consists of two subunits S1 and S2, S1 interacts with the surface receptor and S2 helps in the fusion of viral and cellular membranes, and subsequent entry of the virus into the host cells. Different coronaviruses, depending upon the viral species, use different receptor binding domains (RBD) present on the S1 subunit to interact with host cell receptors [8,9]. While RBD of MERS-CoV recognizes non-acetylated sialoside attachment receptors (human dipeptidyl peptidase 4) [14,15], SARS-CoV interacts with ACE2 receptors [16]. However, SARS-CoV-2, as compared to SARS-CoV, contains different amino acids (five amino acids out of six are mutated) composition in its RBD that are crucial for receptor binding (ACE2) with high affinity [17], and has a functional polybasic furin site at S1-S2 boundary that may have a role in defining the viral infectivity and host range [18,19]. The furin cleavage site is not reported in SARS-CoV, and its fusion to membrane involves either direct receptor-mediated fusion or receptor-mediated endocytosis [20]. The high-affinity binding of SARS-CoV-2 with ACE2 receptor along with the presence of furin and TMPRSS2 (at S2′) cleavage sites explain its rapid spread and strong ability for human to human transmission [21].

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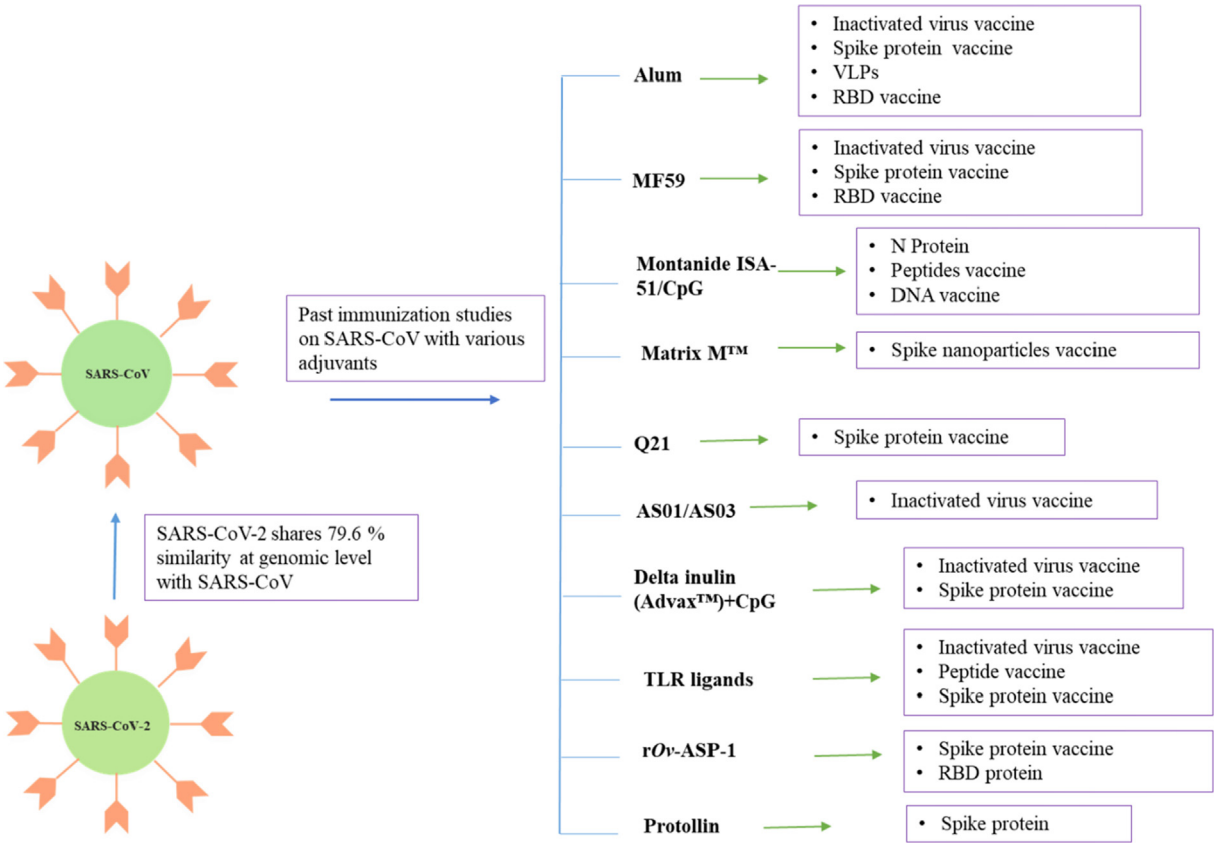

Fig. 1. As SARS-CoV-2 is highly similar to SARS-CoV at the genomic level, the outcomes of the past vaccine studies may expedite the development of a vaccine against COVID-19.

Currently, there is no specific anti-viral medicine or vaccine available for the disease; however, many studies and clinical trials are being undertaken to assess the efficacy and safety of various drugs and vaccine candidates [22]. As SARS-CoV-2 share significant similarities with SARS and MERS coronaviruses and use similar spike protein for receptor binding, the key information from the past vaccine studies with such similar viruses, may help accelerate the development of an effective therapy/vaccine against COVID-19.

Vaccine candidates against SARS-CoV have been tested in many studies and include inactivated whole virus vaccine, recombinant spike (S) protein preparations, virus-like particles (VLPs), plasmid DNA, and several viral vectors containing genes for SARS-CoV proteins [Fig. 1] [23-25]. Many of such vaccine candidates efficiently induced the production of neutralizing antibodies. Such antibodies target the spike protein of the coronavirus, so it cannot bind to its cellular receptor, and, consequently, it cannot enter the cell. However, there are a few basic constraints to vaccine development. The key factor in vaccine design is ensuring the efficacy of the vaccine while reducing the potential risks associated with it. The inactivated virus vaccine may not elicit an ideal immune response to confer protective immunity and may require multiple booster doses. Similarly, new generation vaccines that contain recombinant viral proteins, need help from immunostimulatory molecules [26,27]. Further, as with other RNA viruses, these CoVs go through frequent recombination; therefore, a live attenuated vaccine carries the inherent risk of reversal to a virulent form and may pose a severe threat to human lives. Ideally, a vaccine against a highly pathogenic virus, including CoVs should be able to generate a protective antibody response to the minimum antigen dose in a quick time with no adverse reactions. The COVID-19 is spreading globally and infecting a large number of people due to the lack of any prophylactic measure. The demand for the vaccine, once available, will be overwhelming, and therefore; highly desirable for a potential vaccine to be able to induce immunity in a short time with the minimum quantity of the antigen required. This will make it affordable and help meet the high global demands [28,29]. Most of the above issues may be resolved by incorporating a suitable adjuvant in vaccine preparations, which will not only help in eliciting a robust immune response but also possibly reduce the antigen quantity and the need for multiple doses of vaccine. Thus, the selection of an effective adjuvant becomes crucial for the development of an adjuvanted vaccine against COVID-19. The past vaccine studies on similar CoVs with various adjuvant combinations may provide key information and help in picking adjuvants which possess a good safety profile and efficacy. Therefore, in this review, we discuss the past adjuvanted vaccine studies and their outcomes, with similar CoVs which might be useful in developing an adjuvanted vaccine against COVID-19. Further, we briefly discuss the current progress in the development of adjuvanted vaccines against COVID-19.

2. Adjuvants

2.1. Aluminum hydroxide

Aluminum hydroxide (alum) is the most commonly used agent as an adjuvant. The mechanism of its adjuvant action is complex and, so far, multiple hypotheses have been put forward to explain its mode of action [152]. It has been shown to act by depot formation at the site of injection allowing for a slow release of antigen. This prolongs the interaction time between antigen and antigen-presenting cells (APCs). Further, it converts soluble antigens into particulate forms which are readily phagocytosed [30]. At the cellular level, aluminum hydroxide directly stimulates monocytes to produce pro-inflammatory cytokines activating T cells. Activated Th2 cells release IL-4, which in turn can induce an increase in the expression of MHC class II molecules on monocytes [31]. Alum has been approved as an adjuvant in multiple

Table 1
Common vaccine adjuvants.

Sr. No.	Adjuvants	Possible adverse reactions	Status/Approval	References
1	Alum	Granulomas, allergenicity, potential neurotoxicity	Approved for many human vaccines such as Hepatitis A, hepatitis B, Haemophilus influenza type b (Hib) diphtheria-tetanus-pertussis (DTaP, Tdap), etc.	[32]
2	MF59™	Injection site pain, local tissue damage inflammatory reactions, sterile granuloma ulceration	Approved for the flu vaccine (FLUAD®)	[125]
3	AS01/AS03/ AS04	Injection site pain, local tissue damage inflammatory reactions, sterile granuloma ulceration	Approved for RZV, Shingrix, influenza, and Cervarix – HPV vaccines, respectively	[126]
4	Montanide ISA-51	Injection site pain, local tissue damage inflammatory reactions, sterile granuloma ulceration	Approved for use in the lung cancer vaccine (CIMAvax EGF) in Cuba	[126]
5	Delta inulin	Safe, mild local reactions	Clinical trial phase-1 for seasonal influenza and hepatitis B	[84,127,128]
6	TLR3	(Poly-ICLC): Transient neutropenia, mild injection site reactions, erythema and induration at the site of injection, transient flu-like symptoms, such as malaise, headache, fever, and chills	Clinical trial phase-1/II for various cancers and HIV vaccines	[129,130]
7	TLR4	MPL: Mild reactions in formulationsGLA: mild reactions including erythematous reactions, tenderness, and fever	MPL: Approved for use in adjuvant systems (AS)GLA: In various phases of clinical trials	[131,132]
8	TLR9	Injection-site reactions or systemic flu-like symptoms may trigger autoimmune disease or inflammation	CpG1018 approved for a Hepatitis B vaccine (HEPLISAV-B)	[131,133]
9	TLR7/8	Resiquimod (oral): Systemic cytokine induction, including fever, headache, shivering, and lymphopeniaImiquimod (topical): Mild adverse reactions	Imiquimod R837 is approved for topical application in basal and squamous cell carcinomas	[131,134]
10	rOv-ASP-1	No adverse reactions in NHPs	Pre-clinical stage vaccine trials against influenza and HIV-1	[102,105,135]
11	Protollin	Rhinorrhoea and mild nasal congestion, malaise, myalgia, and headache	Pre-clinical stage vaccine trials against influenza and SARS, measles, respiratory syncytial virus (RSV)	[110,136-138]
12	CoVaccine HT™	No local or systemic adverse events were detected in macaques, temporary elevations in neutrophil count and drop in free serum iron in Rhesus monkeys	Phase 1 clinical trials	[NCT01015703] [139,140]
13	Matrix M™	Local reaction including pain, tenderness, swelling, and erythema at the injection site. The mild systemic reactions such as headache, myalgia, and fatigue	Phase I clinical trial for H5N1 vaccine	[141,142]

licensed vaccines for human use in the majority of the countries worldwide (Table. 1) [32,33]. Mice immunized with UV-inactivated SARS-CoV particles elicited a high level of humoral immunity that resulted in the generation of long-term antibody-secreting and memory B cells. Further, the addition of alum to the vaccine formula significantly augmented serum IgG and reached a level similar to that found in hyper-immunized mice [34]. The antibodies elicited in mice recognized both the spike and nucleocapsid proteins and neutralized the virus. Tang et al [35] using inactivated SARS-CoV with or without alum adjuvant in female BALB/c mice, demonstrated that the antibodies to SARS-CoV were induced by the adjuvanted vaccine were higher than those without adjuvant. In non-human primates (NHPs), purified inactivated SARS-CoV vaccine with and without alum when administered in Cynomolgus and Rhesus macaques, protected the monkeys from the challenge of SARS-CoV without causing any adverse reactions (Table. 2). Similarly, an inactivated MERS-CoV vaccine adjuvanted with alum plus CpG protected the Ad5-hDPP4 transduced mice [36-38]. Though studies reported the safe use of inactivated viral particles as a vaccine, a few reports observed the occurrence of antibody-dependent enhancement of disease (ADE) and immunopathological changes in the lungs in response to live viral challenge after immunization with inactivated virus vaccine [39-41]. Further studies attributed the occurrence of immunopathological changes or ADE to the presence of anti-N antibodies or non-neutralizing antibodies, antibody isotype, low affinity, or suboptimal levels of neutralizing antibodies induced by immunization with an inactivated virus vaccine alone or with alum [41-44]. Inclusion of an adjuvant that favors a predominantly Th1 response to the whole virus inactivated vaccine may alleviate the issue of Th2-type immunopathology [38,41,45]. These points must be taken into consideration while proceeding with the development of the adjuvanted COVID-19 vaccine using the inactivated virus.

Recombinant subunit or newer generation vaccines have drawn a lot of attention in recent times. A truncated version of SARS-CoV S glycoprotein, containing only the ectodomain that was cloned and expressed in a serum-free insect cell line, when injected into the mice along with alum, induced significantly higher levels of viral neutralizing antibodies with fewer doses and a lower concentration of S glycoprotein [46,47]. Full-length recombinant spike protein plus alum preparations were as immunogenic as Virus-like particles (VLPs) containing the spike protein and the influenza M1 protein, both vaccines resulted in the induction of strong neutralizing antibodies that protected against lethal challenge in a mouse model [48]. Chimeric VLPs consist of SARS-CoV S protein and E, M, and N proteins of mouse hepatitis virus were prepared and tested in Balb/c mice against the SARS-CoV challenge [49]. The addition of alum adjuvant in the chimeric VLPs enhanced the induction of neutralizing antibodies and protected the mice from the SARS-CoV challenge. MERS-CoV and SARS-CoV's full-length spike nanoparticles, in combination with adjuvants (Alum/Matrix M™), were able to produce high titers of neutralizing antibodies in mice [50]. The RBD protein variants in deglycosylated form, to scale-up production of the RBD vaccine candidate, were expressed as recombinant proteins in yeast. The alum adjuvanted preparation induced significantly stronger RBD-specific antibody responses and a higher level of neutralizing antibodies against SARS-CoV [51]. Similarly, alum adjuvanted MERS-CoV nanoparticles and recombinant MERS-CoV RBD protein vaccines could elicit neutralizing antibodies and protect the Rhesus macaques (Table. 3) [52,53]

2.2. MF59

MF59 is an oil-in-water type immunogenic adjuvant which consists of squalene and two surfactants, Tween 80 and Span 85. The MF59 is a safe, well-tolerated, and potent adjuvant that has been used in pandemic (Focetria® and Celtura®) and seasonal influenza (Fluad®) vaccines in many countries (Table. 1) [54,55]. The inclusion of the MF59 adjuvant in the influenza vaccine has been reported to induce higher

Table 2
Important vaccine studies on SARS-CoV.

Sr. No.	Adjuvant	Vaccine candidate	Animal model	Results	References
1	Alum	Inactivated SARS-CoV vaccine (BJ-01 strain)	Cynomolgus macaques	Vaccine induced high levels of neutralizing antibodies. Prevented the replication of SARS-CoV in monkeys Protected monkeys on live viral challenge. ADE was not observed	[36]
2	Alum	Double-inactivated SARS-CoV	Mice	SARS-CoV vaccine induced antibody and protection against infection with SARS-CoV. On challenge, Th2-type immunopathology was observed	[40,41]
3	Alum	A recombinant SARS-CoV spike (S) vaccine (truncated version)	Mice	Alum adjuvanted group produced significantly enhanced immune responses. Fewer doses and a lower concentration of vaccine induced higher viral neutralizing antibodies in adjuvanted groups	[46]
4	Alum	Chimeric VLPs carrying SARS-CoV S protein	Mice	Induced high levels of SCoV-specific neutralizing antibodies. Protected the animals on live viral challengeThe study did not observe ADE	[48,49]
5	Alum/Matrix-M™	SARS-CoV S and MERS-CoV S nanoparticles vaccine	Mice	Alum adjuvanted both vaccines SARS-CoV and MERS-CoV induced high neutralizing antibody titers; however, Matrix-M™ induced higher antibody levels than alum	[50]
6	MF59	Inactivated SARS-CoV vaccine	Mice	Elicited high titers of neutralizing antibodies, and protected the mice from lethal viral challenge	[60]
7	Montanide ISA-51/CpG	SARS-CoV S protein peptides	Mice, guinea pigs rabbits	Antibodies inhibited SARS/HIV pseudovirus entry into HepG2 cells, One of the peptides (D2) tested could induce neutralizing antibodies as well as T cell response	[72]
8	Montanide ISA-51/CpG	DNA vaccine coding N protein of SARS-CoV/ Recombinant N protein	Mice	Mice receiving DNA vaccine plus recombinant N protein along with adjuvants produced high IgG antibodies specific to N protein	[71,73]
9	AS01/AS03	Inactivated whole SARS-CoV vaccine	Mice/Hamster	The adjuvanted vaccine provided better protectionThe AS01 adjuvanted vaccine induced higher neutralizing antibodies than AS03. The ADE of disease was not observed in hamster	[82]
10	Delta inulin/Alum/CpG	Recombinant SARS-CoV spike protein / Inactivated whole SARS-CoV	Mice	The adjuvanted vaccines induced a high concentration of neutralizing antibodies and protected the animals from wild viral challenge. The alum adjuvanted vaccine triggered lung eosinophilic immunopathology which was not observed with delta inulin adjuvanted vaccine	[45]
11	LPS/poly(U)/poly(I·C)	Whole UV-inactivated SARS-CoV	Mice	The inactivated vaccine with alum induced lung eosinophilic immunopathology; However, the addition of TLR ligands reduced lung eosinophilic immunopathology significantly	[91]
12	CpG	Inactivated SARS-CoV vaccine	Mice	The adjuvanted vaccine produced serum neutralizing antibodies as well as specific IgA in tracheal-lung wash fluids	[93,94]
13	PIKA (TLR3)	Inactivated SARS-CoV vaccine	Mice	The adjuvanted vaccine elicited both systemic and mucosal neutralizing humoral responses via both the routes	[95]
14	CpG ODN/ poly(I:C)/ R848	HLA-A*0201 restricted SARS-CoV S peptides	Mice	The vaccine alone elicited antigen-specific $CD8^+$ T cells responses which were further augmented by CpG adjuvant. The elicited $CD8^+$ T cells carried the memory T cell phenotype	[97]
15	CpG/Alum	SARS-CoV RBD vaccine	Mice	The alum adjuvanted vaccine induced high anti-RBD neutralizing antibodies; however, the addition of CpG caused the production of IgG2a antibodies and high IFN-γ secretion. The studies demonstrated carbohydrate to be important for immunogenicity of peptide	[98]
16	rOv-ASP-1	SARS-CoV RBD protein vaccine	Mice/ Rhesus macaques	The adjuvanted vaccine in mice induced Anti-RBD specific neutralizing antibodies; however, also produced anti-ASP-1 antibodies though it did not affect its adjuvanticityThe vaccine also produced significant neutralizing antibodies in NHPs but did not produce much antibodies against protein adjuvant	[100,102]
17	rOv-ASP-1	SARS-CoV S peptide vaccine	Mice	Demonstrated that rOv-ASP-1 was a better adjuvant than alum or MPL + TDM. It enhanced production Th-1 associated IgG2a isotype. The adjuvanted vaccine produced high antibody responses to SARS peptide	[105]
18	Protollin	SARS-CoV spike protein	Mice	The adjuvanted vaccine elicited high levels of antigen-specific IgG in serum. The antibodies were neutralizing in nature. It also elicited antigen-specific IgA levels. The vaccine protected mice on the viral challenge.	[112]

Table 3
Important vaccine studies on MERS-CoV.

Sr. No.	Adjuvant	Vaccine candidate	Animal model	Results	References
1	Alum + CpG	Inactivated whole MERS-CoV	Ad5-hDPP4 transduced mice	The adjuvant group showed increased protective immunity. The study did not observe enhanced pulmonary immunopathology	[38]
2	Alum/MF59	Inactivated MERS-CoV vaccine	hCD26/DPP4 transgenic mice	Adjuvant groups showed increased antibody responses which were protective; however, the study observed hypersensitivity-type lung immunopathologic reaction on viral challenge	[39]
3	Alum	MERS-CoV VLPs	Rhesus macaques	Induced viral neutralizing antibodies, RBD specific IgG antibodies. Also elicited Th1 cell-mediated immunity	[52]
4	Alum	MERS-CoV RBD vaccine	Rhesus macaques	Robust induction of neutralizing antibodies. Vaccination decreased the viral load of lung, trachea, and oropharyngeal swabs of monkeys. Provided partial protection on viral challenge	[53]
5	Alum	MERS-CoV spike protein nanoparticles/ Ad5/MERS (S)	Mice	Heterologous as well as spike nanoparticle homologous vaccination induced neutralizing antibodies and protected the animals on the viral challenge. The heterologous strategy could elicit both Th1 and Th2 responses.	[148]
6	Poly(I:C)/Alum	Chimeric VLPs expressing RBD of MERS-CoV	Mice	Poly(I:C) group showed higher neutralizing antibodies than that of alum. RBD-specific humoral and cellular immune response. Antisera prevented pseudotyped MERS-CoV entry into susceptible cells	[96]
7	MF59	MERS-CoV Trimeric RBD vaccine	hDPP4 transduced mice	Induced robust RBD-specific neutralizing antibodiesProtected the animals from lethal viral challenge	[64]
8	Alum/CpG	A recombinant N-terminal domain (rNTD) of MERS-CoV spike protein vaccine candidate	Ad5-hDPP4 transduced mice	Induced high titers of neutralizing antibodies. Inclusion of adjuvants (alum + CpG) caused activation of both Th1 and Th1 responses. The vaccine protected the animals on viral challenge	[99]
9	MF59	MERS-CoV RBD fused with human Fc	Mice	Potent induction of viral neutralizing antibodies. Dose as low as 1 μg was sufficient to induce strong humoral and cellular immune responses	[58,149]
11	Montanide ISA51	MERS-CoV RBD	Mice	Induced neutralizing antibodies. Neutralized MERS-CoV infection in cell culture	[74]
15	Matrix™ M	MERS-CoV S nanoparticles vaccine	Mice	Vaccine elicited strong anti-S neutralizing antibodies and protected the mice from viral infection	[150]

levels of antigen-specific antibody levels, higher HAI titers (hemagglutination inhibiting), and better protection [56-58]. Though it favors Th2-type response like alum, MF59 activates more CD4 T cells and produces higher antibody tiers against the HA (hemagglutinin) of the influenza virus [59]. The MF59 has been tested as an adjuvant with many other vaccines against bacteria and viruses, including SARS-CoV. Immunization with inactivated SARS-CoV vaccine adjuvanted with MF59 protected mice from challenge with the virus. The vaccine was able to elicit neutralizing antibodies after only 2 doses [60]. Subunit vaccines based on S1 protein and RBD epitopes of MERS-CoV when co-administered with MF59, significantly augmented their immunogenicity resulting in strong induction of IgG and neutralizing antibody response. The preparations protected the mice from lethal virus challenge [61-63]. Many MERS-CoV RBD candidate vaccines adjuvanted with MF59 were developed and tested in mice. These vaccines elicited neutralizing antibodies and protected the hDPP4 transduced mice from the lethal challenge (Table. 3) [63,64].

2.3. Montanide ISA-51

Montanide is a water-in-oil (w/o) emulsion that contains mineral oil and surfactant from mannide monooleate family. It possesses immunostimulatory activity and works by the depot formation at the site of injection resulting in a slower release of antigens, local inflammation, and recruitment of antigen-presenting cells (APCs). It enhances antibody titer as well as cytotoxic T-lymphocyte (CTL) activity against the antigen it is formulated with [65,66]. It is well tolerated in humans as compared to FIA (Freund's Incomplete Adjuvant) and has been tested in vaccines against AIDS (Acquired Immunodeficiency Syndrome), malaria, cancer, and influenza with varying degree of success [67-70]. The combination of Montanide ISA-51 and CpG ODN (oligodeoxynucleotide) has been used widely in many vaccines as the combination exerts a synergistic effect in boosting the Th1 immune response. Montanide ISA-51 and CpG were used to emulsify the recombinant N protein of SARS-CoV. The formulation elicited strong Th1 immune responses in mice and macaques [71]. The synthetic peptides of the spike protein of SARS-CoV formulated with an adjuvant Montanide ISA-51/CpG combination, elicited strong antibody titer in immunized animals that were capable of inhibiting SARS-CoV pseudovirus entry into HepG2 cells [72]. A DNA vaccine encoding SARS-CoV N protein elicited a potent N protein-specific immune response when co-administered with Montanide ISA-51/CpG adjuvants [73]. The recombinant RBD of MERS-CoV when adjuvanted with Montanide ISA-51, induced high titers of serum antibodies that could neutralize the virus in cell culture [74]. The above pieces of evidence from the previous studies indicate that Montanide ISA-51 may increase the immunogenicity of the vaccines based on recombinant spike protein or synthetic peptides corresponding to RBD of S protein. Further, it may be used along with CpG ODN to induce an overall Th1 biased immune response as a predominantly Th2 immune response seems to cause ADE on the viral challenge following immunization.

2.4. $AS01_B$ and $AS03_A$

These are oil-in-water adjuvant systems developed by GlaxoSmithKline (GSK). The AS01 (adjuvant system 01) is a liposome-based adjuvant that is consist of monophosphoryl lipid A (MPL) and a saponin molecule (QS-21), whereas AS03 (adjuvant system 03) is an α-tocopherol and squalene-based adjuvant that has been used in GSK's A/H1N1 pandemic flu vaccine Pandemrix®. The MPL is extracted from *Salmonella minnesota* and QS-21 is purified from the bark of the South American tree *Quillaja saponaria* Molina. The MPL signals through Toll-like receptor-4 (TLR4), which results in the activation of APCs and the production of cytokines and interferons (IFNs). Q-21 is reported to induce antigen-specific antibody response as well as cell-mediated immunity [75,76]. When co-administered with recombinant SARS-CoV S

protein, Q-21 induced high titers of antigen-specific serum antibodies and protected from viral infection [77]. The AS01 system has been used in recently developed RTS,S/AS01 malaria vaccine as well as tested in Herpes zoster subunit vaccine, polyprotein HIV-1 vaccine and tuberculosis (Mtb72F/AS02 candidate) vaccine [78-81]. Further, these adjuvants have also been used with inactivated SARS-CoV vaccine preparation in mice and hamsters. The $AS01_B$-adjuvanted vaccine was slightly more immunogenic than the $AS03_A$-adjuvanted vaccine. The vaccine in animals immunized with adjuvant provided better protection than the non-adjuvanted vaccine. Importantly, the study did not observe enhance disease (ADE) in the lungs or liver of hamsters following SARS-CoV challenge [82].

2.5. Delta inulin (Advax™)

Delta inulin (DI) is one of the many isoforms of inulin polysaccharide, which is stable at a higher temperature and has better immune potency. It activates the complement system and, when co-administered with antigen, helps mount a robust antigen-specific adaptive immune response consisting of both antibody and cell-mediated immunity (a Th0 adjuvant property) [83-85]. A balanced enhancement of both Th1 and Th2 responses was observed when DI (Advax™) adjuvant was co-administered with an inactivated JEV (Japanese Encephalitis Virus) and influenza vaccines, which conferred the protection against the viral challenges [83,86]. Similarly, when recombinant spike protein or inactivated SARS-CoV vaccine was co-administered with delta inulin plus CpG oligonucleotide as adjuvants, the combination significantly enhanced serum neutralizing antibody titers without causing lung immunopathology, which was not the case when alum was used instead of delta inulin [45].

2.6. TLR agonist adjuvants

TLRs are pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs). These are present on cell surfaces as well as in endosomal compartments. Interaction with the appropriate ligand triggers the release of proinflammatory cytokines and type −1 IFNs (Interferons), which activates the cells of the innate and adaptive immune system leading to antigen-specific enhanced humoral and cell-mediated responses [87]. Therefore, TLR ligands have been widely studied and tested as immune adjuvants in many human and veterinary vaccine preparations against infectious diseases [88-90]. Different TLR ligands induce a different type of immune response (Th1/Th2/Th0) depending on the signaling pathway involved. The TLRs that have been widely used in vaccine adjuvant studies and their ligands are as follow: TLR3 (dsRNA), 4 (LPS), 5 (Flagellin), 7 (ssRNA), 8(ssRNA) and 9 (unmethylated CpG oligonucleotide) [88]. Out of these, only TLR4 ligand which is MPL has been approved for use in human vaccine formulations such as Human papillomavirus vaccine (Cervarix™), hepatitis (Fendrix®, GSK Biologicals) and malaria (RTS, S/AS01 or Mosquirix). Nevertheless, many other TLR adjuvants have shown promising results and may make their way to human vaccine formulation in time to come.

TLR ligands have been tested with the inactivated SARS-CoV vaccines since an inactivated vaccine with alum adjuvant has been reported to cause eosinophil infiltration in the lungs of immunized animals following live viral challenge. The TLR ligands [lipopolysaccharide, poly(U), and poly(I:C)], and the vaccine combination protected the animals with significantly reduced eosinophil infiltration in the lungs [91]. Intranasal administration of a TLR3 agonist, poly(I:C), induced IFN-β and IFN-γ production and protected the animals from SARS-CoV infection. Therefore, the poly(I:C) as a prophylactic measure should be further evaluated for use in aged or high-risk individuals [92]. There are many ways an intranasal application of vaccines may benefit. The mucosal immune response generated following intranasal vaccine application, may block the viral entry, and alleviate the concern associated with systemic administration of inactivated vaccine i.e. immunopathology in the lungs. Mice immunized with inactivated SARS-CoV and CpG resulted in the local production of specific IgA and neutralizing antibodies in serum. This strategy may offer double protection; once at the site of entry and the second when the virus enters the circulation [88]. Similar results were observed by another lab that reported induction of significant levels of SARS-CoV specific IgG antibodies in sera, and a detectable amount of IgA antibodies in sera and mucosal secretions when an inactivated vaccine adjuvanted with CpG ODN 2006 was administered intranasally [94]. A TLR3 ligand, PIKA (Polyinosinic-Polycytidylic Acid Based Adjuvant) which a stabilized derivative of poly(I:C), also produced similar results when administered with inactivated SARS-CoV vaccine. Both, the intranasal and intraperitoneal, administration of PIKA with inactivated SARS-CoV induced significant levels of both mucosal and serum antibodies [95]. Moreover, poly(I:C) was superior to alum with a Chimeric VLPs expressing MERS-CoV RBD protein vaccine when administered to mice. Poly(I:C) elicited stronger neutralizing antibody as well as cell-mediated responses that prevented pseudotyped virus entry into susceptible cells [96].

The CpG ODN, poly(I:C), and R848(TLR7/8) ligands when used with HLA-A*0201 restricted SARS-CoV S epitopes, all three agonists enhanced the epitope-specific $CD8^+$ T cells, though the effect was more pronounced with CpG ODN. The immune response induced was stated to carry a memory cell phenotype that had long-term survival ability [97]. Further, there are concerns the spike protein subunit vaccine adjuvanted only with alum (favors Th2 response) might trigger immunopathology in the lungs, therefore; inclusion of a TLR ligand that favors Th1 response may provide dual benefits, reduction in ADE as well as the activation of both the arms of the immune system. Further, a combination of either a recombinant S protein of SARS-CoV or an N-terminal domain (rNTD) of MERS-CoV with alum and CpG, induced high IgG2a antibody titers and IFN-γ production [98,99].

2.7. Recombinant Onchocerca volvulus activation associated protein-1 (rOv-ASP-1)

Many helminth-derived molecules have been shown to have potent immunostimulatory effects, including ASP-1. The ASP-1 is present in helminth *Onchocerca volvulus*. A recombinant ASP-1 (r*Ov*-ASP-1) is reported to induce a mix of Th1 and Th2 responses when included with vaccine antigen; however, depending on the antigen, it could also activate a predominantly Th1 type of immune response [100,101]. Therefore, it can be beneficial in a condition where bits of help from both the arms of the immune response is needed to clear the infection. Further, the immune response induced against the ASP-1 protein adjuvant did not affect its adjuvant activity on subsequent boost [102]. ASP-1 has been shown to improve IgG1 and IgG2a antibody levels against commercially available vaccines such as influenza and rabies, and the adjuvant effect observed was higher than either traditional adjuvant alum, or MPL plus trehalose dicorynomycolate (TDM) [100,103,104]. A recombinant ASP-1 protein when included with recombinant SARS-CoV spike protein antigen or an RBD containing vaccine, the combinations induced a mixed immune response with Th1 dominance [100]. Similar findings were observed when r*Ov*-ASP-1 was used as an adjuvant with either S protein of SARS-CoV or HIV-1gp120 [105]. Further, immunization with RBD of SARS-CoV plus r*Ov*-ASP-1 in NHPs resulted in a high titer of neutralizing anti-RBD antibodies with only low levels of anti- r*Ov*-ASP-1 antibodies [102].

2.8. Protollin

A complex of Proteosomes with LPS derived from *Shigella flexneri* is named as Protollin, whereas Proteosomes itself contain hydrophobic outer membrane proteins derived from *Neisseria meningitides*. Protollin exerts its immunostimulatory effect via TLR2 and TLR4 signaling

resulting in the production of cytokines, IFNs, and the activation of APCs. Intranasal administration of Protollin with influenza antigen (split or recombinant HA) induced both serum IgG and mucosal secretory IgA that protected the mice from lethal virus challenge [106,107]. Further, its application was shown to be effective in reducing allergic asthma in a mouse model [108], protective against the development of pneumonia in a murine model of Shigella infection [109] and safe and well-tolerated in healthy adults [110]. Protollin was an effective, safe, and immunogenic and induced protective antibody response consisting of both serum IgG and mucosal IgA in humans when administered intranasally with trivalent influenza vaccine [111]. Protollin when administered intranasally with recombinant SARS-CoV S protein, induced significant levels of antigen-specific serum IgG and mucosal IgA antibodies as compared alum plus recombinant S protein administered via intramuscular route, and protected the mice from the live viral challenge [112]. Therefore, as evidence reflects, Protollin adjuvant appears to be safe and well-tolerated in human, more importantly, its ability to induce both serum and mucosal immunity might prove to be beneficial and should be explored further in SARS-CoV-2 vaccine development.

3. Antibody-dependent enhancement of disease (ADE)

Many investigations have revealed an over activation of the immune system when an animal is challenged with the live virus following immunization. It causes immunopathological changes because of the excessive release of pro-inflammatory cytokines (cytokine storm) such as IL-2, IL-7, IL-8, IL-9, IL-10, IL-17, IL-1β, G-CSF, GM-CSF, and TNFα. [153-155]. ADE or immune enhancement occurs when a virus gains access into the immune cells while adhered to the antibodies, leading to an increase in infectivity and virulence of the virus. Once inside the cells, which aren't the usual target, the virus multiplies and releases its progenies infecting more cells [156]. Many viruses such as dengue, yellow fever, HIV, and CoVs have been reported to induce ADE [157]. Most pieces of evidence suggest viruses exploit the Fc receptor (FcR) on the immune cells to gain entry while being attached to the antibody-binding site of the antibodies. The Fc region of the antibody has a sequence that binds to complement system or immune cells expressing FcR receptors [158,159]. The presence of high levels of non-neutralizing antibodies appears to be the primary reason that triggers ADE [43]. Other factors that contribute include low-affinity or sub-optimal levels of neutralizing antibodies and isotype of antibodies induced [42,43]. The presence of circulating antibodies against related epitopes from different strains or related viruses may also contribute to the enhancement of disease [160]. Though data is conflicting, it has been observed in many inactivated virus vaccine experiments on SARS and MERS-CoVs [36,39-41]. However, incorporation of a predominantly Th1 inducing adjuvant seemed to address the issue [45]. There is no evidence yet that antibody-dependent disease enhancement could be associated with the SARS-CoV-2 vaccine, rather recent experiments with SARS-CoV-2 vaccines have shown promising results without ADE in rodents and macaques [113,115,161].

4. Ongoing developments

Recent data available on vaccine studies with SARS-CoV-2 in NHPs appears promising. An inactivated vaccine (PiCoVacc) adjuvanted with alum, elicited spike, and RBD protein-specific neutralizing antibodies and protected the macaques on the viral challenge. Importantly, the study did not observe ADE in immunized animals (Table. 4) [113]. The recombinant S1 protein of the virus fused with Fc and adjuvanted with saponin microemulsion, when administered to macaques, induced a strong anti-S1 neutralizing antibodies [114]. The Oxford University's vaccine candidate, ChAdOx1 nCoV-19 encoding the S protein of SARS-CoV-2, protected rhesus monkeys from developing pneumonia. The vaccine significantly reduced the viral loads in bronchoalveolar lavage

Table 4
Recent studies on SARS-CoV-2 vaccine candidates.

Sr. No.	Vaccine candidate	Adjuvant	Animal model	Outcomes	References
1	Inactivated SARS-CoV-2 virus vaccine candidate (PiCoVacc)	Alum	Rhesus macaques	The vaccine induced spike and RBD protein-specific neutralizing antibodies. Immunized macaques were protected from the lethal viral challenge. No ADE was observed	[113]
2	SARS-CoV-2 S1-Fc vaccine candidate	Saponin-based microemulsion	Cynomolgus monkeys	The monkeys developed high titers of S1-specific neutralizing antibodies	[114]
3	SARS-CoV-2 RBD vaccine candidate	MPLA and QuilA	Rodents	Vaccine elicited potent neutralizing antibodies. ADE was not observed.	[115]
4	ChAdOx1 nCoV-19	–	Rhesus macaques	A single of ChAdOx1 nCoV-19 induced both humoral and cellular immune responses. The vaccine significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue. The study observed no pneumonia and immune-enhanced disease in vaccinated rhesus macaques following viral challenge	[161]
5	DNA vaccine encoding S protein(INO-4800)	–	Mice/Guinea pigs	The vaccine induced T-cell and neutralizing antibody responses. The antibodies blocked virus entry in vitro	[162]
6	DNA vaccine encoding S protein	–	Rhesus macaques	The vaccinated animals developed humoral and T-cell responses. The neutralizing antibodies titers were comparable to convalescent humans and macaques. The vaccine reduced the viral loads and protected the animals on challenge.	[163]
7	Ad5-nCoV expressing S protein	–	Phase I	The vaccine found to be safe, well-tolerated and immunogenic at 28 days post-vaccination.Antibody responses peaked at day 28 post-vaccination and specific T-cell responses from day 14 post-vaccination.	[164]

Table 5
SARS and MERS-CoV vaccine candidates that entered clinical trials.

Sr. No.	Vaccine	Type	Target	Developer	Status	Outcomes	Reference
1	DNA plasmid encoding S protein	DNA vaccine	MERS-CoV	Inovio pharmaceuticals	Phase I	The study did not observe vaccine-induced serious adverse events. Mild systemic reactions occurred such as headache, myalgia, and fatigue. The dose-independent immune responses were detected in more than 85% of participants after two vaccinations. The vaccine induced both humoral and cell-mediated responses.	[143]
2	MVA-MERS-S	Non-replicating viral vector	MERS-CoV	IDT Biologika GmbH	Phase I	No serious events were recorded. Mild swelling, induration, fatigue, headache, and malaise were observed in some participants.Both the humoral and cell-mediated immune responses were induced following homologous prime-boost immunization.	[144]
3	ChAdOx1 MERS	Non-replicating viral vector	MERS-CoV	University of Oxford	Phase I	The vaccination induced mild adverse events that were self-limiting. No serious reactions or events were recorded. A single dose could elicit both humoral as well as cell-mediated immunity	[145]
4	DNA vaccine encoding S protein	DNA vaccine	SARS-CoV	National Institute of Allergy and Infectious Diseases (NIAID)	Phase I	The vaccine was safe and well-tolerated. It induced neutralizing antibodies in all the participants and a CTL response in about 20% of participants.	[146]
5	Inactivated vaccine	Inactivated vaccine SARS-CoV vaccine (ISCV)	SARS-CoV	Sinovac Biotech Ltd, China	Phase I	The vaccine was safe and well-tolerated, some symptoms such as diarrhea and an increase in serum ALT were observed in some participants which were transient. The vaccine produced neutralizing antibodies against SARS-CoV.	[147]

Table 6
SARS-CoV-2 vaccine candidates in clinical trials*.

Sr No	Vaccine candidates	Type	Adjuvant	Developer	Status	Reference
1	ChAdOx1 nCoV-19	Viral vector encoding SARS-CoV-2 spike protein	No	University of Oxford, Uk	Phase II/III	NCT04400838
2	Encapsulated mRNA-1273	Spike protein	No	Moderna, Inc., USA	Phase I/II	Phase I: NCT04283461Phase II: NCT04405076
3	Inactivated SARS-CoV-2 vaccine	Inactivated virus vaccine	Alum	Sinovac Research and Development Co., Ltd., China	Phase I/II	Phase I/II NCT04383574 NCT04352608
4	SARS-CoV-2 rS	Recombinant Spike Protein Nanoparticle vaccine	Matrix-M™	Novavax	Phase I	NCT04368988
5	BNT162a1/b1/b2/c2	RNA vaccines	No	Biontech SE	Phase I/II	NCT04368728
6	Ad5-nCoV	Viral vector encoding spike protein	No	CanSino Biologics Inc./ Insitute of Biotechnology, Academy of Military Medical Sciences, PLA of China	Phase II	NCT04341389ChiCTR2000031781
7	bacTRL-Spike	Engineered *Bifidobacterium longum* encoding SARS-CoV-2 S	No	Symvivo Corporation	Phase I	NCT04334980
8	Inactivated SARS-CoV-2	Inactivated virus vaccine	No	Wuhan Institute of Biological Products/Sinopharm	Phase I/II	ChiCTR2000031809
9	Inactivated SARS-CoV-2	Inactivated virus vaccine	No	Beijing Institute of Biological Products/Sinopharm	Phase I/II	ChiCTR2000032459
10	DNA plasmid vaccine with electroporation (INO-4800)	DNA vaccine	No	Inovio Pharmaceuticals	Phase I	NCT04336410
11	Inactivated SARS-CoV-2 vaccine	Inactivated vaccine	No	Chinese Academy of Medical Sciences	Phase I/II	NCT04412538

*As on 30/05/2020.

Table 7
SARS-CoV-2 adjuvanted vaccine candidates in pre-clinical/developing stages.

Sr. No.	Vaccine candidates in developing/pre-clinical stage	Adjuvant	Developer(s)	References
1	Recombinant spike protein (S-Trimer)	CpG 1018	Clover Biopharmaceutics, China & Dynavax Technologies Corp., USA	[116]
2	Recombinant protein vaccine	AS03	Xiamen Innovax Biotech & GlaxoSmithKline	[117]
3	Recombinant protein vaccine	AS03	Sanofi & GlaxoSmithKline	[117]
4	Recombinant protein vaccine	MF59 adjuvant	Commonwealth Serum Laboratories, Australia & Seqirus, Germany	[118]
5	SARS-CoV-2 spike protein nanoparticles	Matrix-M™	Novavax, (USA)	[119]
6	Vaccine candidate	CoVaccine HT	Soligenix, Inc., & BTG Speciality Pharmaceutics	[120]
7	SARS-CoV-2 VLP vaccine	Yes	iBIO & Infectious Disease Research Institute (IDRI, Seattle, US)	[123]
8	Inactivated virus vaccine	CpG 1018	Sinovac/Dynavax	[151]
9	Inactivated virus vaccine	CpG 1018	Valneva/Dynavax	[151]
10	SARS-CoV-2 protein subunit (RBD)	Yes	Biological E Ltd	[151]
11	Recombinant spike protein	Advax™ (delta inulin)	Vaxine Pty Ltd	[151]

fluid and respiratory tract tissue without causing disease enhancement in vaccinated monkeys [161]. Further, a recently concluded Phase I clinical trial with Ad5-nCoV (adenovirus Type 5 vector) expressing S protein vaccine reported promising findings. The vaccine has been found to be safe, well-tolerated and induced both humoral and cellular immunity [164]. Encouraging results have also been received from a recently concluded Phase I clinical trial for the MERS-CoV vaccine [Table. 5]. An MVA-MERS-S (MVA: modified vaccinia virus Ankara) DNA vaccine was safe, well-tolerated, and produced both humoral and cell-mediated immune responses in 87% of the participants after the second dose [144]. The MVA vector technology may be repurposed to develop a COVID-19 vaccine by incorporating SARS-CoV-2 S protein. Further, to expedite the development of the SARS-CoV-2 vaccine, many leading vaccine manufacturers are coming together and collaborating. This has enabled them to share each other's proprietary molecular compounds which already have some degree of safety approval. To date, 10 vaccine candidates have entered either phase I or II of clinical trials (Table. 6), and many are in preclinical or developing stages (Table. 7). Out of these, Ad5-nCoV (CanSino Biologics, Inc) is advancing quickly and has reached the second phase of clinical trials. Many of these are using the established adjuvant system with their COVID-19 vaccine candidates. An adjuvant based on TLR9 agonists (CpG 1018), which has been developed by Dynavax Technologies Corp., USA, is being used in a recombinant spike protein (S-Trimer) vaccine candidate against COVID-19 announced by Clover Biopharmaceutics, China [116]. The CpG 1018 adjuvant has been used in an FDA (Food and Drug Administration) -approved hepatitis B vaccine (HEPLISAV-B®). GlaxoSmithKline has also teamed up with many firms such as Xiamen Innovax Biotech and Sanofi, to make its adjuvant technology available to the collaborators for their vaccine candidates [117]. Both Sanofi and Xiamen Innovax Biotech are using GSK's AS03 adjuvant (squalene-based) in their recombinant protein vaccine candidates. Similarly, CSL (Commonwealth Serum Laboratories, Australia) and Seqirus, Germany have tied up to use the MF59 adjuvant, which employs novel molecular-clam technology, in their COVID-19 vaccine candidate [118]. Novavax, (USA), on the other hand, is using its proprietary Matrix-M™ adjuvant for a vaccine candidate (NVX-CoV2373), consisting of nanoparticles carrying SARS-CoV-2 spike protein antigens [119]. Another Biopharma company, Soligenix, Inc., has collaborated with BTG Speciality Pharmaceutics. Soligenix will use a novel vaccine adjuvant (CoVaccine HT) from BTG, with its COVID-19 vaccine candidate [120]. CoVaccine HT is an oil-in-water emulsion consisting of sucrose fatty acid sulfate easter (SFASE) and squalene, which has been reported to induce both humoral as well as cell-mediated immunity [121,122]. iBio has signed up an agreement with Infectious Disease Research Institute (IDRI, Seattle, US) in an effort to utilize their novel adjuvants such as GLA (Glucopyranosyl Lipid Adjuvant), a synthetic analogue of the MPL, for its SARS-CoV-2 VLP vaccine development [123,124].

5. Conclusions

The experience and knowledge generated from the past vaccine studies with different adjuvants against similar CoVs may expedite the development of an adjuvanted vaccine against COVID-19. The inclusion of an adjuvant may cut down the amount of antigen significantly in a vaccine, especially when vaccine candidates are recombinant spike/RBD protein. This could help address an overwhelming demand for the vaccine in a pandemic in a short time. So far, there is no firm evidence whether an antibody response is sufficient or a T-cell response is also critical for recovery from SARS-CoV-2 infection. Past studies on similar CoVs have observed that T-cell immunity also plays a crucial role in clearing the viral infection. Therefore, the inclusion of an adjuvant with a recombinant spike/RBD protein vaccine candidate, which stimulates a mixed immune response, such as delta inulin, CoVaccine HT, Matrix-M™, MF59®, AS03, and rOv-ASP-1, might be appropriate in such a scenario. However, selecting an adjuvant that has a proven safety profile and efficacy may be beneficial for obtaining quick clearance from the regulatory bodies. Though delta inulin, CoVaccine HT, Matrix-M™, and rOv-ASP-1 have shown good safety and efficacy in pre-clinical and clinical trials, CpG 1018, MF59® and AS03 are already approved for human vaccines and their inclusion may expedite the vaccine development process. Further, Protollin has shown promising results in pre-clinical studies in inducing both systemic and mucosal immune responses against viruses affecting the respiratory system and should be further evaluated with SARS-CoV-2. A few past studies with inactivated SARS-CoV and MERS-CoV vaccines alone or with adjuvant have been reported to cause antibody-mediated enhancement of disease on the live viral challenge following immunization, however, so far, we have no conclusive evidence that the same phenomenon will happen with SARS-CoV-2. In fact, a recent study on rhesus macaque with alum adjuvanted inactivated vaccine revealed the development of protective immune responses without causing disease enhancement [113]. Nevertheless, other studies have suggested the use of a Th1 favoring adjuvant such as CpG 1018, might help ease such concerns.

Conflict of interest

None

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