

REVIEW



## SARS-CoV-2 subunit vaccine adjuvants and their signaling pathways

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### ABSTRACT

**Introduction:** Vaccines are the agreed upon weapon against the COVID-19 pandemic. This review discusses about COVID-19 subunit vaccines adjuvants and their signaling pathways, which could provide a glimpse into the selection of appropriate adjuvants for prospective vaccine development studies.

**Areas covered:** In the introduction, a brief background about the SARS-CoV-2 pandemic, the vaccine development race and classes of vaccine adjuvants were provided. The antigen, trial stage, and types of adjuvants were extracted from the included articles and then assimilated. Finally, the pattern recognition receptors (PRRs), their classes, cognate adjuvants, and potential signaling pathways were comprehended.

**Expert opinion:** Adjuvants are unsung heroes of subunit vaccines. The *in silico* studies are very vital in avoiding several costly trial errors and save much work times. The majority of the (pre)clinical studies are promising. It is encouraging that most of the selected adjuvants are novel. Much emphasis must be paid to the optimal pairing of antigen-adjuvant-PRRs for obtaining the desired vaccine effect. A good subunit vaccine/adjuvant is one that has high efficacy, safety, dose sparing, and rapid seroconversion rate and broad spectrum of immune response. In the years to come, COVID-19 adjuvanted subunit vaccines are expected to have superior utility than any other vaccines for various reasons.

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## 1. Introduction

### 1.1. SARS-CoV-2 and vaccine status

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiology of coronavirus disease 2019 (COVID-19), is ravaging the human race regardless of political and geographic boundary [1,2]. As of 10 July 2021, with the lowest estimate, infection and fatality surpassed 186 million and four million, respectively. The pandemic is now broadening its target demography and geography through mutations on key structural genes [3–5].

Following the SARS-CoV-2 outbreak in Wuhan, China, century-old containment measures such as lockdown, physical/social distancing, and school closing were applied. However, these measures were found to be costly and collaterally damage the global economy [6]. Vaccines are the ideal weapon against SARS-CoV-2 for bringing back life like the pre-pandemic situation [7,8]. As such, several studies investigated the aftermath of countries' routine immunization for repurposing existing vaccines [9–14]. However, major biases could not be excluded by the majority of the studies [15] and are accompanied with conflicting reports [16]. Currently, the globe is rolling out mRNA and vector vaccines [17–21]. Additionally, several new vaccines are under different stages

of scrutiny [22]. The complete list of the candidate vaccines are available at the WHO website [23]. Of the listed candidate vaccines, the subunit protein vaccine shared 34% of the COVID-19 vaccine research and is the highest in proportion compared with other forms of vaccines [23].

By virtue of the presence of a heterogeneous mixture of structures and genetic materials that can function as an intrinsic adjuvant, live-attenuated vaccines are relatively more effective compared with subunit vaccines [24,25]. Conversely, purified subunit vaccines lack pathogen-associated molecular patterns (PAMPs) and such types of vaccines are barely immunogenic unless supplemented with adjuvants [24,26]. Synthetic DNA-based vaccines targeting the S protein of SARS-CoV-2 exhibited promising results on animal model experiments [27–29]. However, besides safety issues, host-related factors in higher animals might delay its translation as can be inferred from other early researches [30,31].

### 1.2. Vaccine adjuvants and their role

Adjuvants are essential molecules for subunit protein vaccines. It is the stimulator of the innate arm of the immune system and the modulator to the adaptive arm of the host defense system [32,33]. The main role of vaccine adjuvants is to (1)

**Article highlights**

- Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an etiological agent of coronavirus disease-2019 (COVID-19), is ravaging the human race regardless of political and geographic boundary.
- Physicochemical characteristics (charge, size, and nature of chemical), immunological correlate of protection (the titer, kinship, isotype, dose sparing and seroconversion rate, and profile of cellular immune components) and safety, reactogenicity, and toxicity are the three-dimensional evaluation criteria employed for qualifying vaccines/adjuvants.
- Among the 52 articles identified and examined, 27 distinct types of adjuvants were identified.
- Defensin, alum, Matrix-M1, CpG, and MF59 are the top five adjuvants on this list. By the same token, Spike and RBD shared 43.6% and 33.8% of the candidate vaccine antigens. Spike proteins are more immunogenic than RBD.
- Novel COVID-19 vaccine adjuvants ensure host resistance by activating endosomal (Toll-like receptor 3/7/8/9) and cytosolic (RLRs and cGAS-STING) receptor signaling pathways.
- Nanoparticulate adjuvants have several important advantages over large molecules including antigen/nucleic acid delivery, limiting bioavailability, and depot effect.
- The effectiveness of subunit vaccines relies on the art of designing a vaccine, which has optimal antigen–adjuvant–PRR synergy pairing.
- Both pre-clinical and clinical trials confirmed the induction of several orders of magnitude of higher titer of neutralizing antibody than the convalescent sera of recovered people.
- The majority of the second generation COVID-19 vaccines might be the subunit vaccine types having the power of protection against the SARS-CoV-2, including emerging variants of concern.

increase the amount of vaccine production using smaller antigen, (2) dose sparing, (3) broaden the profile of adaptive immune components, and (4) hasten seroconversion rates [25,34]. These come through its depot effect, activation of PRR-mediated innate immune signaling, enhancing the activities of antigen presenting cells (APC) and activation of inflammasomes [33,35].

Vaccine adjuvants are classified into (1) mineral salts (examples: aluminum/alum, manganese), (2) emulsions (Freund's complete and incomplete adjuvants, Montanide, Ribi), (3) pathogen-associated molecular pattern (Cholera toxin, CpG DNA, Escherichia heat labile toxin, Monophosphoryl lipid A: MPL, Defensin), (4) hormones and cytokines (granulocyte macrophage colony stimulating factor, IFN, IL), and (5) synthetic adjuvants (imidazoquinolines, multi-antigen peptide system, polymerization of haptenic peptides, peptide linkage to epitopes) [36–45].

The immunological function of mineral salts is by their depot effect, complement activation, and inflammasome and tissue damage for releasing damage-associated molecular patterns (DAMPs) [36–38]. Emulsion groups have good antigen bioavailability. However, they are relatively toxic and are associated with delayed-type hypersensitivity. Unlike mineral salts that are biased toward Th2 immune response, emulsions groups activate the Th1 pathways [36,37,40]. The PAMP, cytokine, hormone, and synthetic adjuvants are considered 'novel adjuvants' by virtue of having cognate receptors for their effector function. However, toxicity is the main limitation for these classes [41–45]. Despite the attempts made to reduce the toxicity issues associated with synthetic adjuvants, they are found to be less bioavailable and remain localized to the

injection site [43–45]. In general, particulate vaccines are easily taken up by APCs than soluble vaccine forms. As such, the efficacy can be increased by modifying the delivery in particulate forms [41].

In situations where measuring clinical correlates of vaccine protections (infection, transmission, or diseases) is difficult for various reasons, the immunological correlates of protections (titer, affinity, isotypes and half-life of the neutralizing antibody, and CD4<sup>+</sup> T cells) are used as surrogate criteria for measuring vaccine/adjuvant efficacy [46,47].

Several lines of evidence revealed that each adjuvant has limitations on one or more of the desired immunological correlates of protection. For instance, alum in the spike protein subunit vaccine study induced an increased B cell and long-lived neutralizing antibody (NA) production. However, alum-S adjuvants failed to induce a remarkable level of cell mediated immunity (Th1CD4<sup>+</sup> T cell and cytotoxic CD8<sup>+</sup> T cells) and are linked to eosinophilic associated lung pathology. The CpG adjuvant is associated with an increased production of CD8<sup>+</sup> T cells and IgG and IgA production, but again the half-life of the produced antibodies is short and skewed toward Th1. Liang and Colleagues considered **ST**imulator of **I**nterferon genes (STING), AS01B, delta inulin microparticles, and matrix M1 adjuvants to be better in terms of inducing long-lived neutralizing antibody and IFN production in the mucosal area [48]. In general, despite the several remarkable success, we are far from identifying and unlocking the magic bullet vaccine adjuvants.

Of note, the year 2020 was the year of human suffering but also the year of breakthrough for mRNA vaccine against the COVID-19 pandemic. Unfortunately, mRNA vaccines need freezers for transportations, which is very challenging in resource-limited countries [49]. Additionally, mRNA vaccines are expensive and unaffordable for nations of the south. Furthermore, the existing vaccines cannot satisfy the global need. Additionally, due to the continued emergency of 'variants of concern,' developing a new generation of vaccine is a top priority of the global health [50]. Hence, effective and safe alternative second and third generation COVID-19 vaccines are urgently needed. Adjuvanted subunit vaccines are the best alternative, and such vaccines are currently under intense research. Thus, the aim of this review is to identify primary articles evaluating the efficacy and safety of the adjuvanted subunit COVID-19 vaccine, give a glimpse into the landscape and immunology of COVID-19 adjuvants, and facilitate the subunit vaccine research arena.

## 2. Literature search methods

A systematic literature search was carried out at the electronic database of PubMed and Scopus with 19/05/2021 as the final search date. The search was carried out using MeSH terms and Boolean operators. The search algorithm at PubMed was (('SARS-CoV-2'[Mesh] OR 'COVID-19'[Mesh]) AND 'Vaccines, Subunit'[Mesh]) OR 'Adjuvants, Immunologic'[Mesh]. Similarly, the literature search at Scopus was formulated as (Title Abs Key (sars cov 2) OR Title Abs Key (covid 19) OR Title Abs Key (severe AND acute AND respiratory AND syndrome AND coronavirus-2) OR Title-Abs-Key (coronavirus AND disease 2019)

AND Title-Abs-Key (“subunit vaccine”) OR Title-Abs-Key (“vaccine adjuvant”) OR Title-Abs-Key (“Recombinant Protein Vaccine”) AND (Limit to (Pubyear, 2021) OR limit to (pubyear, 2020)) AND (Limit-To (Language, “English”)) .The identified articles were imported into the EndNote library, and eligible articles were filtered out following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram. The complete article selection strategy is given in the supplementary material (S1).

### 3. Body of the review

#### 3.1. Antigen profile of COVID-19 subunit vaccine trials

The systematic literature search gave a total of 1062 articles (S1). After screening of the 1062 articles, 52 were identified, which fulfilled the eligibility criteria. Of these, 24 articles were in silico experiments, 25 articles were animal model studies, and the remaining three articles were clinical trial reports. Articles evaluating the efficacy of two or more adjuvants were treated as separate studies. Hence, 71 adjuvant experiments/evaluations were identified inside 52 included articles; 38 of the adjuvant evaluations were at pre-clinical, 29 in silico, and 4 clinical trials (Figure 1a).

The complete data set of the review including the summary of key findings of each study is found in Table S2. The full spike protein and its subunits are the leading antigens used by SARS-CoV-2 structural immunologists. For instance, out of 71 adjuvant/vaccine evaluations, 15 (21%) and 14 (19.7%) employed the spike protein and receptor binding domain (RBD), respectively. However, when derivatives and modification such as (S1, S2, S-2P, S-Trimer) and (RBD-NG, RBD-mFc, RBD-Fc, RBD-NP) are included, their role increased to 31 (43.6%) and 24 (33.8%), respectively. Similarly, around 15 subunit vaccine trials used multi-epitopes (MEs) (Figure 1b). Table

1 below depicts the antigens, adjuvants, and stages of the COVID-19 subunit vaccine trials (Table 1).

#### 3.2. COVID-19 subunit vaccine adjuvants

Overall, our review identified 27 distinct types of adjuvants. However, this list might not be comprehensive due to the fact that our literature search was limited to two databases. Human beta defensin, alum, matrix-M, CpG, and MF59 are the common and top five adjuvants employed by COVID-19 subunit vaccine researchers (Table 1 & Figure 2). As shown in Figure 2 below, most of the adjuvants are PAMP and small molecules that have known receptors.

The review captured several insightful findings. For instance, immunoinformatics-based vaccine construction contributed the lion share in vaccine epitope search. This in silico strategy helps predict the antigenicity, allergenicity, and toxicity of the construct. Additionally, it will predict the physical properties such as the molecular weight, half-life, and hydrophobicity nature of the predicted subunit vaccine. These collectively save resources and time and reduce the costly trial errors [86]. For instance, B-defensin adjuvanted multi-epitope (28 epitopes including 3 from replicase, 3 from NSp1, 2 from envelope, 5 from membrane, 6 from nucleocapsid, and 9 from spikes proteins) subunit vaccines have been constructed. The molecular docking demonstrated excellent affinities against TLR3 and TLR8 [87]. Such types of vaccines might have broad spectrum action including against the emerging variants of concern (VOC).

Titan *et al* using the full S-Matrix-M adjuvanted vaccine (NVXCoV2373) elicited high titer anti-S IgG, polyfunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells, follicular CD4<sup>+</sup> Th, and germinal center B cells in the spleen of mice [88]. A phase 1 subunit COVID-19 vaccine (SCB-2019) assessed the safety, efficacy, and tolerability using S-AS03, S-CpG/alum, and placebo groups at 3, 9, and

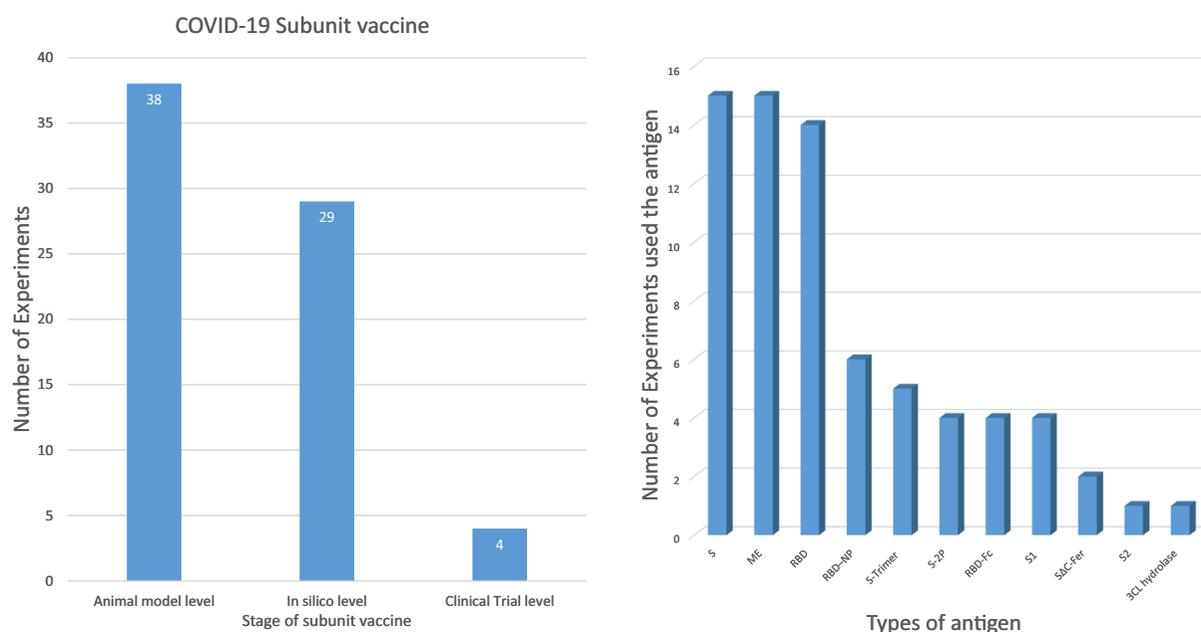


Figure 1. COVID-19 subunit vaccine trial stages (a) and (b) antigens, 19 May 2021.

S: spike protein; ME: multi-epitope (antigen); RBD: receptor binding domain; S-2p: spike prefusion protein 2; RBD-mFc: RBD-mouse IgG1 Fc domain

**Table 1.** The COVID-19 subunit vaccine epitopes, adjuvants, and stage of the trials, 19 May 2021.

Study	Vaccine epitope	Adjuvant	Stage of the trial
[51]	RBD	50S R-protein L2	In silico
[90]	dRBD	Alum	Phase 1/2
[108]	S-2P	Alum	Mice
[52]	S-2P	MF59	Hamster
[87]	ME1	β-defensin	In Silico
[53]	RBD	Mn nano particle	Mice
[54]	S1 Vs RBD	Alum	Mice
[88]	S	Matrix-M	Mice
[55]	S Vs RBD	Addavax	Mice
[56]	S1-4	Alum	Mice
[99]	RBD	AMP-CpG	Mice
[57]	RBD	Zn-chitosan, Alhydrogel, Adju-Phos	Animal
[89]	S-Trimer	AS03 or CpG/Alum	Phase 1
[58]	S, N, E, M	β-defensin 2	In Silico
[94]	SΔC-Ferritin	Quil-A MPLA	Mice
[59]	S	Matrix-M	Mice & Macaques
[60]	RBD	MF59	Guinea pig
[61]	S-2P	CpG 1018 and alum	Hamster
[62]	S-Trimer	AS03/CpG 1018 + alum	Rhesus macaques
[86]	S, M, N	β-defensin2 2/3	In Silico
[63]	S-Trimer	IMDQ-PEG-CHOL	Mice
[64]	RBD	Alum	Mice
[65]	S	OmpA	In Silico
[66]	Struc.& NS proteins	β-defensin	In Silico
[67]	S	Matrix-M	In Silico
[68]	RBD – NP	AS03, AS37, TLR7, CpG1018/alum, alum	R. macaques
[69]	S,E,M	β-defensin	In Silico
[70]	RBD	β-defensing 3	In Silico
[71]	S, M, E and N	50S rRNA L7/L12	In Silico
[72]	S, N, M, ORF3a	β-defensin, L7/L12 rRNA & HABA protein	In Silico
[73]	S,N,M,E	β-defensin-3	In Silico
[74]	nsp7-12, nsp14	β-defensin	In Silico
[92]	RBD-mFc	Alum, Freund's adjuvant (FA)	Mice
[91]	r RBD	PAPE	Animal
[75]	S	RS09	In Silico
[76]	nsp	B-defensin 3	In Silico
[77]	S1 and S2	RS09, TR-433, MTB-Hsp70	In Silico
[98]	RBD-Fc	FCA and FIA	Mice
[78]	RBD w/wout IgG1-FC	MF59	Mice
[115]	S-2P	CpG 1018	Mice
[79]	S	CTB & TTFrC	In Silico
[80]	S,E,N	β-defensin 1	In Silico
[93]	S	Matrix-M1	Phase1-2
[100]	S,N,M	β-defensin 1	In Silico
[81]	3CL hydrolase	β-defensin-3	In Silico
[109]	S1	CoVaccine HT, Alum	Mice
[82]	S	Matrix-M	C. macaques
[83]	S	CTB	In Silico
[125]	RBD-NG	Alum	Mice
[84]	S2	β-defensin	In Silico
[102]	S,E,N,M	β-defensin	In Silico
[85]	S	Matrix-M	In Silico

**RBD:** receptor binding domain; **drRBD:** dimeric RBD; **s-2p:** spike prefusion protein 2; **ME1:** 28 epitopes including 3 replicase, 3 NSp1, 2 envelope, 5 membrane, 6 nucleocapsid, and 9 spikes; **S:** spike protein; **CAF01:** cationic liposome-based adjuvant; **MnARK:** manganese nanoadjuvant; **N:** nucleocapsid; **E:** envelope; **M:** membrane protein; **OmpA:** outer membrane protein A; **RBD-mFc:** RBD-mouse IgG1 Fc domain; **PAPE:** packed, forming an alum-stabilized Pickering emulsion; **CpG:** unmethylated cytidine phosphate guanosine; **IMDQ-PEGCHOL:** imidazoquinoline cholesteryl-polyethylene glycol; **RBD-NG:** RBD-nanogel; **CTB:** cholera toxin subunit-B; **TTFrC:** tetanus toxin fragment-C; **FA:** Freund's adjuvant; **AMP-CpG:** Amphiphile AMP-CpG, **NSP:** non-structural protein – CoVaccine HT is an oil-in-water (O/W) emulsion of hydrophobic, negatively charged sucrose fatty acid sulfate esters with the addition of squalane

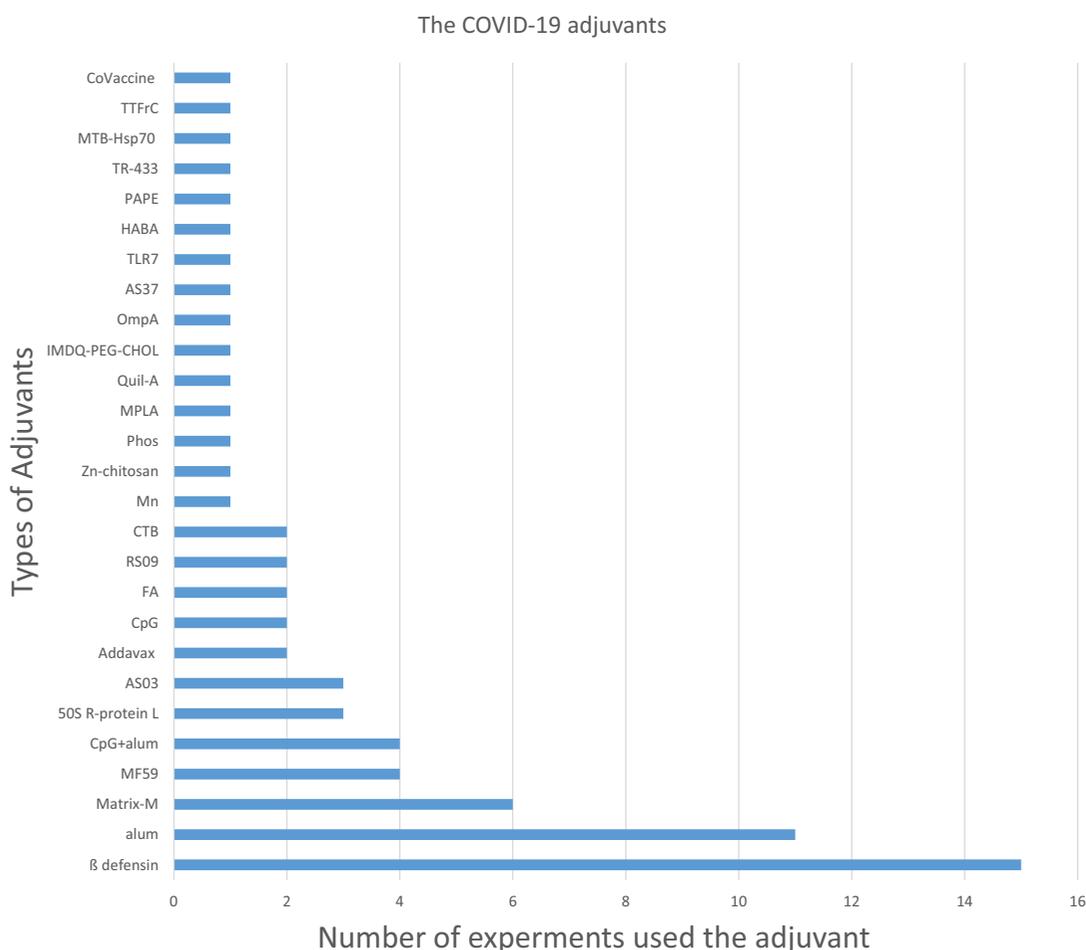
30 µg doses at 21 days interval. In terms of safety, CpG is relatively safe compared with AS03. Both S-AS03 and S-CpG/alum induce neutralizing antibody (NA) production. However, rapid neutralizing antibody was produced by S-AS03 than by the S-CpG/alum group, showing the distinct qualities of the adjuvants. S-protein specific Th-1-biased immune responses could be induced in the two adjuvanted groups but none in the non-adjuvanted S-trimer COVID-19 vaccine. This dose finding study concluded that 9 µg S-trimer-AS03 and 30 µg S-trimer- CpG/alum were the preferred candidates [89].

A phase 1 and 2 subunit vaccine study was carried out by Yang *et al* (2021). In both phase 1 and phase 2, adverse events were mild to moderate. In phase 2, 14 days after the second dose, the seroconversion rates of NA were 76% and 72% in the 25 µg and 50 µg dose groups, respectively. In the 3<sup>rd</sup> dose schedule, after 14 days, the seroconversion rates reached 97% and 93% in the 25 µg and 50 µg groups, respectively. Hence, within 14 days intervals, three consecutive shoots of 25 µg dose were found to be safe and effective [90]. The adjuvant inside this vaccine is alum. From these reports, broad dimensions of the adjuvant function can be appreciated. The titer and durability of the produced antibody are revealed. Moreover, the types of cells in the adaptive immune system and dose sparing effect of adjuvants are clearly seen.

Besides finding new adjuvants, researchers are also modifying the existing adjuvants for enhancing the immune inducing ability and reducing toxicity. For instance, the main limitation of alum adjuvants was the inability to induce Th1 cellular immunity. As a solution, Peng and colleagues packed alum on the squalene–water interface for forming *Particulate Alum via Pickering Emulsion (PAPE)*. The finding showed that six times higher order of NA and three times more IFN-γ producing T cells were produced [91]. By the same fashion, modification at the epitope also further increases the immunogenicity of subunit vaccines. For instance, fusion of RBD with IgG Fc increased the half-life, stability, solubility, and uptake power of APCs, which collectively increase the Th1 response [92].

Multiple subunit vaccine reports claimed a higher order magnitude of NA production against two dose vaccine shots compared with the antibody titer of convalescent sera. For instance, according to Keech *et al* (2020), the geometric mean titer (GMT) levels of 5 and 25 µg doses of vaccine are nearly four times higher than those in symptomatic COVID-19 patients [93]. Additionally, two-fold higher convalescent sera was induced by single immunization with spike-*Helicobacter pylori* ferritin particles [94]. Collectively, higher titer of NA is induced through vaccination than natural infection.

In a phase three trial of a matrix-M1 adjuvanted subunit vaccine, the overall efficacy of 96.4% was recorded against common SARS-CoV-2 strains, 86.3% efficacy against B.1.1.7 (alpha), and 51% against B.1.351 (beta) variants [95,96]. These variants of concern are now threatening all the first generation vaccines [97]. Liu and colleagues did an experiment to generate strong and broad NA using a subunit vaccine of RBD-Fc adjuvanted with FA/FIA. The experimental vaccine sera collected from immunized mice effectively neutralized seven mutant SARS-CoV-2 strains 35 days post first immunization [98].



**Figure 2.** Types of adjuvants and the number of experiments that used the adjuvant.

**TTFrC:** tetanus toxin fragment-C; **MTB-Hsp70:** mycobacterial heat shock proteins of 70 kDa; **TR-433:** human TLR4-derived 20-residue peptide, **PAPE:** particulate alum via Pickering emulsion; **HABA:** hydroxylaminobenzene mutase HabA [*Mycobacterium tuberculosis*], **TLR7:** toll-like receptor 7; **AS37:** adjuvant system 37 (a TLR-7 agonist adsorbed to alum), **OmpA:** outer membrane protein A; **IMDQ-PEGCHOL:** imidazoquinoline cholesteryl-polyethylene glycol; **Quil-A:** Quillaja saponin adjuvants derived from Quillaja saponaria Molina tree; **MPLA:** monophosphoryl lipid A (synthetic, TLR4/Th1, vaccine adjuvant); **phos:** phosphorus; **Mn:** Manganese; **CTB:** cholera toxin B subunit; **RS09:** synthetic TLR-4 agonist peptides; **FA:** Freund's adjuvant; **CpG:** unmethylated cytidine phosphate guanosine; **AddaVax:** a squalene-based oil-in-water nano-emulsion with a formulation similar to that of MF59, **AS03:** adjuvant system 03 ( $\alpha$ -tocopherol, squalene, and polysorbate 80 in an oil-in-water emulsion); MF59: is an oil-in-water emulsion composed of squalene and two surfactants (Tween 80 and Span 85).

T cell response is the hall mark and preferred immune response than humoral immune response in viral infection. Optimal SARS-CoV-2 subunit vaccines must produce mainly Th1-skewed immune response across age groups. However, achieving this desired outcome is not straightforward and innovative approach is required either to the adjuvant, antigen, or the delivery system. Steinbuck *et al* designed a subunit vaccine composed of an amphiphile (AMP)-CpG (diacyl lipid with modified CpG) mixed with S-RBD. Animal experiments in young and aged mice showed greater than 25-fold higher epitope-specific and Th1 skewed polyfunctional cell induction. The induced NA reached 265-fold higher titers than convalescent sera, with higher efficiency in terms of neutralization capacity. Additionally, higher order of cellular and humoral immunity was also induced among aged mice. This is due to the art of adjuvant modification; AMP modification adroitly distributes CpG to lymph nodes [99]. The summary of the key findings of the included articles is given in S2.

Additionally, a brief account has been given below for some of the common adjuvants used in the COVID-19 subunit vaccine study.

### 3.2.1. B-defensin

B-defensin is a TLR3 agonist used by several SARS-CoV-2 subunit vaccine studies [100–102]. Defensins are cationic peptides found from human innate and epithelial cells, serving as antimicrobials and signaling molecules [103,104]. Among  $\alpha$ ,  $\beta$ , and  $\theta$  defensins,  $\beta$ -defensin is the most abundant antimicrobial in most cells [103]. Three  $\beta$ -defensins; human  $\beta$ -defensin-1 (hBD1), hBD2, and hBD3; have been identified in human epithelial cells [104]. The hBD3 played a role in dendritic cell and T cell activation, migration, and polarization [105]. It activates the IFN- $\gamma$  and plays a role in the integration of innate and adaptive immune responses [106]. A study evaluated the adjuvant role of hBD2 and

demonstrated increased expression levels of antiviral molecules [107].

### 3.2.2. Alum, emulsion, and liposome

Cationic adjuvant formulations (CAF01) are a liposome adjuvant containing a cocktail of dimethyldioctadecyl ammonium bromide (DDA) as a delivery vehicle and synthetic mycobacterial cord factor as an immunomodulator. Worzner *et al* [108] evaluated the efficacy of alum, squalene oil in water emulsion system (SE), and CAF01 and spike protein antigen in mice. The finding confirmed that CAF01 induced a higher level of B, Th, and CD4 + T cells than alum [108]. In a similar study, while CAF01 induced higher titer of IFN- $\gamma$  and IL-17, alum adjuvanted vaccines skewed toward IL-5, IL-10, and IL-13 [108]. Studies confirmed that pre-fusion stabilized spike protein (S-2P), S1, and RBD based subunit vaccines produced NA regardless of adjuvants [108,109]. A study using S1 as the antigen evaluated the titer of neutralizing antibody and found that CoVaccine adjuvanted S1 protein subunit vaccines produced more neutralizing IgG antibodies than aluminum adjuvanted S1 protein vaccines [109].

### 3.2.3. CpG adjuvant

Cytosine phosphate guanidine oligodeoxy nucleotides (CpG ODNs) are a popular novel adjuvant that contain unmethylated CG motifs. This adjuvant activates B lymphocytes and plasmacytoid dendritic cells and presents antigens through TLR9 [110]. It enhances the production of Th1 and proinflammatory cytokines. The adjuvant properties of CpG ODNs are improved when the vaccine antigen and ODN are in close proximity. Structurally, three distinct classes of synthetic CpG ODNs have been described [111]; namely, 'K/B,' 'D/A,' and 'C' type ODNs. Each class activates distinct immunoglobulin types [111–113]. Collectively, CpG ODN is a novel and recommended adjuvant that functions through enhancing the TNF- $\alpha$  and IL-6 production. Additionally, CpG is known to augment the surveillance power of antigen presenting cells. The utility of CpG ODNs is further increased by their dual abilities of raising mucosal and systemic immunity [111–113]. The frequency of the CpG motif in the genome of SARS-CoV-2 is rare, and the microevolution is toward fewer CpG genomes. The lower CpG motif might be associated with the high rate of asymptomatic and mild cases. Hence, using CpG ODN as an adjuvant might be a good approach for enhancing immunogenicity with reduced toxicity [114].

A preclinical COVID-19 subunit vaccine study was carried out to determine the efficacy and safety of the SARS-CoV-2 S-2P antigen combined with CpG and/or aluminum hydroxide. The finding showed that the induction of NA is higher when CpG 1018 and aluminum hydroxide are combined than being individual adjuvants. Addition of CpG 1018 to alum suppressed the expression levels of Th2 cytokines (IL-5 and IL-6). However, CpG is associated with liver toxicity, spleen and lymph node enlargement, and inflammation [42]. Taken together, CpG 1018 is a more potent neutralizing antibody and Th1 inducer than the alum adjuvant [115].

### 3.2.4. Saponin-based matrix M

The matrix is a cocktail of two individually formed saponin matrix particles: a highly active saponin adjuvant (Fraction-C saponin) and a safe and weak saponin adjuvant (Fraction A). The admixture generated a new potent adjuvant with dose sparing nature. The matrix M adjuvant is a nanoparticulate adjuvant containing a heterogeneous mixture of saponin, cholesterol, and phospholipid [116]. This is the adjuvant of the potent COVID-19 subunit vaccine recently released [93] (Table 1). Matrix M is known to induce high titer and durable NA and multifunctional cell mediated immunity [116].

### 3.2.5. Nano-adjuvants

Several types of vaccines including COVID-19 mRNA vaccines are designed at the nanoscale [117–120]. The architecture and application of nano-adjuvants are reviewed elsewhere [121]. Nanomaterials have several important functions, including antigen/nucleic acid delivery, limiting bioavailability, and depot effect among others [122]. For instance, according to Sun *et al* (2020), the nanodepot of manganese is found to be effective and safe as treatment and vaccine adjuvants compared with free Mn<sup>2+</sup>. NanoMn treatment increased the CD8 + memory T cell population and polarized macrophages into M1 types and increased the serum IgG, TNF $\alpha$ , and IFN $\gamma$  concentrations. Pharmacokinetic and safety evaluation data demonstrated reduced neural inflammation. These collectively make nano-manganese as a safe and effective adjuvant for COVID-19 [123]. Another experiment was performed for evaluating the adjuvant nature of cationic nanocarriers: polyethyleneimine (PEI), DOTAP, and chitosan. The experiment compared these candidate cationic nanocarrier adjuvants with other anionic and neutral nanocarriers controls. An ELISA serum antibody titer showed the PEI adjuvanted subunit vaccine induced a significantly higher titer of NA than control nanocarriers [124]. Nanoparticles are more membrane penetrating and are able to reach and accumulate inside DC and macrophages. These phenomena enhance the innate immune response power of DC and macrophages [125]. Contrary to these claims, a systematic review by Hoseini *et al* (2021) concluded against the effectiveness of metal nano-adjuvants [126]. Several studies in the literature and our synthesis confirmed the superior value of nano-particulate adjuvants than other forms of the same adjuvant.

## 3.3. Signaling through pattern recognition receptors

The innate immune cells sense the entry of invading pathogens by targeting PAMP and damage associated molecular patterns (DAMP). The nature of the danger is investigated, weighted, and immediately confronted by the innate immune system. The adaptive immune defense is a learned effector of the message encoded by innate immune signaling products [127,128]. Thus, it is the strength and type of PRR-PAMP/DAMP interaction that determines the nature of downstream signaling pathways across the PRR for controlling infection. Adjuvants derived from PAMP/DAMP enhance and modulate innate immunological signal transduction pathways. Whether

the SARS-CoV-2 genome contains potential PAMP adjuvants or not is the future area of security. A recent immunoinformatic study identified motifs having high affinity to TLR7 and TLR8 [129].

Pattern recognition receptors are diverse. These include toll-like receptors (TLR), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), the family of absent in melanoma 2 (AIM2)-like receptors (ALRs), cyclic guanosine monophosphate (cGMP)-adenosine monophosphate (AMP) (cGAMP) synthase (cGAS), complement receptors, scavenger receptors, C-type lectin receptors (CLR), nucleotide-binding oligomerization domain, leucine-rich repeat-containing protein receptors (NLRs), Fc gamma receptors, mannose receptors, and scavenger receptors [130–133]. Based on their site of expression in the cell, these PRRs are usually classified into cell surface, endosomal, and cytosolic PRRs. For instance, TLR 1/2/4/5/6/10 and CLR are cell surface sensors; TLR3/7/8/9/13 are endosomal receptors; and RLRs, cGAS, and AIM2 are cytosolic sensors (Table S3). The different PAMP-PRR complex activates common adaptor proteins and downstream signaling pathways for the production of pro-inflammatory cytokines and IFN [134] (Figures 3–4). Cell surface PRRs are mainly important for bacterial and fungal resistance, and host innate defense against viruses including SARS-CoV-2 mainly activates the endosomal and cytosolic PRR signaling pathways. A comprehensive list of PRR and their cognate PAMP along with expression products (cytokine) is summarized and given in the supplementary material, S3 [135–138].

### 3.3.1. TLR 3/7/8/9

The TLR is the prototype and well-studied PRR [139]. The discovery of TLR is considered as one of the turning points that brings a paradigm shift in the innate immune system biology [135]. Hitherto, 13 mammalian TLRs types have been identified. The TLR is a type I transmembrane glycoprotein helix containing three domains: an agonist-binding ectodomain, a transmembrane region, and a cytoplasmic Toll/interleukin-1 receptor (TIR) structure [131,138,140,141]. During interaction of TLR with their cognate antigen, TLR undergoes homo/heterodimerization. The ectodomain portion of the TLR contains leucine rich repeat (LRR) 'LxxLxLxxN' motifs of 20–30 amino acid length. Binding of PAMP causes rearrangement of the TLR-PAMP complexes and calls specific adaptor proteins to the cytoplasmic TIR domains [131]. This interaction follows two pathways: myeloid differentiation primary-response protein 88 (MYD88) and TIR domain-containing adaptor-inducing interferon- $\beta$  (TRIF) dependent pathways [139]. The MYD88-dependent pathways activate the MAL, MYD88, IL-1 R-associated kinases (IRAKs), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) [137,141,142]. Examples to this pathways include TLR7/8/9 [143]. The TRIF pathways activate dendritic cells, NF- $\kappa$ B, and induced IFN- $\beta$  [139]. The TLR3 [143] signaling pathways follow the TRIF adaptor signaling pathway (Figure 3).

### 3.3.2. Retinoic acid-inducible gene-I-like receptors (RLR)

The RLR, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) are members of RNA helicase and sensors of RNA of the pathogen source in the cytoplasm [130,144]. The interaction of RLR–

RNA activates type I interferons (IFN- $\alpha$ ) and proinflammatory cytokines, which are known effectors of the innate immune system [144,145]. However, the RLR signaling pathway is very prone to overactivate and leads to autoimmunity. Hence, it is under strict regulation to keep the immune homeostasis [146] (Figure 3). Signaling pathways by RLR are employed by several viruses including SARS-CoV-2 [147].

### 3.3.3. The cGAS-STING signaling axis

Cyclic GMP-AMP (cGAMP) synthase (cGAS) is one of the cytoplasmic sensors of cytosolic DNA either directly or indirectly through second messenger cGAMP [148,149]. The binding of cGAMP with STING adaptor protein at the surface of endoplasmic reticulum (ER) pushed the complex into the Golgi complex for further recruiting of the interferon regulatory factor 3 (IRF3), IKK, and TANK-binding kinase 1 (TBK1) complex. This complex formation followed by phosphorylation and dimerization leads to the production of type I interferon (IFN- $\alpha$ ) and the type I IFN/ NF- $\kappa$ B dependent proinflammatory cytokines [134,148,150,151].

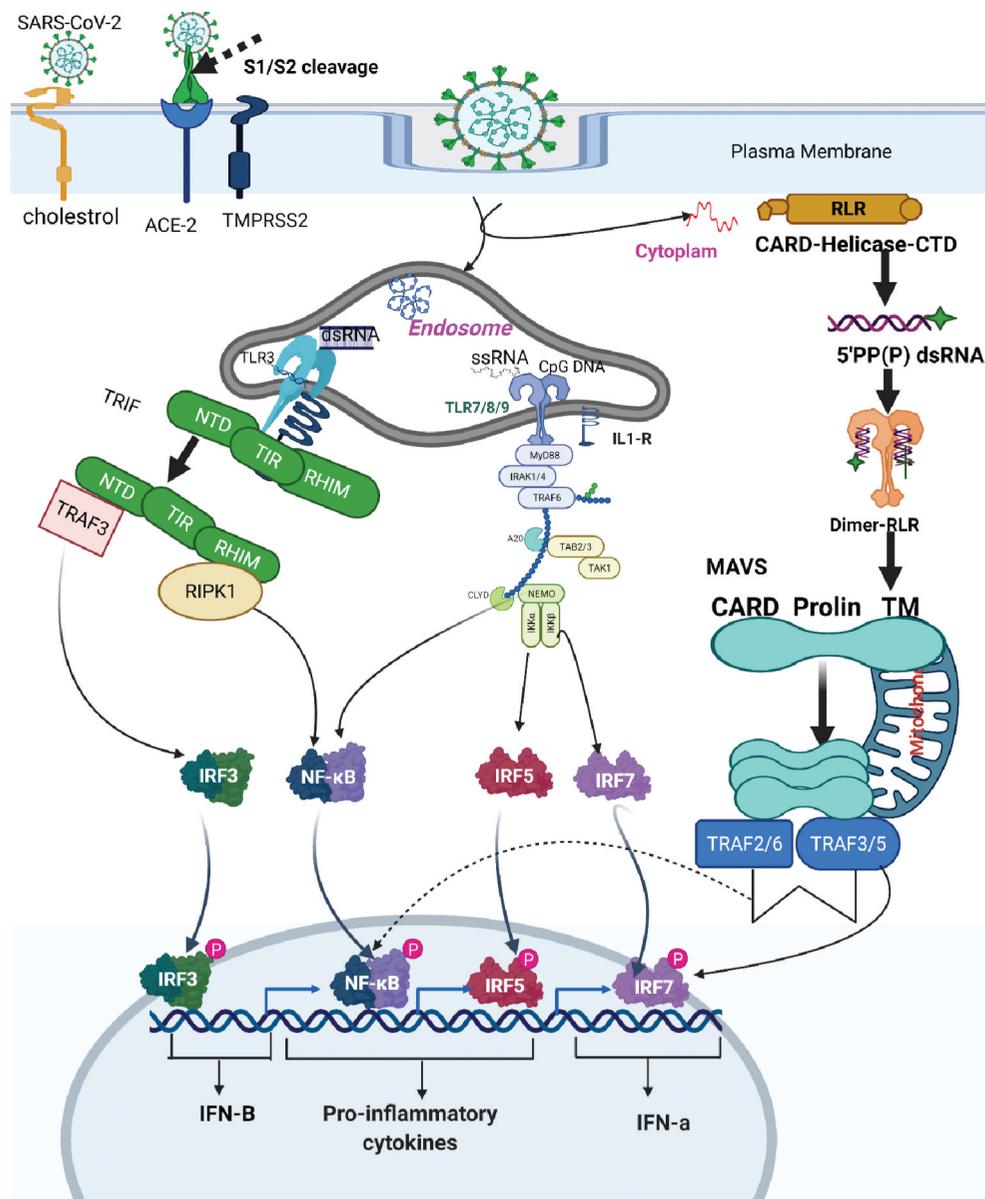
Recent evidences proposed that, besides DNA viruses, RNA viral infection could also activate the cGAS-STING signaling pathways through DAMPs released from mitochondria. An indirect cGAS-STING signaling pathway inhibition experiment confirmed the upregulation of this pathways in SARS-CoV-2 infection [152]. Another study further identified the specific cGAS-STING signaling pathways leading to antiviral resistance. Based on this study, SARS-CoV-2 antiviral resistance in the cGAS-STING pathways is through selective activation of NF- $\kappa$ B pathways. The IRF3 pathways are suppressed [153] (Figure 4).

## 4. Conclusions

Several *in silico* and (pre)clinical studies evaluated different types of adjuvanted COVID-19 subunit vaccines. The current COVID-19 subunit vaccine development researches included several 'novel adjuvants,' which have known PRR receptors. Of these, defensins, alum, matrix-M, and CpG are the most utilized adjuvants. Despite some controversy, nanoparticulate adjuvants are found to be superior to larger size/form of adjuvants. Novel SARS-CoV-2 adjuvants activate the innate immune defense system either through endosomal (TLR3/7/8/9/13) and/or cytosolic (RLRs, cGAS, and AIM2) sensors. The effectiveness of subunit vaccine relies on the art of designing vaccines, which have optimal antigen–adjuvant–PRR blending. As such, like epitopes, in-depth structural and molecular characterizations of candidate adjuvants are equally important for rational selection of adjuvants. Available evidence showed that the world would have several alternative COVID-19 vaccine adjuvants in the coming few years.

## 5. Expert opinion

Subunit vaccines are a state of the art and modern biotechnology products. In the immunoinformatics stage, sequence identification from the database, prediction of epitope allergenicity and toxicity, adjuvant and linker selection,



**Figure 3.** SARS-CoV-2 entry and predicted endosomal TLR and cytoplasmic RLR signaling pathways.

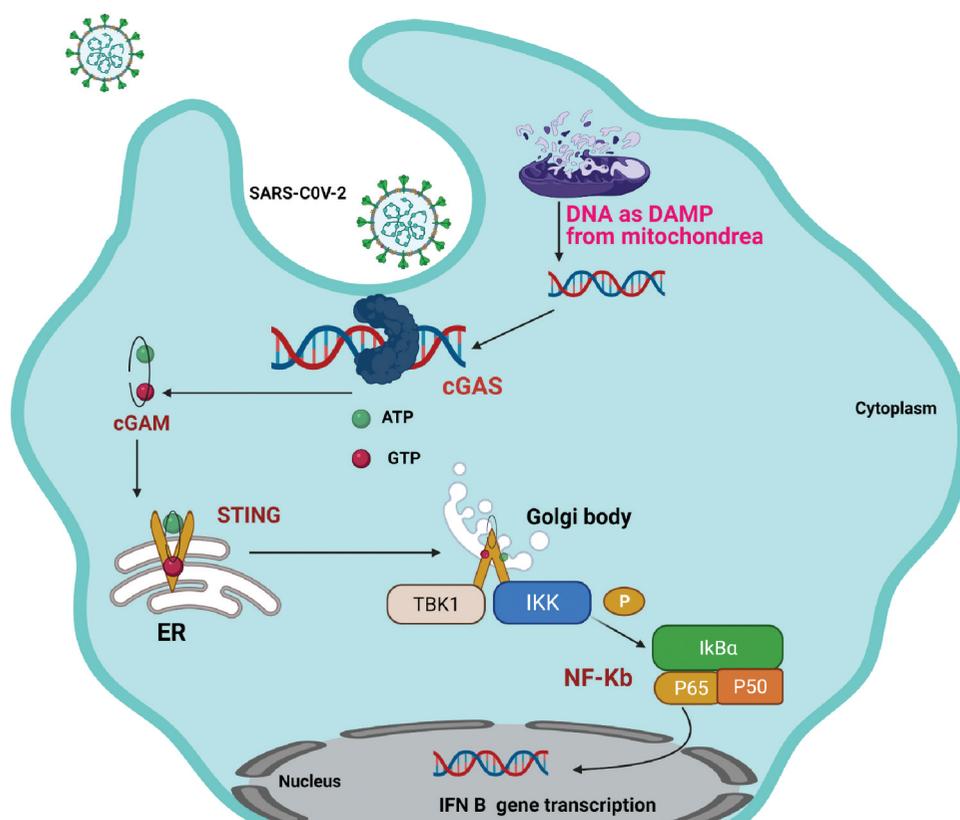
**TLR:** Toll-like receptor; **RLR:** retinoic acid inducible gene 1 like receptor. Figure created with BioRender.com

construction, molecular docking, and physico-chemical characterization are key upstream research activities. The animal model experiment is a dose finding and safety evaluation stage. As such, selection of an appropriate lab animal followed by inoculation and measurement of the safety and efficacy of the vaccine are the key tasks. The clinical trial phase is the measure of safety, efficacy, and correlate of protection using clinical and immunological variables.

Different types of molecular adjuvants have been applied in COVID-19 subunit vaccine development. Across the three stages (in silico, pre-clinical, and clinical), safe and effective adjuvants (adjuvanted vaccine) have been characterized in terms of their physicochemical nature, size, depot and dose sparing effect, speed in seroconversion rate, and ability to induce broad spectrum immune response. All published

COVID-19 subunit clinical studies demonstrated excellent efficacy and safety profile [89,90,93]. Many more clinical trials are running against time and the pandemic (NCT04783311, NCT04780035, and NCT04813562) for producing second and third generation vaccines. However, the number of clinical trials are a few compared with that of upstream experiments. This might be due to failure in defining the appropriate immunological product profile and subsequent selection of antigens and adjuvants with synergistic immunological effect.

Both (pre)clinical trials confirmed the production of several orders of magnitude higher antibody than the convalescent sera of recovered people. The reason behind this scenario is unknown, but it is likely due to the persistent stimulation and dose sparing nature of vaccines. Additionally, as immune



**Figure 4.** Predicted SARS-CoV-2 cytosolic cGAS-STING signaling pathways.

**GMP:** guanosine monophosphate; **AMP:** adenosine monophosphate; **cGAMP:** cyclic GMP-AMP monophosphate; **cGAS:** GMP-AMP monophosphate synthase; **STING:** stimulator of interferon genes. Figure created with BioRender.com

evasion mechanisms, pathogens such as the SARS-CoV-2 tend to cover their antigen with carbohydrates through glycosylation. This might lower the pathogen immunogenicity and subsequent host induction of NA.

The most commonly used COVID-19 adjuvants include  $\beta$ -defensin, alum, M1 matrix protein, MF59, and CpG. However, this does not guarantee their superiority in terms of efficacy and safety. For instance, all  $\beta$ -defensin adjuvanted experiments are at in silico stage. Despite that, it is a good step that the majority of researches are now using PAMP/small molecule adjuvants that have known PRRs. Additionally, several improved results were obtained through modification of the existing classical adjuvants and antigens. Such a strategy must be expanded. It is likely that more potent and safe adjuvants will be identified from the study of PRR's signaling pathways. Currently, major new fields are also being exploited for the identification of metabolic, cell death, and epigenetic adjuvants [38].

The finding of safe and effective adjuvants must go down to the nanoscale size and nanoparticulate form. This is because of the fact that nanomaterials concentrate the antigen and display antigens in prolonged patterns and help APCs co-localize antigens and adjuvants [154]. The smaller the size, the more inflammatory response formation. On the other hand, nanoscale materials are associated with toxicity by different mechanism than bulky materials. In nanomaterial, the toxicity has been thought to originate from nanomaterials' size and surface area, composition, and shapes [155].

Summing up, the nanoparticulate adjuvant is the future promising area of vaccine research.

Safety is the single most important issue when we talk about adjuvants. The surrogate makers of the correlate of protection such as titer, durability, class switching, rate of seroconversion, and dose sparing are more common among major adjuvants and these differences are as such insignificant. Rather, the major differences are with regard to the ability of the adjuvant to induce cell-mediated immunity (polyfunctional ThCD4<sup>+</sup> and CTH8+ cells), the balance of Th1/Th2, induction of life-long memory cells, etc. Future vaccine research must focus on identifying adjuvants that could have the potential of shortening the number of vaccine shoot/individuals and are able to induce tissue resident memory T cells and long lived plasma cells [38]. Taken together, adjuvants in subunit COVID-19 are the unsung heroes that give the most controlled, efficacious, and safe vaccines.

Our review showed that the search for effective and safe subunit vaccines is broadening with unprecedented depth and speed. The search spans from modification of the existing adjuvants to mining of OMICS sciences. The results of new formulations of the existing adjuvants are astonishing. The continued spillover of pandemic infectious disease is leveraging the vaccine research arena and is expected to boost the biomedical and vaccine research funding. Hence, the search for biological adjuvants is an untouched area of innovation.

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## Author contributions

D Mekonnen set the outline and subtopics, collected literatures, reviewed evidences, and drafted the manuscript, HM Mengist reviewed the draft and edited the manuscript. T Jin conceived the review topic, supervised the review process, reviewed, investigated, and validated the final manuscript. All authors read and approved the final manuscript.

## Data availability statement

All datasets presented in this study and its supplementary materials are included in the submission.

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