Liposomes as vaccine delivery systems. A review of the recent advances.

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Abstract:	Liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carriers systems in vaccine development and the interest for liposome-based vaccines has markedly increased. A key advantage of liposomes, archaeosomes and virosomes in general, and liposome-based vaccine delivery systems in particular, is their versatility and plasticity. Liposome composition and preparation can be chosen to achieve desired features such as selection of lipid, charge, size, size distribution, entrapment and location of antigens or adjuvants. Depending on the chemical properties, water soluble antigens (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space of liposomes, whereas lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) are intercalated into the lipid bilayer and antigens or adjuvants can be attached to the liposome surface either by adsorption or stable chemical linking. Co-formulations containing different types of antigens and/or adjuvants can be combined with the parameters mentioned to tailor liposomal vaccines for individual applications. Special emphasis is given in this review to cationic adjuvant liposome vaccine formulations. Examples of vaccines made with CAF01, an adjuvant composed of the synthetic immune stimulating mycobacterial cordfactor glycolipid trehalose-dibehenate (TDB) as immunomodulator and the cationic membrane forming molecule dimethyl-dioctadecylammonium DDA are presented. Other vaccines such as cationic liposome-DNA complexes (CLDC) and other adjuvants like muramyl dipeptide, monophosphoryl lipid A and listeriolysin O are mentioned as well. The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent and relevant studies emphasizing current reports dealing with the most studied antigens and adjuvants and pertinent

	examples of vaccines. Studies on liposome-based veterinary vaccines and experimental therapeutic cancer vaccines are also summarized.

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Abstract

Liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carriers systems in vaccine development and the interest for liposome-based vaccines has markedly increased. A key advantage of liposomes, archaeosomes and virosomes in general, and liposome-based vaccine delivery systems in particular, is their versatility and plasticity. Liposome composition and preparation can be chosen to achieve desired features such as selection of lipid, charge, size, size distribution, entrapment and location of antigens or adjuvants. Depending on the chemical properties, water soluble antigens (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space of liposomes, whereas lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) are intercalated into the lipid bilayer and antigens or adjuvants can be attached to the liposome surface either by adsorption or stable chemical linking. Co-formulations containing different types of antigens and/or adjuvants can be combined with the parameters mentioned to tailor liposomal vaccines for individual applications.

Special emphasis is given in this review to cationic adjuvant liposome vaccine formulations. Examples of vaccines made with CAF01, an adjuvant composed of the synthetic immune stimulating mycobacterial cordfactor glycolipid trehalose-dibehenate (TDB) as immunomodulator and the cationic membrane forming molecule dimethyl-dioctadecylammonium DDA are presented. Other vaccines such as cationic liposome-DNA complexes (CLDC) and other adjuvants like muramyl dipeptide, monophosphoryl lipid A and listeriolysin O are mentioned as well.

The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent and relevant studies emphasizing current reports dealing with the most studied antigens and adjuvants and pertinent examples of vaccines. Studies on

liposome-based veterinary vaccines and experimental therapeutic cancer vaccines are also summarized.

Keywords: Liposomes, archaeosomes, virosomes, vaccines, antigens, adjuvants, veterinary vaccines, therapeutic cancer vaccines

Introduction (7011 words)

Classical vaccines rely on the use of whole killed or attenuated pathogens. Today, research is focused on the development of subunit vaccines because they are better defined, easier to produce and safer. Vaccines are manufactured on the basis of well characterized antigens, such as recombinant proteins and peptides. However, due to their synthetic nature, their immune response is often weak which is largely related to the inability of the antigens to induce maturation of dendritic cells (DC), the primary antigen presenting cells (APC) that react to foreign pathogens and trigger the immune response [Moser *et al.*, 2010, Reed *et al.*, 2013].

The immune system is composed of the innate and the adaptive systems. The first is responsible for first line host defense, rapidly recognizing and responding to foreign pathogens. The complement system and phagocytic cells belong to this defense system which depends on pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Toll like receptors (TLR) present on APCs are the receptors for pathogens containing PAMPs. TLR activation is the hallmark of innate immune response. The second defense line, the adaptive immune system, mounts specific responses against molecular determinants on pathogenic agents. These responses are initiated by antigen-mediated triggering of T cells, the $CD4^+$ T helper (T_H) cells, the $CD8^+$ cytotoxic T lymphocytes (CTL) and B lymphocytes carrying antigen-specific surface receptors. T_H cells have subpopulations, of which T_H1 and T_H2 are the most important [Nordly *et al.*, 2009, Kawai *et al.*, 2010].

The diverse mechanisms by which nanoparticles induce immune responses are summarized in Figure 1. Activation of PRRs triggers the initiation of the innate immune response. Activated CTLs recognize peptides bound to the major histocompatibility complex class I and II molecules (MHC-I, MHC-II) which express antigenic peptides on APCs and bind to T cells via the T cell receptor. A co-stimulatory signal is needed for full CTL and T_H cell activation which differentiate into T_H1 or T_H2 and other T-helper lineages that produce cytokines. T_H cells provide help to antigen-specific B cells, resulting in antibody production [Lin *et al.*, 2010, Chen *et al.*, 2013]. Each invasion of a foreign antigen requires activation of a specific type of adaptive immune response for efficient control and elimination. Thus, vaccine formulations should be designed rationally to induce specific protective responses. This includes the choice of antigen and adjuvant(s) and their pharmaceutical formulation.

Adjuvants

The ability to enhance the immune response of vaccines by certain compounds was first demonstrated with aluminium salts, - termed “adjuvants” -, added to killed or attenuated pathogens. Their functions were related to the ability to form a depot which prolonged antigen exposure to APCs. However, efficient adjuvants also stimulate the immune system by direct interaction with APCs. The nature of immune adjuvants is large and heterogeneous. Adjuvants are divided into immunostimulants and delivery systems. Immunostimulants interact with specific receptors, like TLRs and others, while delivery systems increase the immune response by multiple mechanisms, depending on their particular characteristics [Leroux-Roels, 2010, Alving *et al.*, 2012]. Thus, modern vaccines comprise adjuvants such as pathogen-derived subcellular components, recombinant proteins, peptides and nucleic acid sequences [Zepp, 2010, Perez *et al.*, 2013, Reed *et al.*, 2013]. In addition, due to better knowledge of the immune system and improvements in formulation technology, effective therapeutic cancer vaccines are developed [Joshi *et al.*, 2012]. Today’s challenges in vaccine development are linked to complex pathogens (e.g. malaria, tuberculosis, HIV) and to antigens susceptible to genetic mutations (e.g. influenza) as well as to subjects with a compromised or dysfunctional immune system [Leroux-Roels, 2010].

Nanoparticulate carriers provide adjuvant activity by enhancing antigen delivery or by activating innate immune responses. Strength and mechanisms of immunostimulation induced by nanocarrier vaccines depend on various factors, such as chemical composition, particle size and homogeneity, charge, nature and location of antigens and/or adjuvants within the carrier and, last but not least, the site of administration (see Fig. 2) [Watson *et al.*, 2012, Brito *et al.*, 2013, Gregory *et al.*, 2013, Smith *et al.*, 2013, Zaman *et al.*, 2013].

Liposomes: ideal carriers for antigens and/or adjuvants

The ability of liposomes to induce immune responses to incorporated or associated antigens was first reported by Gregoriadis and Allison [Allison *et al.*, 1974, Allison *et al.*, 1976]. Since then, liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carrier systems and the interest for liposome-based vaccines has markedly increased. The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent reports highlighting the most studied antigens and

adjuvants in pertinent examples of vaccines, including summaries of veterinary and experimental therapeutic cancer vaccines. Other nanoparticulate vaccines based on lipoplexes, niosomes, virus-like particles, solid lipid nanoparticles and nanoemulsions are not covered in this review.

A key advantage of liposomes, archaeosomes and virosomes in general, and liposome-based delivery systems in particular, is their versatility and plasticity (see Table 1). Liposome composition and preparation can be chosen to achieve desired features such as lipid composition, charge, size, size distribution, entrapment and location of antigens or adjuvants. Depending on the chemical properties, water soluble compounds (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space, whereas lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) are intercalated into the lipid bilayer and antigens can be attached to the liposome surface either by adsorption or stable chemical linking [Torchilin, 2005, Watson *et al.*, 2012]. Co-formulations containing different types of antigens and/or adjuvants can be combined to tailor liposomal vaccines for individual applications (see Figure 2).

Liposome-based antigens

Liposome-mediated effects of antigen uptake, trafficking, processing, and presentation

As the majority of vaccines are administered by intramuscular or subcutaneous injection, liposome properties play a major role in local tissue distribution, retention, trafficking, uptake and processing by APCs. Earlier studies showed clear size dependent, but not unambiguous charge or lipid composition dependent effects at the injection site [Oussoren *et al.*, 1997]. Newer studies with the cationic liposome formulation dimethyl-dioctadecylammonium (DDA) plus trehalose-dibehenate (TDB) (DDA/TDB, CAF01) showed no differences in liposome draining or antigen release from the injection site. However, differences in movement to regional lymph nodes (LN) were noted [Henriksen-Lacey *et al.*, 2010, Henriksen-Lacey *et al.*, 2011]. A cationic liposome pDNA vaccine of 500 nm and 140 nm size with encapsulated ovalbumin (OVA) encoding pDNA as antigen showed strongest retention at large vesicle size. Addition of poly(ethyleneglycol) (peg) coating resulted in enhanced lymphatic drainage, without improved immune response [Carstens *et al.*, 2011]. Other pegylated DDA/TDB-liposomes reduced the depot effect and altered the immune response confirming these results [Kaur *et al.*, 2012]. Badiee *et al.* evaluated liposomes of

different sizes containing the surface glycoprotein of *Leishmania* (rgp63). Immunization with small liposomes induced a T_H2 response, whereas large liposomes induced a T_H1 response, higher IFN-γ levels and IgG2a/IgG1 ratios [Badiee *et al.*, 2012]. Adjuvant effects of neutral, positive or negative liposomes were evaluated when admixed with OVA, cationized OVA (cOVA) or *Bacillus anthracis* antigen by Yanasarn *et al.* Immunization with OVA admixed with different liposomes generated different antibody responses. Interestingly, OVA admixed with negative 1,2-dioleyl-*sn*-glycero-3-phosphatidic acid (DOPA) liposomes was as immunogenic as OVA admixed with positive 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) liposomes. The cOVA antigen showed comparable adjuvant activities in all liposomes [Yanasarn *et al.*, 2011]. Neutral phosphatidylcholine (PC)/cholesterol small unilamellar vesicles (SUV) proved also to be effective vaccine carriers. We evaluated a vaccine with peptides derived from the glycoprotein of the lymphocytic choriomeningitis virus (LCMV). Liposome-encapsulated peptides were highly immunogenic and elicited protective antiviral immunity by *in vivo* antigen loading of DCs. Encapsulated cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpGs) further enhanced immune activation [Ludewig *et al.*, 2000]. We also used the vaccine to prime a $CD8^+$ T cell response against 10 different hepatitis C virus (HCV) epitopes resulting in strong CTL responses. Challenge experiments with *Vaccinia* virus expressing HCV epitopes emphasized the utility of neutral liposomes as HCV vaccine [Engler *et al.*, 2004, Schwendener *et al.*, 2010]. Moon *et al.* describe novel interbilayer-crosslinked multilamellar vesicles (MLV) formed by crosslinking adjacent lipid bilayers within MLVs. These vesicles entrapped protein antigens in their core and lipid-based immunostimulatory molecules in the bilayers, forming a potent vaccine, eliciting strong T-cell and antibody responses [Moon *et al.*, 2011].

Investigation of hemagglutinin (HA) adsorption versus encapsulation and co-encapsulation of CpGs in 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol) liposomes showed that adsorbed HA was more immunogenic than encapsulated HA. Cholesterol enhanced the adjuvant effect and CpG-loaded liposomes were highly efficient at enhancing HA-specific humoral responses [Barnier Quer *et al.*, 2012, Barnier-Quer *et al.*, 2013]. Covalent attachment of protein antigens to nanocarriers can disrupt protein structure and mask epitopes, altering the antibody response. Watson *et al.* used metal chelation via nitrilotriacetic acid (NTA) to attach antigens to liposomes. OVA and a HIV-1 gp41 (N-MPR) peptide were attached via NTA or covalent linkage. Attachment of N-MPR, but not OVA,

elicited stronger antibody responses than antigen admixed with liposomes and covalent attachment was superior to NTA-anchored antigens [Watson *et al.*, 2011].

Mannose receptors (MR) expressed on macrophages and APCs mediate endocytosis and cooperate in antigen capture and presentation. MR recognize carbohydrate moieties of many pathogens. Thus, targeting of glycosylated antigens or carrier systems to MR is a method to develop vaccines [Irache *et al.*, 2008]. To establish a human papilloma virus 16 (HPV16) cancer vaccine, Mizuuchi *et al.* generated oligomannose-liposomes containing HPV16-E6 plasmid antigens (OML-HPV). HPV16-E6-specific CTLs were generated from HPV16-positive cervical carcinoma patients with OML-HPV, but not with standard liposomes [Mizuuchi *et al.*, 2012]. OMLs in combination with entrapped dsRNA to induce anti-human parainfluenza virus-3 (HPIV3) immunity were studied by Senchi *et al.* [Senchi *et al.*, 2013]. Hemagglutinin-neuraminidase (HN) antigen was co-encapsulated with adjuvant poly(I:C) into OMLs. Systemic and mucosal immune responses were generated and immune sera suppressed viral infection *in vitro*. Finally, Li *et al*. constructed a mannosylated liposome/protamine/DNA (Man-LPD) vaccine. Man-LPD exhibited higher intracellular uptake and transfection *in vitro* and induction of co-stimulatory molecules on BMDCs [Li *et al.*, 2013].

Peptides and proteins as antigens

The antigen location in liposomes influences immunogenicity. Both, entrapped or surface-attached antigens induce T cell responses, the latter having advantages of availability for antibody or B cell recognition, whereas encapsulated antigens require vesicle disruption to be accessible. The necessity of $CD4^+$ T cells to induce memory $CD8^+$ T cells was investigated in mice immunized with liposome surface-coupled OVA peptides. CTL responses were induced and confirmed in mice lacking $CD4^+$ T cells, suggesting that $CD4^+$ T cells were not required for memory $CD8^+$ T cell generation [Taneichi *et al.*, 2010]. Phosphatidylserine (PS)-liposome conjugated antigens were efficiently captured by APCs resulting in T_H cell stimulation, validating PS as adjuvant for peptide vaccines [Ichihashi *et al.*, 2013]. Takagi *et al.* coupled several HCV peptides to liposomes. One D^b-restricted and three HLA-A(*)0201-restricted peptides conferred complete protection to immunized mice and long-term memory [Takagi *et al.*, 2013].

Liposome-encapsulated protein antigens have been used frequently in earlier work. More recently, Nagill *et al.* compared encapsulated 78kDa antigen of *Leishmania donovani* with antigen plus monophosphoryl lipid A (MPLA) resulting in decreased parasite burden after challenge [Nagill *et al.*, 2010]. In another study Bal *et al.* co-encapsulated OVA and the TLR ligand Pam_3CysSK_4 or CpGs in DOTAP-liposomes. Encapsulation of both ligands did not obstruct activation of TLR-transfected cells and OVA/CpG-liposomes shifted the IgG1/IgG2a balance to IgG2a, whereas Pam_3CysSK_4 was less efficient [Bal *et al.*, 2011]. Hepatitis B surface antigen (HBsAg) encapsulated liposomes coupled with *Ulex europaeus* agglutinin-1 were developed by Gupta *et al*. Lectinized liposomes were predominantly targeted to M-cells on intestinal Peyer's patches after oral immunization, yielding high antibody titers in mucosal secretions [Gupta *et al.*, 2011]. Another mucosal vaccine was described by Figueiredo *et al.* who encapsulated *Streptococcus equi* antigens in PC/cholesterol/stearylamine-liposomes or chitosan nanoparticles. Intranasal immunization of mice elicited mucosal, humoral and cellular responses with higher sIgA levels of the chitosan nanoparticles, due to enhanced mucoadhesive properties [Figueiredo *et al.*, 2012]. Liposomes modified with pH-sensitive 3-methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG) were used to encapsulate OVA. MGlu-HPG-liposomes induced a strong immune response which was suppressed with anti-MHC-I/MHC-II antibodies [Hebishima *et al.*, 2012]. Ding *et al.* developed so called RAFTsomes by isolating membrane microdomains containing MHC-I and $I\text{-}A_b$ restricted epitopes from OVA primed DCs and reconstituted them on liposome surfaces. RAFTsome immunization gave high anti-OVA IgG1 levels and protection against OVA expressing EG.7 tumor challenge [Ding *et al.*, 2013].

Liposomal DNA vaccines

Nucleic acid vaccines are an alternative to attenuated bacterial antigens or protein or peptide vaccines. MLVs as inexpensive carriers were used by Rodriguez *et al.* to deliver DNA to mice with plasmids encoding bovine herpesvirus type-1. Vaccinated mice developed specific IgG responses [Rodriguez *et al.*, 2013]. The M1 gene of influenza A virus was used by Liu *et al.* to construct a cationic liposome/DNA vaccine with a M1-encoding plasmid for oral vaccination, resulting in M1 gene expression in intestines of vaccinated mice and strong immune responses and protection against challenge infection [Liu *et al.*, 2013]. Liposomes were also used to deliver plasmid DNA encoding hsp65 to treat the pulmonary fungal

infection *Paracoccidiomycosis* resulting in protective immune response and reduced fungal burden [Ribeiro *et al.*, 2013]. Amidi *et al.* proposed liposomes as artificial microbes that can be programmed to produce specific antigens for vaccination. A bacterial transcription and translation system together with a gene construct encoding β-galactosidase or a luciferase-NP fusion epitope as antigens were entrapped in liposomes. Vaccination of mice showed that such antigen-producing liposomes elicited higher specific immune responses against the produced antigen than control vaccines [Amidi *et al.*, 2011, Amidi *et al.*, 2012].

Liposomal messenger RNA vaccines

The immune system is naturally activated by foreign nucleic acids by inducing specific immune responses. Lack of persistence, genome integration and auto-antibody induction are advantages of mRNA and siRNA vaccines. Currently, mRNA vaccines are developed to treat various diseases including cancers. Pichon *et al.* loaded mannosylated nanoparticles with mRNA encoding a melanoma antigen [Pichon *et al.*, 2013]. The mRNA was formulated with histidylated liposomes promoting endosome destabilization, allowing cytosolic nucleic acid delivery which enhanced anti-B16F10 melanoma vaccination in mice. A liposome encapsulated double-stranded RNA (LE-PolyICLC) was tested in the influenza (H5N1-HPIV) model by Li *et al*. Intranasal LE-PolyICLC inhibited virus replication, reduced viral titers, increased survival of infected mice and attenuated pulmonary fibrosis [Li *et al.*, 2011].

The MUC-1 (BLP25) antigen

The MUC1 glycoprotein is often overexpressed and hypoglycosylated in tumor cells of cancers making it an attractive target for immunotherapy (for other examples see Tab. 2) [Acres *et al.*, 2005, Roulois *et al.*, 2013]. MUC1 variable number tandem repeats (VNTRs) conjugated to tumor-associated carbohydrate antigens (TACAs) break self-tolerance in humanized MUC1 transgenic mice. Sarkar *et al.* formulated an anti-cancer vaccine composed of a MUC1 glycopeptide containing a GalNAc-O-Thr (Tn) TACA conjugated to a TLR ligand. Additional surface-displayed l-rhamnose (Rha) epitopes were included in 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-choline (DPPC) liposomes. Mice were immunized with a Rha-Ficoll conjugate, followed by the vaccine resulting in a >8-fold increase in anti-MUC1-Tn, anti-Tn antibody titers and increased T cell proliferation [Sarkar *et al.*, 2013]. Another liposome

vaccine containing the immunoadjuvant Pam_3CysSK_4, a T_H peptide epitope and a glycosylated MUC1 peptide was reported by Lakshminarayanan *et al.* Covalent surface-linkage of all three components was essential for maximum efficacy [Lakshminarayanan *et al.*, 2012]. The BLP25 liposome (L-BLP25) vaccine which targets MUC1 extended survival of patients with non-small cell lung cancer (NSCLC) and showed promise in prostate cancer [North *et al.*, 2005, North *et al.*, 2006]. Butts *et al.* conducted phase II/IIB studies to evaluate L-BLP25 in patients with stage IIIA/IIIB NSCLC. Patients received either L-BLP25 plus best supportive care (BSC) or BSC alone. Survival time and rates were longer in patients receiving the combination compared to BSC alone [Butts *et al.*, 2010, Butts *et al.*, 2011]. Wu *et al.* conduct an ongoing L-BLP25 study (INSPIRE) in NSCLC patients of East-Asian ethnicity which is the first large therapeutic cancer vaccine study in an East-Asian population [Wu *et al.*, 2011]. Accordingly, a L-BLP25 study was conducted in Japanese NSCLC patients showing consistency with studies of Caucasian patients [Ohyanagi *et al.*, 2011].

Liposomes as carriers for adjuvants

Liposomal DNA as adjuvant

CpGs are adjuvants composed of un-methylated CpG dinucleotide sequences similar to those found in bacterial DNA. They trigger TLR9, activate DC maturation, increase antigen expression and induce T_H1 immune responses [Shirota *et al.*, 2014]. Antigens and CpGs must be co-localized in one APC to generate optimal immune responses [Krishnamachari *et al.*, 2009]. CpG-encapsulation in liposomes of different properties altered antigen encapsulation efficiency, release and delivery rates, thus influencing the immune response. OVA/CpG co-encapsulation augmented T_H1 and cell mediated immune response [Erikci *et al.*, 2011]. Co-encapsulation of OVA and Pam_3CysSK_4 and/or CpGs in cationic liposomes shifted the IgG1/IgG2a balance to IgG2a, showing that antigen/adjuvant co-encapsulation shapes the type of immune response [Bal *et al.*, 2011]. Nuclease-resistant phosphorothioate CpGs (PS-CpGs) or sensitive phosphodiester CpGs (PO-CpGs) were used by Shargh *et al.* in a *Leishmaniasis* model. PO-CpGs or PS-CpGs were encapsulated in DOTAP-liposomes for protection against nuclease degradation. Mice immunized with liposomal soluble *Leishmania* antigens (SLA) co-incorporated with PO-CpGs or PS-CpGs showed no significant difference in immune response. Thus, nuclease-sensitive PO-CpGs can be used as adjuvants [Shargh *et al.*,

2012]. Finally, CpGs incorporated in cationic DOTAP-liposomes but not in neutral 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes provided complete protection against challenge with *Burkholderia pseudomallei* in a mouse model [Puangpetch *et al.*, 2012].

Cationic liposome adjuvant vaccines

The introduction of positively charged compounds is a common method used to alter liposome properties. Cationic liposomes are frequently used as cell transfection reagents and vaccine adjuvants. Most cationic lipids form bilayer liposomes but often additional lipids are needed. The high surface density of positive charges increases liposome adsorbtion on negatively charged cell surfaces. Cationic liposomes penetrate into cells through specific mechanisms and activate different cellular pathways depending on cell type, cationic lipid nature, but also on formulation types and liposome size [Korsholm *et al.*, 2012, Lonez *et al.*, 2012].

The cationic adjuvant CAF01

CAF01 is a novel adjuvant composed of the synthetic immunostimulating mycobacterial cordfactor glycolipid TDB and the cationic membrane forming molecule DDA. TDB induces strong T_H1 and T_H17 immune responses and the C-type lectin Mincle is the receptor for APC activation. The adjuvant effect also requires MyD88 and Schweneker *et al.* identified the Nlrp3 inflammasome as mediator for TDB triggered induction of immune response [Werninghaus *et al.*, 2009, Desel *et al.*, 2013, Schweneker *et al.*, 2013]. Properties of cationic liposome forming lipids were studied with rigid DDA- or fluid dimethyldioleoylammonium (DODA) liposomes. When the antigen Ag85B-ESAT-6 was mixed with DDA/TDB or DODA/TDB liposomes, DDA-liposomes formed a depot resulting in continuous activation of APCs, whereas DODA-liposomes were rapidly cleared [Christensen *et al.*, 2012]. Milicic *et al.* explored modifications of DDA/TDB-liposomes such as size, antigen association and addition of TLR agonists to assess their activity using OVA as antigen. SUV without TLR agonists showed higher antigen-specific antibody responses than MLVs. Addition of TLR3 and TLR9 agonists increased the adjuvant effects of MLVs but not of SUVs. The ability of DDA/TDB-SUVs to induce $CD8^+$ CTL responses without immunostimulators could avoid safety risks

associated with TLR agonists [Milicic *et al.*, 2012]. Yu *et al.* tested several adjuvants, including DDA-monophosphoryl lipid A (DDA-MPLA), DDA-TDB (CAF01) and DDA-monomycolyl glycerol (DDA-MMG, CAF04). *Chlamydia* antigens were used in a mouse genital tract infection model. DDA-MPLA and DDA-TDB elicited the best protective immune responses, characterized by $CD4^+$ T cells co-expressing IFN-γ and TNF-α and by significantly reduced infection [Yu *et al.*, 2012]. Ingvarsson *et al.* studied the parameters of CAF01 spray dried powder formulations using lactose, mannitol or trehalose as stabilizers. Immunization of mice with the tuberculosis antigen H56 demonstrated that spray drying with trehalose resulted in best preservation of adjuvant activity [Ingvarsson *et al.*, 2011, Ingvarsson *et al.*, 2013]. Lindenstrom *et al.* showed that CAF01 vaccination in mice led to establishment of T_H17 memory cells by retaining phenotypic and functional properties for 2 years. Challenge with *Mycobacterium tuberculosis* 2 years later induced T_H17 memory cells at levels comparable to T_H1 memory cells [Lindenstrom *et al.*, 2012]. A trivalent influenza vaccine (TIV) with CAF01 enhanced the immune response determined by HA inhibition and antibody titers, promoting strong T_H1 responses. Maintenance of the T_H1/T_H17 cytokine profile over 20 weeks resulted in complete survival of H1N1 challenged mice [Rosenkrands *et al.*, 2011]. A commercially available TIV was compared with the same vaccine mixed with CAF01 in ferrets. CAF01 induced increased influenza-specific IgA and IgG levels and promoted immunity and protection against challenge with H1N1. [Martel *et al.*, 2011]. The combination of cationic liposomes and immunopotentiators such as MPL with DDA/TDB-liposomes was tested in mice using OVA as antigen. DDA/TDB/MPL-liposomes induced antigen-specific $CD8^+$ T-cell and humoral responses [Nordly *et al.*, 2011]. CAF01 was also used in a phase I trial with a therapeutic HIV-1 peptide vaccine. Safety and immunogenicity were assessed in untreated HIV-1-infected individuals. Vaccine-specific T cell responses were induced in 6/14 individuals, showing that therapeutic immunization with CAF01-adjuvanted HIV-1 peptide in humans is feasible [Roman *et al.*, 2013]. In another clinical trial the potential of inducing T-cell immunity during chronic HIV-1 infection was investigated. Treatment-naive HIV-1-infected individuals were immunized with peptides/CAF01. Specific $CD4^+$ and $CD8^+$ T-cell responses were induced in all individuals [Karlsson *et al.*, 2013]. Kamath *et al.* reported that physical linkage between antigens and immunomodulators is required to elicit T_H1/T_H17 responses. Separate same-site administration of a mycobacterial fusion antigen and CAF01 failed to elicit T_H1/T_H17 responses. Tracking experiments showed that

separate same-site administration elicited an early antigen-positive/adjuvant-negative DC population. Antigen targeting of LN DCs prior to their activation generated non-activated antigen-pulsed DCs that recruited antigen-specific T cells and triggered proliferation, but interfered with T_H1 induction in a dose-dependent manner. Thus, synchronization of DC targeting and activation is a critical determinant for T_H1/T_H17 adjuvanticity [Kamath *et al.*, 2012].

In summary, CAF01 adjuvant liposomes prove to be a valuable vaccine formulation for different antigens.

CLDC adjuvant liposomes

Another widely studied cationic liposome complex contains the cationic lipid 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium-chloride (DOTIM) and cholesterol. Cationic liposome-DNA complexes (CLDC) are prepared by mixing liposomes with DNA. CLDC (JVRS-100, Juvaris BioTherapeutics, Burlingame, CA) is a lyophilized powder composed of selected plasmid DNA complexed with liposomes. CLDCs facilitate APC uptake, activate TLRs and IFN production and stimulate the adaptive immune response. Several CLDC vaccines have been tested in various models. Gowen *et al.* analyzed liposomal delivery and CpG content of plasmid DNA with CLDCs. CpG-free or CpG-containing plasmids with and without liposomes, as well as poly(I:C) were evaluated to elicit protection against lethal Punta Toro virus (PTV) challenge in hamsters. CLDC-containing CpG-plasmid significantly improved survival, decreased viral loads and reduced liver damage. [Gowen *et al.*, 2009]. CLDC enhanced anti-SIV immune responses induced by SIV vaccines. CLDC immunized rhesus macaques developed stronger SIV-specific T and B cell responses compared to controls, resulting in persistence and better memory responses [Fairman *et al.*, 2009]. As no vaccines are available for common herpes simplex virus (HSV) infections CLDCs were evaluated for a HSV gD2 vaccine in a genital herpes guinea pig model. The CLDC/gD2 vaccine significantly decreased duration of acute and recurrent disease compared to gD2 alone. However, when evaluated as therapeutic vaccines they were ineffective, suggesting that such HSV-2 vaccines need improvement [Bernstein *et al.*, 2010, Bernstein *et al.*, 2011]. The protective effects of CLDCs against encephalitic arboviral infection were investigated in a Western equine encephalitis virus (WEEV) model. CLDC vaccinated mice were challenged with virulent WEEV. CLDC pre-treatment provided increased survival and higher cytokine levels, strong T_H1

activation and protective immunity against lethal WEEF [Logue *et al.*, 2010]. An influenza A virus vaccine adjuvanted with CLDC or alum was tested by Hong *et al.* CLDC induced more robust adaptive immune responses with higher levels of virus-specific IgG2a/c and $CD4^+$ and $CD8^+$ T cells plus cross-protection from lethal viral challenges. [Hong *et al.*, 2010]. In another influenza A vaccine study, Dong *et al.* showed that addition of CLDC (JVRS-100) to a H5N1 split vaccine induced higher virus-specific responses than adjuvant-free formulations. CLDC vaccinated mice challenged with H5N1 had mild illness, very low viral titers, 100% survival and long lasting protective immunity [Dong *et al.*, 2012]. Strategies to improve influenza vaccine efficacy in elderly individuals are needed. Thus, Carroll *et al.* determined whether CLDC (JVRS-100) could improve the efficacy of the influenza vaccine Fluzone in elderly rhesus macaques. Vaccination with Fluzone with or without CLDC and challenge with human H1N1 influenza virus showed that only the Fluzone/CLDC-vaccinated animals had lower virus replication. Thus, CLDC enhances immunogenicity and efficacy of a licensed vaccine in immunosenescent monkeys [Lay *et al.*, 2009, Carroll *et al.*, 2014]. CLDC (JVRS-100) was also evaluated as adjuvant for HBsAg in mice expressing hepatitis B virus (HBV). HBsAg+JVRS-100 elicited T and B cell responses, whereas HBsAg elicited only a B cell response. However, the response by HBsAg+JVRS-100 was not sufficient to cause destruction of infected liver cells, but it suppressed HBV DNA non-cytolytically [Morrey *et al.*, 2011]. Similar results were obtained using the woodchuck model of HBV. HBV infection induced T cell responses to WHsAg and selected WHs peptides and expression of $CD8^+$ CTL and T_H1 cytokines. WHsAg plus CLDC elicited antibodies earlier, in more woodchucks and with higher titers than WHsAg and alum. [Cote *et al.*, 2009]. There is a need for mucosal vaccines for pulmonary *Yersinia pestis* infections. The ability of an oral CLDC adjuvanted vaccine against lethal pneumonic plague was investigated by Jones *et al.* Oral immunization with *Y. pestis* F1 antigen combined with CLDC produced high titers of anti-F1 antibodies and long lasting $CD4^+$ T cell dependent protection from lethal pulmonary challenge with *Y. pestis* [Jones *et al.*, 2010].

Other cationic lipid complexes

Several other cationic lipid adjuvant complexes were evaluated in various vaccine models. Phillips *et al.* tested an alphavirus vaccine comprised of cationic lipid nucleic acid complexes (CLNCs) and the ectodomain (E1ecto) of WEEV. Interestingly, CLNC alone had therapeutic efficacy, as it increased survival of mice following lethal WEEV infection. Immunization with

the CLNC/WEEV/E1ecto mixture provided full protection after challenge. Passive serum transfer from immunized to naïve mice conferred protection to challenge, indicating that antibody is sufficient for protection. [Phillips *et al.*, 2014]. Liposomes containing different cationic compounds and neutral DPPC were loaded with influenza HA by adsorption. DC-chol/DPPC-liposomes with a high amount of DC-chol had stronger immunogenicity compared to less DC-chol and elicited higher antibody titers compared to the other compounds and non-adjuvanted HA. Liposome-adsorbed HA was more immunogenic than encapsulated HA and incorporation of cholesterol in DC-chol-liposomes as well as CpGs enhanced adjuvancy [Barnier-Quer *et al.*, 2013]. A similar study by Ma *et al.* showed that DOTAP/DOPC-liposome regulated immune responses relied on surface charge density and might occur through reactive oxygen species (ROS) signaling. High charge density liposomes potently enhanced DC maturation, ROS generation, antigen uptake and production of IgG2a and IFN-γ, whereas low-charge density liposomes failed to promote immune responses [Ma *et al.*, 2011]. Lipid assemblies composed of a polycationic sphingolipid (ceramide carbamoyl-spermine, CCS) are effective adjuvants/carriers for several vaccines when complexed with cholesterol (CCS/C, VaxiSome). Ferrets immunized intranasally with CCS/C-influenza vaccine produced higher HI antibody titers compared to controls. Following viral challenge the vaccine reduced the severity of infection. Biodistribution studies showed that lipids and antigens are retained in nose and lung, increasing cytokine levels and expression of co-stimulatory molecules [Even-Or *et al.*, 2011]. Chen *et al.* developed a cationic lipopolymer, the liposome-polyethyleneglycol-polyethyleneimine complex (LPPC) adjuvant for surface-adsorbtion of antigens or immunomodulators. LPPC enhanced presentation on APCs, surface marker expression, cytokine release and activated T_H1-immunity. With LPS or CpGs, LPPC dramatically enhanced the IgA or IgG2A proportion of total Ig, demonstrating host immunity modulation [Chen *et al.*, 2012]. Effects of pegylation of cationic DOTAP liposome vaccines on LN targeting and immunogenicity were studied by Zhuang *et al*. Peg-DOTAP-liposomes accelerated drainage into LNs, prolonged retention and APC uptake, increased anti-OVA antibody responses and modulated their biodistribution which improved vaccine efficiency [Zhuang *et al.*, 2012]. The activity of cationic vaccines can be hampered by immobilization in the extracellular matrix caused by electrostatic interactions. Thus, Van den Berg *et al.* found that surface-shielding of DOTAP-liposomes by pegylation improved antigen expression drastically. Mice vaccinated with pegylated pVAX/Luc-NP antigen containing liposomes

elicited T cell responses comparable to naked DNA, suggesting that charge shielding improves dermally applied vaccines [Van Den Berg *et al.*, 2010].

Other adjuvants

Muramyl dipeptide (MDP)

MDP originates from a bacterial peptidoglycan cell wall fragment and is responsible for the activity of Freund's complete adjuvant (FCA). After phagocytosis by APCs, MDP is detected by the NOD2 receptor that activates the immune response. Numerous MDP derivatives have been synthesized to evaluate their immunostimulatory effects and adjuvant activity [Traub *et al.*, 2006, Ogawa *et al.*, 2011]. It was early recognized that liposomes were ideal carriers for MDP and its derivatives [Alving, 1991]. For example, the lipophilic MDP analogs MDP-PE and MDP-glycerol-dipalmitate (MDP-GDP) were added to liposomal HBsAg formulations both of which induced higher antibody titers, T_H1 response and IFN-γ levels [Jain *et al.*, 2009]. Masek *et al.* used small Ni-chelating liposomes to attach His-tagged *Candida albicans* heat shock protein (hsp90-CA) as antigen and to co-incorporate the MDP derivative C18-O-6-norAbuMDP as adjuvant. The immune response was of T_H1 and T_H2 type, comparable to FCA, but without side effects [Masek *et al.*, 2011]. Liposomes formulated as MDP with 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-ethanolamine (DPPE) are called Mifamurtide which is an adjuvant to standard chemotherapy for osteosarcoma. A study by Anderson *et al.* in patients with metastatic osteosarcoma showed that Mifamurtide had a manageable safety profile but that a randomized clinical trial is required to further determine its utility [Anderson *et al.*, 2014].

Monophosphoryl lipid A (MPLA)

Monophosphoryl lipid A (MPLA) is the active moiety of the bacterial endotoxin lipopolysaccharide (LPS). TLR4 is the receptor for LPS forming a complex with MD2, representing the main LPS binding component. LPS supports the development of diverse T_H cell types, depending on the tissue microenvironment. For instance, peripheral immunization with LPS drives T_H1 priming in lymphoid tissue and T_H17 priming in the intestinal mucosa [Mcaleer *et al.*, 2010]. Several lipopeptides derived from microbial origin convey self-adjuvanting activity by TLR2 signaling recognizing many PAMPs [Kawai *et al.*,

2010]. MPLA-liposomes have considerable potency and safety with a variety of candidate vaccines, including malaria, HIV-1 and several types of cancer [Alving *et al.*, 2012, Zaman *et al.*, 2013]. Combination of MPLA with the saponin QS-21 and immunostimulants in various adjuvant formulations forms the basis of Adjuvant Systems (AS, GlaxoSmithKline-Biologicals) to promote protective immune responses. MPLA and aluminum salts are present in AS04, and both MPLA and QS-21 are present in AS01 and AS02 which are liposome- and emulsion-based formulations [Garcon *et al.*, 2011]. Rizwan *et al.* analyzed the immunological activity of cubosomes containing MPLA and imiquimod. Cubosomes, a novel variation of MLVs, are composed of highly twisted lipid bilayers and non-intersecting water channels, providing increased encapsulation rates of lipophilic compounds. In MPLA-cubosomes sustained release of OVA was observed with induction of OVA-specific antibodies and $CD8^+$ and $CD4^+$ T cell proliferation at higher efficiency than liposomes plus adjuvants and alum [Rizwan *et al.*, 2013]. Immune response and protection induced by liposomal soluble leishmanial antigen (SLA) formulated with lipid A-trehalose dicorynomycolate (MPLA-TDM) was evaluated by Ravindran *et al.* This vaccine induced strong and long lasting protection against experimental visceral *Leishmaniasis* [Ravindran *et al.*, 2012]. An influenza vaccine with MPLA was developed by Adler-Moore *et al.* using the ectodomain of the M2e channel protein as a universal vaccine candidate. A liposomal M2e vaccine elicited anti-M2e antibodies that inhibited viral cell lysis and conferred complete protection to mice challenged with H1N1. Lymphocyte depletion markedly decreased protection, suggesting a primarily T_H2 mediated immune response [Adler-Moore *et al.*, 2011].

Listeriolysin

Cytolysins are virulence factors of various pathogenic bacteria. They form pores in target cell membranes, degrade membrane lipids or solubilize cell membranes. Bacteria use cytolysins to either inhibit functions of host immune cells or to gain access to intracellular niches. The bacterium *Listeria monocytogenes* can escape host immune defenses by lysis of the phagosomal membrane by use of listeriolysin O (LLO). LLO is used as vaccine adjuvant to provide cytosolic access for antigens in APCs [Dietrich *et al.*, 2001]. LLO based vaccines were reported by Mandal *et al.* who prepared OVA/LLO-liposomes. OVA immunization resulted in higher CTL activity and high IFN-γ production. The vaccine also conferred protection to mice from lethal challenges with antigen-expressing tumor cells [Mandal *et al.*, 2002]. LLO-

liposomes were also used to deliver the LCMV nucleoprotein (NP) to stimulate a NP-specific CTL response. Immunized mice generated high frequencies of NP-specific $CD8^+$ T cells and full protection against a lethal intracerebral challenge with virulent LCMV [Mandal *et al.*, 2004]. An anionic liposome-polycation-DNA complex combined with LLO was used as vaccine by Sun *et al.* to deliver OVA-cDNA. This formulation produced an enhanced $CD8^+$ T cell response, higher CTL frequency and IFN-γ production after stimulation by an OVA-specific peptide [Sun *et al.*, 2010]. Andrews *et al.* analyzed whether encapsulating CpGs in LLO-liposomes would enhance cell-mediated immune response and skew T_H1-type responses in a protein antigen-based vaccine utilizing LLO-liposomes. Co-encapsulation of CpGs in LLO-liposomes activated the NFκB pathway, maintaining cytosolic delivery of antigen mediated by co-encapsulated LLO. Immunization with OVA and CpG-LLO-liposomes showed enhanced T_H1 immune responses. [Andrews *et al.*, 2012].

Currently, 26 clinical trials are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org with the search terms liposome AND vaccine).

Veterinary vaccines

Knowledge of molecular details of immune mechanisms is relatively scarce for veterinary and pet animals and special concerns regarding the use of vaccine adjuvants must be considered. Demands like compatibility to human consumption, animal production, costs, challenges met by different species, vaccine administration for large numbers of animals and others must be evaluated [Heegaard *et al.*, 2011, Underwood *et al.*, 2012]. Table 2 summarizes some of the most recent experimental studies of liposome-based veterinary vaccines.

Therapeutic cancer vaccines

Although most cancers modify host proteins that can function as antigens the development of effective vaccines against such antigens is hampered by the weak immune response and the immunosuppressive effects induced by cancers. Tumor-associated antigens include viral proteins (e.g. HPV), chromosomal translocation products (e.g. bcr/abl), over-expressed

proteins likeHER2/neu, telomerase, MUC-1 and others [Kozako *et al.*, 2012]. In Table 3 some recent examples of experimental liposome-based cancer vaccines are listed.

Archaeosomes

Archaebacteria (Archaea) were discovered and classified by Woese and Fox as new group of prokaryotes, besides the Eubacteria (Bacteria) [Woese *et al.*, 1977]. Archaea contain DNA-dependent RNA polymerases and proteinaceous cell walls that lack peptidoglycan. Their cell membranes are composed of L-glycerol ether lipids with isoprenoid chains instead of D-glycerol ester lipids with fatty acid chains [Spang *et al.*, 2013]. Archaeal lipids are uniquely constituted of ether-linked isoprenoid phytanyl-archaeol (diether) and/or caldarchaeol (tetraether) cores conferring high membrane stability. Archaeosomes are liposomes prepared with archaeal glycerolipids. The head groups displayed on the glycerol-lipid cores of archaeosomes interact with APCs and induce T_H1, T_H2 and $CD8^+$ T cell responses to the entrapped antigen. The immune responses are persistent and subject to strong memory responses [Krishnan *et al.*, 2008, Benvegnu *et al.*, 2009]. The polar lipid from the archaeon, *Methanobrevibacter smithii* has been well characterized for its adjuvant potential. It contains archaetidyl-serine, promoting interaction with a PS receptor on APCs. These archaeosomes mediate MHC-I cross-priming and promote co-stimulation by APCs without inflammatory cytokine production. [Krishnan *et al.*, 2000]. Patel *et al.* showed that archaesomes prepared from *Methanobrevibacter smithii* lipids were suitable adjuvants for multivalent mucosal vaccines. Archaeosomes containing the encapsulated antigens OVA, bovine serum albumin and hen egg lysozyme conferred strong and sustained specific antibody responses to all three antigens [Patel *et al.*, 2004]. Intranasal immunization of mice with the archaeal lipid mucosal vaccine adjuvant and delivery (AMVAD) system, obtained by interaction of archaeosomes/antigens with multivalent cations induced robust mucosal antigen-specific IgA responses. AMVAD formulations are stable, safe and show protective efficacy in murine models of infection/challenge [Patel *et al.*, 2010]. Archaeosomes prepared from lipids of the non-pathogenic bacteria *Leptospira biflexa* (leptosomes) and *Mycobacterium smegmatis* (smegmosomes) were used as adjuvants. Both vesicles caused strong APC activation, cytokine release and expression of co-stimulatory signals which was significantly higher for smegmosomes compared to leptosomes. APC activation by both formulations induced immune responses in mice to entrapped OVA [Faisal *et al.*, 2009, Faisal *et al.*, 2011]. Borrero *et al.* studied immune response and cross reactivity against

Mycobacterium tuberculosis (MTB) with archaeosomes containing a mixture of cell wall glycolipids such as phosphatidylinositol mannosides of *M. smegmatis* (Ms) and DSPC/cholesterol. Ms containing liposomes induced a specific IgG response and recognition of MTB surface antigens, showing that immunogenic Ms-glycolipids could enhance subunit vaccines against tuberculosis [Borrero *et al.*, 2013]. The relation between archaeal lipid structures and their activity was explored by synthesizing novel head groups linked to archaeol. Archaeosomes consisting of various combinations of synthesized lipids with entrapped OVA antigen were used to immunize mice. Addition of the glycolipids gentio-triosyl-archaeol, mannotriosyl-archaeol or maltotriosyl-archaeol to archaetidylglycero-phosphate-O-methyl (AOM) archaeosomes significantly enhanced $CD8^+$ T cell responses, but diminished antibody titers. All three triglycosyl archaeols combined with AOM resulted in additive $CD8^+$ T cell responses [Sprott *et al.*, 2012]. Ansari *et al.* showed that archaeosome-entrapped secretory antigens (SAgs) of *L. monocytogenes* resulted in upregulation of T_H1 cytokines and boosted protective effects by reducing listerial burden in infected mice. Archaeosome-entrapped SAgs enhanced CTL response and increased survival of immunized animals [Ansari *et al.*, 2012]. Finally, Singha *et al.* used *E. coli* lipid liposome (escheriosome) based DNA delivery to induce superoxide dismutase (SOD) and IL-18 specific immune responses in murine *Brucellosis*. Escheriosome-mediated delivery of SOD- and IL-18-encoding DNA induced specific immune responses in immunized mice. Co-expression of SOD+IL-18 resulted in stronger IgG2a type response compared free SOD-DNA [Singha *et al.*, 2011].

Currently, no clinical trials with archaeosomal vaccines are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org, search terms archaeo-some AND vaccine).

In summary, vaccines prepared with archaeal lipids, the archaeosomes, represent a new interesting and promising alternative to classical liposomes and virosomes.

Virosomes

Virosomes are liposomes prepared by combining natural or synthetic phospholipids with virus envelope phospholipids, viral spike glycoproteins and other viral proteins. First virosomes were prepared and characterized by Almeida *et al.* [Almeida *et al.*, 1975], followed by Helenius *et al.* who incorporated Semliki Forest virus glycoproteins in liposomes

[Helenius *et al.*, 1977, Balcarova *et al.*, 1981]. Significant progress was made with virosomes termed "immunopotentiating reconstituted influenza virosomes" (IRIVs). IRIVs are SUVs with spike projections of the influenza surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). The fusogenic properties of HA are their primary features. IRIVs allow antigen presentation in the context of MHC-I and MHC-II and induce B- and T-cell responses [Gluck, 1992, Gluck *et al.*, 2005]. The first virosome-based influenza vaccine used in humans was Inflexal V™ which has an excellent tolerability profile and immunogenicity in healthy and immunocompromized persons [Herzog *et al.*, 2009]. Another virosome vaccine containing inactivated hepatitis A virus (HAV), Epaxal™, was developed as hepatitis A vaccine. It is excellently tolerable and highly immunogenic, conferring protection of at least 9-11 years in vaccinated individuals [Ambrosch *et al.*, 1997, Gluck *et al.*, 2000, Bovier *et al.*, 2010]. Immunogenicity and safety of Epaxal™ was evaluated in Thai children with HIV infection. Prevalence of HAV protective antibodies was 100% after vaccination, showing that Epaxal™ is an effective HAV vaccine for HIV-infected children [Saksawad *et al.*, 2011]. Another vaccine contains an aspartyl proteinase-2 (Sap-2) of *Candida albicans* incorporated into IRIVs. Following intravaginal administration anti-Sap2 antibodies were detected in vaginal fluids of rats inducing long-lasting protection [De Bernardis *et al.*, 2012]. Walczak *et al.* demonstrated that a heterologous prime-boost with Semliki Forest virus (rSFV) encoding a fusion protein of E6 and E7 of HPV16 and virosomes containing the HPV16-E7 protein resulted in higher numbers of antigen-specific CTL in mice than homologous protocols [Walczak *et al.*, 2011]. Today, a second generation of influenza virosomes has evolved for various preclinical and clinical stage vaccine candidates. Additional components are included to optimize particle assembly and stability and to enhance immunostimulatory effects [Moser *et al.*, 2013]. GPI-0100, a saponin derivative, enhanced immunogenicity and protective efficacy of a virosomal influenza vaccine, providing full protection of infected mice at extremely low antigen doses [Liu *et al.*, 2013]. A combination of reconstituted respiratory syncytial virus (RSV) envelopes with incorporated MPLA (RSV-MPLA) virosomes was studied by Kamphuis *et al.* in enhanced respiratory disease (ERD)-prone rats. Vaccination with RSV-MPLA induced higher antibody levels and protection against infection [Kamphuis *et al.*, 2013]. Jamali *et al.* developed a DNA vaccine using cationic influenza virosomes (CIV). CIV-delivered epitope-encoding DNA induced equal numbers of IFN-γ and granzyme B-producing T cells than a 10-fold higher dose of naked pDNA [Jamali *et al.*, 2012]. Another DNA/virosome vaccine was reported by Kheiri

et al. who prepared a vaccine complex containing an influenza nucleoprotein encoding plasmid that induced much higher T cell responses and protection as plasmid alone [Kheiri *et al.*, 2012]. In clinical trials, IRIVs have shown vast potential for delivery of peptides derived from *Plasmodium falciparum* antigens [Peduzzi *et al.*, 2008]. An IRIV-formulated fusion protein composed of two malaria antigens was described by Tamborrini *et al.* Compared to other vaccines the adjuvant-free formulation elicited specific IgG1 antibody profiles in mice and cross-reactivity with blood-stage parasites [Tamborrini *et al.*, 2011]. Virosomes containing surface HIV-1 gp41-derived P1 lipid conjugated peptides (MYM-V101) as prophylactic HIV-1 vaccine were prepared. MYM-V101 was safe and well-tolerated administered by intramuscular and intranasal routes in healthy women. P1-specific serum IgGs and IgAs were detected in all recipients but P1-specific T_H1 responses were not found [Leroux-Roels *et al.*, 2013].

Currently, several clinical trials with virosome vaccines are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org, search terms virosome AND vaccine).

Conclusions

The enormous versatility of liposomes and the related archaeosomes and virosomes endows them as highly valuable carrier systems for vaccines. Besides improving antigen stability and presentation to immunocompetent cells, depending on their specific properties as composition, size and surface properties, these nanocarriers also possess the ability to overcome biological barriers such as skin and mucosa and provide controlled and slow release of antigens. Together with the ability to induce strong immune responses provided by co-formulated adjuvants, liposome-based vaccines provide properties that are fundamental for the development of modern vaccine formulations. It is predictable, that these delivery systems will be increasingly applied in the near future with success, leading to major improvements in vaccine development.

Declaration of Conflicting Interests

The author declares that there is no conflict of interest.

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References

Acres, B. and Limacher, J.M. (2005) Muc1 as a Target Antigen for Cancer Immunotherapy. *Expert Rev Vaccines* 4: 493-502.

Adler-Moore, J., Munoz, M., Kim, H., Romero, J., Tumpey, T., Zeng, H. *et al.* (2011) Characterization of the Murine Th2 Response to Immunization with Liposomal M2e Influenza Vaccine. *Vaccine* 29: 4460-4468.

Allison, A.C. and Gregoriadis, G. (1976) Liposomes as Immunological Adjuvants. *Recent Results Cancer Res*: 58-64.

Allison, A.G. and Gregoriadis, G. (1974) Liposomes as Immunological Adjuvants. *Nature* 252: 252.

Almeida, J.D., Edwards, D.C., Brand, C.M., and Heath, T.D. (1975) Formation of Virosomes from Influenza Subunits and Liposomes. *Lancet* 2: 899-901.

Alving, C.R. (1991) Liposomes as Carriers of Antigens and Adjuvants. *J Immunol Methods* 140: 1-13.

Alving, C.R., Peachman, K.K., Rao, M., and Reed, S.G. (2012) Adjuvants for Human Vaccines. *Curr Opin Immunol* 24: 310-315.

Alving, C.R., Rao, M., Steers, N.J., Matyas, G.R., and Mayorov, A.V. (2012) Liposomes Containing Lipid A: An Effective, Safe, Generic Adjuvant System for Synthetic Vaccines. *Expert Rev Vaccines* 11: 733-744.

Ambrosch, F., Wiedermann, G., Jonas, S., Althaus, B., Finkel, B., Gluck, R. *et al.* (1997) Immunogenicity and Protectivity of a New Liposomal Hepatitis a Vaccine. *Vaccine* 15: 1209-1213.

Amidi, M., De Raad, M., Crommelin, D.J., Hennink, W.E., and Mastrobattista, E. (2011) Antigen-Expressing Immunostimulatory Liposomes as a Genetically Programmable Synthetic Vaccine. *Syst Synth Biol* 5: 21-31.

Amidi, M., Van Helden, M.J., Tabataei, N.R., De Goede, A.L., Schouten, M., De Bot, V. *et al.* (2012) Induction of Humoral and Cellular Immune Responses by Antigen-Expressing Immunostimulatory Liposomes. *J Control Release* 164: 323-330.

Anderson, P.M., Meyers, P., Kleinerman, E., Venkatakrishnan, K., Hughes, D.P., Herzog, C. *et al.* (2014) Mifamurtide in Metastatic and Recurrent Osteosarcoma: A Patient Access Study with Pharmacokinetic, Pharmacodynamic, and Safety Assessments. *Pediatr Blood Cancer* 61: 238-244.

Andrews, C.D., Huh, M.S., Patton, K., Higgins, D., Van Nest, G., Ott, G. *et al.* (2012) Encapsulating Immunostimulatory Cpg Oligonucleotides in Listeriolysin O-Liposomes Promotes a Th1-Type Response and Ctl Activity. *Mol Pharm* 9: 1118-1125.

Ansari, M.A., Zubair, S., Tufail, S., Ahmad, E., Khan, M.R., Quadri, Z. *et al.* (2012) Ether Lipid Vesicle-Based Antigens Impart Protection against Experimental Listeriosis. *Int J Nanomedicine* 7: 2433-2447.

Badiee, A., Khamesipour, A., Samiei, A., Soroush, D., Shargh, V.H., Kheiri, M.T. *et al.* (2012) The Role of Liposome Size on the Type of Immune Response Induced in Balb/C Mice against Leishmaniasis: Rgp63 as a Model Antigen. *Exp Parasitol* 132: 403-409.

Bal, S.M., Hortensius, S., Ding, Z., Jiskoot, W., and Bouwstra, J.A. (2011) Co-Encapsulation of Antigen and Toll-Like Receptor Ligand in Cationic Liposomes Affects the Quality of the Immune Response in Mice after Intradermal Vaccination. *Vaccine* 29: 1045-1052.

Balcarova, J., Helenius, A., and Simons, K. (1981) Antibody Response to Spike Protein Vaccines Prepared from Semliki Forest Virus. *J Gen Virol* 53: 85-92.

Barnier-Quer, C., Elsharkawy, A., Romeijn, S., Kros, A., and Jiskoot, W. (2013) Adjuvant Effect of Cationic Liposomes for Subunit Influenza Vaccine: Influence of Antigen Loading Method, Cholesterol and Immune Modulators. *Pharmaceutics* 5: 392-410.

Barnier Quer, C., Elsharkawy, A., Romeijn, S., Kros, A., and Jiskoot, W. (2012) Cationic Liposomes as Adjuvants for Influenza Hemagglutinin: More Than Charge Alone. *Eur J Pharm Biopharm* 81: 294-302.

Benvegnu, T., Lemiegre, L., and Cammas-Marion, S. (2009) New Generation of Liposomes Called Archaeosomes Based on Natural or Synthetic Archaeal Lipids as Innovative Formulations for Drug Delivery. *Recent Pat Drug Deliv Formul* 3: 206-220.

Bernstein, D.I., Earwood, J.D., Bravo, F.J., Cohen, G.H., Eisenberg, R.J., Clark, J.R. *et al.* (2011) Effects of Herpes Simplex Virus Type 2 Glycoprotein Vaccines and Cldc Adjuvant on Genital Herpes Infection in the Guinea Pig. *Vaccine* 29: 2071-2078.

Bernstein, D.I., Farley, N., Bravo, F.J., Earwood, J., Mcneal, M., Fairman, J. *et al.* (2010) The Adjuvant Cldc Increases Protection of a Herpes Simplex Type 2 Glycoprotein D Vaccine in Guinea Pigs. *Vaccine* 28: 3748-3753.

Borrero, R., Garcia Mde, L., Canet, L., Zayas, C., Reyes, F., Prieto, J.L. *et al.* (2013) Evaluation of the Humoral Immune Response and Cross Reactivity against Mycobacterium Tuberculosis of Mice Immunized with Liposomes Containing Glycolipids of Mycobacterium Smegmatis. *BMC Immunol* 14 Suppl 1: S13.

Bovier, P.A., Bock, J., Ebengo, T.F., Frosner, G., Glaus, J., Herzog, C. *et al.* (2010) Predicted 30-Year Protection after Vaccination with an Aluminum-Free Virosomal Hepatitis a Vaccine. *J Med Virol* 82: 1629-1634.

Brignole, C., Marimpietri, D., Di Paolo, D., Perri, P., Morandi, F., Pastorino, F. *et al.* (2010) Therapeutic Targeting of Tlr9 Inhibits Cell Growth and Induces Apoptosis in Neuroblastoma. *Cancer Res* 70: 9816-9826.

Brito, L.A., Malyala, P., and O'hagan, D.T. (2013) Vaccine Adjuvant Formulations: A Pharmaceutical Perspective. *Semin Immunol* 25: 130-145.

Butts, C., Maksymiuk, A., Goss, G., Soulieres, D., Marshall, E., Cormier, Y. *et al.* (2011) Updated Survival Analysis in Patients with Stage Iiib or Iv Non-Small-Cell Lung Cancer Receiving Blp25 Liposome Vaccine (L-Blp25): Phase Iib Randomized, Multicenter, Open-Label Trial. *J Cancer Res Clin Oncol* 137: 1337-1342.

Butts, C., Murray, R.N., Smith, C.J., Ellis, P.M., Jasas, K., Maksymiuk, A. *et al.* (2010) A Multicenter Open-Label Study to Assess the Safety of a New Formulation of Blp25 Liposome Vaccine in Patients with Unresectable Stage Iii Non-Small-Cell Lung Cancer. *Clin Lung Cancer* 11: 391-395.

Carroll, T.D., Matzinger, S.R., Barry, P.A., Mcchesney, M.B., Fairman, J., and Miller, C.J. (2014) Efficacy of Influenza Vaccination of Elderly Rhesus Macaques Is Dramatically Improved by Addition of a Cationic Lipid/DNA Adjuvant. *J Infect Dis* 209: 24-33.

Carstens, M.G., Camps, M.G., Henriksen-Lacey, M., Franken, K., Ottenhoff, T.H., Perrie, Y. *et al.* (2011) Effect of Vesicle Size on Tissue Localization and Immunogenicity of Liposomal DNA Vaccines. *Vaccine* 29: 4761-4770.

Chen, C.H., Lin, Y.L., Liu, Y.K., He, P.J., Lin, C.M., Chiu, Y.H. *et al.* (2012) Liposome-Based Polymer Complex as a Novel Adjuvant: Enhancement of Specific Antibody Production and Isotype Switch. *Int J Nanomedicine* 7: 607-621.

Chen, L. and Flies, D.B. (2013) Molecular Mechanisms of T Cell Co-Stimulation and Co-Inhibition. *Nat Rev Immunol* 13: 227-242.

Cheng, G., Zhao, X., Yan, W., Wang, W., Zuo, X., Huang, K. *et al.* (2007) Alpha Interferon Is a Powerful Adjuvant for a Recombinant Protein Vaccine against Foot-and-Mouth Disease Virus in Swine, and an Effective Stimulus of in Vivo Immune Response. *Vaccine* 25: 5199-5208.

Christensen, D., Henriksen-Lacey, M., Kamath, A.T., Lindenstrom, T., Korsholm, K.S., Christensen, J.P. *et al.* (2012) A Cationic Vaccine Adjuvant Based on a Saturated Quaternary Ammonium Lipid Have Different in Vivo Distribution Kinetics and Display a Distinct Cd4 T Cell-Inducing Capacity Compared to Its Unsaturated Analog. *J Control Release* 160: 468-476.

Christensen, D., Korsholm, K.S., Andersen, P., and Agger, E.M. (2011) Cationic Liposomes as Vaccine Adjuvants. *Expert Rev Vaccines* 10: 513-521.

Cote, P.J., Butler, S.D., George, A.L., Fairman, J., Gerin, J.L., Tennant, B.C. *et al.* (2009) Rapid Immunity to Vaccination with Woodchuck Hepatitis Virus Surface Antigen Using Cationic Liposome-DNA Complexes as Adjuvant. *J Med Virol* 81: 1760-1772.

Cruz, L.J., Rueda, F., Simon, L., Cordobilla, B., Albericio, F., and J, C.D. (2013) Liposomes Containing Ny-Eso-1/Tetanus Toxoid and Adjuvant Peptides Targeted to Human Dendritic Cells Via the Fc Receptor for Cancer Vaccines. *Nanomedicine (Lond)*:

De Bernardis, F., Amacker, M., Arancia, S., Sandini, S., Gremion, C., Zurbriggen, R. *et al.* (2012) A Virosomal Vaccine against Candidal Vaginitis: Immunogenicity, Efficacy and Safety Profile in Animal Models. *Vaccine* 30: 4490-4498.

Desel, C., Werninghaus, K., Ritter, M., Jozefowski, K., Wenzel, J., Russkamp, N. *et al.* (2013) The Mincle-Activating Adjuvant Tdb Induces Myd88-Dependent Th1 and Th17 Responses through Il-1r Signaling. *PLoS One* 8: e53531.

Dietrich, G., Hess, J., Gentschev, I., Knapp, B., Kaufmann, S.H., and Goebel, W. (2001) From Evil to Good: A Cytolysin in Vaccine Development. *Trends Microbiol* 9: 23-28.

Ding, Q., Chen, J., Wei, X., Sun, W., Mai, J., Yang, Y. *et al.* (2013) Raftsomes Containing Epitope-Mhc-Ii Complexes Mediated Cd4+ T Cell Activation and Antigen-Specific Immune Responses. *Pharm Res* 30: 60-69.

Dissanayake, D.R., Wijewardana, T.G., Gunawardena, G.A., and Poxton, I.R. (2010) Potential Use of a Liposome-Encapsulated Mixture of Lipopolysaccharide Core Types (R1, R2, R3 and R4) of Escherichia Coli in Controlling Colisepticaemia in Chickens. *J Med Microbiol* 59: 100-107.

Dong, L., Liu, F., Fairman, J., Hong, D.K., Lewis, D.B., Monath, T. *et al.* (2012) Cationic Liposome-DNA Complexes (Cldc) Adjuvant Enhances the Immunogenicity and Cross-Protective Efficacy of a Pre-Pandemic Influenza a H5n1 Vaccine in Mice. *Vaccine* 30: 254-264.

Engler, O.B., Schwendener, R.A., Dai, W.J., Wolk, B., Pichler, W., Moradpour, D. *et al.* (2004) A Liposomal Peptide Vaccine Inducing Cd8+ T Cells in Hla-A2.1 Transgenic Mice, Which Recognise Human Cells Encoding Hepatitis C Virus (Hcv) Proteins. *Vaccine* 23: 58-68.

Erikci, E., Gursel, M., and Gursel, I. (2011) Differential Immune Activation Following Encapsulation of Immunostimulatory Cpg Oligodeoxynucleotide in Nanoliposomes. *Biomaterials* 32: 1715-1723.

Even-Or, O., Joseph, A., Itskovitz-Cooper, N., Samira, S., Rochlin, E., Eliyahu, H. *et al.* (2011) A New Intranasal Influenza Vaccine Based on a Novel Polycationic Lipid-Ceramide Carbamoyl-Spermine (Ccs). Ii. Studies in Mice and Ferrets and Mechanism of Adjuvanticity. *Vaccine* 29: 2474-2486.

Fairman, J., Moore, J., Lemieux, M., Van Rompay, K., Geng, Y., Warner, J. *et al.* (2009) Enhanced in Vivo Immunogenicity of Siv Vaccine Candidates with Cationic Liposome-DNA Complexes in a Rhesus Macaque Pilot Study. *Hum Vaccin* 5: 141-150.

Faisal, S.M., Chen, J.W., Mcdonough, S.P., Chang, C.F., Teng, C.H., and Chang, Y.F. (2011) Immunostimulatory and Antigen Delivery Properties of Liposomes Made up of Total Polar Lipids from Non-Pathogenic Bacteria Leads to Efficient Induction of Both Innate and Adaptive Immune Responses. *Vaccine* 29: 2381-2391.

Faisal, S.M., Yan, W., Mcdonough, S.P., Chang, C.F., Pan, M.J., and Chang, Y.F. (2009) Leptosome-Entrapped Leptospiral Antigens Conferred Significant Higher Levels of Protection Than Those Entrapped with Pc-Liposomes in a Hamster Model. *Vaccine* 27: 6537-6545.

Fan, Y., Wang, D., Hu, Y., Liu, J., Han, G., Zhao, X. *et al.* (2012) Liposome and Epimedium Polysaccharide-Propolis Flavone Can Synergistically Enhance Immune Effect of Vaccine. *Int J Biol Macromol* 50: 125-130.

Figueiredo, L., Cadete, A., Goncalves, L.M., Corvo, M.L., and Almeida, A.J. (2012) Intranasal Immunisation of Mice against Streptococcus Equi Using Positively Charged Nanoparticulate Carrier Systems. *Vaccine* 30: 6551-6558.

Garcon, N. and Van Mechelen, M. (2011) Recent Clinical Experience with Vaccines Using Mpl- and Qs-21-Containing Adjuvant Systems. *Expert Rev Vaccines* 10: 471-486.

Gluck, R. (1992) Immunopotentiating Reconstituted Influenza Virosomes (Irivs) and Other Adjuvants for Improved Presentation of Small Antigens. *Vaccine* 10: 915-919.

Gluck, R., Burri, K.G., and Metcalfe, I. (2005) Adjuvant and Antigen Delivery Properties of Virosomes. *Curr Drug Deliv* 2: 395-400.

Gluck, R. and Walti, E. (2000) Biophysical Validation of Epaxal Berna, a Hepatitis a Vaccine Adjuvanted with Immunopotentiating Reconstituted Influenza Virosomes (Iriv). *Dev Biol (Basel)* 103: 189-197.

Gowen, B.B., Fairman, J., Dow, S., Troyer, R., Wong, M.H., Jung, K.H. *et al.* (2009) Prophylaxis with Cationic Liposome-DNA Complexes Protects Hamsters from Phleboviral Disease: Importance of Liposomal Delivery and Cpg Motifs. *Antiviral Res* 81: 37-46.

Gregory, A.E., Titball, R., and Williamson, D. (2013) Vaccine Delivery Using Nanoparticles. *Front Cell Infect Microbiol* 3: 13.

Gupta, P.N. and Vyas, S.P. (2011) Investigation of Lectinized Liposomes as M-Cell Targeted Carrier-Adjuvant for Mucosal Immunization. *Colloids Surf B Biointerfaces* 82: 118-125.

Hansen, J., Lindenstrom, T., Lindberg-Levin, J., Aagaard, C., Andersen, P., and Agger, E.M. (2012) Caf05: Cationic Liposomes That Incorporate Synthetic Cord Factor and Poly(I:C) Induce Ctl Immunity and Reduce Tumor Burden in Mice. *Cancer Immunol Immunother* 61: 893-903.

Hebishima, T., Yuba, E., Kono, K., Takeshima, S.N., Ito, Y., and Aida, Y. (2012) The Ph-Sensitive Fusogenic 3-Methyl-Glutarylated Hyperbranched Poly(Glycidol)-Conjugated Liposome Induces Antigen-Specific Cellular and Humoral Immunity. *Clin Vaccine Immunol* 19: 1492-1498.

Heegaard, P.M., Dedieu, L., Johnson, N., Le Potier, M.F., Mockey, M., Mutinelli, F. *et al.* (2011) Adjuvants and Delivery Systems in Veterinary Vaccinology: Current State and Future Developments. *Arch Virol* 156: 183-202.

Helenius, A., Fries, E., and Kartenbeck, J. (1977) Reconstitution of Semliki Forest Virus Membrane. *J Cell Biol* 75: 866-880.

Henriksen-Lacey, M., Bramwell, V.W., Christensen, D., Agger, E.M., Andersen, P., and Perrie, Y. (2010) Liposomes Based on Dimethyldioctadecylammonium Promote a Depot Effect and Enhance Immunogenicity of Soluble Antigen. *J Control Release* 142: 180-186.

Henriksen-Lacey, M., Christensen, D., Bramwell, V.W., Lindenstrom, T., Agger, E.M., Andersen, P. *et al.* (2011) Comparison of the Depot Effect and Immunogenicity of Liposomes Based on Dimethyldioctadecylammonium (Dda), 3beta-[N-(N',N'-Dimethylaminoethane)Carbomyl] Cholesterol (Dc-Chol), and 1,2-Dioleoyl-3-Trimethylammonium Propane (Dotap): Prolonged Liposome Retention Mediates Stronger Th1 Responses. *Mol Pharm* 8: 153-161.

Herzog, C., Hartmann, K., Kunzi, V., Kursteiner, O., Mischler, R., Lazar, H. *et al.* (2009) Eleven Years of Inflexal V-a Virosomal Adjuvanted Influenza Vaccine. *Vaccine* 27: 4381-4387.

Hiszczynska-Sawicka, E., Li, H., Boyu Xu, J., Akhtar, M., Holec-Gasior, L., Kur, J. *et al.* (2012) Induction of Immune Responses in Sheep by Vaccination with Liposome-Entrapped DNA Complexes Encoding Toxoplasma Gondii Mic3 Gene. *Pol J Vet Sci* 15: 3-9.

Hong, D.K., Chang, S., Botham, C.M., Giffon, T.D., Fairman, J., and Lewis, D.B. (2010) Cationic Lipid/DNA Complex-Adjuvanted Influenza a Virus Vaccination Induces Robust Cross-Protective Immunity. *J Virol* 84: 12691-12702.

Ichihashi, T., Satoh, T., Sugimoto, C., and Kajino, K. (2013) Emulsified Phosphatidylserine, Simple and Effective Peptide Carrier for Induction of Potent Epitope-Specific T Cell Responses. *PLoS One* 8: e60068.

Ingvarsson, P.T., Schmidt, S.T., Christensen, D., Larsen, N.B., Hinrichs, W.L., Andersen, P. *et al.* (2013) Designing Caf-Adjuvanted Dry Powder Vaccines: Spray Drying Preserves the Adjuvant Activity of Caf01. *J Control Release* 167: 256-264.

Ingvarsson, P.T., Yang, M., Nielsen, H.M., Rantanen, J., and Foged, C. (2011) Stabilization of Liposomes During Drying. *Expert Opin Drug Deliv* 8: 375-388.

Irache, J.M., Salman, H.H., Gamazo, C., and Espuelas, S. (2008) Mannose-Targeted Systems for the Delivery of Therapeutics. *Expert Opin Drug Deliv* 5: 703-724.

Jain, V., Vyas, S.P., and Kohli, D.V. (2009) Well-Defined and Potent Liposomal Hepatitis B Vaccines Adjuvanted with Lipophilic Mdp Derivatives. *Nanomedicine* 5: 334-344.

Jamali, A., Holtrop, M., De Haan, A., Hashemi, H., Shenagari, M., Memarnejadian, A. *et al.* (2012) Cationic Influenza Virosomes as an Adjuvanted Delivery System for Ctl Induction by DNA Vaccination. *Immunol Lett* 148: 77-82.

Jones, A., Bosio, C., Duffy, A., Goodyear, A., Schriefer, M., and Dow, S. (2010) Protection against Pneumonic Plague Following Oral Immunization with a Non-Replicating Vaccine. *Vaccine* 28: 5924-5929.

Joshi, M.D., Unger, W.J., Storm, G., Van Kooyk, Y., and Mastrobattista, E. (2012) Targeting Tumor Antigens to Dendritic Cells Using Particulate Carriers. *J Control Release* 161: 25-37.

Kamath, A.T., Mastelic, B., Christensen, D., Rochat, A.F., Agger, E.M., Pinschewer, D.D. *et al.* (2012) Synchronization of Dendritic Cell Activation and Antigen Exposure Is Required for the Induction of Th1/Th17 Responses. *J Immunol* 188: 4828-4837.

Kamphuis, T., Shafique, M., Meijerhof, T., Stegmann, T., Wilschut, J., and De Haan, A. (2013) Efficacy and Safety of an Intranasal Virosomal Respiratory Syncytial Virus Vaccine Adjuvanted with Monophosphoryl Lipid a in Mice and Cotton Rats. *Vaccine* 31: 2169-2176.

Kamphuis, T., Stegmann, T., Meijerhof, T., Wilschut, J., and De Haan, A. (2013) A Virosomal Respiratory Syncytial Virus Vaccine Adjuvanted with Monophosphoryl Lipid a Provides Protection against Viral Challenge without Priming for Enhanced Disease in Cotton Rats. *Influenza Other Respir Viruses* 7: 1227-1236.

Karlsson, I., Brandt, L., Vinner, L., Kromann, I., Andreasen, L.V., Andersen, P. *et al.* (2013) Adjuvanted Hla-Supertype Restricted Subdominant Peptides Induce New T-Cell Immunity During Untreated Hiv-1-Infection. *Clin Immunol* 146: 120-130.

Kaur, R., Bramwell, V.W., Kirby, D.J., and Perrie, Y. (2012) Pegylation of Dda:Tdb Liposomal Adjuvants Reduces the Vaccine Depot Effect and Alters the Th1/Th2 Immune Responses. *J Control Release* 158: 72-77.

Kawai, T. and Akira, S. (2010) The Role of Pattern-Recognition Receptors in Innate Immunity: Update on Toll-Like Receptors. *Nat Immunol* 11: 373-384.

Kheiri, M.T., Jamali, A., Shenagari, M., Hashemi, H., Sabahi, F., Atyabi, F. *et al.* (2012) Influenza Virosome/DNA Vaccine Complex as a New Formulation to Induce Intra-Subtypic Protection against Influenza Virus Challenge. *Antiviral Res* 95: 229-236.

Korsholm, K.S., Andersen, P.L., and Christensen, D. (2012) Cationic Liposomal Vaccine Adjuvants in Animal Challenge Models: Overview and Current Clinical Status. *Expert Rev Vaccines* 11: 561-577.

Kozako, T., Arima, N., Yoshimitsu, M., Honda, S.I., and Soeda, S. (2012) Liposomes and Nanotechnology in Drug Development: Focus on Oncotargets. *Int J Nanomedicine* 7: 4943-4951.

Krishnamachari, Y. and Salem, A.K. (2009) Innovative Strategies for Co-Delivering Antigens and Cpg Oligonucleotides. *Adv Drug Deliv Rev* 61: 205-217.

Krishnan, L., Dicaire, C.J., Patel, G.B., and Sprott, G.D. (2000) Archaeosome Vaccine Adjuvants Induce Strong Humoral, Cell-Mediated, and Memory Responses: Comparison to Conventional Liposomes and Alum. *Infect Immun* 68: 54-63.

Krishnan, L. and Sprott, G.D. (2008) Archaeosome Adjuvants: Immunological Capabilities and Mechanism(S) of Action. *Vaccine* 26: 2043-2055.

Lakshminarayanan, V., Thompson, P., Wolfert, M.A., Buskas, T., Bradley, J.M., Pathangey, L.B. *et al.* (2012) Immune Recognition of Tumor-Associated Mucin Muc1 Is Achieved by a Fully Synthetic Aberrantly Glycosylated Muc1 Tripartite Vaccine. *Proc Natl Acad Sci U S A* 109: 261-266.

Lay, M., Callejo, B., Chang, S., Hong, D.K., Lewis, D.B., Carroll, T.D. *et al.* (2009) Cationic Lipid/DNA Complexes (Jvrs-100) Combined with Influenza Vaccine (Fluzone) Increases Antibody Response, Cellular Immunity, and Antigenically Drifted Protection. *Vaccine* 27: 3811-3820.

Leroux-Roels, G. (2010) Unmet Needs in Modern Vaccinology: Adjuvants to Improve the Immune Response. *Vaccine* 28 Suppl 3: C25-36.

Leroux-Roels, G., Maes, C., Clement, F., Van Engelenburg, F., Van Den Dobbelsteen, M., Adler, M. *et al.* (2013) Randomized Phase I: Safety, Immunogenicity and Mucosal Antiviral Activity in Young Healthy Women Vaccinated with Hiv-1 Gp41 P1 Peptide on Virosomes. *PLoS One* 8: e55438.

Li, P., Chen, S., Jiang, Y., Jiang, J., Zhang, Z., and Sun, X. (2013) Dendritic Cell Targeted Liposomes-Protamine-DNA Complexes Mediated by Synthetic Mannosylated Cholesterol as a Potential Carrier for DNA Vaccine. *Nanotechnology* 24: 295101.

Li, Y., Hu, Y., Jin, Y., Zhang, G., Wong, J., Sun, L.Q. *et al.* (2011) Prophylactic, Therapeutic and Immune Enhancement Effect of Liposome-Encapsulated Polyiclc on Highly Pathogenic H5n1 Influenza Infection. *J Gene Med* 13: 60-72.

Lin, Y., Slight, S.R., and Khader, S.A. (2010) Th17 Cytokines and Vaccine-Induced Immunity. *Semin Immunopathol* 32: 79-90.

Lin, Y.F., Deng, M.C., Tseng, L.P., Jiang, P.R., Jan, T.R., Hsieh, F.I. *et al.* (2011) Adjuvant Effect of Liposome in Chicken Result from Induction of Nitric Oxide. *Biomed Mater* 6: 015011.

Lindenstrom, T., Woodworth, J., Dietrich, J., Aagaard, C., Andersen, P., and Agger, E.M. (2012) Vaccine-Induced Th17 Cells Are Maintained Long-Term Postvaccination as a Distinct and Phenotypically Stable Memory Subset. *Infect Immun* 80: 3533-3544.

Liu, H., De Vries-Idema, J., Ter Veer, W., Wilschut, J., and Huckriede, A. (2013) Influenza Virosomes Supplemented with Gpi-0100 Adjuvant: A Potent Vaccine Formulation for Antigen Dose Sparing. *Med Microbiol Immunol*:

Liu, J., Wu, J., Wang, B., Zeng, S., Qi, F., Lu, C. *et al.* (2013) Oral Vaccination with a Liposome-Encapsulated Influenza DNA Vaccine Protects Mice against Respiratory Challenge Infection. *J Med Virol*:

Logue, C.H., Phillips, A.T., Mossel, E.C., Ledermann, J.P., Welte, T., Dow, S.W. *et al.* (2010) Treatment with Cationic Liposome-DNA Complexes (Cldcs) Protects Mice from Lethal Western Equine Encephalitis Virus (Weev) Challenge. *Antiviral Res* 87: 195-203.

Lonez, C., Vandenbranden, M., and Ruysschaert, J.M. (2012) Cationic Lipids Activate Intracellular Signaling Pathways. *Adv Drug Deliv Rev* 64: 1749-1758.

Ludewig, B., Barchiesi, F., Pericin, M., Zinkernagel, R.M., Hengartner, H., and Schwendener, R.A. (2000) In Vivo Antigen Loading and Activation of Dendritic Cells Via a Liposomal Peptide Vaccine Mediates Protective Antiviral and Anti-Tumour Immunity. *Vaccine* 19: 23-32.

Ma, Y., Zhuang, Y., Xie, X., Wang, C., Wang, F., Zhou, D. *et al.* (2011) The Role of Surface Charge Density in Cationic Liposome-Promoted Dendritic Cell Maturation and Vaccine-Induced Immune Responses. *Nanoscale* 3: 2307-2314.

Mandal, M., Kawamura, K.S., Wherry, E.J., Ahmed, R., and Lee, K.D. (2004) Cytosolic Delivery of Viral Nucleoprotein by Listeriolysin O-Liposome Induces Enhanced Specific Cytotoxic T Lymphocyte Response and Protective Immunity. *Mol Pharm* 1: 2-8.

Mandal, M. and Lee, K.D. (2002) Listeriolysin O-Liposome-Mediated Cytosolic Delivery of Macromolecule Antigen in Vivo: Enhancement of Antigen-Specific Cytotoxic T Lymphocyte Frequency, Activity, and Tumor Protection. *Biochim Biophys Acta* 1563: 7-17.

Martel, C.J., Agger, E.M., Poulsen, J.J., Hammer Jensen, T., Andresen, L., Christensen, D. *et al.* (2011) Caf01 Potentiates Immune Responses and Efficacy of an Inactivated Influenza Vaccine in Ferrets. *PLoS One* 6: e22891.

Masek, J., Bartheldyova, E., Turanek-Knotigova, P., Skrabalova, M., Korvasova, Z., Plockova, J. *et al.* (2011) Metallochelating Liposomes with Associated Lipophilised Norabumdp as Biocompatible Platform for Construction of Vaccines with Recombinant His-Tagged Antigens: Preparation, Structural Study and Immune Response Towards Rhsp90. *J Control Release* 151: 193-201.

Mcaleer, J.P. and Vella, A.T. (2010) Educating Cd4 T Cells with Vaccine Adjuvants: Lessons from Lipopolysaccharide. *Trends Immunol* 31: 429-435.

Milicic, A., Kaur, R., Reyes-Sandoval, A., Tang, C.K., Honeycutt, J., Perrie, Y. *et al.* (2012) Small Cationic Dda:Tdb Liposomes as Protein Vaccine Adjuvants Obviate the Need for Tlr Agonists in Inducing Cellular and Humoral Responses. *PLoS One* 7: e34255.

Miyazaki, J., Nishiyama, H., Yano, I., Nakaya, A., Kohama, H., Kawai, K. *et al.* (2011) The Therapeutic Effects of R8-Liposome-Bcg-Cws on Bbn-Induced Rat Urinary Bladder Carcinoma. *Anticancer Res* 31: 2065-2071.

Mizuuchi, M., Hirohashi, Y., Torigoe, T., Kuroda, T., Yasuda, K., Shimizu, Y. *et al.* (2012) Novel Oligomannose Liposome-DNA Complex DNA Vaccination Efficiently Evokes Anti-Hpv E6 and E7 Ctl Responses. *Exp Mol Pathol* 92: 185-190.

Moon, J.J., Suh, H., Bershteyn, A., Stephan, M.T., Liu, H., Huang, B. *et al.* (2011) Interbilayer-Crosslinked Multilamellar Vesicles as Synthetic Vaccines for Potent Humoral and Cellular Immune Responses. *Nat Mater* 10: 243-251.

Morrey, J.D., Motter, N.E., Chang, S., and Fairman, J. (2011) Breaking B and T Cell Tolerance Using Cationic Lipid--DNA Complexes (Cldc) as a Vaccine Adjuvant with Hepatitis B Virus (Hbv) Surface Antigen in Transgenic Mice Expressing Hbv. *Antiviral Res* 90: 227-230.

Moser, C., Muller, M., Kaeser, M.D., Weydemann, U., and Amacker, M. (2013) Influenza Virosomes as Vaccine Adjuvant and Carrier System. *Expert Rev Vaccines* 12: 779-791.

Moser, M. and Leo, O. (2010) Key Concepts in Immunology. *Vaccine* 28 Suppl 3: C2-13.

Nagill, R. and Kaur, S. (2010) Enhanced Efficacy and Immunogenicity of 78kda Antigen Formulated in Various Adjuvants against Murine Visceral Leishmaniasis. *Vaccine* 28: 4002-4012.

Nakamura, T., Yamazaki, D., Yamauchi, J., and Harashima, H. (2013) The Nanoparticulation by Octaarginine-Modified Liposome Improves Alpha-Galactosylceramide-Mediated Antitumor Therapy Via Systemic Administration. *J Control Release* 171: 216-224.

Nishimura, M., Kohara, J., Kuroda, Y., Hiasa, J., Tanaka, S., Muroi, Y. *et al.* (2013) Oligomannose-Coated Liposome-Entrapped Dense Granule Protein 7 Induces Protective Immune Response to Neospora Caninum in Cattle. *Vaccine* 31: 3528-3535.

Nordly, P., Agger, E.M., Andersen, P., Nielsen, H.M., and Foged, C. (2011) Incorporation of the Tlr4 Agonist Monophosphoryl Lipid a into the Bilayer of Dda/Tdb Liposomes: Physico-Chemical Characterization and Induction of Cd8+ T-Cell Responses in Vivo. *Pharm Res* 28: 553-562.

Nordly, P., Madsen, H.B., Nielsen, H.M., and Foged, C. (2009) Status and Future Prospects of Lipid-Based Particulate Delivery Systems as Vaccine Adjuvants and Their Combination with Immunostimulators. *Expert Opin Drug Deliv* 6: 657-672.

North, S. and Butts, C. (2005) Vaccination with Blp25 Liposome Vaccine to Treat Non-Small Cell Lung and Prostate Cancers. *Expert Rev Vaccines* 4: 249-257.

North, S.A., Graham, K., Bodnar, D., and Venner, P. (2006) A Pilot Study of the Liposomal Muc1 Vaccine Blp25 in Prostate Specific Antigen Failures after Radical Prostatectomy. *J Urol* 176: 91-95.

Ogawa, C., Liu, Y.J., and Kobayashi, K.S. (2011) Muramyl Dipeptide and Its Derivatives: Peptide Adjuvant in Immunological Disorders and Cancer Therapy. *Curr Bioact Compd* 7: 180-197.

Ohyanagi, F., Horai, T., Sekine, I., Yamamoto, N., Nakagawa, K., Nishio, M. *et al.* (2011) Safety of Blp25 Liposome Vaccine (L-Blp25) in Japanese Patients with Unresectable Stage Iii Nsclc after Primary Chemoradiotherapy: Preliminary Results from a Phase I/Ii Study. *Jpn J Clin Oncol* 41: 718-722.

Onuigbo, E.B., Okore, V.C., Ofokansi, K.C., Okoye, J.O., Nworu, C.S., Esimone, C.O. *et al.* (2012) Preliminary Evaluation of the Immunoenhancement Potential of Newcastle Disease Vaccine Formulated as a Cationic Liposome. *Avian Pathol* 41: 355-360.

Oussoren, C., Zuidema, J., Crommelin, D.J., and Storm, G. (1997) Lymphatic Uptake and Biodistribution of Liposomes after Subcutaneous Injection. Ii. Influence of Liposomal Size, Lipid Compostion and Lipid Dose. *Biochim Biophys Acta* 1328: 261-272.

Pang, Y., Zhang, Y., Wang, H., Jin, J., Piao, J., Piao, J. *et al.* (2013) Reduction of Salmonella Enteritidis Number after Infections by Immunization of Liposome-Associated Recombinant Sefa. *Avian Dis* 57: 627-633.

Patel, G.B. and Chen, W. (2010) Archaeal Lipid Mucosal Vaccine Adjuvant and Delivery System. *Expert Rev Vaccines* 9: 431-440.

Patel, G.B., Zhou, H., Kuolee, R., and Chen, W. (2004) Archaeosomes as Adjuvants for Combination Vaccines. *J Liposome Res* 14: 191-202.

Peduzzi, E., Westerfeld, N., Zurbriggen, R., Pluschke, G., and Daubenberger, C.A. (2008) Contribution of Influenza Immunity and Virosomal-Formulated Synthetic Peptide to Cellular Immune Responses in a Phase I Subunit Malaria Vaccine Trial. *Clin Immunol* 127: 188-197.

Perche, F., Benvegnu, T., Berchel, M., Lebegue, L., Pichon, C., Jaffres, P.A. *et al.* (2011) Enhancement of Dendritic Cells Transfection in Vivo and of Vaccination against B16f10 Melanoma with Mannosylated Histidylated Lipopolyplexes Loaded with Tumor Antigen Messenger Rna. *Nanomedicine* 7: 445-453.

Perez, O., Romeu, B., Cabrera, O., Gonzalez, E., Batista-Duharte, A., Labrada, A. *et al.* (2013) Adjuvants Are Key Factors for the Development of Future Vaccines: Lessons from the Finlay Adjuvant Platform. *Front Immunol* 4: 407.

Phillips, A.T., Schountz, T., Toth, A.M., Rico, A.B., Jarvis, D.L., Powers, A.M. *et al.* (2014) Liposome-Antigen-Nucleic Acid Complexes Protect Mice from Lethal Challenge with Western and Eastern Equine Encephalitis Viruses. *J Virol* 88: 1771-1780.

Pichon, C. and Midoux, P. (2013) Mannosylated and Histidylated Lpr Technology for Vaccination with Tumor Antigen Mrna. *Methods Mol Biol* 969: 247-274.

Puangpetch, A., Anderson, R., Huang, Y.Y., Sermswan, R.W., Chaicumpa, W., Sirisinha, S. *et al.* (2012) Cationic Liposomes Extend the Immunostimulatory Effect of Cpg Oligodeoxynucleotide against Burkholderia Pseudomallei Infection in Balb/C Mice. *Clin Vaccine Immunol* 19: 675-683.

Ravindran, R., Maji, M., and Ali, N. (2012) Vaccination with Liposomal Leishmanial Antigens Adjuvanted with Monophosphoryl Lipid-Trehalose Dicorynomycolate (Mpl-Tdm) Confers Long-Term Protection against Visceral Leishmaniasis through a Human Administrable Route. *Mol Pharm* 9: 59-70.

Reed, S.G., Orr, M.T., and Fox, C.B. (2013) Key Roles of Adjuvants in Modern Vaccines. *Nat Med* 19: 1597-1608.

Ribeiro, A.M., Souza, A.C., Amaral, A.C., Vasconcelos, N.M., Jeronimo, M.S., Carneiro, F.P. *et al.* (2013) Nanobiotechnological Approaches to Delivery of DNA Vaccine against Fungal Infection. *J Biomed Nanotechnol* 9: 221-230.

Rizwan, S.B., Mcburney, W.T., Young, K., Hanley, T., Boyd, B.J., Rades, T. *et al.* (2013) Cubosomes Containing the Adjuvants Imiquimod and Monophosphoryl Lipid a Stimulate Robust Cellular and Humoral Immune Responses. *J Control Release* 165: 16-21.

Rodriguez, A.E., Zamorano, P., Wilkowsky, S., Torra, F., Ferreri, L., Dominguez, M. *et al.* (2013) Delivery of Recombinant Vaccines against Bovine Herpesvirus Type 1 Gd and Babesia Bovis Msa-2c to Mice Using Liposomes Derived from Egg Yolk Lipids. *Vet J* 196: 550-551.

Roman, V.R., Jensen, K.J., Jensen, S.S., Leo-Hansen, C., Jespersen, S., Da Silva Te, D. *et al.* (2013) Therapeutic Vaccination Using Cationic Liposome-Adjuvanted Hiv Type 1 Peptides Representing Hla-Supertype-Restricted Subdominant T Cell Epitopes: Safety, Immunogenicity, and Feasibility in Guinea-Bissau. *AIDS Res Hum Retroviruses* 29: 1504-1512.

Rosenkrands, I., Vingsbo-Lundberg, C., Bundgaard, T.J., Lindenstrom, T., Enouf, V., Van Der Werf, S. *et al.* (2011) Enhanced Humoral and Cell-Mediated Immune Responses after Immunization with Trivalent Influenza Vaccine Adjuvanted with Cationic Liposomes. *Vaccine* 29: 6283-6291.

Roulois, D., Gregoire, M., and Fonteneau, J.F. (2013) Muc1-Specific Cytotoxic T Lymphocytes in Cancer Therapy: Induction and Challenge. *Biomed Res Int* 2013: 871936.

Saksawad, R., Likitnukul, S., Warachit, B., Hanvivatvong, O., Poovorawan, Y., and Puripokai, P. (2011) Immunogenicity and Safety of a Pediatric Dose Virosomal Hepatitis a Vaccine in Thai Hiv-Infected Children. *Vaccine* 29: 4735-4738.

Sarkar, S., Salyer, A.C., Wall, K.A., and Sucheck, S.J. (2013) Synthesis and Immunological Evaluation of a Muc1 Glycopeptide Incorporated into L-Rhamnose Displaying Liposomes. *Bioconjug Chem* 24: 363-375.

Schwendener, R.A., Ludewig, B., Cerny, A., and Engler, O. (2010) Liposome-Based Vaccines. *Methods Mol Biol* 605: 163-175.

Schweneker, K., Gorka, O., Schweneker, M., Poeck, H., Tschopp, J., Peschel, C. *et al.* (2013) The Mycobacterial Cord Factor Adjuvant Analogue Trehalose-6,6'-Dibehenate (Tdb) Activates the Nlrp3 Inflammasome. *Immunobiology* 218: 664-673.

Senchi, K., Matsunaga, S., Hasegawa, H., Kimura, H., and Ryo, A. (2013) Development of Oligomannose-Coated Liposome-Based Nasal Vaccine against Human Parainfluenza Virus Type 3. *Front Microbiol* 4: 346.

Shargh, V.H., Jaafari, M.R., Khamesipour, A., Jaafari, I., Jalali, S.A., Abbasi, A. *et al.* (2012) Liposomal Sla Co-Incorporated with Po Cpg Odns or Ps Cpg Odns Induce the Same Protection against the Murine Model of Leishmaniasis. *Vaccine* 30: 3957-3964.

Shirota, H. and Klinman, D.M. (2014) Recent Progress Concerning Cpg DNA and Its Use as a Vaccine Adjuvant. *Expert Rev Vaccines* 13: 299-312.

Singha, H., Mallick, A.I., Jana, C., Fatima, N., Owais, M., and Chaudhuri, P. (2011) Co-Immunization with Interlukin-18 Enhances the Protective Efficacy of Liposomes Encapsulated Recombinant Cu-Zn Superoxide Dismutase Protein against Brucella Abortus. *Vaccine* 29: 4720-4727.

Smith, D.M., Simon, J.K., and Baker, J.R., Jr. (2013) Applications of Nanotechnology for Immunology. *Nat Rev Immunol* 13: 592-605.

Spang, A., Martijn, J., Saw, J.H., Lind, A.E., Guy, L., and Ettema, T.J. (2013) Close Encounters of the Third Domain: The Emerging Genomic View of Archaeal Diversity and Evolution. *Archaea* 2013: 202358.

Sprott, G.D., Yeung, A., Dicaire, C.J., Yu, S.H., and Whitfield, D.M. (2012) Synthetic Archaeosome Vaccines Containing Triglycosylarchaeols Can Provide Additive and Long-Lasting Immune Responses That Are Enhanced by Archaetidylserine. *Archaea* 2012: 513231.

Sun, X., Provoda, C., and Lee, K.D. (2010) Enhanced in Vivo Gene Expression Mediated by Listeriolysin O Incorporated Anionic Lpdii: Its Utility in Cytotoxic T Lymphocyte-Inducing DNA Vaccine. *J Control Release* 148: 219-225.

Takagi, A., Kobayashi, N., Taneichi, M., Uchida, T., and Akatsuka, T. (2013) Coupling to the Surface of Liposomes Alters the Immunogenicity of Hepatitis C Virus-Derived Peptides and Confers Sterile Immunity. *Biochem Biophys Res Commun* 430: 183-189.

Tamborrini, M., Stoffel, S.A., Westerfeld, N., Amacker, M., Theisen, M., Zurbriggen, R. *et al.* (2011) Immunogenicity of a Virosomally-Formulated Plasmodium Falciparum Glurp-Msp3 Chimeric Protein-Based Malaria Vaccine Candidate in Comparison to Adjuvanted Formulations. *Malar J* 10: 359.

Taneichi, M., Tanaka, Y., Kakiuchi, T., and Uchida, T. (2010) Liposome-Coupled Peptides Induce Long-Lived Memory Cd8 T Cells without Cd4 T Cells. *PLoS One* 5: e15091.

Thakur, A., Aagaard, C., Stockmarr, A., Andersen, P., and Jungersen, G. (2013) Cell-Mediated and Humoral Immune Responses after Immunization of Calves with a Recombinant Multiantigenic Mycobacterium Avium Subsp. Paratuberculosis Subunit Vaccine at Different Ages. *Clin Vaccine Immunol* 20: 551-558.

Thomann, J.S., Heurtault, B., Weidner, S., Braye, M., Beyrath, J., Fournel, S. *et al.* (2011) Antitumor Activity of Liposomal Erbb2/Her2 Epitope Peptide-Based Vaccine Constructs Incorporating Tlr Agonists and Mannose Receptor Targeting. *Biomaterials* 32: 4574-4583.

Torchilin, V.P. (2005) Recent Advances with Liposomes as Pharmaceutical Carriers. *Nat Rev Drug Discov* 4: 145-160.

Traub, S., Von Aulock, S., Hartung, T., and Hermann, C. (2006) Mdp and Other Muropeptides--Direct and Synergistic Effects on the Immune System. *J Endotoxin Res* 12: 69-85.

Tseng, L.P., Liang, H.J., Deng, M.C., Lee, K.M., Pan, R.N., Yang, J.C. *et al.* (2010) The Influence of Liposomal Adjuvant on Intranasal Vaccination of Chickens against Newcastle Disease. *Vet J* 185: 204-210.

Underwood, C. and Van Eps, A.W. (2012) Nanomedicine and Veterinary Science: The Reality and the Practicality. *Vet J* 193: 12-23.

Van Den Berg, J.H., Oosterhuis, K., Hennink, W.E., Storm, G., Van Der Aa, L.J., Engbersen, J.F. *et al.* (2010) Shielding the Cationic Charge of Nanoparticle-Formulated Dermal DNA Vaccines Is Essential for Antigen Expression and Immunogenicity. *J Control Release* 141: 234-240.

Walczak, M., De Mare, A., Riezebos-Brilman, A., Regts, J., Hoogeboom, B.N., Visser, J.T. *et al.* (2011) Heterologous Prime-Boost Immunizations with a Virosomal and an Alphavirus Replicon Vaccine. *Mol Pharm* 8: 65-77.

Wang, C., Zhuang, Y., Zhang, Y., Luo, Z., Gao, N., Li, P. *et al.* (2012) Toll-Like Receptor 3 Agonist Complexed with Cationic Liposome Augments Vaccine-Elicited Antitumor Immunity by Enhancing Tlr3-Irf3 Signaling and Type I Interferons in Dendritic Cells. *Vaccine* 30: 4790-4799.

Watson, D.S., Endsley, A.N., and Huang, L. (2012) Design Considerations for Liposomal Vaccines: Influence of Formulation Parameters on Antibody and Cell-Mediated Immune Responses to Liposome Associated Antigens. *Vaccine* 30: 2256-2272.

Watson, D.S., Platt, V.M., Cao, L., Venditto, V.J., and Szoka, F.C., Jr. (2011) Antibody Response to Polyhistidine-Tagged Peptide and Protein Antigens Attached to Liposomes Via Lipid-Linked Nitrilotriacetic Acid in Mice. *Clin Vaccine Immunol* 18: 289-297.

Werninghaus, K., Babiak, A., Gross, O., Holscher, C., Dietrich, H., Agger, E.M. *et al.* (2009) Adjuvanticity of a Synthetic Cord Factor Analogue for Subunit Mycobacterium Tuberculosis Vaccination Requires Fcrgamma-Syk-Card9-Dependent Innate Immune Activation. *J Exp Med* 206: 89-97.

Woese, C.R. and Fox, G.E. (1977) Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms. *Proc Natl Acad Sci U S A* 74: 5088-5090.

Wu, Y.L., Park, K., Soo, R.A., Sun, Y., Tyroller, K., Wages, D. *et al.* (2011) Inspire: A Phase Iii Study of the Blp25 Liposome Vaccine (L-Blp25) in Asian Patients with Unresectable Stage Iii Non-Small Cell Lung Cancer. *BMC Cancer* 11: 430.

Yaguchi, K., Ohgitani, T., Noro, T., Kaneshige, T., and Shimizu, Y. (2009) Vaccination of Chickens with Liposomal Inactivated Avian Pathogenic Escherichia Coli (Apec) Vaccine by Eye Drop or Coarse Spray Administration. *Avian Dis* 53: 245-249.

Yanasarn, N., Sloat, B.R., and Cui, Z. (2011) Negatively Charged Liposomes Show Potent Adjuvant Activity When Simply Admixed with Protein Antigens. *Mol Pharm* 8: 1174-1185.

Yu, B., Mao, Y., Bai, L.Y., Herman, S.E., Wang, X., Ramanunni, A. *et al.* (2013) Targeted Nanoparticle Delivery Overcomes Off-Target Immunostimulatory Effects of Oligonucleotides and Improves Therapeutic Efficacy in Chronic Lymphocytic Leukemia. *Blood* 121: 136-147.

Yu, H., Karunakaran, K.P., Jiang, X., Shen, C., Andersen, P., and Brunham, R.C. (2012) Chlamydia Muridarum T Cell Antigens and Adjuvants That Induce Protective Immunity in Mice. *Infect Immun* 80: 1510-1518.

Yu, Y., Wang, D., Abula, S., Hu, Y., Zhao, X., Huang, Y. *et al.* (2013) The Immunological Adjuvant Activity of Gypenosides Liposome against Newcastle Disease Vaccine. *Int J Biol Macromol* 60: 116-121.

Zaman, M., Good, M.F., and Toth, I. (2013) Nanovaccines and Their Mode of Action. *Methods* 60: 226-231.

Zepp, F. (2010) Principles of Vaccine Design-Lessons from Nature. *Vaccine* 28 Suppl 3: C14-24.

Zhao, X., Fan, Y., Wang, D., Hu, Y., Guo, L., Ruan, S. *et al.* (2011) Immunological Adjuvant Efficacy of Glycyrrhetinic Acid Liposome against Newcastle Disease Vaccine. *Vaccine* 29: 9611-9617.

Zhong, Z., Wei, X., Qi, B., Xiao, W., Yang, L., Wei, Y. *et al.* (2010) A Novel Liposomal Vaccine Improves Humoral Immunity and Prevents Tumor Pulmonary Metastasis in Mice. *Int J Pharm* 399: 156-162.

Zhuang, Y., Ma, Y., Wang, C., Hai, L., Yan, C., Zhang, Y. *et al.* (2012) Pegylated Cationic Liposomes Robustly Augment Vaccine-Induced Immune Responses: Role of Lymphatic Trafficking and Biodistribution. *J Control Release* 159: 135-142.

Table 1: Characteristics of Liposome-, Archaeosome-, and Virosome-Vaccines

Vesicle Type	Composition	Characteristics	References
Liposome Neutral or anionic	Neutral and/or anionic lipids (PC, PG, PS, cholesterol) plus immunomodulators (MPLA, CpG, lipopeptides, glycolipids, etc.) and antigens (OVA, plasmids, mRNA, etc.)	Flexible compositions and antigen or adjuvant incorporation (encapsulation, adsorption, covalent surface attachment) T_H1 and cell-mediated immune responses	[Torchilin, 2005, Watson *et al.*, 2012]
Liposome Cationic	Cationic lipids (DDA, DC-chol, DOTAP, etc.) plus neutral phospholipids, cholesterol plus immunomodulators (TDB, MPLA, CAF01, etc.)	Long depot effect at site of injection. Strong electrostatic interactions with APCs and strong T_H1 and T_H17 mediated immunostimulatory effects.	[Christensen *et al.*, 2011, Korsholm *et al.*, 2012]
Archaeosome	Polar glycerolipids from Archaea and other bacteria plus phospholipids, cholesterol and antigens (OVA, plasmids)	Very stable formulations due to ether lipid bilayers. Archaeal glycerolipids are strong adjuvants mediating T_H1 and cellular immune responses without need of TLR agonists.	[Krishnan *et al.*, 2008, Patel *et al.*, 2010]
Virosome	Vesicles reconstituted from virus membranes (Influenza, Semliki Forest, respiratory syncytial virus) and phospholipids. Hemaglutinin (HA), Neuraminidase (NA)	Strong binding to cells and high immunogenicity induced by HA and NA. Human influenza and hepatitis A vaccines (Inflexal, Epaxal)	[Gluck *et al.*, 2005, Herzog *et al.*, 2009, Kamphuis *et al.*, 2013]

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine.

Table 2: Examples of liposomal Veterinary vaccines

Type of liposome	Antigen Vaccine	Adjuvant or Challenge	Animal Species Application	Outcome	Reference
PC/cholesterol	Newcastle Disease vaccine	Gypenoside-saponin (GPS) encapsulated in liposomes	Chickens, oral	Enhanced immune response with GPS-liposomes; T-, B-cell proliferation	[Yu *et al.*, 2013]
PC/cholesterol, sonicated SUV	Newcastle Disease vaccine	Glycyrrhetinic acid (GA) encapsulated in liposomes	Chickens	Enhanced immune response with GA-liposomes; Higher IgG, IgM antibody titers; T-, B-cell proliferation	[Zhao *et al.*, 2011]
DPPC/cholesterol, MLV	Newcastle Disease (La Sota) encapsulated in MLV	Newcastle Disease, Sato strain	Chickens, intranasal	High mucosal anti-NDV IgA, high serum IgG; high HA inhibition and survival rate; macrophage activation via ERK 1/2 and NFκB	[Lin *et al.*, 2011]
DOTAP/cholesterol, catioinic MLV	Newcastle Disease (La Sota) encapsulated in MLV	Newcastle virus (Herts 33)	Chickens, oral	Higher antibody titers, 100% survival	[Onuigbo *et al.*, 2012]
DPPC/cholesterol, DPPS/cholesterol, SA/cholesterol, MLV	Newcastle Disease (La Sota) encapsulated in MLV	Newcastle Disease, Sato strain	Chickens, intranasal	DPPC/Chol-liposomes 320-fold higher HA-inhibition than SA/Chol liposomes; 90% survival with DPPC/Chol vaccine	[Tseng *et al.*, 2010]
PC/cholesterol/ α-Tocopherol SUV	Newcastle Disease vaccine	Epimedium polysaccharide-propolis flavone (EP), EP liposomes; Challenge: NDV $F_{48}E_9$ strain	Chickens, intramuscular	High protection, high T-, B-cell proliferation; low mortality	[Fan *et al.*, 2012]
PC/bovine brain PS/cholesterol, MLV	Non endotoxic LPS core types R1-R4	Non endotoxic LPS core types R1-R4 encaps. in MLV Challenge : virulent *E. coli* strain O78	Chickens, intramuscular	Increased antibody response and lower lesion scores with MLV	[Dissanayake *et al.*, 2010]
PC/cholesterol/DDA Cationic SUV	Inactivated avian pathogenic *E. coli* vaccine (APEC)	Inactivated APEC adsorbed to SUV Challenge: APEC (PDI-386 strain O78)	Chickens, eye drops or coarse spraying	Higher mucosal and serum antibodies, reduced bacteria in blood	[Yaguchi *et al.*, 2009]
Liposome-associated SEF14	*S. enteritidis* fimbrial protein, SEF14	Live *S. enteritidis*	Chickens, oral	High IgG, IgA in intestinal mucus and serum. Low bacterial and low excretion of *S. enteritidis* in feces.	[Pang *et al.*, 2013]
DOTAP/DOPE Cationic MLV	n.a.	Plasmid DNA of microneme *Toxoplasma gondii* protein, MIC3 adsorbed to MLV	Sheep, intramuscular	High IgG2 and IgG1 serum levels, high anti-MIC3 antibodies.	[Hiszczynska-Sawicka *et al.*, 2012]
DSPC/DOPE/DOTAP Cationic SUV	Recombinant Foot-and-Mouth-disease protein vaccine (IgG-FMDV)	Porcine IFN-α DNA adsorbed to SUV Challenge: FMDV isolate HKY 2002	Swine, intramuscular	Strong inflammatory cytokine production and T_H1 response, high IFN-α, no viremia or lesions	[Cheng *et al.*, 2007]
DPPC/cholesterol/ mannotriose-DPPE-liposomes	M3-NcGRA7	Dense granule protein 7 of *Neospora caninum* (M3-NcGRA7) encapsulated in liposomes Challenge: Tachyzoites of *N. caninum*	Cattle, subcutaneous	Decreased parasite load	[Nishimura *et al.*, 2013]
DDA/TDB-liposomes (CAF01)	rec. *M.avium paratuberculosis* proteins	CAF01, rec. *M.avium paratuberculosis* proteins	Calves of different age, subcutaneous	Age dependent IFN-γ response, humoral response age independent	[Thakur *et al.*, 2013]

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidyl glycerol; DPPS, 1,2-dipalmitoyl-phosphatidylserine; DOPE, 1,2-dioleyl-*sn*-glycero-3-phosphatidyl ethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; pegDSPE, pegylated 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; DCPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine.

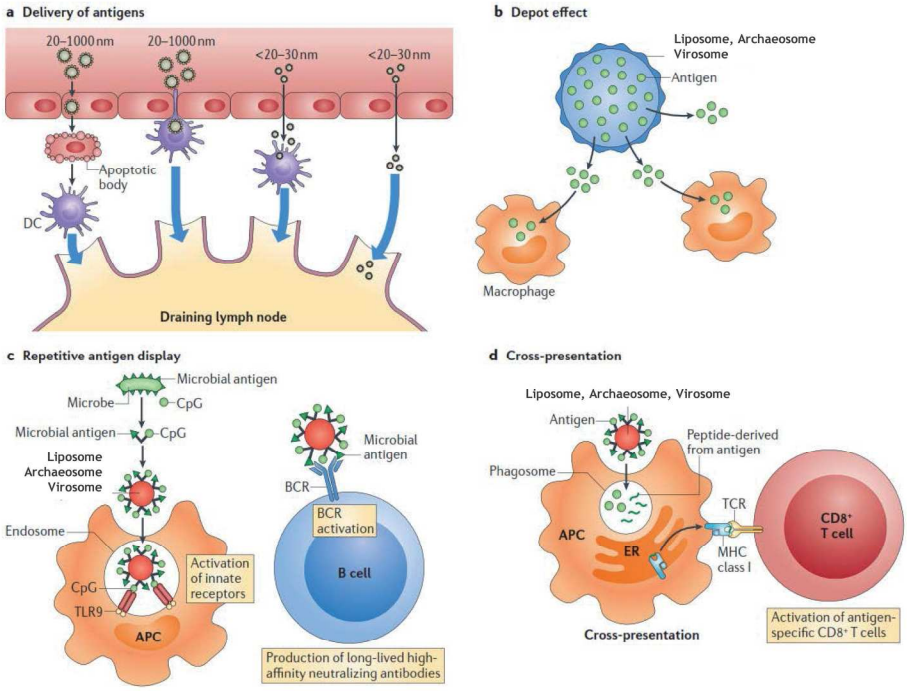
Table 3: Examples of liposomal therapeutic cancer vaccines

Type of liposome	Antigen	Adjuvant/ Treatment	Tumor model	Outcome	Reference
DC-chol/PC/peg-DSPE/ protamine SUV, surface-linked anti-CD20 (rituximab)	Encapsulated Bcl-2 antisense oligonucleotide (G3139)	Bcl-2-targeted antisense G3139 as antisense therapeutic	Selective B cell targeting in Raji B cell lymphoma in NOD-SCID mice	Reduced adverse immuno-stimulatory effects of G3139. 80% survival due to inhibition of TLR9-driven immuno-stimulation and Bcl-2 downregulation	[Yu *et al.*, 2013]
Pegylated PC/cholesterol liposomes with octa-arginine-SA + α-GC	α-galactosyl-ceramide (α-GC), lipid antigen	Activation of NK T cells by α-GC, spleen targeting	B16 melanoma lung metastases	65% inhibition of lung metastases	[Nakamura *et al.*, 2013]
Nanoliposomes	Encapsulated tumor associated antigen ESO-1 + tetanus toxoid helper peptide	Co-administration of palm-IL-1 and MAP-IFN-γ as adjuvants	In vitro targeting to Fcγ receptors on DCs	Highest immune response with nanoliposomes compared to soluble antigens	[Cruz *et al.*, 2013]
DOTAP-PIC-liposome complex (200 -500 nm)	Hepa 1-6 cell lysates	polyriboinosinic: polyribocytidylic acid (poly I:C, PIC)	Hepa 1-6, subcutaneous	High tumor-specific CTL response and IFN-γ levels; significant tumor growth inhibition with DOTAP-PIC + antigen	[Wang *et al.*, 2012]
Cationic TDB/DDA liposomes (CAF01)	Ovalbumin and HP16 E7 protein	Prophylactic and therapeutic vaccinations with CAF01 + poly I:C (= CAF05)	1) Lung B16-OVA 2) TC-1 expressing HP16 E7 protein, s.c.	Significant reduction of tumor growth with CAF05 vaccine by CD8 T cell induced target cell lysis	[Hansen *et al.*, 2012]
PC/PG/cholesterol + $DOG(Man)_2$ liposomes + mannosylated ligands	ErbB2 CTL and influenza virus HA T_H-peptide epitopes	TLR2/1 (Pam_3CAG), TLR2/6 (Pam_2CAG, Pam_2CGD) agonists	Mice bearing RenCa-lacZ/ErbB2 tumors	100% cures after vaccination with mannosylated liposomes + TLR2 ligand Pam_3CAG	[Thomann *et al.*, 2011]
Mannosylated and histidylated lipopolyplexes with MART-1 mRNA (Man(11)-LPR100)	MART-1 (MelanA) mRNA	Immunization with Man(11)-LPR100 followed by challenge with B16F10 cells.	Delivery of mRNA to splenic DCs in vivo and anti-B16F10 melanoma vaccination in mice.	Immunization with Man(11)-LPR100 gave better transfection of DCs and increased survival	[Perche *et al.*, 2011]
PC/cholesterol/octaarginine SUV liposomes with cell wall skeleton extracts of BCG (R8-liposome-BCG-CWS)	*M. bovis* bacillus Calmette-Guerin (BCG) liposome-incorporating cell wall skeleton (BCG-CWS)	Rats treated with BCG-CW or R8-liposome-BCG-CWS intravesically once/week for 8 weeks	Fisher-344 rats with nitrosamine-induced bladder cancer	R8-liposome-BCG-CWS treated rats had significantly lower numbers of tumors	[Miyazaki *et al.*, 2011]
DOTAP/DOPE (SUV) + MPLA and surface-adsorbed bFGF	bFGF as angiogenesis stimulator	Human bFGF plus monophosphoryl lipid A (MPLA)	C57 mice vaccinated with liposomal bFGF and challenged with Lewis lung carcinoma cells (LL-2)	Significant Inhibition of lung metastases in vaccinated mice	[Zhong *et al.*, 2010]
Anti-GD_2-targeted CpG encapsulated in stealth liposomes (TL-CpG)	Neuroblastoma (NB) growth inhibiting CpGs	TLR9 dependent growth inhibition of NB induced by TL-CpG	HTLA-230 neuroblastoma expressing high levels of GD_2 and TLR9	TL-CpG inhibited proliferation of TLR9-expressing NB cells, induced apoptosis and prolonged survival of NB tumor xenografts.	[Brignole *et al.*, 2010]

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; DPPS, 1,2-dipalmitoyl-phosphatidylserine; PG, phosphatidyl glycerol; DOPE, 1,2-dioleyl-*sn*-glycero-3-phosphatidyl ethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; pegDSPE, pegylated 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; DCPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine.

Figure 1. Mechanisms by which nanoparticles alter the induction of immune responses. The immunostimulatory activity of nanocarriers such as liposomes, archaeosomes and virosomes depends on diverse mechanisms: antigen delivery, particle size-dependent tissue penetration and access to the lymphatics (a); depot effects, promoting persistence, stability, conformational integrity and gradual release of vaccine antigens (b); and antigen display facilitating B cell receptor (BCR) co-aggregation, triggering and activation (c). Additional mechanisms include TLR-dependent and TLR-independent signal transduction (not shown); cross-presentation into MHC-I pathways, caused by nanoparticle-mediated leakage of antigens into the cytosol after phagosome uptake (d); and release of cytokines, chemokines and immunomodulatory molecules that regulate the immune response (not shown). APC, antigen presenting cell; DC, dendritic cell; ER, endoplasmic reticulum; TCR, T cell receptor. Reproduced and modified by permission [Smith *et al.*, 2013].

Figure 2. Schematic representation of a small unilamellar liposome showing the versatility of incorporation of various compounds either by encapsulation in the aqueous inner space or by integration in the bilayer or surface-attachment on the lipid bilayer membrane. Reproduced and modified by permission [Heegaard *et al.*, 2011].



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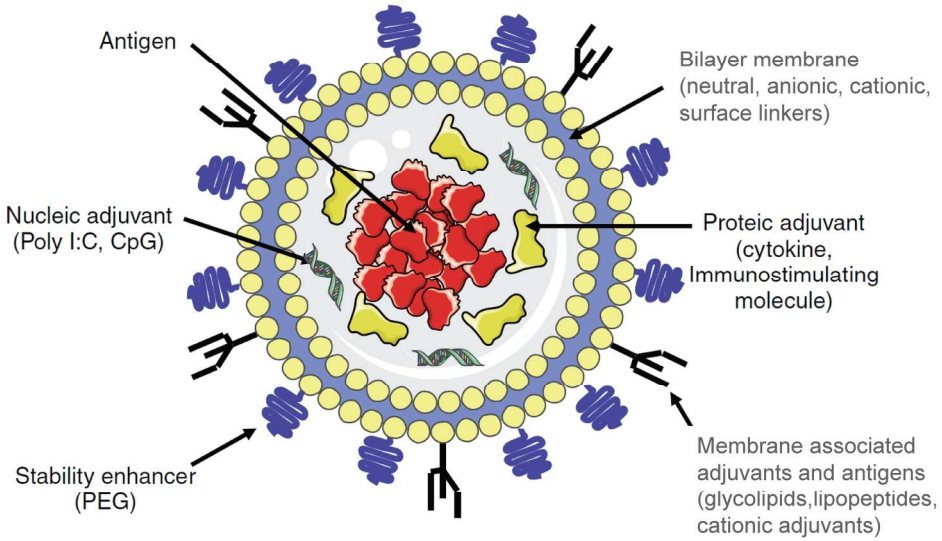

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