# Recent Advances of Vaccine Adjuvants for Infectious Diseases

*Sujin Lee[1,2]\* and Minh Trang Nguyen[1,2]*

[1]Department of Pediatrics, Emory University, School of Medicine, [2]Children's Healthcare of Atlanta, Atlanta, Georgia 30322, USA

Vaccines are the most effective and cost-efficient method for preventing diseases caused by infectious pathogens. Despite the great success of vaccines, development of safe and strong vaccines is still required for emerging new pathogens, re-emerging old pathogens, and in order to improve the inadequate protection conferred by existing vaccines. One of the most important strategies for the development of effective new vaccines is the selection and usage of a suitable adjuvant. Immunologic adjuvants are essential for enhancing vaccine potency by improvement of the humoral and/or cell-mediated immune response to vaccine antigens. Thus, formulation of vaccines with appropriate adjuvants is an attractive approach towards eliciting protective and long-lasting immunity in humans. However, only a limited number of adjuvants is licensed for human vaccines due to concerns about safety and toxicity. We summarize current knowledge about the potential benefits of adjuvants, the characteristics of adjuvants and the mechanisms of adjuvants in human vaccines. Adjuvants have diverse modes of action and should be selected for use on the basis of the type of immune response that is desired for a particular vaccine. Better understanding of current adjuvants will help exploring new adjuvant formulations and facilitate rational design of vaccines against infectious diseases.

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## VACCINES

Infectious diseases remain the second leading cause of death worldwide after cardiovascular disease, but the leading cause of death in infants and children (1). Vaccination is the most efficient tool for preventing a variety of infectious diseases. The ultimate goal of vaccination is to generate a pathogen-specific immune response providing long-lasting protection against infection (2). Despite the significant success of vaccines, development of safe and strong vaccines is still required due to the emergence of new pathogens, re-emergence of old pathogens and suboptimal protection conferred by existing vaccines. Recent important emerging or re-emerging diseases were severe acute respiratory syndrome (SARS) in 2003, the 2009 H1N1 influenza pandemic, and Ebola virus in 2014 (3). Last year, the most widespread epidemic of Ebola virus caused significant mortality in several West African countries (4). As a result, we are aware of pursuing a new approach towards the rapid development of vaccines against emerging diseases.

Three different types of vaccine are currently used in humans: live-attenuated vaccines, inactivated vaccines and subunit vaccines (5). Many of the most effective vaccines in use are live-attenuated vaccines. As an attenuated vaccine is composed of a virus or bacterium that can replicate within the host, this type of vaccine elicits robust humoral and cell-mediated immunity (CMI). Examples of live-attenuated vaccine include MMR (Measles, Mumps, Rubella), chicken pox, oral polio (Sabin), influenza (the seasonal flu nasal spray and the

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\*Corresponding Author. Sujin Lee, Department of Pediatrics, Emory Children's Center, Emory University, 2015 Uppergate Dr. NE Rm 512, Atlanta, GA 30322, United States of America. Tel: 404-712-1637; Fax: 404-727-9223; E-mail: Sujin.lee@emory.edu

Abbreviations: CMI, cell-mediated immunity; DC, dendritic cell; IRIV, immunopotentiating reconstituted influenza virosomes; PRR, pattern recognition receptors; ODN, oligodeoxynucleotides; poly I:C, polyriboinosinic acid-polyribocytidylic acid; ISCOM, immune stimulating complexes

2009 H1N1 nasal spray), rotavirus and yellow fever vaccine. Inactivated (killed) vaccines (e.g. inactivated polio - Salk, Hepatitis A) are either heat-inactivated or chemically inactivated particles of the pathogen. Although these vaccines are safe and non-infectious, they stimulate only weak, short-lived and often insufficient immunity. Thus, large and multiple doses of inactivated vaccine are required to confer protective immunity (6). In contrast to live-attenuated vaccines, inactivated vaccines elicit mainly humoral immunity, with little to no induction of CMI.

Purified or recombinant subunit vaccines derived from non-living vaccine antigens are poorly immunogenic and require the addition of some components to help stimulate protective immunity. In some cases, these vaccines utilize epitopes recognized and bound by antibodies or T-cells. Because subunit vaccines contain only an essential part of the antigen instead of the entire microbe, the chances of adverse reactions to the vaccine are relatively low (7). Subunit vaccines have been made for hepatitis B virus (HBV), influenza virus (injection) and pertussis (part of DTaP combined immunization). Recently developed subunit vaccines are less immunogenic and reactogenic than traditional vaccines such as live-attenuated, and inactivated vaccines. Thus, repeated boost immunizations or the addition of adjuvant are necessary to enhance the efficacy of subunit vaccines.

## NEED FOR ADJUVANTS

The word "adjuvant" is derived from the Latin *adjuvare*, meaning "to help" or "to aid". Adjuvants have been defined as agents added to vaccine formulations that enhance the immunogenicity of antigens and induce protection against infection. Vaccines made from live-attenuated or inactivated pathogens can elicit robust protective immune responses because those vaccines contain naturally occurring adjuvants. In contrast, protein-based vaccines in most cases have limited immunogenicity although they have some advantages in terms of safety and cost-effectiveness. Thus, adjuvants are necessary to help these proteins become effective vaccines by inducing strong and long-lasting protective immune responses. Indeed, some protein-based vaccines have been successfully developed in use for human vaccines by mixing with aluminium salts (alum). However, new vaccine targets will require not only strong antibody responses but also robust CMI including T helper (Th) cells and cytotoxic T lymphocytes (CTL). Alum alone will be insufficient for such cases

because it is a poor inducer of T cell responses. The use of appropriate adjuvants will allow for vaccine formulations that selectively trigger innate immunity and/or adaptive immunity to obtain a desired type of antigen-specific immune responses. We also describe the practical and functional reasons for why adjuvants are needed as a component in vaccines in Table I.

## ADJUVANTS APPROVED FOR HUMAN VACCINES

Licensed adjuvants in use for human vaccines are listed in Table II.

### Aluminium Salts (Alum)
In 1926, Glenny et al. reported the adjuvant activity of aluminium compounds utilizing a suspension of alum-precipitated diphtheria toxoid (DT) (8). Aluminium salts are the most widely used adjuvants in human vaccines. These adjuvants have been used in practical vaccination for more than 80 years and are generally considered stimulators of Th2 immunity (9,10). Until 2009 aluminium salt (referred to as "alum") adjuvants were the only ones contained in vaccines licensed for human use in the United States. Alum is a component of licensed human vaccines such as Hepatitis A virus (HAV), Hepatitis B virus (HBV), human papilloma virus (HPV), diphtheria, tetanus, Haemophilus Influenzae Type b (Hib) and meningococcal. Although there are a number of adjuvants more potent than alum, they have not been used for human vaccine formulations due to high levels of toxicity. Surprisingly, despite the wide use of alum adjuvants in licensed human vaccines, the mechanisms of action are not well characterized. The most well-known mechanism of action of alum is the "depot effect", first proposed by Glenny in 1925, whereby depot formation was cited to facilitate con-

**Table I.** The benefits of adjuvants

1. Decrease the dose of antigen needed (dose sparing)
2. Decrease the number of vaccine doses needed
3. Enhance vaccine efficacy in infants, the elderly and immunocompromised people
4. Increase functional antibody titer
5. Induce more rapid and long-lasting immune responses
6. Induce robust cell-mediated immunity
7. Provide broad protection (cross-reactivity)
8. Facilitate mucosal immunity
9. Overcome antigen competition in combination vaccines

**Table II.** Licensed vaccine adjuvants

| Adjuvant name (year licensed) | Class | Manufacturer | Description | References |
|---|---|---|---|---|
| Alum (1926) | Mineral Salt | Various | Improves humoral immune responses and antigen stability. Antigens are adsorbed to the surface. The adjuvant in > 80 % of vaccines licensed for human use. Th2 type immune responses. | (44, 45) |
| MF59 (1997) | Oil-in-water emulsion | Novartis | Improves humoral and cell-mediated immunity. Used in influenza vaccines. Antigen delivery. | (21, 46) |
| AS03 (2009) | Oil-in-water emulsion | GSK | Improves humoral and cell-mediated immunity. Used in influenza vaccine during 2009 H1N1 pandemic. | (47, 48) |
| Virosome (2000) | Liposome | Berna Biotech (Crucell) | Improves humoral and cell-mediated immunity. A virosome is the reconstituted membrane of an enveloped virus. The vaccines for influenza and for Hepatitis A are approved products | (49, 50) |
| AS04 (2005) | Alum-adsorbed TLR4 agonist | GSK | Improves humoral and cell-mediated immunity. Combination of aluminum adjuvant with monophosphoryl lipid A (MPL) co-adsorbed. Used for HPV and HBV vaccine. | (30, 51) |

tinuous antigen release from the injection site (11). Even though depot formation still remains somewhat controversial, recent studies have clearly demonstrated that depot formation is not required for alum adjuvanticity (12-14). Alum has been shown to facilitate humoral immunity via Th2 type immune responses (IgG1, IgE, IL-4, IL-5 and eosinophil) (10,15). The advantages of alum are high safety record, antigen stabilization and augmentation of high and long-lasting antibody titer. However, alum does not have the ability to elicit Th1 type immunity or cytotoxic T cell responses and vaccines containing alum adjuvant cannot be sterilized by filtration, frozen or lyophilized (16)

## Oil-in-water (o/w) emulsions: MF59 and adjuvant system 03 (AS03)

Emulsions are unstable two-phase systems consisting of at least two immiscible liquids, combined with a surfactant for stabilization. The major benefits of using emulsions are antigen dose sparing and enhancement of antibody titer. Both MF59 (Novartis) and AS03 (GlaxoSmithKline) are squalene based oil-in-water emulsions (5,17). MF59 has been approved for the H5N1 pandemic influenza vaccine (Fluad) and also for the H1N1 influenza vaccine (Focetria and Celtura) in Europe (18,19). It recruits monocytes and macrophages into injection sites by the induction of local chemokine secretion (17). MF59 can also augment antigen uptake by dendritic cells (DCs) and activate CD4 T cells (20). As a result, MF59

generates high antibody titers with balanced IgG1:IgG2a responses. MF59 has been evaluated in conjunction with Herpes simplex virus (HSV), Human immunodeficiency virus (HIV), HBV and Cytomegalovirus (CMV) vaccine trials (21).

AS03 is included in licensed H5N1 and H1N1 pandemic influenza vaccines. Although both MF59 and AS03 contain squalene oil, they have different compositions. AS03 contains $\alpha$-tocopherol. Moral et al. demonstrated that AS03 induced a non-specific activation of the immune system in mice in the presence of $\alpha$-tocopherol (22). Unfortunately, recent studies have reported a possible association between narcolepsy and the use of AS03 adjuvanted H1N1 influenza vaccine (17,22).

Although oil-in-water emulsions seem to be very effective and promising adjuvants, further detailed characterization and analysis of components used in emulsion preparations need to be examined.

## Virosomes

A virosome is a reconstituted viral envelope possessing membrane lipids and viral glycoproteins, but devoid of viral genetic information (23). The virosome vaccine for influenza virus (Inflexal V) is approved in Europe and Hepatitis A virus (Epaxal) vaccine is approved in Asia, Europe and South America (5). Both vaccines utilize virosomes derived from influenza virus represented by Immunopotentiating reconstituted influenza virosomes (IRIV) harboring the influenza hemagglutinin (HA) protein (24). Inflexal V is the only viroso-

mal adjuvanted influenza vaccine licensed for all age groups including children, adults and the elderly. As virosomal adjuvants present antigen via both major histocompability complex (MHC) I and MHC II, virosomes are able to induce both humoral immunity and CMI (25,26). Major advantages of using virosomes in vaccines are: 1) high quality and long-lasting antibody responses, 2) conformational stabilization of antigen, 3) protection of antigen from degradation, 4) excellent safety profile, 5) suitability to specific populations such as infants, immunocompromised patients, and the elderly (5).

## Monophosphoryl lipid A (MPL) and adjuvant system 04 (AS04)

Toll-like receptors (TLR) are transmembrane signaling proteins, comprising a family of pattern recognition receptors (PRR) (27). TLR agonists, the natural ligands which activate TLRs, are immunostimulatory adjuvants. Advances in the design of efficient adjuvants based on the use of TLR agonists have been promising and some of these adjuvants have already been licensed for human vaccines. MPL, a TLR4 agonist, is a chemically detoxified derivative of the parent lipopolysaccharide (LPS) from *Salmonella Minnesota* R595 strain (28). MPL increases the production of pro-inflammatory cytokines such as IL-2 and IFN-$\gamma$, resulting in the generation of Th1 immune responses (29). AS04 is composed of MPL adsorbed to aluminium salts (30). Two AS04-adjuvanted vaccines are licensed for human use: the HPV vaccine (Cervarix) and HBV vaccine (Fendrix) for haemodialised patients (31,32). Since MPL still retains the ability to activate innate immunity by interaction with TLR4, it leads to activation of NF-$\kappa$B signaling and production of pro-inflammatory cyto-

kines and chemokines. Subsequently, chemokines such as CCL2 and CCL3 recruit monocytes and macrophages, and activate dendritic cells (DCs) at the injection site (33). Mature DCs that have migrated to the draining lymph node can interact with T-cells to stimulate CMI. A benefit of using AS04 adjuvant in human vaccines is the effective induction of robust Th1-type immune responses by promoting IL-2 and IFN-$\gamma$ production, which cannot be achieved by using alum alone. A recent study showed that the antigen and AS04 should be co-localized in lymph nodes in order to elicit an adjuvant effect on antigen presenting cells (33).

## ADJUVANTS IN CLINICAL DEVELOPMENT

Table III summarizes a subset of the adjuvants that have been tested in human clinical trials. All adjuvants listed in Table III are known as "immunostimulators" or "immune potentiators".

### TLR agonists

TLRs provide a bridge between innate and adaptive immunity. A new class of effective vaccine adjuvant is based on the TLR pathway. Here, we will focus on TLR 3, 5 and 9 which are in clinical trials of vaccines against infectious pathogens. TLR 9 is one of the more advanced adjuvant candidates among TLR agonists (34). Unmethylated CpG oligodeoxynucleotides (ODN), a type of TLR 9 agonist, enhance antigen-specific immune responses and induce proinflammatory cytokines such as TNF-$\alpha$, IL-1, IL-6 and IFN-$\gamma$. CpG ODN are an example of immunostimmulatory sequences (ISS) currently being evaluated for HBV vaccine (HEPLISAV-B, Dynavax) (35).

**Table III.** Classes of clinically tested vaccine adjuvants

| Adjuvant name | Class | Description | Clinical phase |
|---|---|---|---|
| CpG | TLR 9 agonist | Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. CpG oligonucleotides. | Phase 3 |
| Flagellin | TLR 5 agonist | Enhances antibody titer, Th1 and Th2 type immunity. Flagellin linked to antigen. | Phase 1 |
| PolyI:C | TLR3 agonist | Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. Double-stranded RNA analogues | Phase 1 |
| AS01 | Combination | Enhances antibody titer, Th1 type immunity and CD8 T cell-mediated immunity. Combined with MPL, QS21 and liposomes. | Phase 3 |
| AS02 | Combination | Enhances antibody titer and Th1 type immunity. Combined with MPL, QS21 and emulsion. | Phase 3 |
| ISCOMs and ISCOMMATRIX | Combination | Enhances antibody titer, Th1 and Th2 type immunity and CD8 T cell-mediated immunity. Combined with saponin and phospholipid. | Phase 2 |

Polyriboinosinic acid-polyribocytidylic acid (poly I:C) mimics viral dsRNA and is a promising candidate for a vaccine adjuvant against intracellular pathogens. Poly I:C binds to TLR3 and enhances robust CMI and potent type I interferon response. However, the major draw-back of stability and toxicity issues need to be addressed before proceeding to clinical application of dsRNAs. Recently, a clinically safe dsRNA, PolyI:C analogue (Ampligen), was investigated as an adjuvant for intranasal H5N1 Influenza virus vaccines (36). Bacterial flagellin, a TLR 5 agonist, is a known immunostimulator that induces high antibody titer, and mixed Th1 and Th2 type immune responses. The D1 portion of flagellin binds to TLR 5 and can be expressed in a fusion protein with selected vaccine antigens. Due to this characteristic of flagellin, a major advantage of the TLR5-dependent adjuvant is that a fusion protein can co-deliver antigen and TLR5 agonist to the APC (37). Thus, flagellin fusion proteins are suitable adjuvants for the development of vaccines to induce robust antigen-specific immune responses. Indeed, a flagellin/ hemagglutinin-based vaccine (VAX128) and a flagellin/matrix protein 2 ectodomain (M2e) vaccine (VAX102) are in clinical trials of vaccines against influenza (38, 39). Although further studies in humans are required, it appears that TLR agonists may be attractive candidates for use in human vaccines.

## Immune stimulating complexes (ISCOM)

ISCOMs are another promising lipid-based adjuvant formation. ISCOMs are spherical and ring-like structures spontaneously formed upon mixing antigens with saponin, cholesterol and phospholipid (40). The compound QS-21, a potent immunostimulatory saponin, was extensively studied as an adjuvant in various vaccines, though it has not yet been approved for human vaccine use due to the toxicity of QS-21. Since ISCOM allows for the reduction in QS-21 dose, it is being considered as a new approach to overcome the issue of toxicity. The second type of ISCOM is called ISCOMMATRIX, which doesn't contain antigen. The major advantage of ISCOM and ISCOMMATRIX is their exceptional stability owing to the high affinity between saponin and cholesterol, therefore allowing them to be effective adjuvants for mucosal vaccines (41). Main benefits of these adjuvants are induction of high and long-lasting antibody titer, induction of balanced Th1 and Th2 type immunity, and induction of CMI including cytotoxic T cell response (42). The adjuvant properties of ISCOM and ISCOMMATRIX are currently being evaluated in clinical trials of influenza, HCV and HPV. Collectively, usage of ISCOM and ISCOMMATRIX as adjuvants could be an alternative approach in vaccine development against infectious pathogens.

## Adjuvant systems (AS)

Adjuvant systems (GSK) refer to various combinations of classical adjuvants such as aluminium salts, o/w emulsions, liposomes and immunostimulators designed to adjust the adaptive immune responses against pathogens (30). The challenge for this strategy is to define the best combination for an effective and safe formulation in which individual components can synergize with one another to elicit a more robust immune response. As described in Table II, AS03 and AS04 have been approved as adjuvants in several human vaccines. Here, we will discuss AS01 and AS02, which are in recent development. AS02 is identical to AS03 ($\alpha$-tocopherol+squalene) with the addition of MPL and QS21. While AS03 induces biased Th2 type immune responses, AS02 induces high antibody titer and dominant Th1 type immune responses owing to the addition of MPL. Although some local and systemic reactogenicity has been reported, AS02 is in clinical trials for various vaccine applications, including malaria, HBV, HPV, tuberculosis, and HIV (15). AS01 combines three components such as liposomes, MPL and QS21 (15). Unlike AS02, AS01 was designed to improve CD8 T cell responses. AS01 induces robust Th1 type immune responses, enhances antigen presentation to APC, and induces high antibody titer. Recent studies demonstrated that an AS01 malaria vaccine induces increased IgG titers and polyfunctional CD4 T cells expressing IL-2, IFN-$\gamma$, TNF-$\alpha$, or CD40L (43). Several clinical trials are in progress with AS01-containing vaccine candidates against infectious pathogens, including HIV, tuberculosis and malaria.

## CONCLUSIONS AND PERSPECTIVES

The ultimate goal of vaccination is to generate potent and long-term protection against diseases. Such protective immunity can be elicited by using vaccine formulations containing appropriate antigens and adjuvants. Adjuvants are important components of vaccines and can affect the outcomes of vaccination. Past approaches of vaccine formulation with adjuvants were focused on single-type adjuvants such as alum or emulsions. However, new vaccine targets require the induction of well-defined CMI in addition to high titer of antibody. Consequently, new immunostimulant adjuvants in vaccine formulations are needed in order to stimulate robust

immune responses including humoral immunity and CMI. As great progress has been made in the field of adjuvant research over last two decades, vaccinologists are now able to select an appropriate adjuvant from classical adjuvants, immunostimulants or combinations thereof to enhance vaccine efficacy. Taken together, recent successful clinical studies conducted with new adjuvants suggest that a panel of novel immunostimulant adjuvants will be utilized for human vaccine formulations in a near future. The availability of these adjuvants in various combinations will facilitate the rational design of vaccines against infectious diseases.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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