Expert Opinion





# Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators

Pernille Nordly, Henriette Baun Madsen, Hanne Moerck Nielsen & Camilla Foged†
*University of Copenhagen, Department of Pharmaceutics and Analytical Chemistry, Universitetsparken 2, DK-2100 Copenhagen O, Denmark*

Vaccines seek to adopt pathogen-like characteristics but not true pathogen characteristics to activate the immune system without causing life-threatening disease. Vaccine formulations are therefore often particulate in nature, with dimensions comparable to pathogens, and often contain highly conserved pathogen-associated molecular patterns as adjuvants stimulating the immune system. Only a few adjuvants have been approved for human use. There is therefore an unmet medical need for the development of effective and safe adjuvants that can stimulate cellular, humoral or mucosal immunity, or combinations thereof, depending on the requirements, to prevent the specific disease. Lipid-based particulate systems are in this respect promising and versatile adjuvants that can be customized rationally towards specific vaccine targets by varying their composition. In this review, current progress in the development of lipid-based vaccine delivery systems is discussed, with a special focus on emulsions, liposomes and immune-stimulating complexes, and their combination with immunostimulatory compounds. Formulations, adjuvant mechanisms and alternative administration routes are highlighted.

**Keywords:** adjuvant, antigen, colloidal carriers, drug delivery, emulsion, immune-stimulating complex, immunostimulator, liposome, particulate delivery system, pathogen-associated molecular pattern, pattern recognition receptors, review, Toll-like receptors, vaccine

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## 1. Introduction

Mass vaccination programs have had a major impact on global health, resulting in the eradication of infectious diseases such as smallpox and a decrease in the incidence of life-threatening conditions such as polio and diphtheria. Despite the general success of vaccination, there is still an urgent demand for new prophylactic and therapeutic vaccines against global killers such as cancer, tuberculosis and malaria. Also, there is a need for improved conventional vaccines for populations with impaired immune function (e.g., the elderly). In vaccine development, there has been a shift from whole-cell and live attenuated vaccines towards the safer split- and subunit vaccines. However, such new vaccine candidates are poorly immunogenic by themselves. Thus, adjuvants are required that can enhance, accelerate and/or prolong a specific immune response.

Adjuvants that are approved or in clinical trials can be subdivided into two classes: delivery systems such as emulsions, liposomes, immune-stimulating complexes (ISCOMs) and mineral salts; and immunostimulators such as Toll-like receptor (TLR) agonists (e.g., the TLR4 agonist monophosphoryl lipid A [MPL]). So far,

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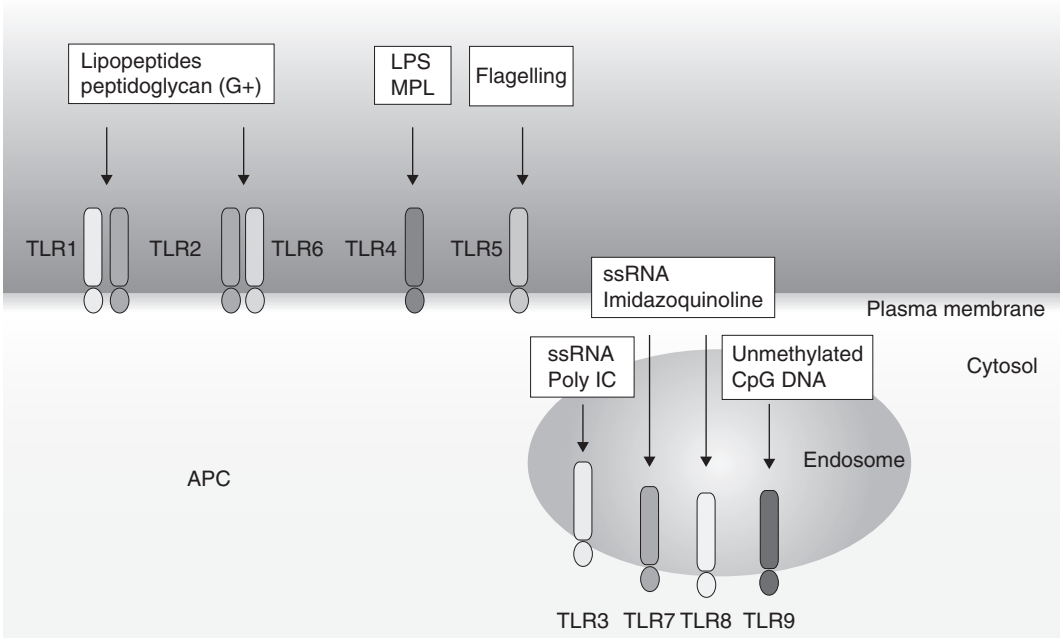


**Figure 1. Toll-like receptors and selected ligands.** TLRs recognize a variety of natural or synthetic ligands, which can be exploited in adjuvant research. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are endosome-located. Only a few selected ligands are depicted, although many more ligands are known (for a more comprehensive review of the function of TLRs and their ligands, see e.g., [58]).

APC: Antigen-presenting cell; G+: Gram-positive bacteria; LPS: Lipopolysaccharide; MPL: Monophosphoryl lipid A; Poly IC: Polyinosinic-polycytidylic acid; TLR: Toll-like receptors.



only a few vaccine adjuvants have gained approval by the authorities. These include the aluminum hydroxide/phosphate salts, also called alum, which have been widely used for vaccines against, for example, diphtheria, tetanus and hepatitis B for many years. Alum is at present the only adjuvant licensed in the US. However, more adjuvants have been licensed in Europe. These include the two oil-in-water emulsions MF59™ (Novartis, Basel, Switzerland) and AS03 (GlaxoSmithKline, London, UK), which are used in seasonal and pandemic influenza vaccines, and AS04 (GlaxoSmithKline), which is alum combined with MPL for vaccination against human papilloma virus (HPV) and hepatitis B virus (HBV) (called Cervarix® and Fendrix®, respectively). Finally, a few virosome-based vaccines are also marketed (e.g., Inflexal® V (Crucell, Leiden, The Netherlands) for influenza vaccination).

In the development of new vaccine formulations, reactogenicity and safety are of major importance. In general, vaccines should elicit a sufficient immune response to induce immunological memory that can protect the host against concomitant infection. The pharmaceutical formulation of the antigen should therefore present the antigen to the immune system in an appropriate way [1]. As alum predominantly induces antibody responses, it is crucial to discover new adjuvants for development of vaccines that require a cell-mediated response. Immune responses towards vaccine formulations are discussed further in the next section. The safety criterion is a very important issue, as vaccination programs encompass healthy people, and the approval of new adjuvants by the regulatory authorities constitutes a major hurdle in vaccine development. Alum has a 75-year record of safety around the world and is generally safe, but causes adverse effects such as reddening, nodules at the injection site and allergic reactions [2]. Furthermore, factors such as the stability of the formulation, the reproducibility of the formulation and the cost of production are decisive for commercialization of a vaccine. Alternatives to the parenteral administration route are highly desired; in particular oral vaccine dosage forms are attractive for reasons of increased patient compliance.

Presented in this review are the status and future prospects of lipid-based particulate delivery systems such as emulsions, liposomes and ISCOMs (Section 2) and their rational combination with immunostimulatory compounds (Section 3) with a special focus on formulation, adjuvant mechanisms (Section 4) and alternative administration routes (Section 5).

### 1.1 Immune responses

The immune system, which is activated on vaccination, is traditionally viewed as having two components: the innate and the adaptive immune systems [3]. The innate immune system constitutes the first line of host defense, which rapidly

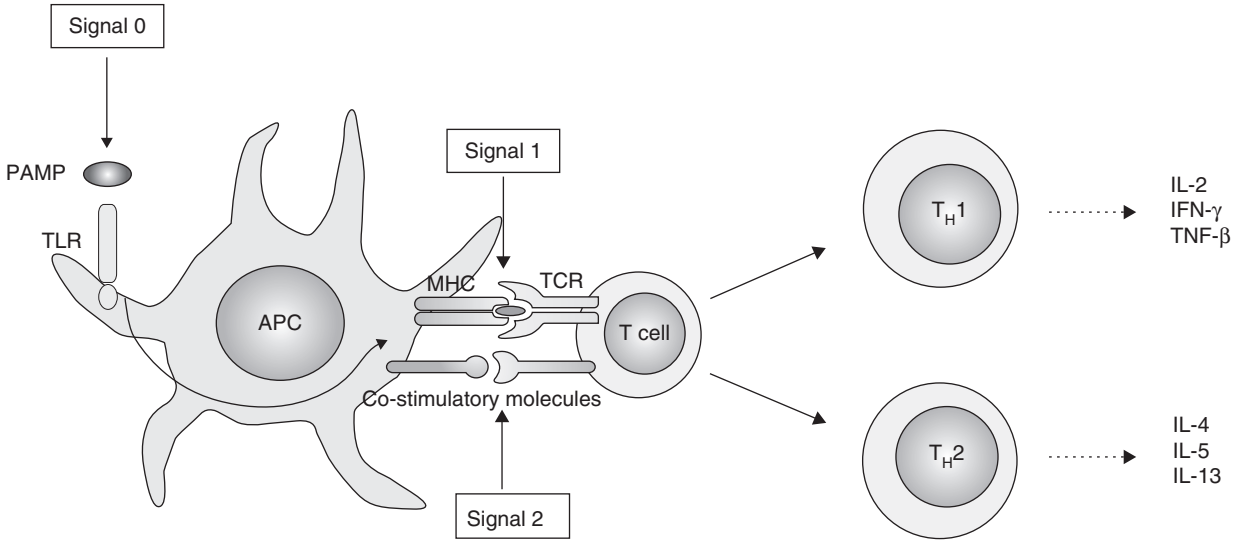


**Figure 2. Initiation of T-cell responses.** The recognition of pattern-associated molecular patterns by pattern recognition receptors, such as Toll-like receptors, present on antigen-presenting cells, induces signal 0 for activation of T cells. Presentation of antigenic peptides on major histocompatibility complex to specific T cells constitutes signal 1, whereas signal 2 is the co-stimulatory signal needed for T cells to become fully activated. Activated T cells differentiate into T-helper 1 or 2 cells, which produce distinct cytokine subsets through which they mediate cellular or humoral immune responses, respectively.

APC: Antigen-presenting cell; IL: Interleukin; IFN-γ: Interferon-γ; MHC: Major histocompatibility complex; PAMP: Pathogen-associated molecular pattern; TCR: T-cell receptor; $T_H$: T-helper: TLR: Toll-like receptors; TNF-β: Tumor necrosis factor-β.



recognizes and responds to a microbial invasion. The innate immune system is mainly composed of the complement system and phagocytic cells that ingest and kill pathogens. The innate immune system depends on pattern recognition receptors (PRRs) that recognize highly conserved pathogen-associated molecular patterns (PAMPs) [4]. TLRs are the best characterized class of PRRs [5,6]. TLRs are membrane-bound and act as PRRs for pathogenic compounds containing microbially unique PAMPs, such as bacterial cell wall components, unmethylated CpG motifs of bacterial D3NA and double-stranded RNA of viruses (**Figure 1**) [7,8]. Engagement of PRRs triggers intracellular signaling cascades that initiate the innate, essentially inflammatory, immune response, also called signal 0 (**Figure 2**) [9]. This results in the destruction of pathogens and in the enhancement of adaptive immune responses, which are required for the specific eradication of microorganisms, as well as for the generation of specific memory responses.

The adaptive immune system constitutes the second line of defense, capable of mounting highly specific responses against molecular determinants on pathogenic agents, which is a process that may proceed over weeks (reviewed in [10]). Specific cell-mediated and humoral immune responses are initiated by antigen-mediated triggering of T and B lymphocytes carrying antigen-specific surface receptors. Two main lineages of T lymphocytes exist: $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTL). Th cells can be divided into subpopulations, of which Th1 and Th2 are the most important (**Figure 2**). The division into Th subsets is largely based on their secretion of different cytokines, and since the identification of Th1/Th2 cells, more Th lineages have been discovered, such as Th3 and Th17 [11]. Th1 cells mediate a cellular immune response by producing cytokines (e.g., interleukin-2 (IL-2), interferon-γ (INF-γ) and tumor necrosis factor-β (TNF-β), which among other mechanisms activate CTLs and enhance phagocytosis. Th2 cells are characterized by the secretion of cytokines such as IL-4, IL-5, IL-10 and IL-13. Th2 cells support the production of circulating antibodies, and are effective against extracellular pathogens. IL-4 production by antigen-presenting cells (APCs) tends to favor a Th2 type of response. CTLs exert their most important effector mechanism by antigen-mediated killing of infected cells [12].

At least two signals are required to activate T lymphocytes to proliferate and differentiate into functional effector cells (**Figure 2**). Specific T cells recognize by means of T-cell receptors (TCR) peptide fragments bound to the major histocompatibility complex (MHC) class I and class II molecules, which display antigenic peptides on the surface of APCs (**Figure 3**). Such an initial recognition event constitutes the so-called activation signal 1 [13]. In addition to the signaling initiated by the specific TCR-recognition of peptides displayed on MHC, T cells need further stimulation to become fully activated (**Figure 2**), often denoted as co-stimulation or signal 2 [14]. Receptors on T cells interact with co-stimulatory molecules on the surface of APCs. Examples of important co-stimulatory molecules on the APC surface are the B7 family and ICOS (inducible co-stimulator) ligands, which

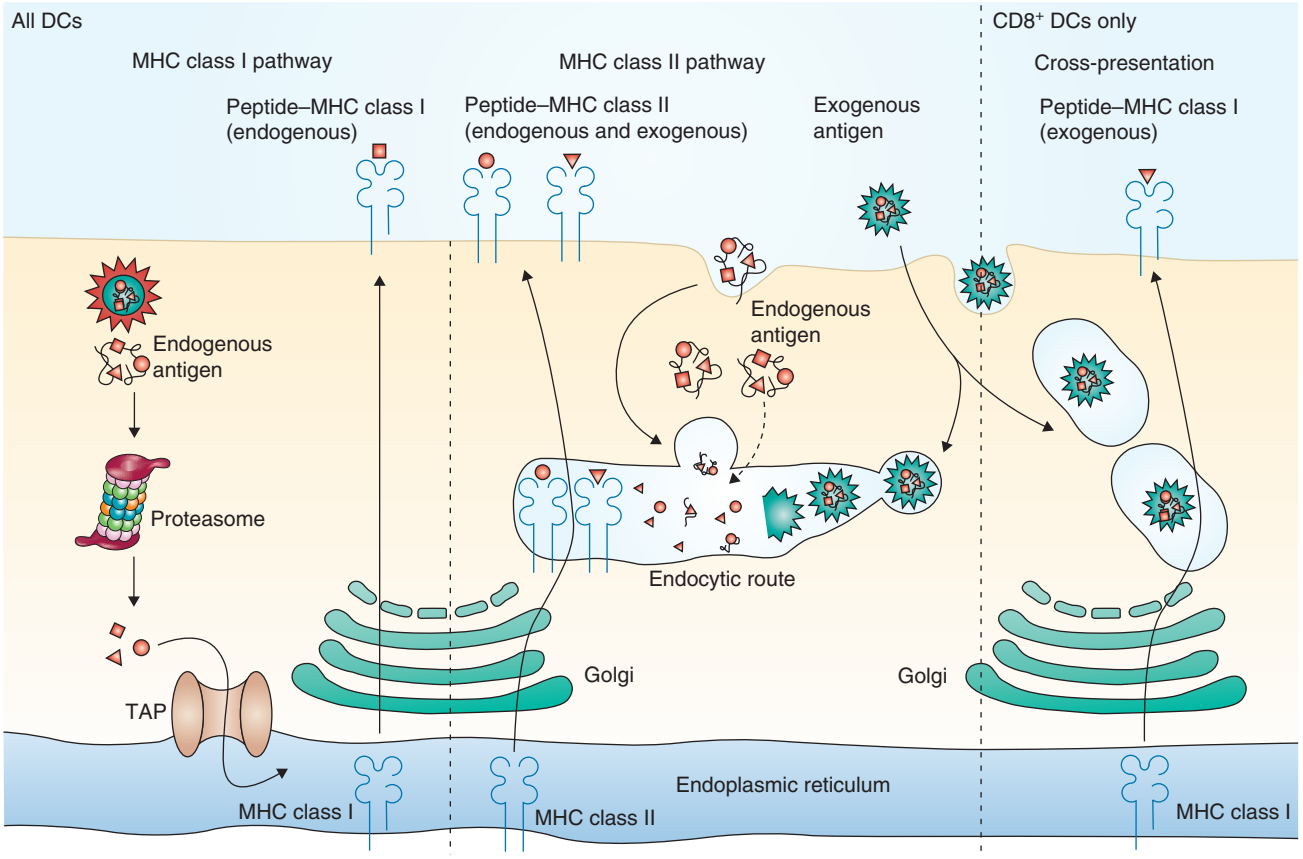


**Figure 3. Antigen presentation pathways in dendritic cells.** MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol. MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material and endogenous components that access the endosomes by autophagy. CD8+ dendritic cells have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway.
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DC: Dendritic cell; MHC: Major histocompatibility complex; TAP: Transporter associated with antigen processing.

are recognized by CD28, cytotoxic T lymphocyte antigen (CTLA)-4 and ICOS. These extra signals activate the priming and differentiation of CTL and Th cells. Th cells subsequently deliver help to antigen-specific B cells, resulting in antibody production.

Each infection requires activation of a specific type of adaptive immune response to control and eliminate the infection efficiently. Vaccine formulations should thus be designed rationally to induce protective immune responses without injuring the healthy tissue when administered in the right place with the right timing. This includes the choice of antigen (signal 1) and adjuvant. It is therefore important initially to identify the immune effector mechanism(s) responsible for protection, although this can be difficult to determine clearly *in vivo*. However, other types of immune response, which differ from the immune response activated by the pathogen itself, can also be efficient in controlling infectious disease. When the appropriate antigen has been identified, a delivery system should be developed that efficiently delivers the antigen to the immune system. The immune system has evolved to protect eukaryotes from pathogens [4,15]. Vaccines seek to adopt pathogen-like characteristics but not true pathogen characteristics to activate the immune system without causing life-threatening disease. Vaccine formulations are therefore often particulate in nature with dimensions comparable to those of pathogens, and may contain highly conserved PAMPs as adjuvants stimulating the immune system by means of PRRs (signal 0). Examples of particulate vaccine formulations are lipid-based delivery systems, such as emulsions, liposomes and ISCOMs.

## 2. Lipid-based particulate delivery systems

Lipid-based particulate systems are versatile adjuvants that can be customized towards specific vaccine targets by varying their composition. Being natural constituents of biomembranes, lipids are attractive components of adjuvant systems in being

**Table 1. Examples of vaccines containing lipid-based formulations, marketed and in clinical trials.**

| Company | Vaccine | Delivery system | Composition of formulation | Status | Target disease | Ref. |
|---|---|---|---|---|---|---|
| Crucell | Epaxal | Virosome | Phospholipids<br>Hemagglutinin<br>Neuraminidase | Marketed | Hepatitis A | [16] |
| Crucell | Inflexal V | Virosome | Phospholipids<br>Hemagglutinin<br>Neuraminidase | Marketed | Influenza | [16] |
| National Cancer Institute, Norris Comprehensive Cancer Center, Ludwig Institute for Cancer | | Emulsion – Montanide ISA 720 | Squalene oil<br>Mannide monooleate | Phase I/II | Cancer | [18] |
| Novartis | Fluad | Emulsion – MF59 | Squalene oil<br>Tween 80<br>Span 85 | Marketed | Influenza | [23] |
| GlaxoSmithKline | Prepandrix | Emulsion – AS03 | Squalene oil<br>Alpha-tocopherol<br>Polysorbate 80 | Marketed | Pre-pandemic influenza | [25] |
| GlaxoSmithKline | | Liposome – AS01 | Liposomes<br>MPL<br>QS21 | Phase II | Malaria | [35] |
| Nasvax | | Liposome – VaxiSome™ | CCS<br>Cholesterol | Phase I/IIA | Influenza | [37] |
| Oncothyreon and Merck KGaA | Stimuvax | Liposome | BLP25 lipopeptide<br>MPL<br>Cholesterol<br>DMPG, DPPC | Phase III | Non-small cell lung cancer | [42] |
| CSL | ISCOMATRIX | ISCOM matrix | Saponin<br>Cholesterol<br>Phospholipid | Phase 1 | Various (HPV, cancer) | [47,48] |
| Ludwig Institute for Cancer Research | ISCOMATRIX | ISCOM matrix | | Phase II | Cancer | |

CCS: Ceramide carbamoyl spermine; DPPC: Dipalmitoyl phosphatidylcholin; DMPG: Dimyristoyl phosphatidylglycerol; HPV: Human papilloma virus; ISCOM: Immune-stimulating complexes; MPL: Monophosphoryl lipid A; QS21: *Quillaja saponaria* 21.



biodegradable, biocompatible and affordable. Some lipid-based particulate delivery systems can be regarded as artificial viruses lacking the infectious part, which causes disease, but with preserved antigenic properties. Viral-vectored vaccines, or live recombinant vaccines, are non-replicating viruses stimulating strong immune responses. Virus-like particles and virosomes possess the structural features of viruses, which stimulate immune responses without the viral genetic material responsible for infecting the host. The virosomes are made of phospholipid vesicles, combined with the influenza virus proteins hemagglutinin and neuraminidase and vaccine-specific antigens [16]. Examples of vaccines based on virus-like particles and virosomes are Recombivax HB® (Merck), Gardasil® (Merck), Epaxal™ (Crucell), Inflexal® (Crucell) and Invivac® (Solvay) (**Table 1**). Viral-vectored vaccines, virus-like particles and virosomes will not be discussed further. Presented below are adjuvant technologies that apply the three lipid-based particulate delivery systems, namely, emulsions, liposomes and ISCOMs (**Figure 4**).

### 2.1 Emulsions

Apart from alum, emulsions are among the most frequently used adjuvants in humans and animals. Well-known examples are Montanide™ (Seppic, Paris, France), MF59 and the Freund's adjuvants (animals only). Emulsions are two-phase systems comprised of a continuous phase and a disperse phase (**Figure 4**). Both oil-in-water and water-in-oil emulsions have been applied as adjuvants, each affecting the immune response towards an antigen in a different way (see Section 4). However, water-in-oil emulsions have been used the most since their broad introduction in 1937 by Freund *et al.* [17]. Being thermodynamically unstable two-phase systems, emulsion adjuvants are usually stabilized by surfactants that

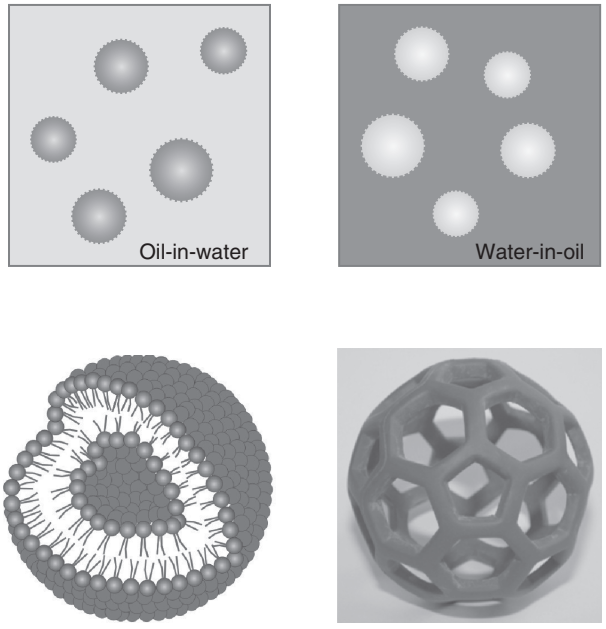


**Figure 4. Schematic illustrations of lipid-based particulate delivery systems.** Emulsions (upper panel) are two-phase systems of various sizes comprising a continuous phase and a disperse phase, usually stabilized by surfactants. Emulsions can be oil-in-water or water-in-oil emulsions. Water phase: light gray. Oil phase: dark gray. Liposomes consist of an aqueous core and one or several concentric phospholipid bilayers. A unilamellar liposome is shown in the lower panel, left. An immune-stimulating complex (lower panel, right) is a more porous, 40 – 50 nm particle and has a characteristic, cage-like structure. The sizes of the particles are not equalized.

form a monolayer on the surface of the droplets. Surfactants contain a polar group that is hydrophilic and a non-polar group that is hydrophobic, and often composed of a fatty acid chain. The antigen is added to the aqueous phase and can be adsorbed to the oil–water interface, thus partly replacing surfactant molecules depending on the interaction forces present in the specific system. As some antigens also possess surfactant properties, they can stabilize or destabilize emulsions, which necessitates further optimization of the formulation. Localization of antigen in adjuvant emulsions is rarely described in the literature, though. Emulsion adjuvants have continuously been developed towards more tolerable mixtures with better reactogenicity profiles by careful selection of the individual components.

Montanide comprises a group of emulsions, including ISA-50V, ISA-51, ISA-206 and ISA-720 [18]. ISA-50V, ISA-51 and ISA-720 are water-in-oil emulsions, whereas ISA-206 is a water-in-oil-in-water double emulsion. ISA-51 is a water-in-mineral oil adjuvant containing white medicinal oil and mannide monooleate as surfactant, and has been tested in many patients, showing a good safety profile. ISA-720 is based on the non-mineral metabolizable oil squalene. This is to improve the safety of the formulation, squalene being a natural product derived from animal or vegetable origin. Droplet size is ~ 1 μm, and the emulsion is physically stable for 1 year at 4°C [19]. Both formulations stimulate humoral and cellular immune responses and are fairly well-tolerated and safe [18]. ISA-206 and ISA-50V have been used only for animal vaccination. ISA-720 is under clinical trials (**Table 1**) for vaccines against for instance cancer, human immunodeficiency virus (HIV) and malaria [18,20,21].

The emulsion MF59 is approved for human use (**Table 1**) and has for a decade been included as adjuvant in a licensed influenza vaccine in Europe (Fluad®, Novartis), but is not yet licensed in the US [22]. MF59 is an oil-in-water emulsion composed of biodegradable squalene oil as the dispersed phase and low ionic strength citrate buffer as the continuous phase. It is emulsified by microfluidization with the two surfactants polysorbate 80 and sorbitan trioleate into a milky white, stable formulation with a particle size of ~ 160 nm [23]. Preclinical data in several animal models have shown that MF59 is a surprisingly potent stimulator of both humoral and cellular immune responses against a variety of antigens, and also that it possess a favorable safety profile [22,23]. Several vaccines containing the MF59 adjuvant have been tested in clinical trials encompassing different patient age groups against viral infections which, in addition to influenza virus, are caused by HIV, herpes simplex virus (HSV), hepatitis B/C virus (HBV/HCV), parvovirus, HPV and cytomegalovirus (CMV). This extensive testing in humans has shown that MF59-adjuvanted antigens elicit a strong humoral immune response, as well as a cellular immune response, and that MF59 has a good safety profile [23,24].

AS03 is also an oil-in-water emulsion consisting of squalene oil, α-tocopherol and Polysorbate 80 [25]. It was approved in 2008 in Europe as a part of the vaccine Prepandrix™ (GlaxoSmithKline) against pre-pandemic influenza strain H5N1 (**Table 1**). Prepandrix is a split virion, inactivated vaccine containing H5 hemagglutinin antigen. Adjuvantation of H5N1 vaccine with AS03 allows for a substantial reduction in the H5 hemagglutinin dose required to elicit an adequate immune response, and vaccination programs with two-dose administration of the adjuvanted vaccine offers full protection against pandemic flu. These advantages of the vaccine offset the disadvantages of higher reactogenicity, but the vaccine is well tolerated and adverse events are transient and predominantly of mild to moderate severity [25].

### 2.2 Liposomes

Liposomes are spherical vesicles composed of lipid bilayers enclosing aqueous compartments (**Figure 4**). Liposomes have been studied extensively as drug delivery systems owing to their ability to encapsulate, embed or associate a wide range of molecules, such as antigens or immunomodulatory compounds, and to facilitate targeted delivery of these to specific immune cells. In addition to enhanced delivery and uptake, as well as protection of antigen, the role of liposomes

in vaccine formulations is also to enhance and/or modulate the immunogenic properties of the vaccine. In some cases, liposomes show only limited immunogenicity, and extra immunostimulatory components are required (Section 4).

The properties of liposomes are highly attributable to their physicochemical characteristics such as vesicle size, surface charge, composition, rigidity of bilayer and method of preparation [26]. The surface charge in particular is of importance. *In vivo* studies demonstrate a superior adjuvant effect of cationic liposomes compared with anionic or neutral liposomes [27]. It has been demonstrated that anionic liposomes interact with a limited fraction of dendritic cells *in vitro*, whereas cationic liposomes interact with a very high percentage of the dendritic cells, probably by means of electrostatic binding to negatively charged surface heparane sulfate proteoglycans, resulting in intracellular localization [28]. Furthermore, the ability of liposomes to stimulate dendritic cells is dependent on the composition of the head group, the length and the saturation of the lipid tail groups [29]. The surface charge of liposomes is also of importance for formulating liposomes with antigens, as most antigens are negatively charged and thus capable of adsorbing to cationic liposomes. Encapsulation of antigens may offer protection and facilitate uptake of antigen by APCs. Association of peptide antigens to liposomes can be improved by utilizing the corresponding lipopeptide. The entrapment efficiency of a peptide antigen derived from the E7 oncoprotein of HPV was thus increased from 25 to 90% compared with the native E7 peptide by incorporation of E7-lipopeptide [30]. Encapsulation may also be improved by optimizing the formulation method. Up to 90% of antigen-encoding DNA may be incorporated using the dehydration–rehydration procedure [31].

Liposomes may in addition be provided with fusogenic properties, which make possible delivery of antigens to the cytosol of APCs. This results in MHC class I presentation of antigenic peptides and subsequent stimulation of CTL responses, which are important for protection against a variety of viral infections [32]. Fusogenic liposomes may be based on lipids derived from lower organisms such as *Saccharomyces cerevisiae* (saccharosomes) or *Escherichia coli* (escheriosomes) that mediate fusion of liposomes with the plasma membrane, enabling access of antigen to the cytosol [33,34].

No liposomal adjuvants have yet reached the market, but several are in clinical trials (**Table 1**). AS01 (GlaxoSmithKline) is an adjuvant based on liposomes, MPL and *Quillaja saponaria* (QS) 21. Immunogenicity of the formulation has been demonstrated in monkeys with malaria vaccine candidates [35], and it is now in Phase II studies for malaria. The same adjuvant system also induced strong humoral and cellular immune responses against hepatitis B surface antigen (HBsAg) in healthy volunteers [36]. Liposomes based on ceramide carbamoyl spermine (CCS) and cholesterol were found to be efficacious in a murine influenza model, and a PhaseI/IIa clinical trial was initiated [37]. The vaccine is evaluated for intramuscular as well as intranasal administration. Liposomes based on the cationic lipid 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) showed potential as an adjuvant *in vivo* [38]. The safety of the formulation was evaluated recently in a Phase I study with a candidate HIV vaccine [39]. Cationic liposomes based on dimethyldioctadecylammonium (DDA) and trehalose 6,6-dibehenate (CAF01, Statens Serum Institut, Copenhagen, Denmark) have shown potential against a range of diseases *in vivo* and are scheduled to enter clinical trials with a tuberculosis vaccine candidate [40]. Different liposome-based therapeutic vaccines have been shown to be efficacious in cancer patients. Stimuvax® (or L-BLP25, Oncothyreon and Merck) is a liposomal vaccine candidate consisting of BLP25 lipopeptide and MPL, as well as cholesterol, dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylcholine formulated into a liposomal product. A Phase IIb trial encompassing patients with stage IIIB and IV non-small cell lung cancer indicated an increased survival time for patients receiving the liposomal vaccine [41]. The vaccine entered Phase III clinical trials in 2007 [42].

### 2.3 Immune-stimulating complexes

ISCOMs are closely related to liposomes in being composed of cholesterol and phospholipids [43]. Furthermore, they contain an extra built-in saponin adjuvant, often Quil-A, which changes the bilayered structure of liposomes into spherical, cage-like hollow assemblies with a diameter of 40 – 50 nm (**Figure 4**). These structures have been reviewed thoroughly elsewhere [44,45]. The saponins are heterogeneous mixtures of sterol glycosides and triterpenoid glycosides, which have been widely used as potent adjuvants, and several purified fractions have been tested, the most common being Quil-A and QS-21. However, it has long been known that saponins interact with lipids of cell membranes and cause cell lysis. ISCOMs have the advantage of combining a powerful immunostimulator (saponin) with a vaccine delivery system, and thereby reducing the cytotoxic effect of the saponins [46].

ISCOMs are generally defined as antigen-incorporated vehicles, whereas the empty ISCOMs are named ISCOM matrices. Various companies are now investigating the use of ISCOMs for vaccination purposes (**Table 1**). CSL has ISCOMATRIX™ adjuvant in preclinical and clinical development, and successful Phase I studies have been published regarding ISCOMATRIX mixed with different antigens, for example, HPV and the cancer testis antigen NY-ESO-1 [47,48]. An ISCOM technology has also been marketed in several veterinary vaccines by the Swedish company Isconova and is now being tested preclinically for influenza vaccination. In animals, ISCOMs have been shown to promote both humoral and cellular immune response with several different antigens such as tumor antigens, viral antigens and ovalbumin. This includes an induction of high antibody titers at low doses of antigen, stimulation of both Th1 and Th2 type T-cell responses [49] and activation of CTL [50].

Conventional ISCOMs are negatively charged at physiological pH, which can present a challenge for association of negatively charged antigens. As ISCOMs are hollow structures,

it is not possible to encapsulate antigens as, for example, liposomes. Alternatively, the antigen can be embedded or anchored into the lipid structure, making it accessible on the surface of the ISCOMs. Antigens that are derived from cell membranes can generally be incorporated directly into the ISCOM structure owing to their hydrophobic transmembrane domains. However, it is often necessary to modify more hydrophilic antigens, yet the effect of this modification on the immunogenicity of the antigen remains to be understood. Alternatively, cationic ISCOMs (PLUSCOMs) have been prepared and tested *in vivo* [51]. This enables electrostatic interaction between negatively charged antigens and the particles. Another variant of this approach is to use a positively charged cholesterol derivative to modify the charge of the ISCOMs. Particles based on this promising principle are called Posintro™ [52].

It is still being debated whether the antigens have to be associated to ISCOM structures at all to activate immune responses. Traditionally, ISCOM matrices with a negative charge (ISCOMATRIX) have been shown to induce an immune response in humans on physical mixing with the chosen antigen [53]. Another study in mice indicated that the antigen dose can be reduced 6 – 10 times when the antigen is associated to ISCOMs as compared with physical mixing [54]. A clinical study has compared influenza antigens in combination with ISCOM matrix or incorporated in ISCOMs. This study did not show any significant difference between the two ISCOM formulations, but both ISCOM-adjuvanted vaccines induced significantly higher antibody responses compared with the marketed influenza vaccines. Furthermore, the influenza ISCOM-based vaccines induced specific CTL response in 25 – 80% of recipients, compared with 5% of those receiving standard influenza vaccine [55,56].

## 3. Immune-stimulating compounds

It is relevant to combine lipid-based particulate adjuvants and immunostimulators to enhance immune responses or to direct immune responses towards the desired pathway of humoral or cellular immunity. The involvement of TLR activation in adaptive immunity has suggested an important role for TLR ligands in vaccine adjuvants (**Figure 1**). Thus, immunostimulators include ligands that target TLRs and other types of PRR, such as the nucleotide-binding oligomerization domain proteins (NOD), NOD-like receptors (NLR) and RIG-like helicases (RLH) expressed by APCs [57]. TLRs recognize a wide range of extracellular PAMPs (**Figure 1**): i) lipopolysaccharide (LPS) and its derivatives (e.g., MPL) are recognized by TLR4; ii) peptidoglycan from Gram-positive bacteria and lipopeptides bind to TLR2; iii) double-stranded RNA is recognized by TLR3; iv) bacterial flagellin signals through TLR5; and v) unmethylated CpG motifs in bacterial DNA are recognized by TLR9 (reviewed in [58]). Different natural or synthetic TLR agonists can induce distinct Th-cell responses. TLR activation generally favors initiation of Th1 responses, but a subset of TLR ligands (e.g., Pam3cys and schistosome egg Ag) favors development of a Th2 response [59].

Only a few reports exist on studies of TLR agonists in combination with emulsions or ISCOMs, whereas several studies have been conducted using liposomes in combination with TLR ligands. ISCOMs have been combined with TLR ligands to increase targeting [60]. The adjuvant emulsion AS02 contains the TLR4 ligand MPL. However, the liposome-based formulation AS01, which also contains MPL, was found to be immunologically superior to AS02 for cellular responses, without sacrificing humoral immunity in Rhesus macaques [35]. Similar results were demonstrated in a comparative challenge study in humans [61]. Also, MPL has been evaluated in combination with other liposomes with subsequent improved immunogenicity. For example, MPL in combination with DDA liposomes mediated an antigen-specific T-cell response and increased protection in a murine tuberculosis challenge model [62].

TLR2 recognizes microbial lipoproteins and lipopeptides, which usually requires formation of a receptor dimer complex of TLR2 with either TLR1 or TLR6 [63]. Lipopeptides enable antigens to be delivered to the cytoplasm of dendritic cells, which is needed for MHC class I presentation and subsequent induction of $CD8^+$ T-cell responses [63]. $CD8^+$ CTL responses were induced after immunization of mice with a lipid-conjugated HSV glycoprotein B epitope incorporated into liposomes. Free antigen did not induce $CD8^+$ CTL responses, whether incorporated into liposomes or not [64]. Other studies of liposomal adjuvants also show an enhanced antigen-specific $CD8^+$ T-cell response by lipopeptide antigen compared with the native antigen [30]. It is unclear, though, whether TLR2 activation is involved or the effect is merely a result of cytoplasmic delivery.

Strong antigen-specific $CD8^+$ T-cell responses are induced by complexes of cationic liposomes with the TLR9 ligands CpG oligonucleotide, and/or plasmid DNA or the TLR3 ligand Poly IC [65]. Other studies also support the tenet that liposomes in combination with CpG oligodeoxynucleotides induce immune responses of the Th1 type [66,67] or mixed Th1/Th2 response [67].

Small interfering RNA (siRNA) mediates immune activation through TLR3 [68] or TLR7 [69] signaling. A new approach is to use siRNA to inhibit a natural inhibitor of TLR and cytokine signaling. By using siRNA to inhibit a suppressor of cytokine signaling (SOCS) 1, the immune response to an HIV antigen was improved in mice [70]. The SOCS1-silencing induced antigen-specific $CD8^+$ CTLs and $CD4^+$ Th cells as well as antibody responses. Stimulation by Poly IC (TLR3 ligand) or R837 (TLR7 ligand) improved the response further. Combining this approach with lipid-based particulate adjuvants has, to the authors' knowledge, not yet been investigated.

Non-TLRs such as the cytosolic NOD, NLR and RLH sense intracellular signals. A well-known example of a NOD ligand is muramyl dipeptide (MDP) from the cell wall component petidoglycan of Gram-negative and Gram-positive bacteria.

MDP is an active but low-toxicity component of complete Freund's adjuvant. MDP has been used as an adjuvant and was suggested to induce humoral immune responses, but recent data suggest that MDP is a poor inducer of antibodies [71]. However, MDP might, in combination with antigen delivery systems, have attractive immune-activating effects. For further description of more ligands for non-TLR innate receptors, refer to, for example [57].

## 4. Adjuvant mechanisms

A rational choice of adjuvant is complicated by the fact that the *in vivo* molecular and cellular mechanisms required for the generation of an effective immune response by adjuvanted antigens are poorly understood, and that the structural requirements for adjuvants are largely unknown [72]. Adjuvants can be classified functionally according to six proposed concepts of immunogenicity [73,74]: i) the paradigm that adjuvants act as signal 0; ii) the geographical concept of immune reactivity (signal 1); iii) the hypothesis that adjuvants play a role as signal 2 molecules; iv) the hypothesis that adjuvants induce or act as danger molecules; v) the theory of depot effect; and vi) the hypothesis that adjuvants can stimulate CTL induction. These concepts are summarized in **Table 2**, and examples of adjuvants belonging to each category are given [73,74].

The first concept suggests that adjuvants mimic PAMPs [75]. Binding to PRRs such as the TLRs can in direct or indirect ways induce signal 2 on APCs [76]. As the PAMP-recognition precedes signal 1 and 2, it is referred to as signal 0. This concept of action seems to explain the adjuvant effect of microbial products such as CpG-rich motifs, LPS, MPL and MDP [57].

Adjuvants that function via the geographical concept of immune reactivity are facilitators of signal 1 by controlling the residence time, the place and the dose of the antigen to maintain immunity levels [77]. It has been proposed that immunization depends on the antigen reaching the lymphoid organs, as initiation of immune responses takes place exclusively in lymphoid organs where antigen-loaded APCs interact with T cells that provide signal 2 [78]. In mice that lack lymph nodes or where the passage to lymph nodes is blocked, no immune response develops [79,80]. Whether adjuvants exert their action at the administration site or in lymphoid organs, as well as the temporal delivery of antigen, is not well understood, but it is hypothesized that adjuvants can initiate or increase the immune response simply by enhancing translocation of antigen from the injection site towards the peripheral draining lymph nodes, thus providing sufficient amounts of antigen to lymphoid tissue to sustain a response [73]. This can be a result of increased attraction of dendritic cells towards the injection site, increased loading of APCs or increased transport of antigen-loaded APCs to lymphoid organs. Increasing the amount of antigen available for the APCs can be mediated by particulate delivery systems such as ISCOMs and liposomes, which are better phagocytosed by APCs, and they thereby facilitate the delivery of antigen to, for example, the dendritic cells [73]. For ISCOMS, it is suggested that uptake in dendritic cells is increased by interaction of the saponin carbohydrates with the cell surface receptor DEC-205, which is capable of binding carbohydrate ligands [81]. Physicochemical characteristics of particulate delivery systems play very important roles for their interaction with APCs and the concomitant induction of immunity [82,83]. Smaller sized particles (< 500 nm) tend to invoke virus-like responses, dominated by Th1 and CTL responses, whereas larger particles (> 500 nm) induce bacterial-like responses, characterized by Th2 and humoral responses [82]. Cationic particles do in general interact more efficiently with APCs than neutral and anionic particles owing to electrostatic binding to cell surface heparane sulfate proteoglycans, enhancing the antigen acquisition by APCs [82,83]. The adjuvant mechanism of the oil-in-water emulsion MF59 is suggested to be the direct stimulation of pro-inflammatory cytokines that can induce recruitment of immune cells from blood to the injection site (intramuscular injection), stimulate monocyte differentiation into dendritic cells, augment antigen uptake and facilitate migration of dendritic cells into tissue-draining lymph nodes to prime adaptive immune responses [22,84].

Adjuvants can also function directly as signal 2 molecules by upregulating co-stimulatory molecules on APCs [85]. Co-delivery of antigens with co-stimulatory molecules thus enhances immune responses by the simultaneous delivery of signal 1 and signal 2 [86]. Inflammatory cytokines produced at the injection site can also induce upregulation of co-stimulatory molecules [73]. These cytokines are produced by macrophages or innate immune cells on stimulation by the adjuvant. The type of inflammatory cytokines produced determines the polarization of the T-cell response. Cytokines by themselves are tested as adjuvants and have been shown to act as such [87].

The 'danger model' of the immune response proposes that signals from damaged or stressed cells can initiate an immune response [88]. According to this model, APCs are activated only in peripheral tissues in situations that are dangerous to the host tissue. The so-called danger signals comprise tissue destruction and necrosis, and these signals upregulate the expression of co-stimulatory molecules (signal 2) on APCs [89]. An adjuvant can thus be defined as a danger (-inducing) signal that, besides tissue destruction and necrosis, also includes infection, cell stress, temperature shifts, hypoxia, trauma, mitochondria and heat shock proteins [90]. These are all signals from damaged or infected cells but not apoptotic cells. It has been suggested that immune responses are proportionally related to the tissue damage evoked by the adjuvant. As an example, adjuvant mineral oils cause necrosis of muscle fibers on injection followed by influx of neutrophils [91].

The concept of the depot effect of adjuvants is based on a prolonged delivery of antigens (signal 1), whereby T cells in the lymph nodes are triggered for a sufficient time period to induce an immune response [73]. Oil-based adjuvants such as the water-in-oil emulsion Montanide

**Table 2. Classification of adjuvants according to the immunological events they induce.**

| Group | Concept of action | Examples of adjuvants | Key event(s) |
|---|---|---|---|
| 1 | Antigen recognition and APC activation (signal 0) | Complement, CpG-rich motifs, LPS (monophosphoryl lipid A), mycobacteria (muramyl dipeptide), yeast extracts, cholera toxin, ISCOMs? | Signaling by PAMPs to PRRs on innate immune cells |
| 2 | Enhancement of antigen uptake, transport and presentation by APCs (signal 1) | ISCOMs, Quil-A, $Al(OH)_3$, liposomes, poly(lactide/glycolic acid) | Antigen localization in the lymph nodes |
| 3 | Recombinant co-stimulation (signal 2) | Cytokines, co-stimulatory molecules | APC polarization, T- and B-cell help, immunomodulation |
| 4 | Danger signal | Oil-emulsion surface-active agents, $Al(OH)_3$, IFNs, heat-shock proteins, hypoxia | Tissue destruction/stress |
| 5 | Depot effect | Water-in-oil emulsions, $Al(OH)_3$?, gels, polymer microspheres, non-ionic block copolymers | Prolonged antigen presentation |
| 6 | CTL induction | Particles that can bind or enclose antigen and fuse with or disrupt cell membrane, e.g., ISCOMs, liposomes | Antigen processed in the cytosol, presentation on MHC class I molecules |

Modified from [73] and [74].
APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; INF: Interferon; ISCOM: Immune-stimulating complexes; LPS: Lipopolysaccharide; PAMP: Pathogen-associated molecular patterns; PRR: Pattern recognition receptor.



maintain antigen at the injection site, where an antigen deposit is formed, and sustain the release of antigen, localized in the water phase, over a prolonged period of time [18,92,93]. Liposomes and ISCOM are also suggested to provide vaccine formulations with depot properties, and in particular cationic particles might aggregate at the injection site when exposed to interstitial fluid following intramuscular or subcutaneous administration.

The last proposed concept of adjuvant activity suggests that some adjuvants can affect intracellular trafficking of antigen in APCs (**Figure 3**) and induce presentation of antigen on MHC class I molecules present on dendritic cells by means of the cross-presentation pathway with subsequent $CD8^+$ T-cell activation. These adjuvants include particulate adjuvants with fusogenic properties, such as liposomes and ISCOMs, which can fuse with cell membranes and introduce their antigen cargo into the cytoplasm of APCs. For ISCOMS, the ability of the saponins in ISCOMs to interact with cholesterol in the cell membrane enhances deposition of the antigen and adjuvant together into the cytosol and increases CTL responses [94,95].

These six immunological concepts are by no means mutually exclusive and some adjuvants may support more than one theory, but in general adjuvant mechanisms are poorly understood. In summary, adjuvants may thus add characteristics to an antigen that include enhancing and sustaining the delivery of antigen to the local lymph nodes, by providing a danger signal or a signal of non-self (signal 0), and by inducing inflammation.

## 5. Alternative administration routes

Most vaccines are still administered by injection. However, in recent years, more focus has been directed towards alternative administration routes to increase patient compliance. These include mucosal and transdermal routes. As most pathogens infect the host by means of the mucosal or dermal epithelium, these are attractive routes for administration of vaccines owing to the importance of controlling and preventing disease at the site of infection. The advantage of alternative administration is targeting of vaccines to local immune-active tissue, for example, the Langerhans cells in the epidermis or the mucosa-associated lymphoid tissue. Mucosal immunization results in induction of local immune responses, where secretion of IgA is an important immune effector mechanism. Below, studies applying alternative administration routes for lipid-based delivery systems are reviewed.

Mucosal (intranasal) administration of MF59-adjuvanted influenza subunit vaccines has been reported in only one study so far [96]. A mucosal IgA response to influenza was developed, and the percentage of subjects with a serum antibody response was only slightly lower, as compared with intramuscular administration. The immune responses to adjuvanted vaccine were not significantly different from those to non-adjuvanted vaccine, which suggests that there is no benefit in using the MF59 adjuvantation. Both vaccines gave more frequent responses than seen in placebo recipients, suggesting a potential benefit of merely using intranasal administration.

Several studies have been performed showing high local and systemic antibody responses following nasal vaccination with ISCOMs (see, e.g., [97]) and effective induction of CTL (see, e.g., [98]). For example, nasal administration of *Echinococcus granulosus* surface antigen in ISCOMs induced higher serum IgA titers in mice in relation to IgG than the subcutaneous route [99]. However, in dogs only a mucosal IgA response, no systemic response, was observed after nasal administration of the same formulation [100]. A comparative study has been performed between the oral route and the nasal route of administration for ISCOMs, indicating that the nasal route induced significantly higher IgG titers than oral immunization [101]. Furrie *et al*. demonstrated recruitment of dendritic cells, activated macrophages and increased amount of absorbed antigen from the intestine after oral administration of ovalbumin-ISCOMs [102]. Furthermore, ovalbumin-ISCOMs have been shown to exert adjuvant activity in the intestinal tract by stimulating secretory IgA in the intestine, spleen and lymph node CTL and serum IgG antibodies [103].

Mucosal administration of liposomal adjuvants has also been shown to possess advantages compared with commonly used invasive routes. Oral and intranasal administration of adjuvant formulations of MPL and liposomes were evaluated in mice with *Streptococcus mutans* crude glucosyltransferase antigen. Higher responses (IgG and IgA) were observed for the intranasally immunized mice than for the orally immunized mice [104]. Nasal delivery of a DNA vaccine in combination with surface-modified liposomes elicited higher IgA responses than intramuscular-administered naked pDNA and alum-absorbed HBsAg [105]. Other studies also show induction of an IgA response after intranasal administration of a liposomal vaccine [106]. Alternative administration routes have been evaluated in a few studies in humans. An HIV vaccine candidate with DC-Chol was administered nasally or vaginally in humans, but antigen-specific activity was not detected despite having been observed previously in Rhesus monkeys [39]. This underlines important species differences with respect to immune activation.

Chin and San Gil investigated intradermal vaccinatiom with a hybrid of liposomes and ISCOMs and concluded that activation of adaptive immunity is dependent on previous activation of the innate immune system [107]. Transcutaneous immunization with ethanol-containing liposomes (ethosomes) and an HBsAg in mice induced higher systemic and mucosal IgA levels compared with intramuscularly alum-absorbed HBsAg [108]. A study of HBsAg plasmid DNA complexed to deformable liposomes administered topically showed comparable serum antibody titers and cytokine levels to a naked plasmid DNA vaccine administered intramuscularly [109].

## 6. Conclusion

Lipid-based particulate delivery systems are promising alternatives to alum. The MF59 emulsion adjuvant is already marketed, and other types of lipid-based particulate system are in clinical development. However, more clinical studies are needed to disclose fully the potential of lipid-based particles. Lipid-based systems are biocompatible and biodegradable, and are versatile with the possibility of incorporating other immunostimulatory agents optimizing the immunological properties of the specific adjuvant. In addition, fusogenic components can make possible cytoplasmic delivery of antigen with cross-presentation by MHC class I and subsequent CTL induction. Further research into adjuvant mechanisms is needed to understand fully the immunostimulatory properties of the lipid-based adjuvants and to enable rational customization of vaccines towards specific immunological profiles. Alternative administration routes are attractive for compliance reasons, although knowledge on important factors such as optimal dosing and reproducibility of administration is still lacking.

## 7. Expert opinion

Vaccination has prevented and treated many diseases since the broad introduction of vaccination programs and is regarded as one of the most cost-effective ways of controlling infectious diseases [110]. Despite this success, there is still an unmet medical need for the development of new vaccines against the global 'killers' such as HIV, tuberculosis and malaria, which cause millions of deaths annually, primarily in the developing world, and against new or emerging diseases such as pandemic influenza, Ebola, cancer and immune disorders.

### 7.1 Vaccine selection criteria

Many promising experimental adjuvants have been reported in the literature for use with subunit antigens, among them lipid-based adjuvants, but only a few of them have been approved for human use. There is thus a need to advance already existing, as well as new, adjuvants into human clinical trials. The main criteria for selection of good vaccine candidates should be improved safety and tolerability profiles, as healthy individuals (often infants) comprise the patient group. Safety-wise, recombinant antigens are preferable in this respect, as compared with whole-cell and live attenuated vaccines, owing to their purity, but also present challenges because their use requires effective adjuvants. Also, adjuvants have to be composed of highly pure components of low toxicity. The individual constituents of the vaccine formulation, as well as the manufacturing process, should be low cost, and the formulation must have adequate stability during production and storage, and must be sufficiently characterized in terms of physicochemical characteristics and stability. In addition, the degree of antigen association might be of importance for a safe and reproducible immunological readout and thus represents an important aspect of the characterization of the formulation.

### 7.2 Adjuvant development

Adjuvant research has been largely empirical for many years, but is now changing direction towards a more rational design of vaccine formulations. This is to obtain customized

immunostimulatory profiles that are more specific and focused, depending on the requirements for humoral, cellular and/or mucosal responses for each specific disease [57]. The increasing knowledge of innate and acquired immune responses is making it easier to take this into account early on in the development phase of new vaccines, with the aim of customizing adjuvants towards the required immunological profile. In this respect, it is important to clarify the mechanism(s) of action of adjuvants, as surprisingly little is known about the mechanism(s) of immune induction of the adjuvants used at present. This includes pharmacokinetic analysis of antigen/adjuvant localization, identification of target cells for the adjuvants and/or the antigen, and the effects the adjuvants exert on them, enabling the identification of efficient adjuvants and the rational design of new and better vaccines. The choice of adjuvant is dependent on the immune response it induces, but also on the specific antigen (associated/co-administered), the administration route and the immunization schedule, allowing the optimal pharmaceutical parameters to be defined on a case-by-case basis.

### 7.3 Customizing immune responses with lipid-based delivery systems and immunostimulators

Lipid-based delivery systems are commonly used adjuvants and also possess a large potential for new vaccine development. They are composed of natural constituents that are favorable because of their biodegradability and biocompatibility, and are versatile systems, the physicochemical and immunoactivating properties of which can be modulated and fine-tuned by changing the composition of the formulation and by adding immunostimulatory compounds. A promising approach seems to be to customize immune responses towards specific immunological profiles required for protection against a given pathological condition, using lipid-based delivery systems and immunostimulators. For each case, the optimal combination of antigen, lipid-based delivery system and immunomodulator should be defined. More specific and customized vaccines might allow for a reduction in the required antigen dose owing to a more efficient adjuvant effect, which is particularly relevant with, for example, pandemic influenza, where vaccine coverage is important. In addition, sustained release and pharmacokinetic/pharmacodynamic properties of particulate delivery systems might allow for better control of the tempospatial requirements of the antigen. ISCOMs and certain types of liposome have attractive fusogenic properties, enabling delivery of antigen to the cytosol of APCs, followed by cross-presentation of antigen and CTL activation for the prevention of, for example, viral diseases and cancer. However, in the literature there is a lack of comparative studies between different formulation types, which makes direct comparison of different adjuvants difficult.

### 7.4 Alternative administration routes

Non-invasive administration techniques are promising alternatives to intramuscular or subcutaneous vaccine delivery in terms of safety (fewer side effects), compliance and the possibility of inducing immunity at the primary site of infection for many pathogens (the mucosa), but present several challenges in terms of device development, keeping costs low and variation in delivery. Airway delivery seems to be the most promising alternative administration route, although this must be applied with caution owing to reported unacceptable neurological side effects associated with retrograde passage of inhaled antigens or adjuvants through the olfactory epithelium [111]. Oral administration and single-dose vaccines have long been desired and remain major challenges in vaccine research.

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## Declaration of interest

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## Affiliation

Pernille Nordly MSc PhD student,
Henriette Baun Madsen MSc Pharm PhD student,
Hanne Moerck Nielsen MSc Pharm PhD Associate Professor & Camilla Foged† MSc PhD Associate Professor
†Author for correspondence
Faculty of Pharmaceutical Sciences,
University of Copenhagen,
Department of Pharmaceutics and Analytical Chemistry,
Universitetsparken 2,
DK-2100 Copenhagen O, Denmark
Tel: + 45 35 33 64 02; Fax: + 45 35 33 60 30;
E-mail: cfo@farma.ku.dk