Expert Opinion

Liposomal vaccine delivery systems

Malou Henriksen-Lacey, Karen Smith Korsholm, Peter Andersen, Yvonne Perrie & Dennis Christensen[†]
[†]*Statens Serum Institute, Department of Infectious Disease Immunology, Copenhagen, Denmark*

Introduction: Liposomes remain at the forefront of drug and vaccine design owing to their well-documented abilities to act as delivery vehicles. Nevertheless, the concept of liposomes as delivery vehicles is not a new one, with most works focusing on their use for the delivery of genes and drugs. However, in the last 10 years a significant amount of research has focused on using liposomes as vaccine adjuvants, not only as an antigen delivery vehicle but also as a tool to increase the immunogenicity of peptide and protein antigens.
Areas covered: This paper reviews liposomal adjuvants now in vaccine development, with particular emphasis on their adjuvant mechanism and how specific physicochemical characteristics of liposomes affect the immune response. The inclusion of immunomodulators is also discussed, with prominence given to Toll-like receptor ligands.
Expert opinion: The use of liposomes as vaccine delivery systems is evolving rapidly owing to the combined increase in technological advances and understanding of the immune system. Liposomes that contain and deliver immunostimulators and antigens are now being developed to target diseases that require stimulation of both humoral and cell-mediated immune responses. The CAF liposomal system, described in detail in this review, is one liposomal model that shows such flexibility.

Keywords: cationic, delivery vehicle, immunostimulatory, liposome, pathogen recognition receptors, physicochemical characteristics, Toll-like receptor

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1. Introduction

Subunit vaccines constituting highly purified peptide or protein antigens are at the forefront of vaccine research owing to their strong safety profile, relative ease in scale-up production, and low production cost as compared with the classical live-attenuated or killed vaccines [1]. However, they generally show poor immunogenicity owing to the lack of pathogen-associated molecular patterns (PAMPs), and rapid degradation of small proteins and peptides *in vivo* reduces the dose-effectiveness of such vaccines. Consequently, delivery systems have been applied in marketed vaccines for over 70 years to protect and carry antigens, as well as acting as adjuvants for otherwise inert molecules [2]. The most commonly used adjuvants in human vaccines are aluminum-based mineral salts, which have an enviable safety record and well-documented ability to adjuvate a range of vaccines. Even though aluminum-based mineral salts have been known as adjuvants for the past 85 years [3], the mechanisms by which they act are still incompletely understood [2,4,5]. Just over 10 years ago, new adjuvants such as virosomes composed of viral proteins inserted into phospholipid bilayers (developed by Crucell (The Netherlands)) and the oil-in-water (o/w) emulsion MF59® developed by Novartis (Switzerland) [6] were licensed. MF59 is now in use in the influenza vaccine Fluad®, and recent successful Phase I clinical trials have established it as a potential adjuvant for vaccination against hepatitis C [7]. More recently licensed human adjuvants are the 'adjuvant

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systems' (AS) AS03 and AS04 developed by GlaxoSmithKline Biologicals (GSK, Belgium) [8]. AS03 is, like MF59, a squalene o/w emulsion and is included in the influenza vaccine Pandemrix® (GSK, Belgium). The AS04 adjuvant is composed of aluminum hydroxide and the Toll-like receptor (TLR) agonist monophosphoryl lipid A (MPL), and is now licensed in vaccines against human papillomavirus (HPV) types 16 and 18 (Cervarix®(GSK, Belgium)) and hepatitis B virus (HBV; Fendrix® (GSK, Belgium)). The efficacy of these adjuvants are, however, limited in many disease targets because oil emulsions and aluminum salts are generally poor inducers of T_H1 and CTL responses, which are vital for the development of strong immunity against most intracellular infections, such as tuberculosis (TB), malaria and HIV, where antibody responses alone are of limited effect. In the last 10 years several studies have shown that liposomes can act not only as vaccine delivery vehicles, but also as tools to increase the poorly immunogenic profiles of peptide or protein antigens, and be modulated to induce both T_H1 and CTL responses. Herein, the adjuvant mechanism of liposomes, and how specific physicochemical characteristics affect the immune response, is reviewed.

1.1 Liposomes as drug and vaccine delivery systems

Although there are a few liposomal formulations already licensed for therapeutic drug delivery and numerous undergoing clinical investigation [9], there is a significant void in the use of liposomes as vaccine delivery systems. Yet, liposomes are effective delivery systems as they render soluble protein antigens into a particulate form thereby lengthening their half-life *in vivo*. Furthermore, liposomal formulations composed of cationic lipids have been shown to be immunostimulatory [10,11].

Liposomes were originally discovered by the late Alec Bangham and co-workers [12], who derived the name from the Greek 'lipos' (fat) and 'soma' (body). Liposome formation is an energy-dependent process that is heavily affected by the geometry of the lipid monomers, which can be quantified by the critical packing parameter (CPP) of the lipids. Lipids expressing large head groups and double hydrocarbon chains have a CPP of < 1, which is required for the formation of bilayered vesicles [13]. The bilayered nature of liposomes makes them attractive delivery systems as both hydrophilic and lipophilic substances can be encapsulated within the liposomes (**Figure 1**). By applying post-formation techniques such as sonication, extrusion or freeze-drying, the natural heterogeneous multilamellar structure of liposomes can be altered to favor a higher encapsulation ratio [14].

The main advantages of using liposomes as drug delivery systems are: i) their ability to entrap and therefore protect drugs from degradation; ii) deliver drugs with otherwise poor solubility; iii) improve the therapeutic index of drugs by shielding and therefore reducing toxicity; and iv) alter the specific targeting/distribution of the drug [9,15,16]. Examples in which these above-outlined liposome attributes are exploited in approved drugs include formulations enhancing the solubility and targeting of amphotercin B (AmBisome®, Astellas Pharma, Canada), and promoting tumor targeting and minimizing nonspecific drug toxicity of anticancer agents such as doxorubicin (Caelyx®, Scheering-Plough, Belgium, Myocet®, Cephalon, USA) and daunorubicin (DaunoXome®, Diatos, France).

Many of the properties leading to successful liposomal drug delivery systems are also valid for vaccine delivery [17]. First, liposomes efficiently protect small peptide/protein antigens from enzymatic breakdown by host cells [18,19]. The bilayered nature of liposomes allows these hydrophilic components to be either encapsulated in or adsorbed onto the liposomal surface while the lipophilic part of the liposomes allows other lipids or apolar polymeric components to be included in the membrane bilayer. Second, depending on the liposome composition, charge and size, liposomes can have different pharmacokinetics and be formulated to obtain optimal retention and presentation of the vaccine antigens [20]. Third, owing to their particulate nature, liposomes are avidly taken up by antigen-presenting cells (APCs). This ability probably represents the most important characteristic of liposomes to be used as vaccine delivery systems [21] because it allows for simultaneously ingested antigen to be processed and presented on MHC molecules.

There is a vast amount of literature on the use of liposomes as vaccine delivery systems. Hence, the focus of this review is the general properties of liposomes favoring their use as vaccine adjuvants.

2. Structural considerations when designing liposomes as vaccine delivery systems

The structural assembly of liposomes comprising monomeric units that form both hydrophilic and hydrophobic domains allows for numerous structural alterations to occur, thereby targeting and interacting with a wide array of cellular and soluble components of the immune system. Among these, size, charge and membrane fluidity are the most commonly studied, and they are discussed in detail below.

2.1 Promotion of cellular uptake by altering vesicle size and surface charge

The range of endocytic routes used by cells to internalize invading and endogenous substances is postulated to be an evolutionary mechanism designed to target different intracellular activation pathways [22]. Concomitantly, if liposomes could be targeted to certain endocytic routes, different immune responses could be stimulated, for example, by expression of certain co-stimulatory molecules or activation of a specific subtype of T cells. This hypothesis has been demonstrated in numerous studies, primarily with the use of size-specific nanoparticles [23-25]. Recently, Mann *et al.* revisited the T_H1/T_H2 paradigm using heterogeneous-sized bilosomes (liposomes incorporating bile salts) for oral delivery in an influenza model [26]. In correlation with previous experiments [23], their findings suggested that smaller vesicles

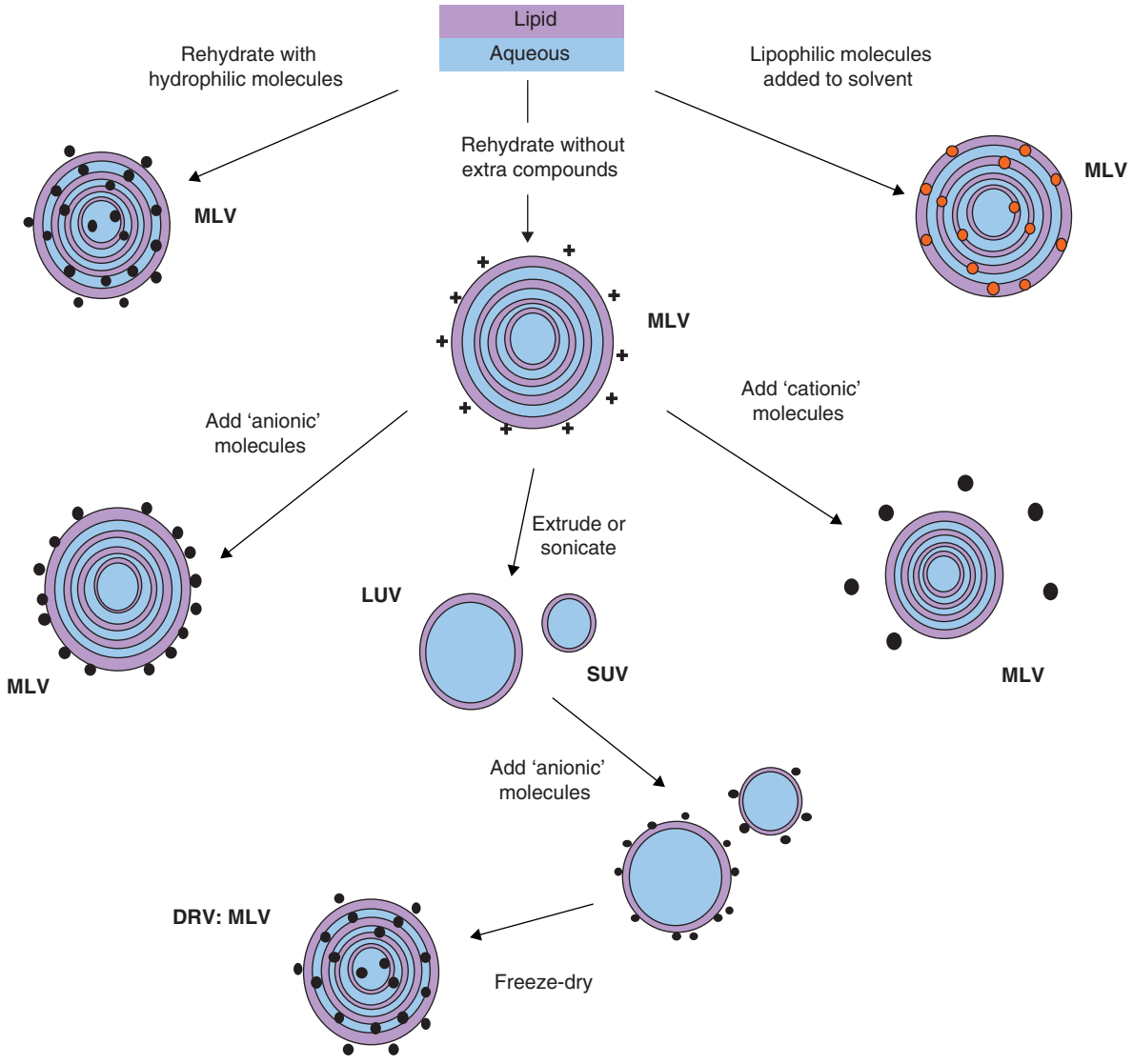

Figure 1. Schematic of liposome types including MLVs, SUVs and LUVs. A further type of liposome can be described as a DRV, which forms after freeze-drying and is used to encapsulate antigen.
DRV: Dehydration–rehydration vesicle; LUV: Large unilamellar vesicle; MLV: Multilamellar vesicle; SUV: Small unilamellar vesicle.

(~ 250 nm) enhanced T_H2 responses whereas larger liposomes (~ 980 nm) induced high levels of IFN-γ and IgG2 antibodies characteristic of T_H1 responses. Importantly, larger liposomes also gave better protection in an influenza challenge model in ferrets [26]. Nevertheless, the interpretation that smaller particles lead to T_H2 and larger ones to T_H1 responses may be an oversimplification because other factors governing immune responses are affected by differences in particle size. Thus, particle size has also been shown to have a significant effect on vesicle trafficking to lymph nodes [27], antigen uptake [28] and processing by APCs [29]. Manolova *et al.* recently described the trafficking of virosomes and nanoparticles of different sizes to the local draining lymph nodes (LNs) after injection into the footpads of mice [30]. Larger particles (0.5 – 2 µm) remained at the site of injection (SOI) and only these larger particles were shown to be taken up by dendritic cells (DCs) at the SOI, whereas small (< 200 nm) particles drained freely to the LNs wherein they were taken up by phagocytic cells (DCs and macrophages), suggesting that the location of particle uptake is highly dependent on the particle size. Consequently, the induction of IgG2 antibodies, B-cell and $CD8^+$ T-cell responses were facilitated when TLR7/8 agonist/antigen particles were small enough to reach the LNs directly, allowing for activation of resident B cells and $CD8^+$ DCs on simultaneous acquisition of native antigen and the TLR agonists [31]. In terms of particulate phagocytosis, previous *in vitro* studies investigating the phagocytic abilities of macrophages versus monocyte-derived DCs have shown that

more efficient phagocytosis occurs if vesicles are large (4.5 μm) and/or positively charged compared with small (1 μm) and/or negatively charged vesicles [32]. Furthermore, higher levels of DC maturation, as measured by CD83 expression, were obtained with cationic vesicles compared with anionic vesicles. Monocyte-derived DCs are noted for their plasticity and ability to accumulate in LNs after infection [33]. Thus, vaccine delivery systems composed of larger cationic liposomes forming a depot at the site of injection and having the ability to mature the monocyte-derived DCs may provide an ideal method to target and stimulate this population of DCs, which can subsequently initiate specific T-cell responses.

Numerous studies have shown that co-administration of antigen with cationic liposomes induces stronger antigen-specific immune responses as compared with neutral/anionic liposomes [34-36]. The authors have shown that cationic but not neutral liposomes are able to cause high levels of antigen retention at the SOI, improved antigen presentation, and strong cell-mediated immune responses characterized by IFN-γ and IL-17 production and proliferation of multifunctional T cells [20]. Indeed, not only cationic liposomes but also cationic structures, such as positively charged immune-stimulating complexes (ISCOMS) termed PLUSCOMS [37,38] and the cationic peptide $KLKL_5KLK$, which is a component of the IC 31® adjuvant (Intercell, Austria) [39], have all been successfully applied as adjuvants in preclinical settings. **Figure 2** lists a variety of cationic vaccine constructs and their antigen/disease models for which they are now in clinical trials or showing promising results in various animal models.

2.2 Effect on immunogenicity by altering the fluidity of liposomal bilayers

Liposomal membrane fluidity is governed by the level of saturation of the hydrocarbon chains and consequently the strength of van der Waals forces that hold adjacent chains in association [9]. The energy required to disrupt these chains and to alter their packing effectiveness and consequently their level of fluidity/rigidity is defined by the main phase transition temperature, T_m, of the lipid. At the T_m the lipid bilayers change from a rigid and ordered structure to a loosely disordered and fluid structure. Decreasing hydrocarbon chain length and introducing C = C bonds, both of which lead to a reduction in the T_m of the liposome, leads to an increase in bilayer fluidity and permeability. Several papers describe how modification of bilayer fluidity can alter the immunogenicity of liposomes. Mazumdar *et al.* have, for example, shown that neutral phosphatidylcholine liposomes in their solid phase at physiological temperatures induce high cell-mediated and humoral immune responses, whereas liposomes in their fluid phase give rise only to humoral immune responses, suggesting that the overall humoral immune response is not significantly affected by changes in fluidity [40]. A recent study investigating influenza vaccines adjuvanted with liposomes composed of a new type of polycationic lipid described no significant changes in immunogenicity (hemagglutination inhibition [HI] titers) on substitution of the saturated lipid chains with monounsaturated analogues [41]. However, no mention was made of the transition temperatures of these liposomes or their membrane state *in vivo*. There are several possible explanations for the alteration of the immune response obtained by changing the liposome fluidity. One explanation could be that the uptake mechanism by APCs varies depending on the fluidity of the liposomes, resulting in different pathways of antigen presentation and immunostimulation of APCs. Another explanation relates to the depot effect, whereby lengthening antigen or adjuvant retention at the SOI leads to increased exposure of APCs to antigen or immunostimulators, respectively. In support of this, the authors' recent research has shown membrane rigidity to have a significant effect on the ability of these liposomal vaccines to be retained at the SOI (paper in preparation). Although liposomes composed of more rigid bilayers were able to form a long-term depot of vaccine components (protein antigen and liposome) at the SOI that drained slowly to the local lymph node, their fluid counterparts were not as strongly retained at the SOI and showed faster draining of liposomal components to the local lymph node. Liposome rigidity corresponded with improved immunogenicity even though both liposomal formulations expressed comparable size and surface charges and showed similar antigen biodistribution and kinetic profiles.

A great number of the studies investigating how modification of bilayer fluidity alters the immunogenicity of liposomes incorporate cholesterol to stabilize lipid bilayers. This, however, adds a level of complexity to the interpretation when comparing, for example, lipids with different chain lengths or degrees of saturation [35,40,42-45]. Rather than altering the gel-to-fluid phase transition as obtained by changing acyl-chain length or degree of saturation of the membrane lipids, cholesterol eliminates the solid ordered and fluid disordered phases by reorganization of the membrane lipids, forming an intermediate fluid ordered phase [46]. In a study by Nakano *et al.*, the humoral immune response mediated by neutral phosphatidylcholine-derived liposomes was shown to be reduced after incorporation of cholesterol [47]. By contrast, Bakouche and Gerlier found that incorporation of cholesterol increased the immunogenicity of the liposomes, suggesting that it is not so much the solid packing of the lipid membrane but rather the ordered conformation of the lipid that is important for the immunogenicity of the liposomes [45]. Van Houte *et al.* observed that incorporation of cholesterol into fluid as well as solid haptenated liposomes increased their immunogenicity, whereas liposomes with an intermediate T_m (~ 40°C) were already highly immunogenic and thus little affected by the incorporation of cholesterol [48]. Based on these results, they concluded that the induction of humoral immune responses by liposomes required an 'intermediate' fluidity of the lipid bilayer. The overall effect of incorporation of cholesterol is therefore not consistent, suggesting that the effect might also be related to other yet unknown factors.

Disease model/agent	Vaccine	Delivery system properties	Antigen	Antigen: delivery system association	Reference
Influenza	'VaxiSome' Liposome composed of CSS and cholesterol helper lipid	Vesicle size: 0.05 – 10 μm Charge: 65 mV	Monovalent subunit/trivalent split virions	> 41 % depending on antigen:lipid ratio	[35]
	Vaxfectin® Liposomes composed of VC1052 lipid and DPyPE helper lipid		Trivalent split influenza vaccine	N/D	[111]
	'CAF01' Liposomescomposed of DDA lipid and TDB immunomodulator	Vesicle size: ~ 500 nm Charge: ~ 50 mV	Vaxigrip influenza split vaccine	N/D	[104]
Chlamydia			MOMP	N/D	[112]
Malaria			Recombinant MSP-1	N/D	[75]
Tuberculosis			Recombinant subunit Ag85B-ESAT-6 protein	98% surface adsorded	[73,75]
	Liposomes composed of DDA lipid and BCG derived lipid 'MMG'	Vesicle size: ~ 400 nm Charge: ~ 70 mV		N/D	[91,92,113,114]
	'IC 31®' Cationic peptide KLKL5KLK and the immunostimulatory oligodeoxynucleotide ODN1a	N/D		High adsorption (SDS-PAGE)	[72]
			Recombinant subunit Ag85B-TB10.4 protein	N/D	[115]
House dust mite allergy	Liposomes composed of DiC14-amidine lipid	N/D	Recombinant proDerp1	90% adsorption	[70,89]
HPV type 16	'LDP' Liposomes composed of DOTAP lipid	Vesicle size: 150 nm Charge: 52 mV	E7 protein	95% entrapment via freeze-drying	[71,116]

Figure 2. Cationic liposomes and constructs of recent interest as vaccine delivery systems.

CSS: *N*-palmitoyl-D erythrosphingosylcarbamoylspermine; DPyPE: 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine; DDA: Dimethyldioctadecylammonium bromide; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; HPV: Human papillomavirus; MMG: Monomycoloyl glycerol; N/D: Not determined/discussed; TDB: Trehalose 6'6-dibehenate; VC1052: (±) *N* (3 aminopropyl)-*N*,*N*-dimethyl-2,3 bis(*cis*-9-tetradecenyloxy)-1-propanaminiumbromide.

It is, however, evident that the changes in the bilayer structure resulting from incorporation of cholesterol can result in changes in immunogenicity.

3. Targeting the immune system with liposomes

The wide variety of adjuvants and mechanisms by which they act makes classification of these systems rather complex. Although there have been numerous notable attempts [49-51], one of the overlapping problems is that adjuvants in general act by several different mechanisms (**Figure 3**). The structural ability to associate with and deliver antigen to APCs can be considered the initial and most basic mechanism that makes liposomes suitable as adjuvants. Also, liposome-cell targeting methods with the use of antibodies or proteins for cellular receptors allow for specific localization and/or uptake accordingly [52-56]. A second mechanism of adjuvant action relies on the incorporation of immunostimulatory components into the liposome systems. These mechanisms are addressed below.

3.1 Liposomal delivery of vaccine antigens

Understanding the interaction between adjuvant and antigen is of considerable importance in vaccine design, not only as it may affect the efficacy of a vaccine, but also because manufacturing considerations, such as formulation stability and inconsistencies in antigen association, may lead to the demise of immunogenically sound vaccines. Most licensed adjuvants associate with antigen externally, either via adsorption or simply mixed together. The sole exception to this is Crucell's adjuvanted influenza vaccine Inflexal® V, composed of virosomes with their antigenic moieties inserted into the membrane [57]. Surface adsorption, resulting from either electrostatic interactions or ligand exchange [58,59], is highly favorable as the processes are relatively easy to undertake (simple mixing of both components is the only thing required), unassociated antigen does not need to be removed and the process is fast. Charged liposomes offer ideal antigen adsorption platforms but their physicochemical flexibility, liposomal charge, buffer pH and antigenic isoelectric point (pI) will all influence the level of antigen adsorption. The anionic nature of nucleic acid antigens (i.e., DNA, mRNA, siRNA) leads to their preferential adsorption to cationic liposomes (often termed lipoplexes), whereas the pI of peptide and protein antigens will determine their adsorption to anionic or cationic liposomes. In contrast to adsorption, antigen entrapment offers the ability to associate similarly charged liposomal and antigenic components, albeit with low entrapment levels often noted [18,60]. However, antigenic entrapment offers improved antigen protection from host enzymatic degradation (required for oral vaccines), and depending on the lipid composition can offer antigen protection from lysosomal enzymes by means of the 'flip-flop' mechanism [19,36,61,62]. A further method of association involves covalent linking of antigen to the adjuvant surface [63-66]. Although this method has numerous benefits, including an extended repertoire of possible antigen–adjuvant combinations, the process is neither fast nor easy and adds an extra layer of complexity to successful vaccine adjuvant formulation.

Figure 4 highlights some recent liposomal formulations that are being tested as experimental vaccines for delivery of nucleic acids. It is interesting to note that liposomal nucleic acid vaccines adopt both entrapment (via freeze-drying) and adsorption approaches. By contrast, protein and peptide-based vaccines are predominantly adsorbed to the surface of liposome formulations owing to difficulties encountered, such as loss of the antigen and its specificity during the preparation process. A further possibility is the simultaneous entrapment of nucleic acid and peptide/proteins antigens, such as the DRV-DNA entrapping ImuXen® (Lipoxen, UK) liposome formulation [67]. Few studies have offered direct comparisons between vaccine efficacy of adsorbed and entrapped peptide/protein formulations (variation in antigen dosage between entrapped and adsorbed formulations complicates matters); however, one such study showed no significant influence on the immunogenicity by entrapping the proteins instead of having them surface adsorbed [68]. Furthermore, the authors' recent work has shown that high levels of antigen adsorption to liposomes need not be necessary for improved antigen delivery, although antigens showing very poor levels of adsorption to liposomes owing to repulsive electrostatic interactions are not retained as long at the SOI [20]. Altogether, it is likely that antigen adsorption to liposomes is, and will remain, more favorable than entrapment.

Many of the antigens investigated for current subunit vaccine strategies express a pI < 7 units and consequently are suitably charged to adsorb externally to cationic liposomes. As noted in **Figure 2**, a variety of antigens can be associated by means of electrostatic interactions with cationic liposomes and most of these formulations describe high levels of antigen association [35,69-74]. Interestingly, regardless of the disease antigen or the route of injection, all these formulations share the ability to induce T_H1-mediated immune responses characterized by IFN-γ production and high IgG2 antibody titers. This is in contrast to the polarized T_H2 immune response induced by aluminum-based mineral salts, which typically elicit IL-4 and IL-5 cytokine production, IgG1 antibodies, and a general bias towards humoral immunity [2,4]. Therefore, although aluminum-based mineral salt adjuvants are suitable and indeed licensed for vaccination against extracellular pathogens causing parasitic diseases (e.g., leishmaniasis) or toxoid-producing pathogens (e.g., diphtheria, tetanus), their use in adjuvanting vaccines targeting intracellular pathogens is often unsuccessful [75].

3.2 Liposomal delivery of immunostimulators

Several studies have shown that liposomes can synergistically increase the immunogenic properties of many immunostimulators and therefore further promote vaccine

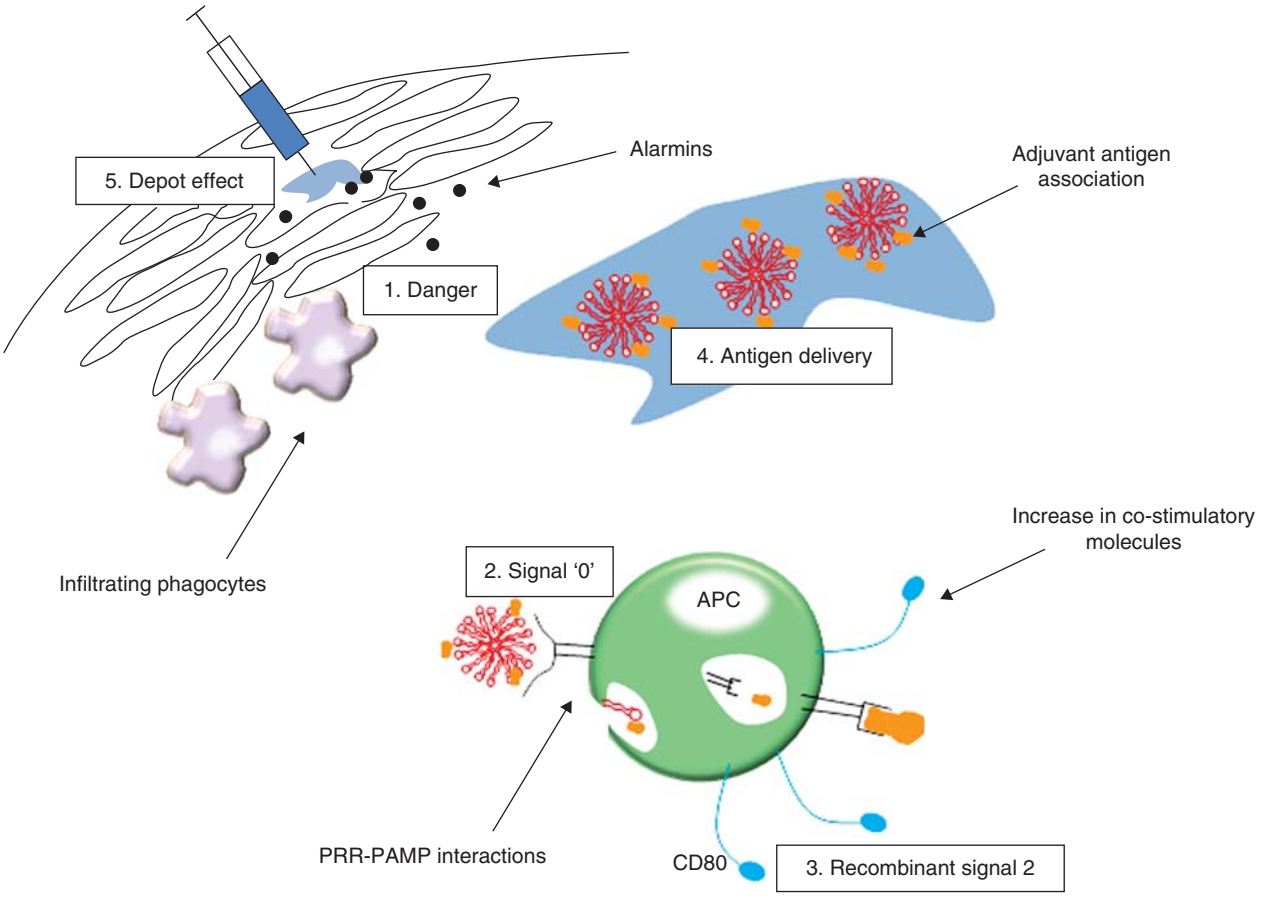

Figure 3. Methods of adjuvant action accepted at present: the five steps of adjuvant action are described in the text.
APC: Antigen-presenting cells; MØ: Macrophage; PAMP: Pathogen-associated molecular patterns; PRR: Pathogen recognition receptors.

uptake and/or activation by APCs. The immunostimulators stem from a wide range of pathogenic (i.e., bacterial, viral) and non-pathogenic (e.g., saponins, lipid) substances, which in most cases act by means of pathogen recognition receptors (PRRs) such as TLRs, Nod-like receptors (NLRs) and C-type lectins. Our understanding of these molecules is increasing at a rapid rate; one such example is the case of the mycobacterial-derived glycolipid trehalose 6′6-dimycolate (TDM), which has been known as a potent immunostimulator for over 40 years [76], yet its cellular receptor remained undetermined until late 2009 when, within 4 months, two separate groups discovered it to be the C-type lectin Mincle [77,78].

PRRs represent a specific component of the innate immune system capable of recognizing PAMPs and their endogenous versions known as alarmins. Liposomes are well suited as delivery systems for these PAMPs owing to their resemblance to the pathogens in size and shape and because the PAMPs can be easily administered and presented to the APCs together with the antigen irrespective of their solubility, size or shape. In addition, liposomes may present the PAMPs to the APCs in a similar manner as if they were presented by the pathogen itself. Other similar adjuvant technologies such as virosomes and archaeosomes aim to mimic the appearance of viruses and archaebacteria, respectively, and in this way activate the immune system as the real pathogen would do. A thorough review of these technologies is beyond the scope of this paper, but can be found elsewhere [79-82].

Of the PRRs, the TLRs are probably the most well studied (**Figure 5**), and there are numerous in-depth reviews regarding their cellular location, their associated PAMPs, as well as downstream intracellular signaling events [83,84]. However, of interest to vaccine development is how TLR agonists can be utilized to achieve a specific immune response, such as a bias towards T_H1 or T_H2 responses. Thus, the US recently licensed the adjuvant AS04 (GSK biologicals) containing aluminum hydroxide associated with synthetic MPL, the latter attributing its immunostimulatory abilities to signaling via TLR4 [85]. The immunostimulatory effect of MPL has also been shown to be significantly increased after incorporation into cationic liposomes [86-88].

DiC14-amidine liposomes represent another TLR agonist adjuvant that has been shown to bind to TLR4 with resulting activation of the MyD88 intracellular signaling pathway [89].

Disease model/agent	Vaccine	Antigen	Relevant points	Ref.
HSV	"CLDC" Liposomes composed of DOTIM lipid and cholesterol helper lipid	HSV gD2	Guinea pig model for genital herpes, s.c injection leads to better protection than Alum/MPL adjuvant and a reduction in viral shedding. Not effective as a therapeutic vaccine	[117]
WEEV		Non-coding empty pMB75.6	Prophylactic/therapeutic challenge study via i.n./s.c. and i.v routes of infection. Greatest survival rates after immunization with CLDCs were noted after s.c viral challenge	[118]
Pneumonic infection with Francisella tularensis			i.n injection/mice; complete protection from lethal infection in challenge studies (no significant protection elicited by s.c/i.v or i.p routes). Protection dependent on NK cells and IFN-γ production	[119]
Influenza		Non-coding empty pMB75.6 and Fluzone® (split trivalent influenza vaccine)	s.c injection/mice; humoral and cell-mediated responses noted with improved protection in challenge studies	[120]
Toxoplasma gondii	"Escort™ Transreagent" Liposomes composed of DOTAP and DOPE	'pVAXGRA7' T.Gondii GRA7 antigen	i.m injection/sheep lead to TH1 responses characterized by IFN-γ and IgG2 antibodies. Vaccine also contains TLR9 agoinst CpG	[93]
JEV	Liposomes composed of DOTAP or DC-Chol with DOPE helper lipid	pCJ3/ME	TCI skin patch/mice; both formulations significantly improved survival rates in a challenge study with predominantly TH2 responses	[121]
TB	Lipofectamine™ 2000	pcDNA3.1+/Ag85A	Oral delivery/mice; elevated secretory IgA and TH1 biased immune responses	[122]
None	"VacciMax" or "DepoVax™" Liposomes composed of phospholipid S100 (Lipoid GmBH) and cholesterol helper lipid	pDNA (IL-12 and GFP) mRNA (GFP) siRNA (IL-12)	s.c.injection/mice; successful delivery of nucleic acids confirmed by IL-12 secretion/GFP expression after pDNA/mRNA immunization, and inhibition of IL-12 secretion after siRNA immunization. Vaccine also contains ICF.	[123]

Figure 4. Liposomal nucleic acid delivery systems in preclinical testing.

CLDC: Cationic liposome-DNA complex; DC-Chol: 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DOPE: Dioleoyl phosphatidylethanolamine; DOTIM: 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride; HEV: Herpes simplex virus; ICF: Incomplete freund's adjuvant; i.n.: Intranasal; i.v.: Intravenous; i.m.: Intramuscular; i.p.: Intraperitoneal; JEV: Japanese encephalitis virus; MPL: Monophosphoryl lipid; NK: Natural killer cells; s.c.: Subcutaneous; TCI: Transcutaneous immunization; WEEV: Western equine encephalitis virus.

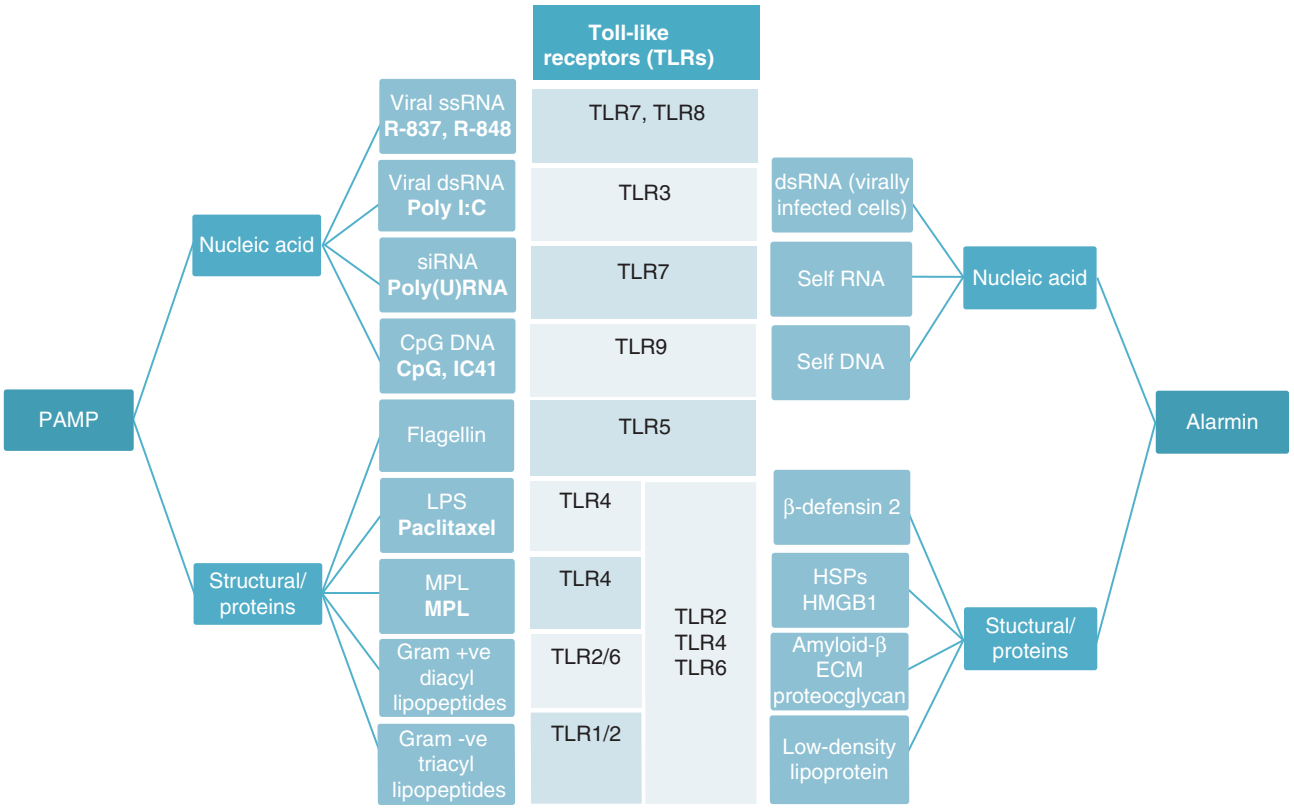

Figure 5. Toll-like receptors and their ligands. Flow chart showing a selection of PAMP and alarmins that are known to bind TLRs. Synthetic analogues known to target specific TLRs are bold and underlined.

ECM: Extracellular matrix; HMGB1: High motility group box 1; HSP: Heat-shock protein; LPS:Lipopolysaccharide; PAMP Pathogen-associated molecular patterns; R-837: Imiquimod; R-838: Resiquimod.

DiC14-amidine liposome is the only liposomal adjuvant known to bind to TLRs, and although there are postulated G protein-coupled receptors (GPCRs) for liposomes composed of DOTAP (and DiC14-amidine), no such receptor has been formally identified [90]. Thus, PRR activation by liposomal adjuvants is commonly attempted by including potential TLR agonists or other PAMPs. One such recently investigated liposome formulation is 'DDA:MMG', which comprises the cationic liposome-forming adjuvant dimethyldioctadecylammonium (DDA) in combination with a synthetic analogue of monomycoloyl glycerol (MMG, an apolar lipid derived from the mycobacterial cell wall) [91]. The presence of MMG in the formulation raises the question of whether DDA:MMG liposomes may interact with cells by means of TLRs, which are known to bind bacterial motifs. Immunization of TLR2/4 knockout mice with DDA:MMG liposomes did not affect cellular responses, suggesting that these particular TLRs do not play a role in the immunostimulating abilities of DDA:MMG liposomes [92], and the supposed PRR specificity of MMG has not yet been described.

Another TLR stimulation strategy focuses on the inclusion of synthetic nucleic acid analogues such as Poly I:C and CpG-containing oligodeoxynucleotides (ODNs), which are known to bind to TLR3 and 9, respectively. A lipoplex formulation containing CpG-ODNs and liposomes composed of DOTAP and DOPE (ESCORT™ Transfection reagent; Sigma, St Louis, USA) was shown to illicit T_H1 responses characterized by IFN-γ production and elevated levels of IgG2 when administered with a DNA vaccine [93]. Although inclusion of CpG-ODN motifs is designed to target TLR9 and therefore the MyD88/NF-κB intracellular signaling pathway, the cationic liposome formulation enhances delivery to the TLR9-expressing endosomal compartment and acts as a transfection agent improving DNA uptake. Similarly, the non-liposomal cationic formulation IC 31 comprising ODNs (non-CpG) and the cationic peptide component $KLKL_5KLK$ have been shown to act in a synergistic way, resulting in TLR9/MyD88-dependent signaling: the $KLKL_5KLK$ component is proposed to initiate cellular uptake whereas the ODNs bind to TLR9 [39]. Importantly, this vaccine combination is successful in stimulating long-term (< 200 days) T_H1-biased immune responses in both young and aged mice [94], and has subsequently moved to Phase IIa trials for tuberculosis vaccines [95] and Phase I trials in a seasonal influenza vaccine (www.intercell.com). With regards to targeting TLR3, some liposomal formulations

containing the TLR3 agonist Poly I:C have the ability to cross-present antigen and therefore stimulate $CD8^+$ T-cell responses [96,97]. Antigen cross-priming holds special importance for vaccines targeting viral pathogens that cannot be controlled solely by neutralizing antibodies as $CD8^+$ T-cell responses are particularly effective at restraining viral load and preventing onset of disease [98].

4. Case study: CAF01

CAF01 is a cationic liposome composed of the lipid DDA in a 5:1 weight ratio with trehalose 6′6-dibehenate (TDB). TDB is a synthetic analogue of mycobacterial cord factor or trehalose dimycolate (TDM) and shows less toxicity *in vivo* while retaining its adjuvant action [99]. CAF01 liposomes are now in Phase I clinical trials as part of a vaccine against TB; they have also been studied in several other disease models, including malaria, chlamydia and hepatitis B [75,100]. A particularly interesting factor of the CAF01 liposomal adjuvant system is its versatility, recently displayed in its ability to induce both cell-mediated and humoral immune responses in malaria, chlamydia and TB models accordingly [75]. In addition, CAF01 liposomes are easy to produce as no sizing restraints (e.g., sonication or extrusion) need be made and they are capable of both antigen entrapment and adsorption [68].

The adjuvant properties of liposomes made solely of DDA have been described since the 1960s [101], although it is only in the past ~ 6 years, since the addition of the synergistic immunostimulator TDB, that the full potential of CAF01 has been shown [74,102]. CAF01 liposomes have been studied extensively with the use of multiparameter flow cytometry to determine the stage of memory and effector T-cell differentiation based on the expression of selected cytokine combinations (see [98] for original report). Phenotyping of cells in this manner gives an indication as to the cellular immune response mounted after immunization.

In the case of administration of CAF01 with the TB subunit antigen Ag85B-ESAT-6, long-term memory is characterized by TNF-α^+ IL-2^+ and/or TNF-α^+ IL-2^+ IFN-γ^+ $CD4^+$ T cells [103,104], which show increased accumulation at the infection site after *Mycobacterium tuberculosis* challenge [105]. These central memory (IFN-γ^+ TNF-α^+ IL-2^+ and TNF-α^+ IL-2^+) and effector memory (IFN-γ^- TNF-α^+) cells (T_{CM} and T_{EM} cells, respectively) are vital for the development of long-term immunity against infection and the ability to reduce bacterial burden in the lung after challenge [106]. CAF01 liposomes also characteristically induce high levels of IFN-γ and production of IgG2 antibodies [20,73,74], both of which are classic indicators of a T_H1-mediated immune response. The CAF01 component DDA does not appear to act through a TLR, but recently the C-type lectin Mincle was discovered to be the receptor for the other component, TDB [77,78], thus activating NF-κB via the syk-CARD9/Bcl10/Malt-1 intracellular pathway, leading to initiation of co-stimulatory and pro-inflammatory molecule transcription [78,107]. This is further supported by immunization studies with CAF01-Ag85B-ESAT-6 liposomes in which upregulation of co-stimulatory molecules including CD40 and CD86 on DCs was noted [103].

More recently, CAF01 has been investigated as a potential mucosal adjuvant administered by means of the intranasal route for influenza vaccination [104]. Both *in vitro* and *in vivo* studies provided promising results, with the immune response characterized by high IFN-γ and total IgG antibody levels, offering significant improvements compared with non-adjuvanted influenza vaccination. The CAF01 adjuvant concept is now being expanded to investigate how incorporation of other immunostimulatory molecules can bias the immune response according to the requirements of specific disease models. One such example is the Poly I:C-containing CAF01 liposomal formulation, termed CAF05, which has been shown to target TLR3, leading to $CD8^+$ T-cell responses [108]. The improved induction of $CD8^+$ T cells conferred by Poly I:C may make the CAF05 formulation more suited to vaccination against viral pathogens.

5. Expert opinion

The development of efficient vaccines with good safety profiles and yet inducing sufficient immunogenicity has resulted in much research based on subunit protein or peptide vaccines. However, with increased safety comes poor immunogenicity and increased exposure of susceptible antigens to biochemical degradation. Liposomes offer an extremely interesting system to overcome these problems as they are capable of providing two important characteristics: antigen delivery and adjuvant properties.

One of the problems facing vaccines, however, is the potential unexpected and unspecific immunostimulatory actions of adjuvants when combined with different antigens. Consequently, adjuvants are at present only licensed combined with specific antigens as a vaccine and not as individual entities. Therefore, the present adjuvant licensing approach relies on testing the adjuvant with each possible antigen and dose combination via selected administration routes. Ultimately, it is not fully understood how adjuvants work – and until it is understood, their outcome cannot be predicted when coupled with particular vaccine antigens. This requires more knowledge on adjuvant mechanisms, and not least the immune system itself.

Exogenous pathogenic stimuli such as lipopolysaccharide (LPS), peptidoglycans, lipopeptides, flagelin and ssRNA have all been recognized as agonists of TLRs, and adjuvant development has naturally leant towards development of synthetic (therefore mass-producible and less toxic) analogues of such molecules. Examples include CpG-ODNs, Poly I:C and MPL. However, the innate immune system does not rely solely on the TLRs, and numerous C-type lectins, including Dectin-1, Mannose, DC-Sign and Mincle receptors, have been described that are all capable of recognizing carbohydrate-like domains of extracellular pathogens. The

complex intracellular pathways by which both TLRs and non-TLRs signal are becoming clearer, with the link between stimulation of the innate and adaptive immune systems becoming increasingly understood.

There have been numerous technological advances in the analytical techniques to determine vaccine effect and efficacy. Among these is the use of knockout mice, which provide great help at increasing our understanding of how liposomes target cells, and the use of multiparameter flow cytometry whereby, for example, the stage of memory T-cell differentiation can be determined based on the cytokine profile of the individual T cells. The use of gene microarrays to study cytokine/chemokine/chemokine receptor expression can also help define the biological processes represented by the expression of selected gene combinations. Examples include: i) injection of the T_H2-stimulating adjuvant aluminum hydroxide versus T_H1-stimulating liposomes composed of DDA and the TLR4-stimulator MPL, where the latter adjuvant was causing higher expression of genes associated with inflammatory and immune responses [87]; and ii) deduction of the mechanisms by which TDB acts as an immunostimulator by using gene profiling of TDB versus TLR9-stimulating CpG in bone marrow-derived macrophages [107].

Overall, one of the principal benefits of using liposomes as adjuvants is their flexibility relating to both physicochemical and immunogenic properties. It is becoming increasingly clear that making small alterations in the lipid monomers, the size of liposomes, or changing the administration route have profound effects on the efficacy of the vaccine, as well as which arm of the immune response is preferentially targeted.

Liposomes offer a substantial advantage over aluminum-based mineral salt and o/w vaccine adjuvants that are licensed at present because they are highly versatile and capable of stimulating both $CD4^+$ (T_H1 and/or T_H2) and $CD8^+$ T cells, depending on the incorporation of TLR-stimulators, immunogenic lipids or bacterially derived molecules.

By developing a profile of different immune responses after immunization with subtly changed liposomal formulations, such as undertaken with the CAF model, it finally seems possible that the methods by which liposomes act as adjuvants will be fully uncovered.

During the last 10 years the battle against Third World diseases has become an ever growing part of the agenda. In the process of designing vaccines against such diseases it is of crucial importance to include production and transportation aspects early in the development phase. In this context, it is therefore encouraging to find that, for example, CAF01 can be freeze-dried and readily reconstituted to the original form, maintaining its immunogenic properties [109]. Furthermore, CAF01 can be subjected to sterile filtration (unpublished data) and γ-sterilization [110]. This new liposome-based adjuvant system is therefore a very promising platform technology fulfilling many of the requirements not only as an adjuvant but also from a more pharmaceutical perspective.

Declaration of interest

The authors employed at Statens Serum Institut are co-inventors of patents relating to cationic liposomes as vaccine adjuvants. All rights have been assigned to the Statens Serum Institut. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. The authors have not received payment in the preparation of this manuscript.

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Affiliation

Malou Henriksen-Lacey[1], Karen Smith Korsholm[2], Peter Andersen[2], Yvonne Perrie[1] & Dennis Christensen[†2]
[†]Author for correspondence
[1]Aston University, School of Life and Health Sciences, Birmingham, B4 7ET UK
[2]Statens Serum Institute, Department of Infectious Disease Immunology, 2300 Copenhagen, Denmark
Tel: +45 32 68 38 04; Fax: +45 32 68 30 35; E-mail: den@ssi.dk