

REVIEW

Vaccine adjuvants: smart components to boost the immune system

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Abstract Vaccination is an effective approach to prevent the consequences of infectious diseases. Vaccines strengthen immunity and make individuals resistant to infections with pathogens. Although conventional vaccines are highly immunogenic, they are associated with some safety issues. Subunit vaccines are safe, but they require adjuvants to stimulate the immune system because of their weaker immunogenicity. Adjuvants are entities incorporated into vaccines to increase the immunogenic responses of antigens. They play a crucial role in increasing the potency and efficacy of vaccines. Different adjuvants have different modes of action; therefore, a better understanding of their immunology could provide guidance for the development of novel adjuvants. Numerous studies have been conducted using different types of adjuvants to characterize their potency and safety; however, in practice, only few are used in human or animal vaccines. This review aims to introduce the different modes of action of adjuvants and give insight into the types of adjuvants that possess the greatest potential for adjuvanticity.

Keywords Vaccines · Adjuvants · Modes of action · Types of adjuvants

Introduction

Vaccination is one of the most effective and cost-efficient ways to prevent many types of infectious diseases. It aims to induce a pathogen-specific immune response and provide long-lasting protection against infection (Lee and Nguyen 2015). Long-lasting and effective stimulation of the immune system can be achieved by conventional vaccines composed of live attenuated or inactive pathogens, but these vaccines are associated with several safety issues including possible mutations that restore pathogenicity or incomplete inactivation of antigens. Meanwhile, subunit vaccines are safer but are less immunogenic. Thus, subunit vaccines need adjuvants to increase their immunogenicity (Park et al. 2016). Adjuvants are commonly defined as entities that are added to formulations of vaccines to increase the immunogenicity of antigens (Lee and Nguyen 2015). They facilitate the use of smaller doses of antigen and permit immunization protocols using fewer doses of vaccine (Coffman et al. 2010). Furthermore, adjuvants help to increase the stability of vaccines by making them less susceptible to degradation that can occur during storage or upon injection. They also influence the onset, strength and duration of the immune responses (Schijns and Lavelle 2011).

This review gives a brief overview of the role of adjuvants in innate and adaptive immune responses, the modes of action and types of adjuvants, the current uses of licensed adjuvants, and obstacles in development of new adjuvants.

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The role of adjuvants in innate and adaptive immune responses

If an antigen enters the body, the immune system responds to remove it (Kleinstein and Seiden 2000). The immune system can respond either quickly or slowly. The fast responding innate immune system acts as the first line of defense in the body, while the adaptive immune system acts slowly and provides long-lasting immune responses (Pashine et al. 2005). The complement system and phagocytic cells are involved in innate immunity. Adaptive immune responses are initiated through antigen-mediated stimulation of T cells and B lymphocytes that have antigen-specific surface receptors. CD4⁺ T-helper (Th) cells and CD8⁺ cytotoxic T lymphocytes (CTLs) are two different types of T cells, and Th cells are further divided into different subpopulations with Th1 and Th2 cells being important (Schwendener 2014). Innate immune cells express pattern-recognition receptors (PRRs), which help to detect infectious agents. C-type lectin-like receptors (CLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs), toll like receptors (TLRs) and retinoic acid-inducible gene-1 (RIG-I) like receptors (RLRs) are different families of PRRs. TLRs and CLRs are present on the surface of antigen presenting cells (APCs), while NLRs and RLRs are located intracellularly (Awate et al. 2013). Pathogen-associated molecular patterns (PAMPs) are molecular patterns present in pathogenic microorganisms like fungi, viruses, bacteria and parasites (Haghighparast et al. 2016). APCs can recognize PAMPs via PRRs before or during the endocytosis of an antigen. After recognition, antigens undergo processing in APCs, and antigen peptides are loaded onto major histocompatibility complex (MHC) molecules. Antigen peptides complexed with MHC class II molecules can activate Th cells, which can result in stimulation of cellular immunity (CTL responses) and/or humoral immunity (opsonic and/or neutralizing antibodies production by B cells). Antigen peptides complexed with MHC class I molecules can directly stimulate CD8⁺ cells leading to cellular responses (Skwarczynski and Toth 2016).

In general, adjuvants activate the innate immune system through PRRs present in immune cells. The majority of immunostimulatory adjuvants act as ligands for PRRs and lead to secretion of cytokines by directly enhancing an activation pathway (Coffman et al. 2010). Receptor-ligand interactions lead to expression of genes that encode the cytokines, chemokines, and costimulatory molecules responsible for priming, expansion, and polarization of immune responses. As a result, components of damaged or dying host cells also contribute to the function of adjuvants due to inflammasomes (Coffman et al. 2010). In particular,

TLR ligands are potent immunomodulators for a variety of immune responses. Different components of bacteria and viruses can be recognized by TLRs. Lipid, protein, and nucleic acid components are different classes of TLR ligands (Akira 2011). When different TLR ligands stimulate cells, distinct patterns of gene expression are induced, demonstrating differences in signaling pathways among TLRs that occur through selective usage of adaptor molecules like MyD88 (myeloid differentiation primary response gene 88) and TRIF (toll/interleukin-1 receptor domain containing adaptor protein inducing interferon- β). MyD88 activates nuclear factor- κ B (NF- κ B) leading to production of inflammatory cytokines, whereas TRIF activates the transcription factor interferon regulatory factor 3 (IRF3) leading to production of type-I interferons (IFN) (Akira 2011). A well-known TLR ligand is lipopolysaccharide (LPS), which can activate TLR4 (Akira 2011). Similarly, poly-I:C (polyinosinic:polycytidylic acid) activates TLR3, flagellin is recognized by TLR5, imidazoquinolines are recognized by TLR7/8, and CpG oligodeoxynucleotides are recognized by TLR9 (Apostólico et al. 2016). Therefore, adjuvants that have structures similar to the various ligands of PRRs can activate their respective receptors, resulting in stimulation of innate immunity.

On the other hand, adjuvants can induce adaptive immune responses either by enhancing T cell responses, by stimulating humoral immunity, or both. Immune-stimulating complexes (ISCOMs) act by inducing antibody production and balanced Th1 and Th2 immune responses (Lövgren Bengtsson et al. 2011), whereas monophosphoryl lipid A (MPL) elicits a Th1 response (Apostólico et al. 2016; Cox and Coulter 1997), and cholera toxin (CT) stimulates a Th2 response (Lee et al. 2009). The Th1 response involves cell-mediated immunity, while Th2 cells activate humoral responses to neutralize extracellular antigens. There are challenges to the development of novel adjuvants that can generate protective CD8⁺ T cell responses. In order to enhance differentiation of functional CD8⁺ T cells, a promising adjuvant should be mixed with an antigen in such a way that facilitates entry of the antigen into the MHC class I processing pathway, triggering dendritic cell (DC) activation and inducing type-I IFN production (Coffman et al. 2010). A study by Bungener et al. demonstrated that ovalbumin delivered by fusion-active virosomes induced a class I MHC-restricted CTL response. They also highlighted the importance of virosomes as an ideal antigen delivery system that can induce cellular immunity against encapsulated protein antigens (Bungener et al. 2005).

Modes of action of adjuvants

Depot formation (Fig. 1), recruitment of immune cells, activation of the inflammasome, enhancement of antigen presentation by MHC molecules, and immunomodulation are different modes of action of adjuvants discussed in this review and these are summarized in Fig. 2.

Depot formation

Adjuvants entrap, adsorb, or aggregate antigens and release them slowly over a long period of time (Bowersock and Martin 1999). This depot effect at the site of injection also prevents the loss of antigens by liver clearance (Cox and Coulter 1997). Liposomes act by the depot effect (Gupta and Siber 1995).

Recruitment of immune cells

Adjuvants create localized pro-inflammatory immune responses at the site of injection leading to recruitment and activation of immune cells. Local inflammation may also precipitate redness, swelling and local pain (Gerds 2015). MF59 acts by recruiting immune cells at the injection site (Seubert et al. 2008).

Activation of the inflammasome

PRRs are recognized and/or are activated by adjuvants. Inflammasome belongs to the NLR family. NOD like receptor protein 3 (NLRP3) is a highly studied inflammasome receptor for adjuvanticity. The NLRP3 inflammasome is intra-cytoplasmic multiprotein complex that contains a NLRP3 receptor, procaspase-1, and an ASC,

which is apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD). Stimulation of the NLRP3 inflammasome results in caspase-1 activation and this leads to cleavage of proforms of interleukin (IL)-1 β , IL-18, and IL-33 into their bioactive forms (Awate et al. 2013). Alum functions as an adjuvant by activating the NLRP3 inflammasome (Awate et al. 2013; Tritto et al. 2009).

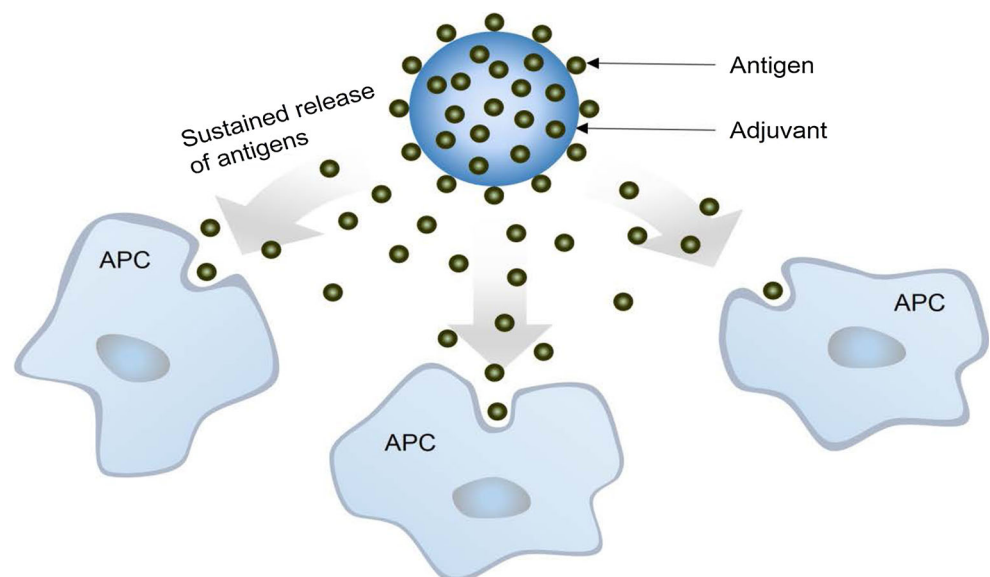
Enhancement of antigen presentation by MHC molecules

Adjuvants like alum or oil-based emulsions are believed to exhibit their adjuvanticity by targeting antigens to APCs, leading to enhancement of antigen presentation by MHC molecules on APCs, and stimulation of adaptive immune responses. However, still the role of adjuvants in the enhancement of antigen presentation by MHC molecules for the development of adaptive immune response has not been clearly evaluated (Awate et al. 2013).

Immunomodulation

Immunomodulatory adjuvants trigger the immune system by modifying the cytokine network. Primarily, adjuvants affect the type of immunity by increasing the concentrations of some cytokines while decreasing concentrations of others. Interferon gamma (IFN- γ), IL-2 and IL-12 are linked with Th1 responses and cell-mediated immunity, whereas IL-4, IL-5, IL-6, IL-13, and possibly IL-10 are linked with Th2 responses and humoral immunity (Spickler and Roth 2003). MPL acts by stimulating Th1 responses (Apostólico et al. 2016; Cox and Coulter 1997).

Fig. 1 Depot formation (redrawn and modified from Smith et al. 2013). Adjuvants entrap, adsorb or aggregate antigens at the site of injection and release them in a sustained manner



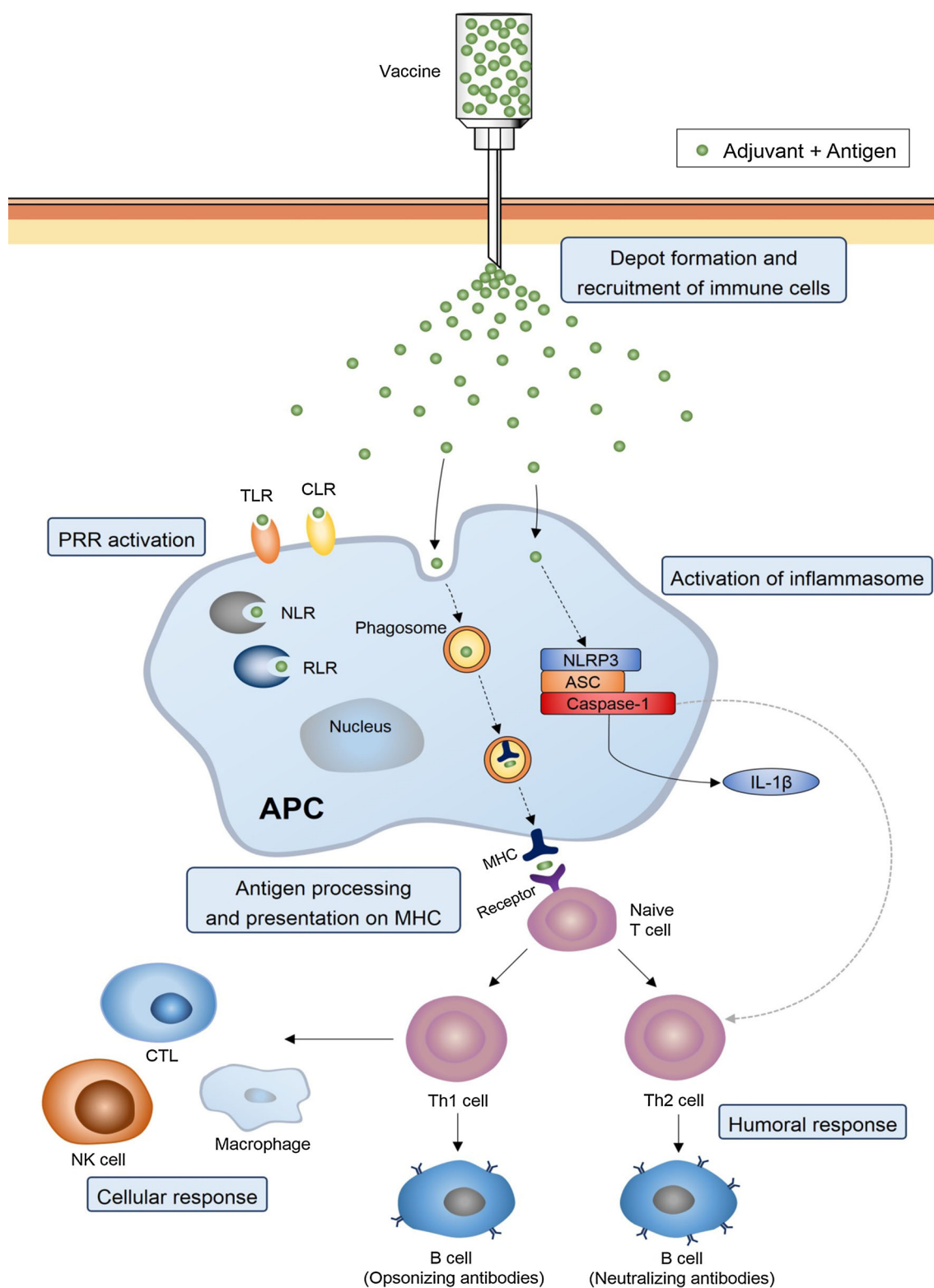


Fig. 2 Modes of action of adjuvants (redrawn and modified from InvivoGen 2011). Adjuvants form depot of antigens and recruit immune cells at the site of injection. They can activate PRRs of recruited APCs before or during endocytosis of antigens, after which antigens are processed and presented to T cells resulting in cellular and/or humoral responses

Types of adjuvants

The modes of action of some common adjuvants are listed in Table 1.

Mineral salts

Mineral salts like calcium phosphate and aluminum salts such as aluminum phosphate and aluminum hydroxide, are commonly used as adjuvants in vaccines. Aluminum phosphate and aluminum hydroxide have different physical and adjuvant properties, but both are imperfectly known as alum. Chemically, alum is potassium aluminum sulfate, and it was initially used for purifying protein antigens like tetanus toxoid and diphtheria toxoid (Sivakumar et al. 2011). Aluminum hydroxide-adsorbed cholera vaccine exhibited higher protection in comparison to cholera

vaccine without adjuvant. In addition, inactivated poliomyelitis vaccine was found to be more immunogenic when adsorbed onto aluminum phosphate and the human diploid cell strain rabies vaccine showed higher immunogenicity when adsorbed onto aluminum hydroxide (Gupta et al. 1993). In a study conducted by Fazeli et al., using aluminum phosphate as an adjuvant, a hepatitis B vaccine formulation exhibited higher adjuvanticity than a preparation with aluminum hydroxide (Fazeli et al. 2008). Thus, the potency of aluminum salts might depend on the type of antigen used in the vaccine formulation.

In the past, aluminum salts were believed to act by forming a depot at the site of injection and by enhancing the recruitment of APCs and uptake by them. Recently, it has been recommended that antigens adsorbed on the surface of aluminum salts should be delivered in particulate multivalent form, which are easily processed by APCs (Leroux-Roels 2010). It also has been shown that

Table 1 Modes of action of some common adjuvants

Adjuvant	Mode of action	References
Aluminium salts	Activation of innate immune responses	Leroux-Roels (2010)
Emulsion (o/w)	Innate inflammatory responses, activation and recruitment of APCs, enhancement of antigen persistence at site of injection and delivery to immune-competent cells	Leroux-Roels (2010)
Emulsion (w/o)	Induction of local inflammation, activation and recruitment of APCs	Leroux-Roels (2010)
Liposomes	Depot formation, presentation of antigens to APCs	Gupta and Siber (1995)
Virosomes	Delivery of antigens to APCs	Copland et al. (2005)
QS21	Presentation of antigens to APCs, induction of CTL production, stimulating both Th1 and Th2 cytokine secretion	Leroux-Roels (2010)
ISCOMs	Induction of balanced Th1 and Th2 responses	Lövgren Bengtsson et al. (2011)
PLGA	Cross-presentation of antigens to CD8 ⁺ T cells	Apostólico et al. (2016)
Chitosan	Translocation of tight junctions of cells	Apostólico et al. (2016)
MPI	Stimulation of both Th1 and Th2 responses	Aguilar and Rodriguez (2007)
IFN- γ	Up-regulation of Th1 responses	Cox and Coulter (1997)
IL-1	Maturation of T and B cells	Cox and Coulter (1997)
IL-2	Up-regulation of Th1 responses	Cox and Coulter (1997)
IL-4	Up-regulation of Th2 responses	Cox and Coulter (1997)
IL-12	Induction of strong Th1 shift	Cox and Coulter (1997)
GM-CSF	Activation and recruitment of APCs	Aguilar and Rodriguez (2007)
VLPs	Direct activation of B cells, stimulation of DCs, induction of cross-presentation to CD8 ⁺ T cells	Apostólico et al. (2016)
CT	Stimulation of Th2 responses	Lee et al. (2009)
CpG motifs	Stimulation of Th1 responses	Aguilar and Rodriguez (2007)
MPL	Stimulation of Th1 responses	Apostólico et al. (2016) and Cox and Coulter (1997)
MDP-lipophilic	Stimulation of Th1 responses	Cox and Coulter (1997)
MDP-hydrophilic	Stimulation of Th2 responses	Cox and Coulter (1997)

aluminum salts exhibit their adjuvanticity by activating the NLRP3 inflammasome (Awate et al. 2013; Tritto et al. 2009). The formation of granulomas, allergenicity, neurotoxicity and stimulation of IgE production are potential side effects of aluminum salts (Petrovsky and Aguilar 2004). Meanwhile, calcium phosphate stimulates IgG production, but does not increase the production of IgE antibodies. It has similar properties to aluminum salts and is a well-tolerated compound in humans (Petrovsky and Aguilar 2004). In France, calcium phosphate had been used regularly in childhood and adult vaccines for many years. However, later, the use of calcium phosphate in vaccines was prohibited because vaccine formulations prepared with calcium phosphate had problems in passing the test for potency of tetanus and diphtheria components in accordance with the regulations of the World Health Organization and European Pharmacopeia (Gupta 1998).

Oil-based emulsions

An emulsion is dispersion of two immiscible phases e.g., water and oil. Surfactants are added to stabilize emulsion systems (Aucouturier et al. 2001). Oil-in-water, water-in-oil, and multiple emulsions including water-in-oil-in-water and oil-in-water-in-oil are different classes of emulsions (Shah et al. 2015). The adjuvanticity of oil-in-water (o/w) emulsions involve innate inflammatory responses and recruitment and activation of APCs. They also facilitate the persistence of antigens at the site of injection and enhance the delivery of antigens to immune-competent cells (Leroux-Roels 2010). Meanwhile, water-in-oil (w/o) emulsions are thought to act by inducing local inflammation and by enhancing recruitment and activation of APCs (Leroux-Roels 2010). Inflammatory reactions, granuloma formation, and ulcers at the site of injection are some common side effects associated with emulsions (Aguilar and Rodriguez 2007).

Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) are w/o emulsions. CFA contains heat-killed mycobacteria, which IFA lacks. CFA induces long-lasting local inflammation, which can cause pain and lead to ulceration at the injection site in animals. Therefore, regulatory guidelines are followed while performing experiments with CFA in experimental animals (Apostólico et al. 2016). MF59 is o/w emulsion, which is safe and highly potent adjuvant allowing for lower concentrations of antigens in vaccines (Schultze et al. 2008). AS03 is o/w emulsion that contains squalene and α -tocopherol, which is the major bioavailable form of vitamin E. Vitamin E has anti-oxidant and immunostimulatory activities. Thus, α -tocopherol must be included in AS03 to obtain high antibody response (Morel et al. 2011). Another example of an oil-based emulsion is Montanide, which is commonly used

in experimental vaccines for rats, mice, dogs, and cats using different kinds of synthetic, recombinant, and natural antigens (Petrovsky and Aguilar 2004).

Several studies have showed synergistic actions of emulsions in combination with other compounds. A study by Bayry et al. suggested that the combination of Montanide ISA 206 and recombinant protein ISCOMs could be a good choice for achieving early protective titers and long-lasting immunity (Bayry et al. 1999). In another study done by Song et al., ginseng stem-leaf saponins (GSLs) and mineral oil adjuvant acted synergistically and promoted both Th1 and Th2 immune responses (Song et al. 2009).

Liposomes, virosomes and niosomes

Liposomes are spherical-shaped vesicles that can be formed from cholesterol and non-toxic natural phospholipids. They may contain one or more lipid bilayers enclosing an aqueous unit. Lipid composition, size, surface charge and preparation methods directly affect the properties of liposomes. Liposomes are biodegradable, biocompatible and less toxic (Akbarzadeh et al. 2013). They can induce both humoral and cell-mediated immune responses. Their adjuvanticity results from the formation of a depot at the injection site and the presentation of antigens to APCs (Gupta and Siber 1995). Liposomes can easily adsorb to mammalian cells and release antigens. In addition, they can easily be phagocytosed by APCs and then can easily release entrapped antigens (Bowersock and Martin 1999). The surface charge of the vesicles, rigidity of the bilayer, epitope density, and the association of antigen with the liposome structure are different factors that influence the immunogenic responses of liposomes (Copland et al. 2005). The immunoadjuvant action of liposomes can be enhanced by different methods including receptor-mediated targeting to macrophages, the use of co-adjuvants, modification of structural characteristics of vesicles and the use of cytokines (Gregoriadis et al. 1996). In a study conducted by Nakanishi et al., positively charged liposomes containing soluble antigens were more potent inducer of antigen-specific cytotoxic T lymphocyte responses and delayed-type hypersensitivity responses compared to neutral and negatively charged liposomes with the same concentrations of antigens (Nakanishi et al. 1999). The high cost, stability and pain at the site of injection are drawbacks associated with the use of liposomes as adjuvant (Sivakumar et al. 2011).

Virosomes are reconstituted viral envelopes composed of membrane lipids and viral glycoproteins. They lack genetic material so they cannot replicate (Apostólico et al. 2016). Immunopotentiating reconstituted influenza virosomes (IRIV) are a widely used virosomal system. These virosomes contain hemagglutinin (HA) and neuraminidase

(NA) proteins, which are intercalated within the lipid membrane (Apostólico et al. 2016). The structure of virosomes is similar to unilamellar liposomes (Copland et al. 2005). Virosomes can deliver antigens into the cytoplasm of APCs either by fusing with the endosomal membrane or by being endocytosed (Copland et al. 2005). In a study conducted on influenza virosomes, Cusi et al. showed that immunization of respiratory syncytial virus-F protein with influenza virosomes in combination with the mucosal adjuvant Escheriagen (*Escherichia coli* heat-labile toxin) in BALB/c mice resulted in a mucosal IgA response, high serum IgG levels and a balanced Th1/Th2 cytokine profile (Cusi et al. 2002).

Niosomes structurally resemble liposomes and have similar physical properties. They are formulated using non-ionic surfactants, which are cheaper and chemically stable (Khan and Irchhaiya 2016). Niosomes also act as adjuvant. For example, if bovine serum albumin (BSA) is incorporated within niosomes, it results in the formulation having an effectiveness equivalent to that achieved by using CFA. Niosomes are safe when administered parenterally (Kersten and Crommelin 1995).

Saponins and immune-stimulating complexes (ISCOMs)

Saponins are extracted from the bark of a tree, *Quillaja saponaria*. The use of crude extracts of plant matter containing saponins is associated with different adverse effects when used in vaccine (Gupta and Siber 1995). The active adjuvant component, known as Quil A, can be partially purified from saponins using methods like dialysis, ion exchange and gel filtration chromatography. Quil A exhibits higher potency and reduced local reactions compared to saponins (Gupta and Siber 1995). QS21 can be obtained by purification of Quil A and acts by enhancing presentation of antigens to APCs and by inducing CTL production, stimulating both Th1 and Th2 cytokine secretion (Leroux-Roels 2010). Quil A has been previously used in veterinary vaccines (Kersten and Crommelin 2003). The Quil A molecule has three distinct polar areas: hydrophilic-hydrophobic-hydrophilic. The hydrophilic areas are composed up of carbohydrates, whereas quillaic acid forms the hydrophobic region (Kersten and Crommelin 2003).

Saponin mixture Quil A, cholesterol and phospholipids are components of ISCOMs. ISCOMs are spherical in shape and have a size about 40 nm (Kersten and Crommelin 2003). Hydrophobic molecules like glycoproteins from the membranes of viruses and bacteria can be incorporated in ISCOMs during their formation, but it is difficult to incorporate purified hydrophilic protein antigens (Smith et al. 1998). Chemical modifications of proteins by acidification to expose hydrophobic groups and addition of

hydrophobic regions by chemical linkage of palmitic acid tails are two different approaches to overcome the problems of incorporating hydrophilic protein antigens in ISCOMs (Smith et al. 1998). An ISCOM-matrix is similar to an ISCOM, but it does not contain antigen (Sjölander et al. 2001). ISCOMs are effective at low antigen concentrations and adjuvant doses, and lead to the induction of long-lasting humoral and cellular immune responses. In comparison to free form of saponins, incorporation of saponins into ISCOMs results in decreased hemolytic activity (Sjölander et al. 2001). ISCOMs act by inducing the production of high concentrations of long-lasting antibodies and by inducing a balanced Th1 and Th2 immune response with multifunctional T cells and CTLs (Lövgren Bengtsson et al. 2011). Rimmelzwaan et al. demonstrated that cynomolgus macaque monkeys immunized with an ISCOM-based vaccine produced high titers of specific IgM, IgA and IgG serum antibodies as well as high titers of haemagglutination-inhibiting and virus-neutralizing serum antibodies in comparison to monkeys immunized with a classical subunit vaccine (Rimmelzwaan et al. 1997).

Polymeric particles

Synthetic polymers like poly(D,L-lactide-co-glycolide) (PLG), poly(D,L-lactic-coglycolic acid) (PLGA), poly(g-glutamic acid), poly(ethylene glycol) and polystyrene are used in vaccines as adjuvant (Zhao et al. 2014). PLG and PLGA nanoparticles are biocompatible and biodegradable (Zhao et al. 2014). PLGA has the ability to cross-present antigens to CD8⁺ T cells as it can easily reach MHC class I molecules (Apostólico et al. 2016). PLG have been used as suture material in humans and animals for many years. They also have been used for the controlled release of drugs in drug delivery systems (Singh and O'Hagan 2003). A study conducted by Eldridge et al. showed that the delivery of staphylococcal enterotoxin B toxoid via poly(D,L-lactide-co-glycolide) microspheres with sizes from 1 to 10 µm in diameter induced an immune response which was approximately 500 times that shown by nonencapsulated toxoid (Eldridge et al. 1991).

Polysaccharide-based natural polymers like chitosan, inulin, pullulan and alginate are also used as adjuvant (Zhao et al. 2014). Chitosan-based nanoparticles are biodegradable, nontoxic, biocompatible, and their shapes and sizes can be easily modified as desired (Zhao et al. 2014). Chitosan is a mucosal adjuvant. It acts in vitro by translocating the tight junctions of cells which enhances the transepithelial transport of antigens and reduces the mucociliary clearance rate, stimulating phagocytosis of antigens (Apostólico et al. 2016). Inulin is a carbohydrate obtained from the roots of plants that belong to the Compositae family. Micro-particulate inulin (MPI) stimulates

both Th1 and Th2 responses but does not induce IgE. Additionally, it does not cause local or systemic toxicity. Inulin gets metabolized into simple sugars, so it does not have the safety issues that are generally associated with alum-based adjuvants (Aguilar and Rodriguez 2007).

Cytokines

Cytokines are proteins released in response to infection. Signals provided by cytokines help to direct immune response towards either cell-mediated or antibody-mediated response in mammals. Thus, they play an important role to control the immune system (Asif et al. 2004). Different cytokines exert their adjuvant activity through different pathways. IFN- γ up-regulates Th1 responses and enhances MHC expression, IL-2 up-regulates Th1 responses, IL-4 up-regulates Th2 responses, IL-1 stimulates maturation of T and B cells, and IL-12 induces a strong Th1 shift (Cox and Coulter 1997). Granulocyte-macrophage colony stimulating factor (GM-CSF) activates and recruits APCs leading to enhancement of primary immune response. The requirement for multiple doses and its toxicity are limitations for practical application of GM-CSF as an adjuvant (Aguilar and Rodriguez 2007). If a large amount of cytokines enters systemic circulation, adverse effects including severe shock or even death, may occur. Cytokines also stimulate autoimmunity. For example, overdose of IL-2 is associated with autoimmune diseases. Use of cytokine inducers like avridine, gliding bacterial adjuvant (GBA) and MPL are an alternative approaches to reduce the toxicity associated with cytokines (Spickler and Roth 2003).

Virus-like particles (VLPs)

VLPs are viral proteins that spontaneously form particles resembling virions. They do not contain viral genes, but retain the authentic conformation of the virus capsid proteins as seen in attenuated and inactivated viral vaccines (Young et al. 2006). Since they mimic the structure of the virus, lower doses of antigen, compared to subunit vaccines, are enough to stimulate a similar protective response (Noad and Roy 2003). Depending on the parental virus, the structures of VLPs can be either enveloped or non-enveloped. Enveloped VLPs contain a host cell membrane (an envelope) and the antigen of interest, whereas non-enveloped VLPs contain only pathogen components with self-assembling capacity (Apostólico et al. 2016). VLPs can directly activate B cells. Additionally, they can stimulate and be taken up by dendritic cells (DCs) resulting in a T cell response. Furthermore, they can induce cross-presentation to CD8⁺ T cells (Apostólico et al. 2016). VLPs are unable to replicate so they are considered safe. They are

alternatives to live, attenuated and inactivated vaccination approaches (Trovato and De Berardinis 2015). Because of their polyvalent structure, they can accommodate multiple copies of antigens. They also can ensure tissues-specific targeting, which depends on the origin of virus (Chroboczek et al. 2014). In a recent study, Fontana et al. demonstrated the ability of rabies virus-like particles (RV-VLPs) to stimulate the production of rabies virus neutralizing antibodies and suggested that RV-VLPs are good rabies vaccine candidate (Fontana et al. 2015).

Bacterial derivatives

Bacterial toxins like CT and *E. coli* heat-labile enterotoxin (LT) are good mucosal adjuvants in experimental animals (Simmons et al. 1999). CT is a major enterotoxin produced by *Vibrio cholera*. It induces a strong Th2 response and suppresses Th1 responses (Lee et al. 2009). Adjuvant properties of CT and LT include the capacity to promote antigen uptake through greater gut permeability, antigen presentation by APCs, and induction of cytokines that lead to immunoglobulin class switching. LT is considered to be safer adjuvant for mucosal vaccination as it is 10-fold less toxic than CT in animals (Katz et al. 1997). To reduce the enterotoxicity of native LT, double mutant LT (dmLT) has been developed (Leach et al. 2012) and is a powerful and safe detoxified enterotoxin that can act as mucosal adjuvant for coadministered antigens (Norton et al. 2011). In different mouse models, dmLT increased immune responses to whole-cell vaccines for enterotoxigenic *E. coli*, *Helicobacter pylori*, and *Streptococcus pneumoniae* (Leach et al. 2012). Ottsjö et al. suggested that dmLT, as an adjuvant, could be included in the mucosal vaccine for *H. pylori* infection (Ottsjö et al. 2013). In another study conducted by Norton et al., incorporation of dmLT in inactivated poliovirus vaccine (IPV) immunizations increased mucosal immunity and the longevity of anti-poliovirus responses (Norton et al. 2015).

Bacterial DNA-based molecules also exhibit immunostimulatory properties. CpG motifs are six base-long DNA sequences, which contain a central CpG dinucleotide. They occur twenty times more within bacterial DNA than within mammalian DNA. In mammalian cells, they are recognized by TLR9 which leads to secretion of type I IFNs (α and β) and IL-12 by cells of the innate immune system, stimulating a Th1 response (Aguilar and Rodriguez 2007).

LPS is also a good adjuvant. Chemically, it has three principal regions. Among them, the Lipid A region functions as both as a mitogen and adjuvant (Skidmore et al. 1975). MPL is a detoxified derivative of LPS obtained from Gram-negative bacteria (*Salmonella minnesota* R595) (Apostólico et al. 2016). MPL induces IL-12 and IFN- γ

production (Apostólico et al. 2016) and stimulates Th1 responses (Apostólico et al. 2016; Cox and Coulter 1997).

GBA is a polymer of amino sugars, which is obtained from the bacterial genus *Cytophaga*. It is a safe and effective adjuvant that can stimulate the release of cytokines in cats, mice and humans (Spickler and Roth 2003).

Muramyl dipeptide (MDP) is an active component of peptidoglycan obtained from mycobacteria (Spickler and Roth 2003). MDP and its different derivatives can induce IL-1 production. Lipophilic derivatives of MDP stimulate Th1 responses, whereas hydrophilic derivatives of MDP stimulate Th2 responses (Cox and Coulter 1997).

Current uses of licensed adjuvants

Alum is widely used and it has been approved in the United States (Mbow et al. 2010). Alum-based vaccines were licensed more than 70 years ago (O'Hagan 2007). In 1997, an influenza vaccine containing an alternative adjuvant known as MF59 was successfully launched in the European market (O'Hagan 2007). In addition, AS04, a combination adjuvant containing MPL adsorbed to alum, has been approved in Europe and licensed in the United States (Mbow et al. 2010). Additionally, Montanide ISA51 is used as an adjuvant in a non-small-cell lung cancer (NSCLC) vaccine, which has been registered in Cuba and Chile (Garçon et al. 2011). Examples of licensed adjuvants with their applications are summarized in Table 2.

Obstacles in adjuvant development

Adjuvanted vaccines show higher reactogenicity at the injection site in comparison to non-adjuvanted vaccines (Leroux-Roels 2010). Thus, adjuvants not only increase the immunogenic responses of antigens, but also can lead to adverse reactions. Local inflammation at the injection site, and granuloma and sterile abscess formation are local adverse reactions associated with adjuvants. Systemic reactions like malaise, fever, adjuvant arthritis and anterior uveitis are also seen in laboratory animals (Vogel 2000).

Although many adjuvant candidates are available, very few of them are licensed and used as successful adjuvants. Safety is the major reason for failure of adjuvants. Generally, short-term safety issues impede the development of adjuvants. Intermediate and long-term safety issues are more difficult to overcome (O'Hagan and De Gregorio 2009). Thus, the field of adjuvant development should not only identify more inflammatory adjuvants, but also should focus on new methods to separate the potency of adjuvants from their reactogenicity (Petrovsky 2013).

Moreover, because of the complex nature of the immune system, there is no in vitro assay that can completely simulate in vivo immune responses. Thus, most preclinical studies are done in animal models. Such studies can provide some information regarding the potency and safety of vaccines, but may not guarantee that similar effects will be observed in humans or veterinary target animals (Schijns and Lavelle 2011).

Table 2 Licensed adjuvants

Adjuvant	Vaccine	References
Alum	Hepatitis A, Hepatitis B, diphtheria/tetanus/pertussis (DTP), human papillomavirus, <i>Haemophilus influenza</i> type B, <i>Pneumococcus</i>	Apostólico et al. (2016)
Virosomes	Influenza, Hepatitis A	Leroux-Roels (2010)
MF59	Influenza (H1N1, H5N1, seasonal)	Garçon et al. (2011)
AS04	Hepatitis B, human papillomavirus	Apostólico et al. (2016)
AS03	Influenza (H5N1, H1N1)	Garçon et al. (2011)
Thermo-reversible oil-in-water emulsion	Influenza (H1N1)	Garçon et al. (2011)
Montanide ISA51 ^a	Cancer	Garçon et al. (2011)

^aMontanide ISA51 is used in cancer vaccine for immunotherapy

Conclusions

Adjuvants enhance the immunogenic responses of antigens and amplify the potency of vaccines. Modes of action include depot formation, recruitment of immune cells, activation of the inflammasome, enhancement of antigen presentation by MHC molecules, and immunomodulation. Advances in immunology have shifted our understanding of the ways through which adjuvants act. This new perspective has led to discovery of novel adjuvants, though only few are considered safe and are currently used in human and animal vaccines. Therefore, more knowledge regarding the side effects and toxicity of adjuvants is essential for the development of safe and effective vaccines. Mineral salts, oil-based emulsions, liposomes, virosomes, niosomes, saponins, ISCOMs, polymeric particles, cytokines, VLPs and bacterial derivatives are different types of adjuvants that are being used or can possibly be used in vaccines for preventing diseases. Therefore, further studies are required to overcome the hurdles of developing novel adjuvants that can adequately boost the immune system without causing side effects and toxicity.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflicts of interest.

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