Vaccine 37 (2019) 3167–3178

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

Vaccine adjuvants: Understanding the structure and mechanism of adjuvanticity

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ARTICLE INFO

Article history:
Received 1 November 2018
Received in revised form 2 April 2019
Accepted 18 April 2019
Available online 29 April 2019

Keywords:
Adjuvant
Aluminum salt
Structure-activity relationship
Immunogenicity

ABSTRACT

In conjugate, inactivated, recombinant, and toxoid vaccines, adjuvants are extensively and essentially used for enhanced and long-lasting protective immune responses. Depending on the type of diseases and immune responses required, adjuvants with different design strategies are developed. With aluminum salt-based adjuvants as the most used ones in commercial vaccines, other limited adjuvants, *e.g.*, AS01, AS03, AS04, CpG ODN, and MF59, are used in FDA-approved vaccines for human use. In this paper, we review the uses of different adjuvants in vaccines including the ones used in FDA-approved vaccines and vaccines under clinical investigations. We discuss how adjuvants with different formulations could affect the magnitude and quality of adaptive immune response for optimized protection against specific pathogens. We emphasize the molecular mechanisms of various adjuvants, with the aim to establish structure-activity relationships (SARs) for designing more effective and safer adjuvants for both preventative and therapeutic vaccines.

Contents

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https://doi.org/10.1016/j.vaccine.2019.04.055
0264-410X/

1. Introduction

Infectious diseases seriously threaten human health. According to the WHO's data in 2018, the spread of influenza results in three to five million cases of severe illness and 290,000–650,000 deaths every year [1]. It is reported that 257 million people are hepatitis B virus surface antigen positive, which resulted in 887,000 deaths in 2015 [2]. In face of these infectious diseases, the introduction of immunization significantly decreased the prevalence and death rate of infectious diseases, and some of them were near eradiation or even eliminated. For example, after vaccination efforts, smallpox was declared eradicated worldwide in 1980. The uses of inactivated oral poliovirus vaccines reduced poliomyelitis worldwide by 99.99% in 2018 [3]. In addition, the deaths caused by tetanus and diphtheria decreased by 83.4% and 73.8% in 2015, respectively. At the early stage of vaccine development, live or attenuated vaccines dominate. For example, rabies vaccines designed from whole viruses were used without adjuvants at the end of the 19th century [4]. The French scientist Louis Pasteur developed the first effective bacterial vaccine against anthrax for use in livestock and humans in 1881. Currently, there were still 17 FDA-approved vaccines that contain live viruses, *e.g.*, BCG vaccine, measles and mumps virus vaccine, rotavirus vaccine, *etc.* The concept of adjuvant was proposed at the beginning of the 20th century when traditional vaccine failed to produce an effective immune response with diphtheria and tetanus purified toxoids. Adjuvant is formulated as a part of a vaccine that aims to enhance a stronger immune response. The components of adjuvants have gone through a process changing from natural ingredients to artificial synthetic compounds, and the discovery of the adjuvant effect of aluminum salts in 1926 is a milestone (Fig. 1). Thus far, adjuvants employed in human vaccines licensed by FDA include aluminum salts, oil-in-water emulsions (MF59 and AS03), AS04 (3′-O-deacylated monophosphoryl lipid A (MPL) plus aluminum salts), CpG ODN and AS01 (MPL and saponin QS-21 formulated in liposomes) (Table 1).

In this paper, we comprehensively reviewed the adjuvants both currently used in FDA-approved vaccines as well as the ones under experimental and clinical investigations, with a focus on the mechanisms regarding how these adjuvants induce immunogenicity. We aim to provide guidance on the design of effective vaccine adjuvants, including optimization of adjuvant formulations, design of new adjuvants and their clinical transformations, which will benefit the development of both preventive and therapeutic vaccines.

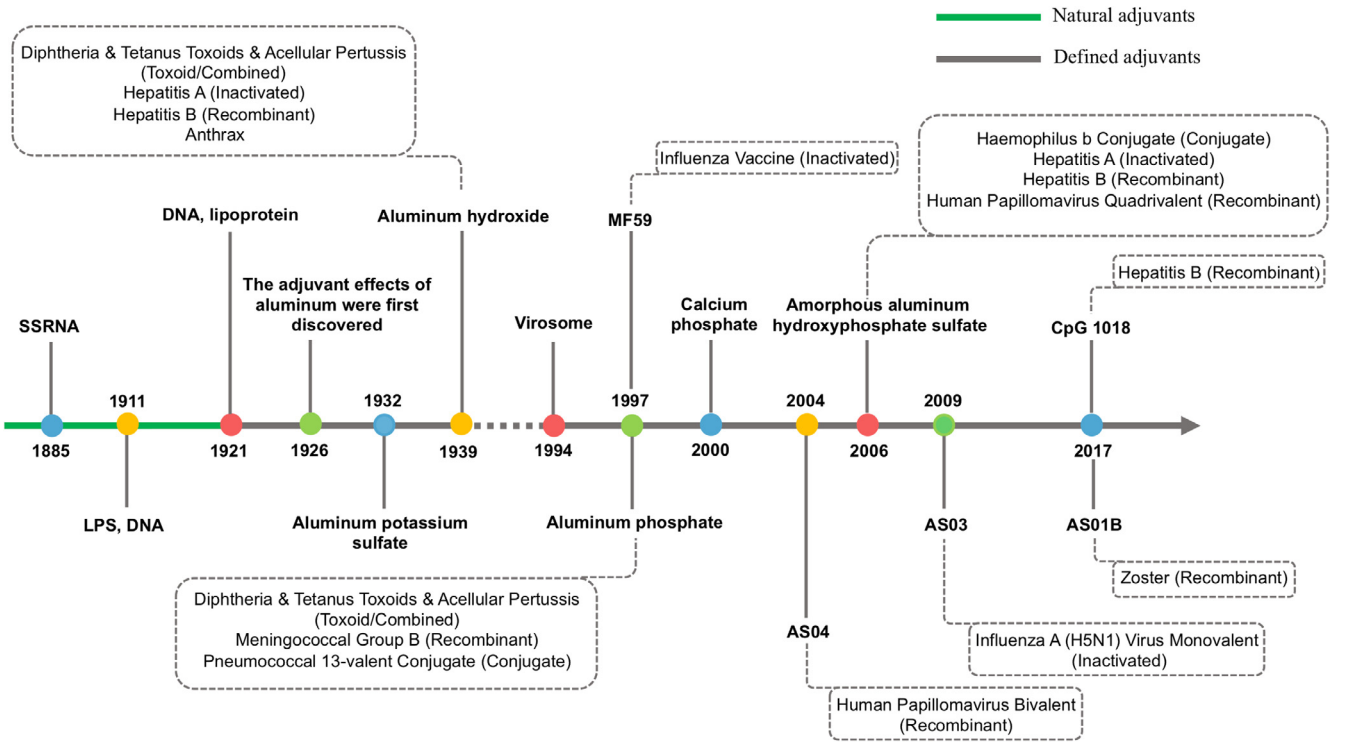

Fig. 1. Timeline of major events in vaccine adjuvants development. The development of vaccines has continuously promoted the advancement of adjuvants from natural components such as RNA and DNA to synthetic engineered vaccine adjuvants.

Table 1
Adjuvants in FDA-approved human vaccines.

	Product Name	Trade Name	Type	Adjuvant	Administration
1	Anthrax Vaccine Adsorbed	Biothrax	N/A	Aluminium hydroxide	Intramuscular/ Subcutaneous
2	Diphtheria & Tetanus Toxoids Adsorbed	N/A	Toxoid	Aluminum phosphate	Intramuscular
3	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	Infanrix	Toxoid	Aluminum hydroxide	Intramuscular
4	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	DAPTACEL	Toxoid	Aluminum phosphate	Intramuscular
5	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed, Hepatitis B (recombinant) and Inactivated Poliovirus Vaccine Combined	Pediarix	Combined (Toxoid, Recombined, Inactivated)	Aluminum hydroxide & Aluminum phosphate	Intramuscular
6	Diphtheria and Tetanus Toxoids and Acellular Pertussis Adsorbed and Inactivated Poliovirus Vaccine	KINRIX	Combined (Toxoid, Inactivated)	Aluminum hydroxide	Intramuscular
7	Diphtheria and Tetanus Toxoids and Acellular Pertussis Adsorbed and Inactivated Poliovirus Vaccine	Quadracel	Combined (Toxoid, Inactivated)	Aluminum phosphate	Intramuscular
8	Diphtheria and Tetanus Toxoids and Acellular Pertussis Adsorbed, Inactivated Poliovirus and Haemophilus b Conjugate (Tetanus Toxoid Conjugate) Vaccine	Pentacel	Combined (Toxoid, Inactivated, Conjugate)	Aluminum phosphate	Intramuscular
9	Haemophilus b Conjugate Vaccine (Meningococcal Protein Conjugate)	PedvaxHIB	Conjugate	Amorphous aluminum hydroxyphosphate sulfate	Intramuscular
10	Hepatitis A Vaccine, Inactivated	Havrix	Inactivated	Aluminum hydroxide	Intramuscular
11	Hepatitis A Vaccine, Inactivated	VAQTA	Inactivated	Amorphous aluminum hydroxyphosphate sulfate	Intramuscular
12	Hepatitis A Inactivated and Hepatitis B (Recombinant) Vaccine	Twinrix	Recombinant (Inactivated)	Aluminum hydroxide & Aluminum phosphate	Intramuscular
13	Hepatitis B Vaccine (Recombinant)	Recombivax HB	Recombinant (subunit)	Amorphous aluminum hydroxyphosphate sulfate	Intramuscular
14	Hepatitis B Vaccine (Recombinant)	Engerix-B	Recombinant	Aluminum hydroxide	Intramuscular
15	Hepatitis B Vaccine (Recombinant), Adjuvanted	HEPLISAV-B	Recombinant	CpG 1018	Intramuscular
16	Human Papillomavirus Quadrivalent (Types 6, 11, 16, 18) Vaccine, Recombinant	Gardasil	Recombinant	Amorphous aluminum hydroxyphosphate sulfate	Intramuscular
17	Human Papillomavirus 9-valent Vaccine, Recombinant	Gardasil 9	Recombinant	Amorphous aluminum hydroxyphosphate sulfate	Intramuscular
18	Human Papillomavirus Bivalent (Types 16, 18) Vaccine, Recombinant	Cervarix	Recombinant	AS04	Intramuscular
19	Influenza A (H5N1) Virus Monovalent Vaccine, Adjuvanted	N/A	Inactivated	AS03	Intramuscular
20	Influenza Vaccine, Adjuvanted	FLUAD	Inactivated	MF59	Intramuscular
21	Japanese Encephalitis Virus Vaccine, Inactivated, Adsorbed	IXIARO	Inactivated	Aluminum hydroxide	Intramuscular
22	Menactra Meningococcal Group B Vaccine	BEXSERO	Recombinant	Aluminum hydroxide	Intramuscular
23	Meningococcal Group B Vaccine	TRUMENBA	Recombinant	Aluminum phosphate	Intramuscular
24	Pneumococcal 13-valent Conjugate Vaccine (Diphtheria CRM197 Protein)	Prevnar 13	Conjugate	Aluminum phosphate	Intramuscular
25	Tetanus & Diphtheria Toxoids, Adsorbed	TDVAX	Toxoid	Aluminum phosphate	Intramuscular
26	Tetanus & Diphtheria Toxoids Adsorbed for Adult Use	TENIVAC	Toxoid	Aluminum phosphate	Intramuscular
27	Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis Vaccine, Adsorbed	Adacel	Toxoid and inactivated toxin	Aluminum phosphate	Intramuscular
28	Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis Vaccine, Adsorbed	Boostrix	Toxoid and inactivated toxin	Aluminium hydroxide	Intramuscular
29	Zoster Vaccine Recombinant, Adjuvanted	SHINGRIX	Recombinant	AS01B	Intramuscular

2. Adjuvants used in FDA-approved vaccines and vaccines under clinical investigation

2.1. Alumnium salt-based vaccine adjuvants

Aluminum-containing adjuvants have been widely used in diphtheria, tetanus, and hepatitis B vaccines, *etc*. Currently, among the human vaccines approved by FDA, there are 25 aluminum-containing vaccines. In the 1920s, the United States used to see as many as 200,000 cases of diphtheria every year, which urged the scientists to develop more effective vaccines to fight against this severe disease [5]. However, the diphtheria toxoid alone could not produce sufficient antibody response, and significant efforts were placed to seek for methods that could induce longer immune responses. Glenny et al. found that adding potassium aluminum sulfate in diphtheria vaccines could significantly improve the effectiveness of vaccines, resulting in longer antigenic effect and stronger antibody production [6]. Since then, aluminum salts have been used in vaccines against various infectious diseases. The types of aluminum salt-based adjuvants in FDA approved vaccines include aluminum hydroxide, aluminum phosphate, and amorphous aluminum hydroxyphosphate sulfate (AAHS). In addition, TWINRIX® and PEDIARIX® contain both aluminum hydroxide and aluminum phosphate (Table 2).

Depending on the types of aluminum salts, they have distinctive physicochemical properties, which have important implications for their immunomodulatory effects [7]. Commercial aluminum hydroxide adjuvant is chemically crystalline aluminum oxyhydroxide (AlOOH), and composed of needle-like nanoparticles with a primary diameter of 20 nm and length of 100–400 nm [7,8]. AlOOH adjuvants form porous and irregular aggregates that can be up to 20 μm in diameter [9]. In contrast, aluminum phosphate is chemically amorphous aluminum hydroxyphosphate, Al(OH)x (PO_4)y. Primary aluminum phosphate particles are a network of platy particles with diameter of 50 nm, and they form 3 μm loose aggregates [7,8]. Another commercially available aluminum-based adjuvant is amorphous aluminum hydroxyphosphate sulfate (AAHS), which is licensed to Merck. AAHS appear as meshes under the transmission electron microscope [10]. Depending on the types of aluminum salts, their surface charges have notable differences.

Table 2
Types of aluminum salts used in human vaccines.

Adjuvant	Product Name	Trade Name
Aluminium hydroxide	Menactra Meningococcal Group B Vaccine	BEXSERO
	Anthrax Vaccine	Biothrax
	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine	Infanrix
	Diphtheria and Tetanus Toxoids and Acellular Pertussis and Poliovirus Vaccine	KINRIX
	Hepatitis A Vaccine	Havrix
	Hepatitis B Vaccine	Engerix-B
	Japanese Encephalitis Virus Vaccine	IXIARO
	Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis Vaccine	Boostrix
AS04	Human Papillomavirus Bivalent (Types 16, 18) Vaccine	Cervarix
Aluminum phosphate	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine	DAPTACEL
	Diphtheria & Tetanus Toxoids	N/A
	Diphtheria and Tetanus Toxoids and Acellular Pertussis and Poliovirus Vaccine	Quadracel
	Diphtheria and Tetanus Toxoids and Acellular Pertussis, Poliovirus and Haemophilus b Conjugate Vaccine	Pentacel
	Meningococcal Group B Vaccine	TRUMENBA
	Pneumococcal 13-valent Conjugate Vaccine	Prevnar 13
	Tetanus & Diphtheria Toxoids for Adult Use	N/A
	Tetanus & Diphtheria Toxoids for Adult Use	TENIVAC
	Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis Vaccine	Adacel
Amorphous aluminum hydroxyphosphate sulfate (AAHS)	Hepatitis A Vaccine	VAQTA
	Haemophilus b Conjugate Vaccine	PedvaxHIB
	Hepatitis B Vaccine	Recombivax HB
	Human Papillomavirus 9-valent Vaccine	Gardasil 9
	Human Papillomavirus Quadrivalent (Types 6, 11, 16, 18) Vaccine	Gardasil
Aluminum hydroxide & Aluminum phosphate	Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine, Hepatitis B and Poliovirus Vaccine	Pediarix
	Hepatitis A and Hepatitis B Vaccine	Twinrix

For example, aluminum hydroxide is positively charged, while aluminum phosphate carries negative charge and AAHS carries scarcely charge in physiological solution (pH = 7.4) [10–12]. The points of zero charge are 5.0, 7.4, and 11.1 for aluminum phosphate, AAHS and aluminum hydroxide, respectively [10,11].

Substantial amounts of mechanistic studies have demonstrated that the aluminum salt-based adjuvants stimulate the Th2 immune responses, with minimum or no Th1 responses [8]. Thus far, several mechanisms were proposed that explain the immunostimulatory activity of aluminum salts [7].

2.1.1. Depot effect

It is traditionally believed that aluminum salt-based adjuvants are typical short-term depots, which could continuously release antigens at the site of injection. Soluble antigens are adsorbed on the surface of adjuvants, which present antigens to immune cells, promote the interaction of antigens with cells, and further enhance the intensity of the immune response [13]. Glenny et al., were the first to propose the formation of depot [14,15]. However, the depot effect has been challenged. Biodistribution studies of 14C-labeled tetanus toxoids adsorbed onto aluminum phosphate showed that the antigen in the injection site was eliminated rapidly within a few hours [16]. Hutchison et al. also reported that antigen-aluminum depot (ear) ablation did not significantly affect antibody responses [17].

2.1.2. Enhanced antigen uptake by antigen presenting cells

Antigens are adsorbed onto the adjuvant, and the binding of antigen to aluminum salts is believed to be a strong electrostatic interaction that enhances antigen uptake and presentation by antigen presenting cells (APCs) [18,19]. Adsorption of antigen enables it to maintain a high local concentration within a certain period of time, which is effective for antigen uptake [20]. Mannhalter et al. demonstrated that aluminum hydroxide adsorbed by tetanus toxoid enhanced antigen uptake and regulated the level of the antigen presentation [21]. Ghimire et al. demonstrated aluminum could prolong the duration and magnitude of antigen presentation by decreasing the degradation rate of internalized antigen [22]. Furthermore, the experimental data of aluminum-containing adjuvants with OVA confirmed that they could enhance uptake of antigen [23].

2.1.3. The NLRP3 inflammasome activation

Aluminum salts could stimulate the NLRP3 inflammasome activation. Studies have demonstrated that aluminum salts were capable of inducing NLRP3 inflammasome activation and stimulating IL-1β and IL-18 productions [9,15], which may explain their ability to induce local inflammation, antigen presenting cell (APC) recruitment, dendritic cell maturation, enhanced antigen uptake, and stimulation and differentiation of T cells [7,24]. Eisenbarth et al. demonstrated that in NLRP3 knockout mice, aluminum adsorbed by ovalbumin (OVA) could not produce antigen-specific antibody [25]. Additionally, studies have shown that the shape, crystallinity and surface functionalization of engineered AlOOH nanoparticles play critical roles in stimulate NLRP3 inflammasome that could quantitatively instruct the adjuvanticity *in vivo*. Further study showed that the NLRP3 inflammasome activation induced by engineered AlOOH involved ROS production, damage of lysosome and release of cathepsin B [24,26]. However, Franchi et al. proposed a different perspective and showed that the induction of antigen-specific IgG production was independent of the NLRP3 inflammasome when adjuvanted with aluminum [27]. The role of NLRP3 inflammasome activation in aluminum's adjuvanticity is still under debating.

2.1.4. Stimulation and differentiation of $CD4^+$ T cells

T cells are one type of lymphocyte that plays a key role in the induction of immunity. T cell differentiation is effectively activated by three molecular signals provided by mature dendritic cells and the local differentiation environment [8]. The first two signals are provided by antigen presentation and costimulatory molecules [7,28]. For the third signal, it is shown that aluminum could induce IL-1β and IL-18 productions in dendritic cells (DCs) [23]. DCs then promote the differentiation of effector T cells [29]. Studies have demonstrated that aluminum-based adjuvants were able to induce the differentiation of $CD4^+$ T cells into Th2 cells [23,30].

2.1.5. Perturbation of dendritic cell membrane

By using atomic force microscopy, Flach et al. demonstrated that aluminum could interact with DC membrane, but are not absorbed. The experimental alum (including CsAl crystal, Imject Alum and Alhydrogel) all enhanced OVA-specific antibody titers in mice [31]. The aluminum salts do not enter the cell, however,

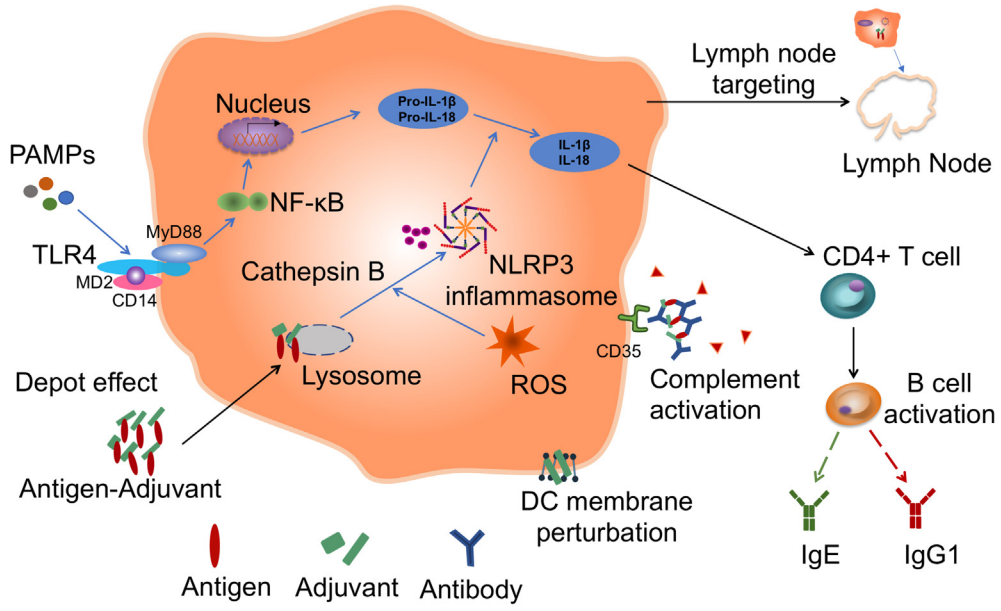

Fig. 2. Acting mechanisms of aluminum salt-based adjuvants. Substantial amounts of mechanistic studies demonstrate that the aluminum salt-based adjuvants stimulate the Th2 immune responses though (1) depot effect; (2) enhanced antigen uptake by antigen presenting cells; (3) the NLRP3 inflammasome activation; (4) stimulation and differentiation of $CD4^+$ T cells; (5) perturbation of dendritic cell membrane; (6) complement activation.

they delivers soluble antigen across the cell membrane. They induce cell membrane lipid reordering, which further lead to antigen uptake and upregulation of $CD4^+$ T cells [31].

2.1.6. Complement activation

The experiment in the guniea pig demonstrated that complement activation by aluminum hydroxide could induce chronic inflammation [32]. Complement factors could regulate immune responses of B cells [33]. Dendritic cells could recruit and deposit complexes of antigen-adjuvant and antibody by CD35 receptors [20]. The receptors on B cells and dendritic cells form antigen-adjuvant-antibody complexes to enhance signal transmission and facilitate immune effects [20]. Therefore, aluminum salts as adjuvants promote complement activation, which enhance immune responses through B cells and dendritic cells [20] (Fig. 2).

2.2. Emulsion adjuvants

2.2.1. MF59

In the late 1980s, new protein antigens could be produced through recombinant DNA technology, however, the majority of recombinant antigens appears to be weakly immunogenic when combined with the traditional aluminum adjuvants. Thus, numerous studies had intended to design water-in-oil adjuvants that could provide potent immunogenicity similar to the Complete Freund's adjuvant (CFA). However, the undesirable side effect of CFA limits its applications [34]. Key turning point occurred when the emulsions used in the adjuvant were changed from water-in-oil to oil-in-water, which not only significantly improved the tolerability of the adjuvant, but also bettered the ease of use due to reduced viscosity [35]. MF59 is composed of squalene, polysorbate 80, sorbitan trioleate and trisodium citrate dehydrate. The oil phase used in the formulation is squalene that is both biodegradable and biocompatible [36]. MF59 became the first oil-in-water adjuvant approved for human vaccine in Italy in 1997 [37]. Currently, MF59 is included in licensed products in 30 countries [35]. MF59 is mainly used in influenza vaccines since studies have found that the addition of MF59 could significantly promote the production of antibody against the antigen and the boost of both Th2 and Th1 immune responses [38,39]. Contrary to the previously belief that MF59 established an antigen depot at injection site [40], it was believed to enhance immune responses by a few factors. It enhances antigen uptake at the injection site and the major target cell types could be monocytes, macrophages, and dendritic cells [41]. These activated cells then could secret a complex of chemokineses to recruit more immune cells to the injection site, which helps to form an immunocompetent environment for enhanced antigen transportation to the draining lymph nodes [42]. A variety of APCs with different antigen processing ways will lead to a more competent immune response, including the increased engulfing of antigen and the accumulation of antigens in the draining lymph nodes contributing to the facilitated transportation of the APCs [43] (Fig. 3).

2.2.2. AS03

AS03 is another oil-in-water emulsion-based adjuvant. The European Commission granted marketing authorization for AS03-adjuvanted Pandemrix in 2009 [44]. AS03-adjuvanted influenza A (H5N1) monovalent vaccine was licensed by FDA in 2013 [45]. Since then, AS03 has begun to play an increasingly important role in influenza vaccines, as well as in malaria vaccines [46,47]. AS03 is composed of surfactant polysorbate 80 and two biodegradable oils, *i.e.*, squalene and DL-α-tocopherol [48]. The use of DL-α-tocopherol, the most bioavailable form of vitamin E, distinguishes AS03 from MF59. In order to elucidate the contribution of DL-α-tocopherol in AS03, studies were designed to compare the effects of AS03 and an equivalent emulsion where DL-α-tocopherol was omitted by using *in vitro* and *in vivo* models. By measuring the secreted cytokines, immune cell recruitments and antigen uptake, it was concluded that the omission of DL-α-tocopherol changed the profile of innate immune response and led to a lower antibody response [49]. Study showed that AS03 could induce cytokine and chemokine productions in muscle and draining lymph nodes (dLN), provoking migration of monocytes, DCs and granulocytes into dLN. In addition, AS03 is found to be able to stimulate $CD4^+$ T cell specific immune response, which might account for persisting neutralizing antibody productions and higher frequency of memory B cells [50] (Fig. 3).

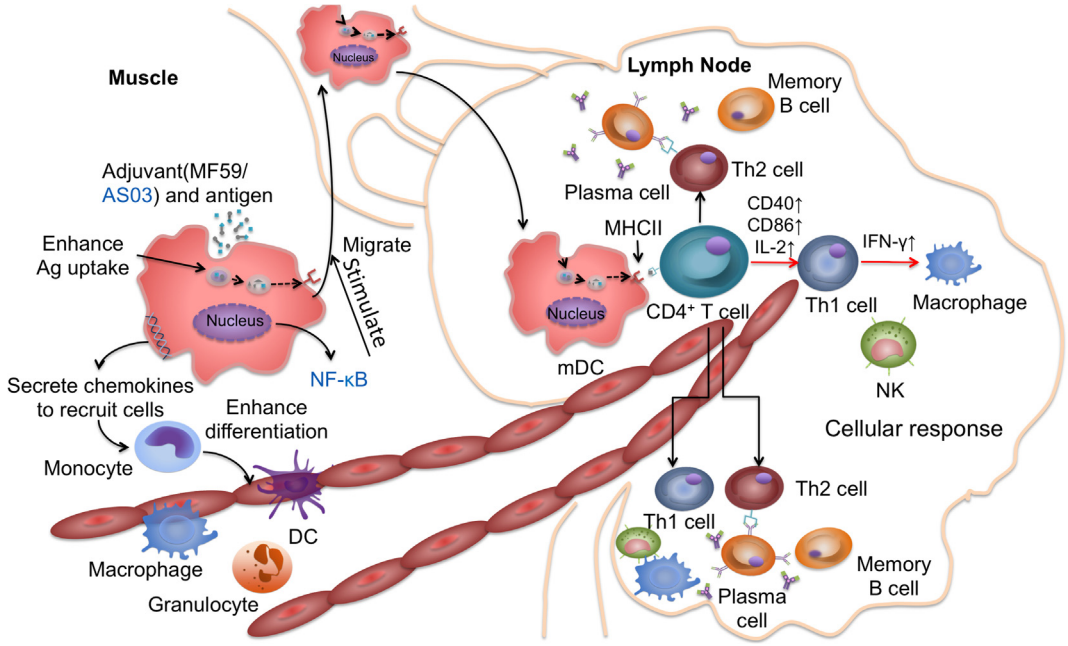

Fig. 3. Models for the activation mechanism of MF59 and AS03. Both MF59 and AS03 create a transient and local immunocompetent environment following injection. They promote cytokine and chemokine productions, and recruitment of cells to injection site. The activated antigen-loaded APCs migrate to draining lymph nodes where APCs could prime naive $CD4^+$ T cells. The chemokine-driven immune cell recruitment is the key characteristic of the mechanism for both MF59 and AS03.

2.3. TLR agonist-based adjuvants

2.3.1. CpG-ODN

After the first report in 1995 showing that cytosine phosphoguanosine (CpG) motifs in bacterial DNA could enhance immune stimulation [51], extensive studies had been performed on bacterial DNA and its synthetic mimic, CpG oligodeoxynucleotides (CpG ODN) [52]. CpG ODNs are synthetic DNA molecules, which are ODNs with phosphorothioate backbone, containing unmethylated CpG motifs [53]. CpG motifs occur at higher frequency in bacterial and viral DNA than vertebrate DNA [54]. Depending on their structure and biological functions, CpG-containing sequences can be divided into different classes, *i.e.*, classes A, B, C, P and S [55]. Among these, CpG-B class, *e.g.*, CpG 1018, 1826, 2007, is the most commonly used one in pre-clinical and clinical trials [56]. HEPLISAV-B, a hepatitis B vaccine approved by the FDA in 2017, is the first vaccine adjuvanted with CpG ODN 1018 [56]. Compared to Engerix-B® that is adjuvanted with aluminum hydroxide, HEPLISAV-B-immunized group induced more rapid and durable antibody responses [56]. CpG ODN 1018 in combination with hepatitis B surface antigen (HBsAg) significantly improves immunogenicity [56]. Moreover, CpG ODN 7909-adjuvanted malaria vaccine and hepatitis B virus vaccine are undergoing phase 1 and 2 trial, respectively [57,58].

CpG ODN is a strong adjuvant for inducing Th1 responses, supporting by its ability to induce strong generation of cytotoxic T lymphocyte (CTL) and IFN-γ secretion [59]. The activation of TLR9 by CpG ODN enhances specific humoral and cellular immune responses to antigens [55,60]. Considering such a unique characteristic, CpG ODN can be used as adjuvant for vaccination *via* intramuscular, subcutaneous, oral and intranasal routes [60,61]. As efficient vaccine adjuvants, CpG ODN can promote the immunostimulatory effect of antigens, activate APC [60], and speed up the immune responses [54]. CpG ODNs could facilitate the expression of MHC, CD40 and CD86 on pDCs and enhance antigen processing and presentation [61]. Sparwasser et al. demonstrated that CpG-DNA could activate immature antigen-presenting DC to professional APC [62]. Another report by Klinman et al. demonstrated that CpG-adjuvanted anthrax vaccine could accelerated serum antibody response and the induction of protective immunity [63] (Fig. 4).

2.3.2. AS04

Aluminum salts have been used as platforms for the discovery of novel adjuvants, consisting of various Toll-like receptor (TLR) agonists adsorbed on them. One of these, known as the adjuvant system 04 (AS04), has been used in HPV and HBV vaccines. Among these, CERVARIX, a human Papillomavirus bivalent vaccine, is the first AS04-adjuvanted vaccine approved by FDA in 2009 [64].

AS04 is prepared from 3′-O-deacylated monophosphoryl lipid A (MPL) and an aluminum salt. MPL is a detoxified lipopolysaccharide (LPS) that has been reported to be a specific agonist of TLR4 [65]. However, MPL's signaling properties through the activation of the TLR4 are not exactly the same as LPS. The difference might be contributed to the absence of the 1-phosphate on the MPL molecule [66]. It has been shown that AS04, compared with an adjuvant containing only aluminum salt, induced a long-term and sustainable effective immune response in the HPV vaccines [67]. Studies have found rapid production of cytokines and recruitment of various immune cells in muscles and draining lymph nodes at the injection site within 3–6 h when adjuvanted with MPL or AS04 [68]. It is demonstrated that MPL is the principal component in the vaccine that mediated the early immune response. Although aluminum salts did not synergize with MPL, its presence prolonged the immune response owing to the function of depot effect [69]. In addition, it's found that AS04 could induce transient and local inflammatory responses, and AS04 must be co-inoculated with the antigen or administered at the same injection site within 1 day of antigen inoculation to achieve superior adjuvant activity [68]. Furthermore, recent study indicates that levels of IFN-γ, a

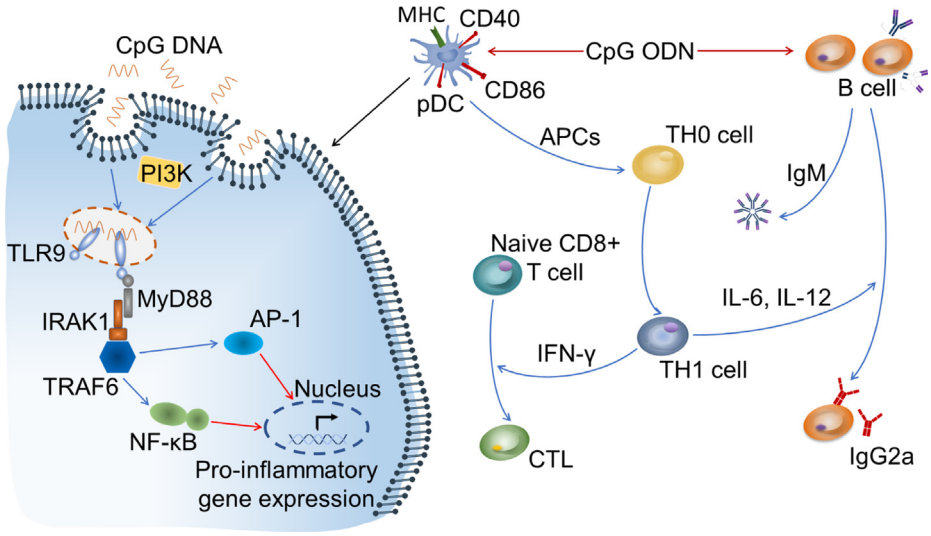

Fig. 4. Acting mechanisms of CpG ODNs. As a new type of adjuvant, synthetic oligodeoxynucleotides (ODNs) that contain immunestimulatory CpG motifs is in favor of Th1 cell responses. After initial CpG ODN uptake inside of the antigen presenting cells, the PI3K facilitates the tranlocation into endosomal vesicles containing TLR9. The interaction between TLR9 and CpG ODN transduces the cytoplasmic activation signal. CpG ODNs directly activate B cells and plasmacytoid dendritic cells, producing proinflammatory- and T helper 1 (Th1) cytokine-rich environment. CpG ODNs could facilitate the pDCs maturation and enhance antigen processing and presentation. CpG ODN induces T cells to promote development of CTL *via* IFN-γ, and increases production of IL-6, IL-12 to support secretion of IgG2a antibodies.

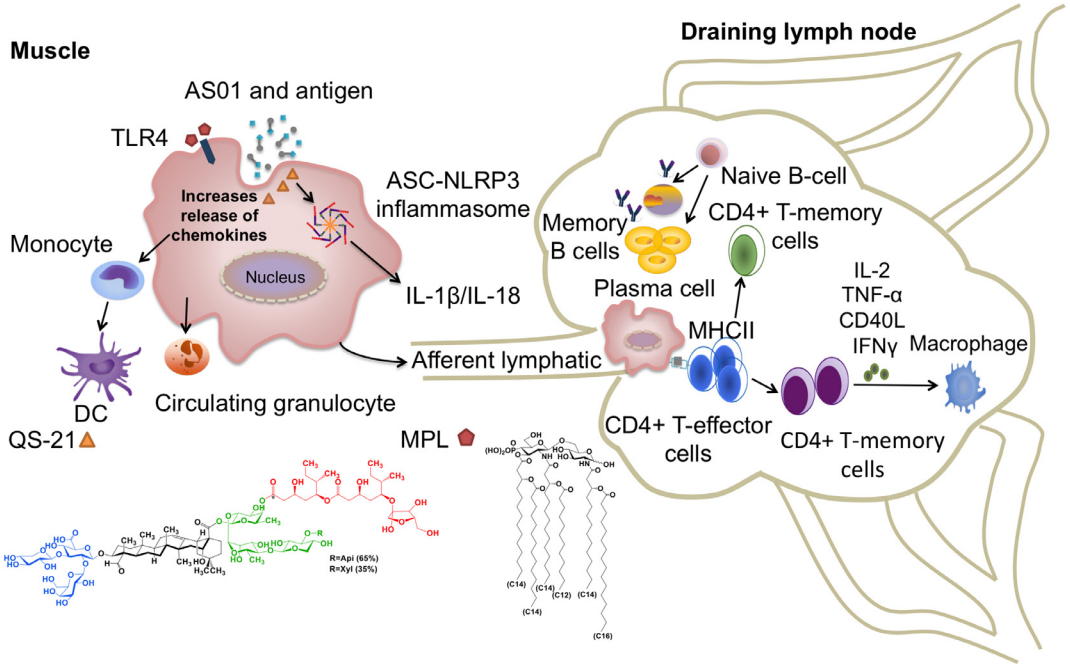

Fig. 5. Acting mechanisms of AS01B. Adjuvant AS01B and antigen are injected into the muscle and taken up by APCs. MPL activates APCs through TLR4. QS-21 activates the NLRP3 inflammasome, resulting in the release of IL-1β and IL-18. MPL and QS-21 act synergistically to increase the release of chemokines, circulate granulocyte and enhance the recruitment of monocytes and dendritic cells. In draining lymph nodes, highly activated dendritic cells efficiently induce naive CD4+ T-cell differentiation into $CD4^+$ T-memory cells and $CD4^+$ T-effector cells. Cytokines secreted by CD4 + T-effector cells such as IL-2, TNF-α, CD40L and IFN-γ could stimulate naive B-cell division into plasma cells and memory B cells.

marker of Th1-biased response, were higher when the HPV-16 and HPV-18 VLPs antigens were adjuvanted with AS04 compared with aluminum hydroxide alone. These results indicate that AS04 is more efficient in inducing the amplification and differentiation of $CD4^+$ T cells and promotes a Th1-biased response [69].

2.4. AS01B

AS01B is an adjuvant developed by GlaxoSmithKline (GSK) Biologicals in recombinant zoster vaccine Shingrix, which was approved by FDA in 2017 [70]. It is formulated in liposome with MPL and QS-21.

MPL is an effective stimulator of T cell and antibody responses, and studies have demonstrated that it could stimulate innate immunity *via* Toll-like activated receptor 4 (TLR4) [71]. QS-21 is purified from the bark of Chilean tree, *Quillaja saponaria* [72]. QS-21 promotes cytotoxic T-lymphocytes (CTLs) production and induces Th1 cytokines, including interleukin-2 (IL-2) and interferon-gamma (IFN-γ), as well as IgG2a antibodies [72]. QS-21 also activates the NLRP3 inflammasome, and promote IL-1β

and IL-18 productions, though its *in vivo* mechanism still remains to be determined [71]. Additionally, MPL and QS-21 could work synergistically to increase the release of chemokines, circulate granulocyte and enhance the recruitment of monocytes and dendritic cells [71].

AS01B-adjuvanted vaccine can induce strong and persistent antigen-specific humoral and cellular immune responses. According to preclinical studies in mice and rhesus monkeys, AS01B is better in inducing antigen-specific CTL and IFN responses than AS02A which is a multicomponent adjuvant system consisting of oil-in-water emulsion, MPL and QS21 [73,74] (Fig. 5).

2.5. Adjuvants under clinical investigation

Limited types of adjuvants with various formulations are being used in human vaccines, however, there is a big demand for novel adjuvants with better potency and longevity. Moreover, owing to the limited understanding of acting mechanisms, the currently available adjuvants are still the compromised results of adequate immunity and low-toxicity [75]. Thus far, various vaccine adjuvants are undergoing different stages of clinical trials.

2.5.1. Lipid-based adjuvants

Lipid-based material has been used as vaccine adjuvant since its unique vesicle structure was proved to be extremely suitable for encapsulating various biologically active materials, such as DNA and fatty acids [76,77]. At the early stage, scientists had been focusing on Lipid A, the hydrophobic anchor of LPS, which was believed to be a potent adjuvant and could remarkably increase both the humoral and cellular immune response. However, Lipid-A based adjuvant was considered not suitable in human vaccines due to high toxicity [78–80]. GLA-SE and GLA-AF are both Lipid A analogues, and are particularly effective in enhancing protection against H5N1 with low toxicity and limited side effects [81–83]. GLA-AF is also tested as an alternative to emulsions in many vaccines, among which the most notable one is pandemic influenza [81,84].

2.5.2. Emulsions

Montanide ISA 51 and Montanide ISA 720 are two types of water-in-oil emulsion adjuvants under clinical investigations. Montanide ISA 51 is a mixture of a mineral oil and a mannide monooleate surfactant, while Montanide ISA 720 is formulated with nonmineral vegetable oil. Moreover, they have different oil to water ratios [85]. Montanide ISA 51 has been assessed in malaria vaccines in phase 1 and influenza vaccines in phase 2 studies [86–88], and it has been licensed in a therapeutic lung cancer vaccine in Cuba [88]. Montanide ISA 720 are tested in malaria vaccine in a phase 2 clinical trial [89]. Studies have shown that both of them are able to promote effective immune response, particularly enhanced specific cytotoxic T-lymphocyte (CTL) response [90]. Although both of them appear to be well tolerated, transient side effects have been observed, such as fever, headache or flu-like symptoms [89].

2.5.3. Saponin

Saponins are natural glycoside compounds. Their ability to activate human's immune system has raised researchers' interest to exploit their applications as vaccine adjuvant [91]. Matrix M™ is next-generation saponin-based adjuvant patented by Novavax [92], powered by a new formulation that provides a potent adjuvant effect and low toxicity. It has been tested in combination respiratory vaccine in preclinical trial [93]. It was shown that Matrix M™ could increase the number of cells in draining lymph nodes (dLNs) and spleen, and activate lymphocytes, DCs and granulocytes in dLNs [93]. AS02, composing of MPL and QS-21, is undergoing phase 2 trial in a malaria vaccine [94].

2.5.4. Nucleotide

Host cells could be invaded by pathogens *e.g.*, lipopolysaccharide, peptidoglycan, flagellin and microbial nucleic acids [95]. When recognizing these molecules, pattern recognition receptors (PRR) of the host will release inflammatory cytokines, and then promote an immune response [96]. Obviously, CpG ODN, dsRNA, IL-12 DNA, and *etc,* are typical nucleotide-based adjuvant components activating through PRR to stimulate immune response. CpG ODN has been discussed in the above section. Double-stranded RNA (dsRNA) could achieve its immune response by secreting cytokines including IFN-α and IFN-γ, signaling through TLR3, MDA-5 and NLRP3 inflammasome [97,98]. It is now undergoing clinical trial in influenza vaccine during phase 1 stage [99] and phase 2 study in rabies vaccine [100]. To provide efficient protection from the wide range of HIV variants, DNA vaccines adjuvanted with IL-12 DNA are introduced [101]. Recent trials demonstrated that IL-12 DNA was able to augment the immune response and proved to be well tolerated [102]. Pika adjuvant is a synthetic analogue of dsRNA stabilized with kanamycin and calcium. Pika adjuvant promotes the production of interferon, IL-2, and IL-12, thus transforming the preventive vaccine, which mainly produces humoral immunity, into a therapeutic vaccine with strong cellular immunity.

2.5.5. Cytokines

Recent studies have demonstrated that molecular adjuvants could stimulate effective immune response, especially in subunit vaccines [103]. Cytokines, *e.g.*, IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), were tested in vaccines against foot and mouth disease [104]. Recently, GM-CSF is assessed for use in both Hepatitis B and HIV vaccines. Its adjuvant effect is believed to be related to its ability to stimulate macrophage differentiation and proliferation, and to activate antigen presenting cells [105]. Additionally, IL-12 and IL-15 are used as adjuvants in HIV vaccine that is undergoing a phase 1 trial. These cytokines were found to have the ability to stimulate both NK cells and T-cell proliferation [106,107].

2.5.6. Others

In addition, several other forms of adjuvants were under clinical investigation. It is worth to mention calcium phosphate. Calcium phosphate, in the form of hydroxyapatite, were licensed in France for use in diphtheria, tetanus, pertussis, and poliomyelitis vaccines [108]. There is a belief that calcium phosphate, as a natural compound in human body and being well tolerated, will become a substitution for aluminum adjuvants to overcome its undesirable side effects [109]. Moreover, bacterial flagellin, a ligand for TLR5, shows its adjuvant effect that leads to mixed Th1 and Th2 responses [110,111]. In a clinical trial, a bivalent *P. aeruginosa* flagella adjuvanted vaccine can help patients with cystic fibrosis to lower the risk for infection with *P. aeruginosa* [112]. Polysaccharide is another potential adjuvant that can enhance the immune response of vaccines and promote cellular immunity, humoral immunity and mucosal immunity [113]. Virosomes are enveloped virus-like particles [114]. Virosomes have been licensed in vaccines against Hepatitis A and influenza in Europe [115], and they have been used in clinical trials in Falciparum Malaria and Chronic Hepatitis C vaccines [116,117]. In addition, there also exist novel adjuvants that are a combination of several types of adjuvants listed above. The effective components synergize to create optimal immune responses. (Table.3)

Table 3
Adjuvants under clinical investigation.

Type of adjuvant	Adjuvant name	Conditions	Clinical phase	Ref.
Lipids	GLA-SE (Lipid A analogue, oil-in-water emulsion)	Malaria	Phase 1	[83]
		Influenza	Phase 2	[82]
	GLA-AF (Lipid A analogue)	Influenza	Phase 1	[81]
		HIV	Phase 1	[84]
	MPL	Hepatitis B	Phase 2	[74]
	CCS/C	Influenza	Phase 2	[118]
Emulsions	Montanide ISA 51	Malaria	Phase 1	[87]
		Influenza	Phase 2	[85]
	Montanide ISA 720	Malaria	Phase 2	[89]
Saponins	Matrix M™	Malaria	Phase 1	[119]
		Respiratory Syncytial Virus F-protein	Phase 3	[93]
		Melanoma	Phase 2	[119]
	Matrix-M1	Malaria	Phase 1	[119]
Nucleotide	CpG 7909	Malaria	Phase 1	[57]
	dsRNA	Influenza	Phase 1	[97]
	IL-12 DNA	HIV	Phase 1	[101]
	Interleukin-2/Immunoglobulin (IL-2/Ig) DNA	HIV	Phase 1	[120121]
		HIV	Phase 1	
	Poly-ICLC	HIV	Phase 1	[122]
Cytokines	GM-CSF	Hepatitis B	Phase 2	[123]
		HIV	Phase 2	[124]
	IL-12	HIV	Phase 1	[102]
	IL-15	HIV	Phase 1	[102]
Others	Advax™ delta inulin adjuvant	Seasonal influenza	Phase 1	[125]
	ViscoGel	Haemophilus influenzae type b	Phase 1/2a	[126]
	Flagellin	Pseudomonas aeruginosa	Phase 3	[112]
	AS01B	Malaria	Phase 2	[127]
	AS02 (MPL, QS21, oil-in-water emulsion)	Malaria	Phase 2	[94]
	Virosomes	Falciparum Malaria	Phase 1	[116]
		Chronic Hepatitis C	Phase 1	[128]
		Hepatitis A	Licensed	[128]
	Alum + TLR7 agonist	Anthrax	Phage 1	[129]

3. Conclusions and perspectives

In this review, we comprehensively discussed the adjuvants used in FDA-approved vaccines, *e.g.,* aluminum salt-based adjuvants, AS01, AS03, AS04, CpG ODN, and MF59 as well as adjuvants under clinical investigations, *e.g.*, lipids, saponins, nucleotide and glycoprotein. We focus on their formulations and potential mechanisms of immunogenicity, understanding of which will help to establish structure-activity relationships that facilitate the design of more effective adjuvants for both preventative and therapeutic vaccines.

In the design and clinical transformation of new adjuvants, many factors affecting the immunogenicity and stability of vaccines should be considered, including route of administration, choices of animal models [130], physicochemical characteristics of adjuvants, *etc*. Depending on the types of adjuvants, the innate immune activation varies that could affect the antigen presentation and further vaccine responses [131]. However, some non-negligible negative situations appear in practical applications. For example, immunostimulants may produce adverse reactions such as autoimmune diseases while effectively inducing an immune response. Systemic adverse reactions include allergies, fever, immunosuppression, and even teratogenic, carcinogenic, and mutagenic risks while local adverse reactions include inflammation, nodules, abscesses, *etc*. In addition, many adjuvants fail the clinical transformation due to complex manufacturing processes, poor stability, and high immune tolerance.

The ideal adjuvants should have a broad-spectrum of safety, ease of production and use, and can effectively activate humoral and cellular immune responses with no adverse reactions. There is need to control and improve the physicochemical properties of the adjuvants and optimize the production process. In-depth study of the action mechanism of adjuvants, comprehensive understanding of their impacts on the immune system and further research on the structure-activity analysis of immunoadjuvant will further paves the way for safe and effective use of adjuvants to enhance the immune effect of vaccines. Additionally, it will promote the development of new and efficient adjuvants and their clinical transformation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31870919), Natural Science Foundation of Liaoning Province (No. 20180550597), and Fundamental Research Funds for the Central Universities (No. DUT17RC(3)021). Bingbing Sun also thank the Recruitment Program of Global Youth Experts for supports.

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