

# New horizons in adjuvants for vaccine development

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**Over the last decade, there has been a flurry of research on adjuvants for vaccines, and several novel adjuvants are now in licensed products or in late stage clinical development. The success of adjuvants in enhancing the immune response to recombinant antigens has led many researchers to re-focus their vaccine development programs. Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunogenic vaccines. For the majority of vaccine researchers this information is not readily available, nor is access to well-characterized adjuvants. In this review, we outline the current state of adjuvant research and development and how formulation parameters can influence the effectiveness of adjuvants.**

## Introduction

Adjuvants are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long lasting immune effects on their own [1]. The addition of adjuvants to vaccines enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines in newborns, elderly or immuno-compromised individuals [2]. Adjuvants have limited or no efficacy unless properly formulated, therefore both adjuvant components and formulation (e.g. oil in water, particle size, charge, etc.) are crucial for enhancing vaccine potency.

Traditional live vaccines based on attenuated pathogens typically do not require the addition of adjuvants. Likewise, vaccines based on inactivated viruses or bacteria are often sufficiently immunogenic without added adjuvants, although some of these (e.g. split flu virus, Hepatitis A virus or whole cell *Pertussis*) can be formulated with adjuvants to further enhance the immune responses. By contrast, protein-based vaccines, although offering considerable advantages over traditional vaccines in terms of safety and cost of production, in most cases have limited immunogenicity and require the addition of adjuvants to induce a protective and long-lasting immune response. Although some recombinant protein-based vaccines, including those for Hepatitis B and human papilloma virus, have been successfully developed to elicit protective

antibody responses using only aluminum salts (Alum) as adjuvant, the next generation of recombinant vaccines, aimed at diseases such as malaria, tuberculosis and HIV and/or AIDS, will require not only very strong and long-lasting antibody responses but also potent cell mediated immunity based on CD4 and CD8 T-cell responses. Alum will be insufficient to trigger such immunity because it is a poor inducer of T-cell responses, and novel adjuvants and formulations will be required.

Recent advances have begun to shed light on the cellular and molecular nature of innate immunity and adjuvant activity [3]. The immune system recognizes pathogen-associated molecular patterns (PAMPs) by means of pathogen-recognition receptors (PRRs), which include the Toll-like receptors (TLRs) [4] (Figure 1), C-type lectin-like receptors [5], cytosolic nucleotide oligomerization domain-like receptors [6] and retinoic acid inducible gene-based-I-like receptors [7,8]. These receptors bind microbial ligands (including cell wall components, lipoproteins, proteins, lipopolysaccharides, DNA and RNA of bacteria, viruses, protozoa and fungi) to trigger different types of immune responses [9,10] (Table 1). These PAMPs, specifically those binding the TLRs, are the basis of many adjuvants [11]. In addition, cytokines, bacterial toxins and glycolipids that alter antigen processing are being used in adjuvants to elicit immune responses (Table 1). Effective adjuvants and adjuvant formulations utilize multiple compounds and mechanisms to achieve the desired immunological enhancement [12]. These mechanisms include the generation of long lasting antigen depots, increased immunological presentation of vaccine antigens by dendritic cells (DC) activated through the engagement of PRR or damage-associated molecular pattern (DAMP) receptors (danger or signal 0) [13] and induction of CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) responses and/or CD4<sup>+</sup> T-helper (Th) lymphocyte responses (Th1 or Th2) [14] (Figure 2).

Adjuvants can be classified according to their component sources, physiochemical properties or mechanisms of action. Two classes of adjuvants commonly found in modern vaccines include:

- Immunostimulants (Table 1) that directly act on the immune system to increase responses to antigens. Examples include: TLR ligands, cytokines, saponins and bacterial exotoxins that stimulate immune responses.
- Vehicles (Table 2) that present vaccine antigens to the immune system in an optimal manner, including controlled release and depot delivery systems to

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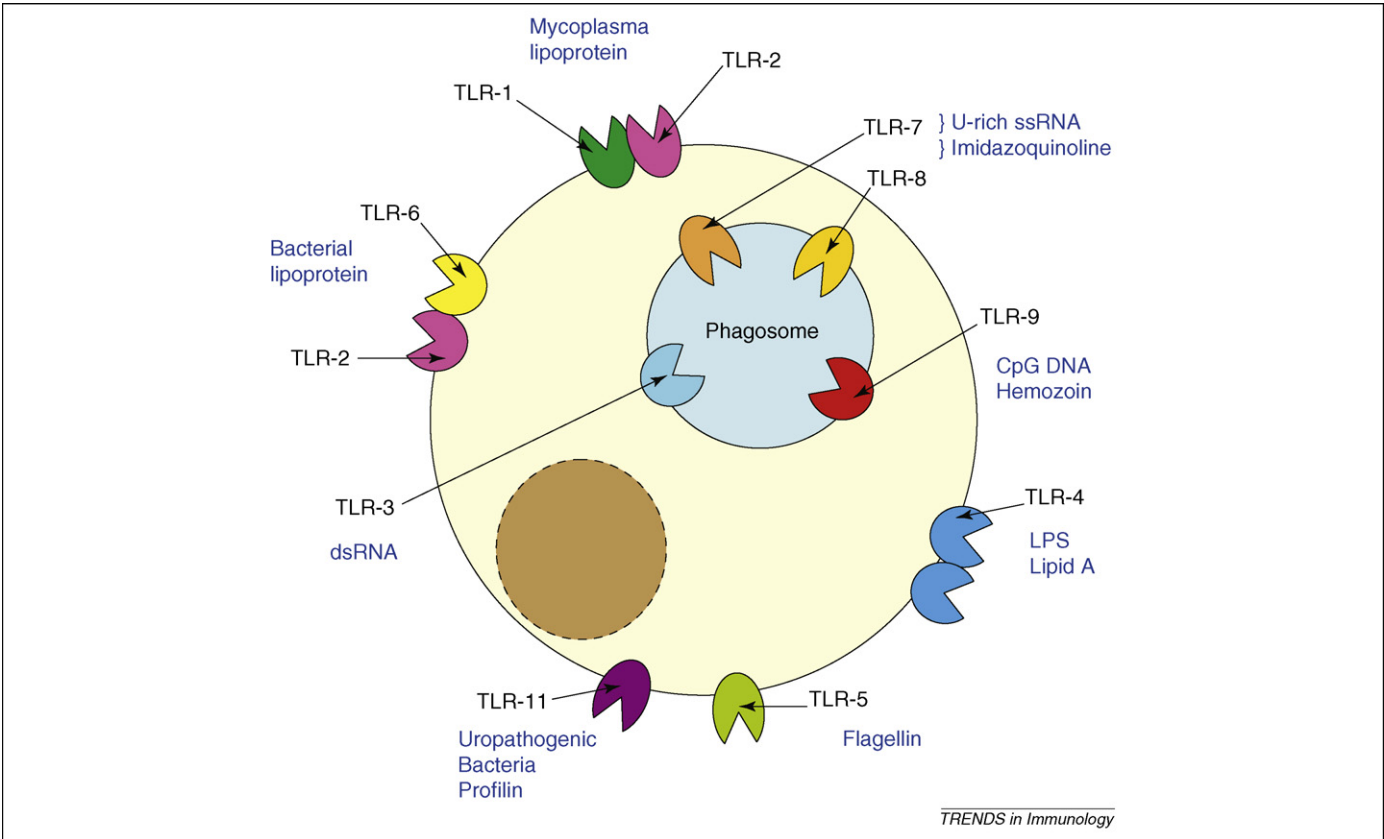


**Figure 1.** TLRs and their ligands. TLRs are present as monomers or heterodimers on the surfaces of certain cells (e.g. TLR 1,2,4,5,6,10,11) or within phagolysosomes (TLR 3,7,8,9) in which they bind a wide variety of microbial components.

increase the specific immune response to the antigen. The vehicle can also serve to deliver the immunostimulants described in the previous point. Examples include: mineral salts, emulsions, liposomes, virosomes (nanoparticles made of viral proteins such as influenza hemagglutinin and phospholipids), biodegradable polymer microspheres and so-called immune stimulating complexes (i.e. ISCOM, ISCOMATRIX<sup>TM</sup>).

**The importance of adjuvant formulation**

Adjuvants must be appropriately formulated for stability and maximum effect. Criteria involved in selecting the formulation for a given vaccine include the nature of the antigenic components, type of immune response desired, preferred route of delivery, avoidance of considerable

adverse effects and stability of the vaccine. The optimally formulated adjuvant will be safe, stable before administration, readily biodegraded and eliminated, able to promote an antigen specific immune response and inexpensive to produce. Furthermore, the ideally formulated adjuvant will be well-defined chemically and physically to facilitate quality control that will ensure reproducible manufacturing and activity.

The importance of formulation can be illustrated with the glycolipid monophosphoryl lipid A (MPL<sup>®</sup>), the first TLR ligand and biological adjuvant approved for human use (i.e. the Hepatitis B vaccine Fendrix<sup>®</sup>). Unformulated MPL<sup>®</sup> is insoluble and prone to aggregation, which adversely affects its bioavailability. Formulations that enhance its solubility, enhance its efficacy and reliability include aqueous phospholipids (MPL<sup>®</sup>-AF) or combining it

**Table 1. Immune responses triggered by immunostimulants**

| Immunostimulant                                                                                          | Cellular interaction     | Type of immune response                  |
|----------------------------------------------------------------------------------------------------------|--------------------------|------------------------------------------|
| <b>TLR ligands</b>                                                                                       |                          |                                          |
| Bacterial lipopeptide, lipoprotein and lipoteichoic acid; mycobacterial lipoglycan; yeast zymosan, porin | TLR-2, 1/2, 2/6          | Th1, antibody (Ab), NK cell              |
| Viral double stranded RNA                                                                                | TLR-3                    | NK cell                                  |
| Lipopolysaccharide, Lipid A, monophosphoryl lipid A (MPL <sup>®</sup> ), AGPs                            | TLR-4                    | Strong Th1, Ab                           |
| Flagellin                                                                                                | TLR-5                    | Th1, CTL, Ab                             |
| Viral single stranded RNA, imidazoquinolines                                                             | TLR-7/8                  | Strong Th1, CTL                          |
| Bacterial DNA, CpG DNA, hemozoin                                                                         | TLR-9                    | Strong Th1, CTL and Ab; NK cell          |
| Uropathogenic bacteria, protozoan profilin                                                               | TLR-11                   | Th1                                      |
| <b>Other</b>                                                                                             |                          |                                          |
| Saponins (Quil-A, QS-21, Tomatine, ISCOM, ISCOMATRIX <sup>TM</sup> )                                     | Antigen processing       | Strong Th1, CTL and Ab; long term memory |
| Cytokines: GM-CSF, IL-2, IFN- $\gamma$ , Flt-3.                                                          | Cytokine receptors       | Th1, Ab                                  |
| Bacterial toxins (CT, LT)                                                                                | ADP ribosylating factors | Ab                                       |

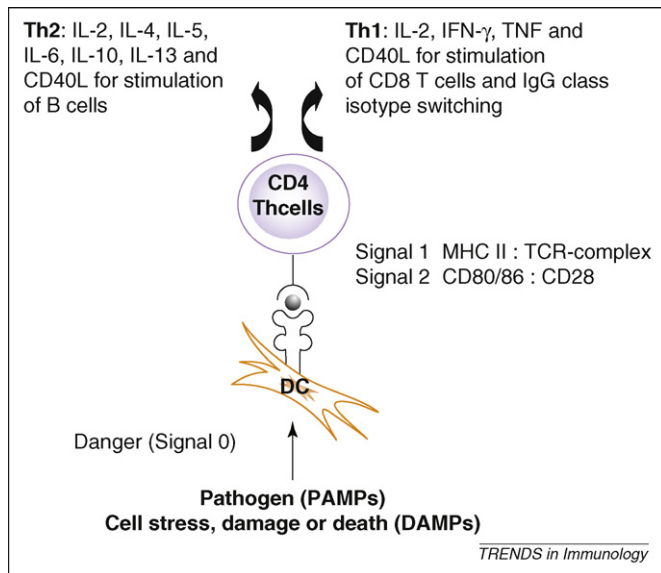


**Figure 2.** CD4 helper T-cell priming. Schematic overview of the major events and signaling during antigen presentation and Th priming. DC, present in all tissues in a non-immunostimulatory state, gather antigens from the local environment. Encounter of PAMPs (stranger model) and/or DAMPs (danger model) induces DC migration to draining lymph nodes, maturation characterized by enhanced presentation of antigenic peptides on MHC I and II molecules (signal 1) and expression of co-stimulatory molecules CD80, CD86 and CD40 (signal 2). Activation of CD4<sup>+</sup> helper T cells results in their secretion of cytokines and chemokines, which can directly affect pathogen survival, or the Th cells can further support the activation of CD8<sup>+</sup> T cells and/or antibody-producing B cells.

with Alum (AS04; GSK Biologicals). Although MPL<sup>®</sup> in aqueous formulation enhances antibody responses, MPL<sup>®</sup> in oil formulation stimulates T-cell responses [15,16]. Moreover, formulations that generate defined structures, such as liposomal AS01B, induce much more potent CTL responses in mice than formulations with similar components but smaller particle size, such as AS02A (GSK Biologicals).

Another illustration of the importance of formulation involves saponin-derived immunostimulants such as Quil-A. Saponins are natural detergents which, when injected in a free form, cause severe reactogenicity and toxicity including hemolysis of red blood cells because of their ability to lower surface tension and interaction with membrane cholesterol which produces destabilization of the membrane and haemolysis [17]. Presumably, cytotoxicity could involve the same mechanism, although some saponins induce an apoptotic process. The mechanism of action of the saponin derivative *Quillaja saponaria* 21 (QS21) is not fully elucidated, but *in vitro* experiments indicate that

QS21 could improve antigen presentation and, therefore, optimize T-cell response. Association of saponin with cholesterol reduces its lytic activity, and enhances its adjuvant effects possibly by improving bioavailability or targeting DC.

Formulation should also be used to complement the inherent immunogenicity of vaccine antigens. For example, small soluble monomeric proteins (such as HIV envelope glycoprotein 120) tend to be poorly immunogenic compared to multimeric proteins that form virus-like particles (VLPs) (e.g. Hepatitis B surface antigen [HBsAg]) [18]. To enhance the immunogenicity of monomeric proteins, a formulation that renders it multimeric (e.g. incorporation into virosomes) might be most appropriate. For multimeric proteins, virosome formulation might not be appropriate, instead adsorption of the protein to mineral salts might enhance its immunogenicity and stability [19]. What seems to be important is the size of the particles themselves (20–100 nm range) and the way they interact with and activate DCs. Hence, virus-sized particles could, by their size alone, act as a form of PAMP [18] to stimulate both cellular and humoral immunity [20].

Unfortunately, decisions by vaccine developers regarding the appropriateness of a particular adjuvant and/or its formulation are often poorly informed and based solely on limited availability or technical knowledge, as opposed to a rational process. These factors often result in testing of sub-optimal vaccines. A good example is the malaria RTS,S antigen which, when mixed with Alum plus MPL<sup>®</sup> (AS04) or oil-in-water emulsion, failed to protect immunized subjects against a *Plasmodium falciparum* challenge, whereas the same antigen in an oil-in-water emulsion containing MPL<sup>®</sup> (AS02) induced protection [21]. Clearly, well-informed and rational selection of adjuvants and formulations will contribute to development of effective new vaccines.

### Adjuvants approved for human vaccines

Adjuvants in approved human vaccines include Alum, MF59<sup>™</sup> (an oil-in-water emulsion), MPL<sup>®</sup> (a glycolipid), VLP, Immunopotentiating Reconstituted Influenza Virosomes (IRIV) and cholera toxin.

### Alum

Aluminum salt based adjuvants, referred to generically as ‘Alum’, are non-crystalline gels based on aluminum oxyhydroxide (referred to as aluminum hydroxide gel), aluminum hydroxyphosphate (referred to as aluminum

**Table 2.** Immune responses triggered by vehicles or delivery systems

| Vehicle or delivery systems                                                                                                                                        | Type of immune response |               |               |                  |                   |                                    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|---------------|---------------|------------------|-------------------|------------------------------------|
|                                                                                                                                                                    | Th1 responses           | Th2 responses | Cross priming | B-cell responses | Mucosal responses | Persistent T- and B-cell responses |
| Mineral Salts (aluminium salts, calcium phosphate, AS04 [Alum+MPL <sup>®</sup> ])                                                                                  | +                       | ++            | –             | +++              | –                 | +                                  |
| Emulsions [MF59 <sup>™</sup> (squalene/water), QS21, AS02 (squalene+MPL <sup>®</sup> +QS21), IFA, Montanide <sup>®</sup> , ISA51, Montanide <sup>®</sup> , ISA720] | ++                      | –             | –             | +++              | –                 | –                                  |
| Liposomes (DMPC/Chol, AS01)                                                                                                                                        | +++                     | –             | +             | +                | –                 | +                                  |
| Virosomes (IRIV), ISCOMs                                                                                                                                           | ++                      | ++            | ++            | +++              | –                 | –                                  |
| DC Chol, mineral oil, IFA, Montanide <sup>®</sup> , squalene                                                                                                       | –                       | ++            | –             | +++              | –                 | –                                  |
| Mucosal delivery systems: Chitosan                                                                                                                                 | –                       | –             | –             | –                | –                 | ++                                 |
| Microspheres                                                                                                                                                       | +                       | –             | ++            | –                | –                 | –                                  |

phosphate gel) or various proprietary salts such as aluminum hydroxy-sulfate. These adjuvants are components of several licensed human vaccines, including diphtheria-pertussis-tetanus, diphtheria-tetanus (DT), DT combined with Hepatitis B (HBV), Haemophilus influenza B or inactivated polio virus, Hepatitis A (HAV), *Streptococcus pneumoniae*, meningococcal and human papilloma virus (HPV) [22]. Formulation is achieved through adsorption of antigens onto highly charged aluminum particles. Depending on the antigen, the appropriate aluminum adjuvant is selected to maintain antigen immunogenicity and to obtain maximum adjuvant effect. The mechanisms of action of the aluminum salts frequently cited include: (i) depot formation facilitating continuous antigen release; (ii) particulate structure formation promoting antigen phagocytosis by antigen presenting cells (APC) such as DC, macrophages and B cells and, (iii) induction of inflammation resulting in recruitment and activation of macrophages, and increased major histocompatibility complex (MHC) class II expression and antigen presentation [23]. Recent reports have established that Alum induces secretion of chemokines such as CCL2, CCL3, CCL4 and CXCL8 by human monocytes and macrophages [24], and CCL2, CXCL1 and CCL11 in mice [25]. Monocytes, defined as  $CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$ , have been shown to be recruited by Alum to the site of injection, and then migrate to draining lymph nodes after antigen uptake, and further differentiate into inflammatory DC [25]. In addition, injection of antigen adsorbed to Alum resulted in priming and persistence of Th2 cells producing IL-4, IL-5 and IL-10. Alum has been shown to boost humoral immunity by providing Th2 cell help to follicular B cells [26]. Finally, the immunostimulatory properties of Alum were linked to an increase in uric acid levels [25], and Nalp3-dependent caspase-1 activation and IL-1 $\beta$  secretion [27]. The advantages of aluminum adjuvants include their safety record, augmentation of antibody responses (i.e. faster, higher antibody titers, longer-lasting antibody responses), antigen stabilization and relatively simple formulation for large-scale production. The major limitations of aluminum adjuvants include their inability to elicit cell-mediated Th1 or CTL responses that are required to control most intracellular pathogens such as those that cause tuberculosis, malaria, leishmaniasis, leprosy and AIDS [28]. Moreover, vaccines containing Alum cannot be frozen because this leads to loss of potency. Accidental freezing is a widespread phenomenon occurring in up to 70% of vaccines in developing countries [29]. Finally, Alum can induce granulomas at the injection site, a concern for vaccines requiring frequent boosts.

#### Oil and water emulsions

MF59<sup>TM</sup> consists of an oil (squalene)-in-water nano-emulsion composed of <250 nm droplets [30] which is used in Europe as an adjuvant in influenza vaccines [31]. MF59<sup>TM</sup> formulation has also been tested with herpes simplex virus (HSV) [32], HBV [33] and HIV [34] antigens. Overall, MF59<sup>TM</sup> has an acceptable safety profile, and with several antigens it generates higher antibody titers with more balanced IgG1: IgG2a responses than those obtained with Alum [35]. In the clinic, strong helper T-cell responses were

also observed as a result of vaccination [36]. MF59<sup>TM</sup> is believed to act through a depot effect and direct stimulation of cytokine [36] and chemokine production by monocytes, macrophages and granulocytes [24].

Like Alum, MF59<sup>TM</sup> does not induce increased CD4<sup>+</sup> Th1 immune responses, but because of its ability to increase the levels of functional haemagglutination inhibiting antibodies and CD8<sup>+</sup> T-cell responses, it has the potential for use in pandemic influenza vaccines [36,37].

Recently, AS03 (GSK Biologicals), a 10% oil-in-water emulsion-based adjuvant, was approved for use in influenza A Prepandrix<sup>TM</sup>.

#### MPL<sup>®</sup>

MPL<sup>®</sup> is a non-toxic derivative of the lipopolysaccharide (LPS) of *Salmonella minnesota* [38], and is a potent stimulator of Th1 responses. LPS consists of two basic structures: a hydrophilic polysaccharide portion and a hydrophobic lipid moiety (called lipid A) [39]. The lipid A portion is thought to be responsible for most of the endotoxic activity of LPS, whereas the polysaccharide portion enhances solubility [40]. Lipid A from *S. minnesota* is highly endotoxic [40] but this can be reduced by defined structural modifications such as the removal of specific phosphate groups or varying the number and length of its acyl chains [41]. Although the mechanisms of action of lipid A endotoxicity are complex, some generalizations can be made. For instance, it has been determined that lipid A derivatives are only biologically active in aggregate forms [41]. Thus, structural modifications to the lipid A molecule alter the shape and structural order of the lipid, which in turn influence its aggregation behavior and resultant biological activity [39,41]. In addition, as a TLR4 agonist, structural alterations of lipid A would presumably influence its binding affinity as a ligand for TLR4. MPL<sup>®</sup> was the first immunostimulant capable of activating T-cell effector responses to be used in a licensed vaccine [38] and is used in the newest HBV vaccine [16,42] and is also part of an HPV vaccine that is anticipated to be licensed shortly. AS04 is an aqueous formulation of MPL<sup>®</sup> and Alum, resulting in higher levels of specific antibody and efficacy with fewer injections. AS04 is a component of a licensed HBV vaccine (Fendrix<sup>®</sup>) and is being assessed in clinical trials evaluating vaccines against HAV and HPV [80]. MPL<sup>®</sup> based adjuvants, including AS01B and AS02A, have been evaluated in clinical trials with vaccines against malaria [15], tuberculosis [43], leishmania [44,45], HIV, vesicular stomatitis virus and cancer [46]. MPL<sup>®</sup> is licensed in Europe for allergy treatment because of its ability to down-modulate Th2 responses to allergens [47]. MPL<sup>®</sup> in several formulations has been given to thousands of individuals, and is a safe, well-tolerated and potent adjuvant component. A newer generation of TLR-4 agonists include aminoalkyl glucosamimide phosphates (AGPs) [48] and glucopyranosyl lipid A (GLA) (patent 11/862 122).

#### VLP and IRIV

VLP are self-assembling particles composed of one or more viral proteins, resulting in the formation of nano-particles 20–100 nm in size. VLP vaccines against HBV and HPV

are commercially available and are based on expression of the HBV surface antigen and HPV major capsid protein L1, respectively. IRIVs are proteoliposomes composed of phospholipids, influenza hemagglutinin (HA) and a selected target antigen [49] that are delivered to APCs that take up the Virosomes by HA receptor-mediated endocytosis. IRIV is registered as a component of the Hepatitis A vaccine in Europe, Asia and South America. In clinical trials, the IRIV vaccine generated a faster immune response and less injection site adverse reactions compared to a conventional Alum-containing vaccine [50]. Both types of particles are taken up by APCs by receptor-mediated endocytosis, and have been shown to stimulate cellular and humoral immune responses [51].

#### *Cholera toxin B subunit*

Cholera toxin B subunit (CTB) is used to enhance mucosal immune responses of orally delivered vaccines. The naturally occurring cholera toxin belongs to the AB class of bacterial toxins. It consists of a pentameric B oligomer that binds to GM-1 receptors (e.g. on the surface of intestinal epithelial cells) and an enzymatically active A subunit that is responsible for the toxicity. The recombinant CTB (rCTB) consists only of the non-toxic B component of the cholera enterotoxin. The rCTB molecule consists of five identical monomers tightly linked into a trypsin-resistant pentameric ring-like structure. CTB can act as a mucosal adjuvant and enhance immunoglobulin A (IgA) levels to co-administered or coupled antigens intranasally [52]. CTB is used to enhance the immune response in a licensed whole-cell orally delivered cholera vaccine [53]. This vaccine has been shown to induce a high level of protection against cholera, but was short-lived [54].

#### **Adjuvants in development**

The development of additional adjuvants has been driven principally by the shortcomings of aluminum adjuvants (failure to stimulate T-cell responses, including CTL, loss of potency if frozen and causing granulomas at injection sites). In many instances, several adjuvants have been combined in one formulation hoping to obtain synergistic or additive effects (Table 3).

#### *Montanides (ISA51 and ISA720)*

Montanides (ISA51 and ISA720) are water-in-oil emulsions containing mannide-mono-oleate as an emulsifier. Montanides, similar in physical character to incomplete Freund's adjuvant (IFA) but biodegradable, have been developed in response to safety concerns with IFA in animal studies [55,56]. Montanides have been used in malaria, HIV and cancer vaccine trials [2]. They induce a strong immune response and are available without requiring a license or contractual agreement. A drawback of Montanides is that they are difficult to formulate because an extensive and costly emulsification procedure is required for each antigen. In several studies, they have produced unacceptable local reactions [57].

#### *Saponins (Quil-A, ISCOM and QS-21)*

Saponins (Quil-A, ISCOM, QS-21) (also included in AS02 and AS01) are triterpene glycosides isolated from plants.

The most widely used in adjuvant research is Quil-A and its derivatives, extracted from the bark of the *Quillaja saponaria* tree [58]. Quil-A is composed of a heterogeneous mixture of triterpene glycosides that vary in their adjuvant activity and toxicity. Saponins have been widely used as an adjuvant in veterinary vaccines. Partially purified fractions of Quil-A have also been used in immunostimulating complexes (ISCOM) composed of antigen, phospholipids, cholesterol and Quil-A fractions. ISCOMs are ~40 nm cage-like particles trapping the protein antigen through hydrophobic interactions, whereas ISCOMATRIX<sup>TM</sup> [59] (pre-formed antigen-free particles) provides for more general applications by later accommodating non-hydrophobic antigens. Because of their particulate nature, ISCOMs are directly targeted to and more efficiently taken up by APC via endocytosis. Saponin-mediated targeting of DEC-205 (a macrophage mannose receptor family of c-type lectin endocytic receptors) on the surface of DC might account for higher uptake and more efficient presentation of antigens to T cells [60,61]. Antigen processing can occur in the endosome for both MHC class II [62] and class I presentation [63], possibly by the recently described cross-presentation pathway [64–66]. ISCOMs have been shown to elicit high titer long-lasting antibodies and strong helper and CTL responses in different models [67–70]. Protective immunity has been generated in a variety of experimental models of infection [71–73], including toxoplasmosis and Epstein-Barr virus-induced tumors. An influenza ISCOM vaccine for horses is licensed in Sweden, and an influenza vaccine for humans containing a less toxic saponin fraction is under development. QS-21 is a purified component of Quil-A that demonstrates low toxicity and maximum adjuvant activity. In a variety of animal models, QS-21 has augmented the immunogenicity of protein, glycoprotein and polysaccharide antigens [74]. QS-21 has been shown to stimulate both humoral and cell-mediated Th1 and CTL responses to subunit antigens [75]. Clinical trials are in progress with QS-21, alone or in combination with carriers and other immunostimulants for vaccines against infections including influenza, HSV, HIV, HBV and malaria, and cancers including melanoma, colon and B-cell lymphoma.

#### *MPL<sup>®</sup> formulations and combinations (MPL<sup>®</sup>-SE, AS01, AS02 and AS04)*

MPL<sup>®</sup>-SE is the result of MPL<sup>®</sup> mixed with squalene oil, excipients (inactive substances used as carriers for the active ingredient) and water to produce a stable oil-in-water emulsion. MPL<sup>®</sup>-SE is an excellent promoter of Th1 responses and is currently being evaluated in several clinical trials to treat and prevent leishmaniasis. The adjuvant system (AS) series of adjuvants are proprietary formulations, several of which contain MPL<sup>®</sup>. AS02 is an oil-in-water emulsion containing MPL<sup>®</sup> and QS-21 that induces both strong humoral and Th1 responses. AS02 is being evaluated in vaccine clinical trials for malaria [15,42], HPV [76], HBV [77,78], tuberculosis [43] and HIV [79]. AS01 is a liposomal formulation containing MPL<sup>®</sup> and it induces potent humoral and cell-mediated responses including CTL responses. AS01 is

Table 3. Adjuvants in development for human vaccines

| Adjuvants                           | Formulation                                                                                    | In pre-clinical or clinical trials                                                                |
|-------------------------------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| Montanides                          | Water-in-oil emulsions                                                                         | Malaria (Phase I), HIV, Cancer (Phase I/II)                                                       |
| Saponins (QS-21)                    | Aqueous                                                                                        | Cancer (Phase II), Herpes (Phase I), HIV (Phase I)                                                |
| SAF                                 | Oil-in-water emulsion containing squalene, Tween <sup>TM</sup> 80, Pluronic <sup>TM</sup> L121 | HIV (Phase I – Chiron)                                                                            |
| AS03                                | Oil-in-water emulsion containing $\alpha$ -tocopherol, squalene, Tween <sup>TM</sup> 80        | Pandemic Flu (GSK)                                                                                |
| MTP-PtdEtn                          | Oil-in-water emulsion                                                                          | HSV                                                                                               |
| Exotoxins                           | <i>P. aeruginosa</i><br><i>E. coli</i> heat-labile enterotoxin LT                              | <i>P. aeruginosa</i> , cystic fibrosis (AERUGEN – Crucell/Berna)<br>ETEC (Phase II – Iomai Corp.) |
| ISCOMs                              | Phospholipids, cholesterol, QS-21                                                              | Influenza, HSV, HIV, HBV, Malaria, Cancer                                                         |
| <b>TLR ligands</b>                  |                                                                                                |                                                                                                   |
| MPL <sup>®</sup> -SE                | Oil-in-water emulsion                                                                          | <i>Leishmania</i> (Phase I/II - IDRI)                                                             |
| Synthetic Lipid A                   | Oil-in-water emulsion                                                                          | Various indications (Avanti/IDRI)                                                                 |
| MPL <sup>®</sup> -AF                | Aqueous                                                                                        | Allergy (ATL); Cancer (Biomira)                                                                   |
| AS01                                | Liposomal                                                                                      | HIV (Phase I), Malaria (AS01, Phase III, GSK)<br>Cancer (Phase II/III, Biomira/MerckKGaA)         |
| AS02                                | Oil-in-water emulsion containing MPL <sup>®</sup> and QS-21                                    | HPV (Cervarix), HIV, Tuberculosis, Malaria (Phase III), Herpes (GSK)                              |
| AS04                                | Alum + aqueous MPL <sup>®</sup>                                                                | HPV, HAV (GSK)                                                                                    |
| AS15                                | AS01 + CpG                                                                                     | Cancer therapy (GSK)                                                                              |
| RC529                               | Aqueous                                                                                        | HBV, pneumovax                                                                                    |
| TLR-9 (CpG)                         | n/a                                                                                            | Cancer (ProMune – Coley/Pfizer)                                                                   |
| TLR-9 ISS series                    | n/a                                                                                            | HCV (ACTILON – Coley)                                                                             |
|                                     |                                                                                                | HIV, HBV, HSV, Anthrax (VaxImmune Coley/GSK/Chiron)                                               |
|                                     |                                                                                                | HBV (HEPLISAV, Phase III - Dynavax)                                                               |
|                                     |                                                                                                | Cancer (Phase II, Dynavax)                                                                        |
| TLR-9 IMO series (YpG, CpR motif)   | n/a                                                                                            | Cancer (IMOXine, Phase I, Hybridon Inc.)<br>Cancer (IMO-2055, Phase II, Idera Pharm.)             |
|                                     |                                                                                                | HIV (Remune, Phase I, Idera/IMNR)                                                                 |
| TLR-9 agonist (MIDGE <sup>®</sup> ) | n/a                                                                                            | Cancer (Phase I, Mologen AG)                                                                      |
| TLR-7/8 (Imiquimod)                 | n/a                                                                                            | Melanoma (3M Pharmaceutical)                                                                      |
| TLR-7/8 (Resiquimod)                | n/a                                                                                            | HIV (preclinical), Leishmaniasis<br>HSV, HCV (Phase II - 3M Pharmaceuticals)                      |

Abbreviations: ETEC, Enterotoxigenic *Escherichia coli*; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HPV, human papilloma virus; HSV, Herpes simplex virus.

being evaluated in clinical trials for malaria. Other AS formulations are being tested in cancer vaccine trials.

Syntex adjuvant formulation (SAF) is an oil-in-water emulsion containing squalene, Tween<sup>TM</sup> 80 and Pluronic<sup>TM</sup> L121 (a nonionic block polymer) in phosphate-buffered saline. SAF or SAF + threonyl-muramyl dipeptide were safe and effective in pre-clinical studies when combined with influenza, HBV, Epstein-Barr virus (EBV), HSV and HIV antigen vaccines [71–73]. SAF elicits both humoral and cell mediated immune responses, but was found to cause severe local adverse reactogenicity in a human HIV clinical trial.

#### Muramyl dipeptide

Muramyl dipeptide (MDP) is the minimal unit of the mycobacterial cell wall complex that generates the adjuvant activity of complete Freund's adjuvant (CFA). Several synthetic analogs of MDP, such as muramyl tripeptide phosphatidylethanolamine (MTP-PtdEtn), have been generated, and they exhibit a wide range of adjuvant potency and side effects. MTP-PtdEtn includes phospholipids that facilitate lipid interactions, whereas the muramyl peptide portion facilitates aqueous interactions. Thus, the MTP-PtdEtn itself is able to act as an emulsifying agent to generate stable oil-in-water emulsions. Nevertheless, MTP-PtdEtn has poor stability [81].

#### Immunostimulatory oligonucleotides

Synthetic oligodeoxynucleotides, containing unmethylated CpG motifs, act through TLR-9 (Figure 1) and induce

activation of DC and secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$ , IL-1 and IL-6. TLR-9 activation also leads to secretion of the pro-inflammatory cytokines interferon (IFN)- $\alpha$ , IFN- $\gamma$  and IL-12. CpGs are extremely efficient inducers of Th1 immunity and CTL responses [82] and induce protection against infectious disease, allergy and cancer in mice and primate models [83]. Ongoing clinical studies indicate that CpGs are relatively safe and well-tolerated in humans [84] but their use has been limited in most cases to therapy rather than prophylactic indications. These are being evaluated both in the absence of antigen, for certain types of cancer therapy, and with allergens. Because of the biological instability of CpG and their resulting short half-life, several approaches have been used to enhance their bioavailability. Replacement of the CpG phosphodiester bonds with phosphothioate bonds enhances the stability and activity of these oligonucleotides, and is the lead CpG candidate. Other stabilizing approaches involve complexing to cationic peptides or cationic carriers, conjugating to the vaccine antigen, or incorporating the CpG into nucleic acids that form double stranded hairpin loops.

#### Other TLR ligands

These include synthetic compounds that induce the maturation and activation of professional APC and the secretion of inflammatory cytokines and chemokines [85]. The small molecule nucleoside analogues imiquimod and resiquimod are ligands for TLR-7 and TLR-7/8, respectively [86]. Imiquimod applied as a topical cream has demonstrated ef-

ficacy in human clinical trials for leishmaniasis [87], and is licensed for treatment of HPV and basal cell carcinoma (BCC). The exact mechanism of action of imiquimod is unknown but it is thought that its activity as a TLR-7 agonist mimics a microbial antigen inducing the expression of different cytokines such as IL-1, IL-6, IL-12, IFN- $\alpha$  and TNF- $\alpha$ , which stimulate or enhance both the innate immune system and the cell-mediated immune response, enhances migration of Langerhans' cells from the dermis to regional lymph nodes, in addition to the stimulation of apoptosis in BCC [88] and diminished pathology associated with *Leishmania* infection [89].

#### *Escherichia coli* heat-labile exotoxin

This is a potent mucosal adjuvant. The native lymphotoxin (LT) is composed of two subunits: LT-A and LT-B. The LT-B subunit has affinity for the GM1 gangliosides of nerves, which is probably responsible for the facial palsy seen when this molecule is administered nasally [90]. Another adjuvant under development for nasal administration contains the fully active LT-A component, with the LT-B component replaced by a LT B-cell binding sequence [91]. Alternatively, recombinant LT, when administered transcutaneously with an influenza vaccine, was shown to be safe and immunogenic in humans. Serological responses were comparable to those observed with an oral challenge that results in protection [92,93].

#### Adjuvants to enable future vaccines

Advances in genomics and proteomics have accelerated the identification of recombinant and synthetic vaccine molecules, but have also heightened the need for improved adjuvants and formulations beyond those currently available. In conjunction with these advances, recent insights into how immune responses are activated have facilitated the discovery of new and improved adjuvants. The activation of DCs is paramount to any effective adjuvant because this results in enhanced antigen uptake, migration to the draining lymph nodes, acquisition of co-stimulatory molecules and presentation of antigenic peptides on MHC class I and II to the TCR (Figure 2). Stimulation of T cells through the TCR-complex in the absence of co-stimulation of CD28 by CD80 or CD86 (signal 2) usually results in T-cell tolerance rather than activation. It is of interest to note that adjuvants possibly engaging DAMPs such as Alum and MF59<sup>TM</sup> tend to induce Th2 and B-cell responses, whereas those containing TLR ligands (PAMPs) tend to favor more Th1 and CTL responses. In addition, the particulate nature of some adjuvants such as virosomes, liposomes and ISCOMs seems to help in antigen cross-presentation and priming of CD8<sup>+</sup> T cells.

An understanding of the mode of action of adjuvants and resulting immune responses will enable the development of vaccines for difficult patient groups such as infants and the elderly who have weaker immune responses. T-cell-independent B-cell (antibody) responses are markedly compromised in the first year of life. T-cell-dependent antibody responses mature much earlier, but neonates and infants can require multiple immunizations to achieve or sustain titers comparable to those in older individuals. Neonates can mount effective antigen-specific T-cell

responses, but CD4 T-cell responses are often slower to develop, less readily sustained and in most cases more easily biased towards a Th2 type response, most probably because of the decreased efficiency of neonatal DC to establish Th1 CD4 T-cell responses [94]. This limitation can be overcome given appropriate stimuli, including adjuvants, delivered in the context of early priming and subsequent boosting.

Aging is associated with declines in immune system function, or 'immunosenescence', leading to progressive deterioration in both innate and adaptive immunity. These changes contribute to the decreased response to vaccines seen in many older adults, and morbidity and mortality from infection [95]. In this regard, increased immunogenicity has been achieved with MF59<sup>TM</sup>-adjuvanted influenza vaccines in the elderly [96], and with MF59<sup>TM</sup>-adjuvanted vaccines against cytomegalovirus and HIV in infants [97,98].

Another important obstacle to the development of any active immunotherapeutic vaccine is the immunosuppressive environment including the induction of tolerogenic DCs and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells, which suppress the development of protective effector T-cell responses. This can be compounded by the use of TLR ligand-containing adjuvants as immunotherapeutics because TLR agonists can generate suppressive and inflammatory responses in innate immune cells and can promote the induction of Treg in addition to effector T cells [99]. Alternatively, manipulating the TLR-activated innate immune responses to selectively blocking Treg recruitment [100] such as has been reported with chemokine (C-C motif) receptor 4 (CCR4) antagonists, might hold the key to enhancing their efficacy as immunotherapeutics and as adjuvants for infectious disease and cancer vaccines.

Several barriers must be overcome to meet the demands for new adjuvants. Unacceptable side effects and toxicity remain barriers for many candidates, particularly for the development of pediatric vaccines. In addition, regulatory standards for adjuvant approval have increased substantially since the approval of Alum.

The following issues remain considerable problems for the development of new adjuvants. (i) Currently, adjuvants do not receive U.S. Food and Drug Administration (FDA) approval as stand alone products, but as part of a registered vaccine adjuvant-antigen combination. Therefore, potential adjuvant-antigen combinations have not been developed because of the huge costs and efforts involved in gaining FDA approval for each adjuvant-antigen combination. (ii) Most for-profit-organizations are unwilling to risk the investment in new vaccines that involve untested antigens and adjuvants. (iii) Most vaccine companies keep their adjuvant formulations proprietary until the adjuvant is registered with a potential vaccine product. This limits the development of the adjuvant for other vaccine applications. (iv) The high cost of developing novel adjuvant formulations makes incorporating proprietary adjuvant formulations into vaccines for neglected diseases prohibitive.

#### Strategy to develop and test new adjuvants and formulations

Today, most researchers working on vaccines are focusing on the antigens, and testing them with the few adjuvants

available that utilize only a single immunostimulant. Lack of either the knowledge or capacity to formulate complex adjuvant systems comprising immunostimulants and delivery vehicles, no readily available published methods for such systems and often difficult access to new immunostimulants because of intellectual property and complicated material transfer agreements are major hurdles for most researchers working on vaccine development. A strategy to solve these important issues needs to address adjuvant access and new adjuvant development.

Adjuvant access would benefit from the creation of an organization that would act as a central resource for adjuvants and formulations, including guaranteed access to licenses for adjuvant systems for developers of vaccines for the public sector. This organization would also create and maintain a public database of formulation procedures and analytical procedures for all adjuvant systems that show promise, enabling diverse laboratories to formulate candidate vaccines in an optimal and reproducible manner.

New adjuvant development is needed to identify novel combinations of adjuvants and formulations capable of inducing strong, long lasting humoral and cellular immune responses in humans. Ideally, these new adjuvants and formulations would generate a protective immune response with a reduced number of administrations. This will result in rational knowledge-based selection of adjuvant systems for the development of new vaccines eliciting either predominantly humoral and/or cellular responses. Finally, the development of alternatives to adjuvants that are largely controlled by large pharmaceutical companies in a manner that does not infringe intellectual property will globally benefit the discovery of novel promising vaccines by providing researchers with the best adjuvants and formulations available to test with their antigens.

Numerous challenges remain related to adjuvant development. In effect, it is unlikely that any single immunostimulant or delivery system will be sufficient to induce the broad and long-lasting immunity that is required for all new vaccines. Effective adjuvant systems are likely to require synergy between one or more immunostimulants, and a carrier or delivery system. In addition, it is often impossible to compare adjuvants analyzed in different laboratories, or even within the same laboratory, because adjuvant formulation and characterization methods are not standardized. Furthermore, each antigen has a different intrinsic immunogenicity and interacts differently with immunostimulants and carriers, and no reliable algorithms exist to permit selection of optimal adjuvants based on physico-chemical or immunological properties of an antigen.

### Final comments

To ensure that new and existing adjuvants will be accessible for use in vaccines and therapeutics, the development path of the adjuvant candidates should include checking for freedom to operate, cost of goods and compliance with current and foreseeable regulatory issues. As lead candidate formulations and active pharmaceutical ingredients emerge, development of candidate adjuvants should focus on establishing modular and transferable standard operating procedures and batch records for processing,

production and fill-finishing, in addition to analytical procedures to evaluate the performance of the process and final product. At the same time, development should also attempt to remove problematic materials such as animal-derived chemicals that might currently or in the near future, raise regulatory and comparability issues. Combining this view of raw material sourcing with attention to cost of goods should allow for the development of sustainable adjuvant formulations that will have long product lifetimes without major changes in manufacturing and sourcing.

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