Lipid-Based Nanoparticles for Vaccine Applications

Rui Kuai, Lukasz J. Ochyl, Anna Schwendeman and James J. Moon

Abstract Currently available vaccine adjuvants are ineffective against a wide range of infectious pathogens as well as cancers. Therefore, there is a critical demand for new vaccine strategies that can elicit potent cellular and humoral immune responses. Liposomes have been widely examined as vaccine delivery systems because of their safety, low toxicity, and ease of scale-up. However, successful clinical translation of liposomal vaccines has been hampered by their limited potency to induce strong T and B cell responses. In this chapter, we will present two classes of lipid-based nanoparticle systems designed to address limitations of liposomal vaccines and discuss their potential as vaccine delivery systems. The first class of lipid-based nanoparticles presented in this chapter is termed interbilayer-crosslinked multilamellar vesicles. These novel vaccine nanoparticles are stable vehicles that can effectively deliver antigens and adjuvant molecules to antigen-presenting cells in lymphoid tissues and induce robust T and B cell immune responses in vivo. The second class of vaccine nanoparticles is lipoproteins composed of endogenous proteins and lipids. Applications of lipoproteins for vaccine delivery have recently gained much attention due to their safety and multi-faceted functions as endogenous drug delivery vehicles. We

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provide an overview on the latest advances in this rapidly evolving interdisciplinary area of research, and we discuss biomaterial-based innovations enabled by nanotechnology for improving vaccine design and development.

Keywords: Biomaterials · Vaccine · Nanoparticles · Nanotechnology · Lipid · Antigen · Adjuvant

List of Abbreviations

α-GC - alpha-galactosyl ceramide; APCs - antigen-presenting cells; CM – chylomicrons; CpG - oligonucleotides with unmethylated CpG motifs; CTL - cytotoxic CD8+ T lymphocyte; DC - dendritic cell; ER - endoplasmic reticulum; FPLC - fast protein liquid chromatography; GGC - galactosyl(α 1-2)galactosyl ceramide; HCV - Hepatitis C virus; HDL - high density lipoproteins; HIV - human immunodeficiency virus; HPV - human papilloma virus; ICMVs - interbilayer-crosslinked multilamellar vesicles; IFNγ - Interferon gamma; LDL - low density lipoproteins; LDLR - low density lipoprotein receptor; MHC - major histocompatibility complex; MPLA - monophosphoryl lipid A; NiNLPs - nickel-chelating nanolipoprotein particles; NKT - natural killer T cells; NLPs - nanolipoprotein particles; OVA – ovalbumin; PAMPs - pathogen-associated molecular patterns; PBAE - poly(β-amino ester); PEG - polyethylene glycol; PLGA - poly(lactide-co-glycolide) acid; PRRs - pathogen recognition receptors; TLR - Toll-like receptor; VLDL - very low density lipoproteins; WNV - West Nile virus

1 Overview of Nanoparticle Vaccine Delivery Systems

Vaccination is considered the most effective form of medical intervention that humans have ever developed, resulting in unprecedented decrease in healthcare costs, human suffering, and death [1]. While advances in vaccine technology have made significant progress in the past, there are numerous other pathogens, especially for intracellular pathogens such as human immunodeficiency virus (HIV) and Hepatitis C virus (HCV), against which there are no available vaccines. In addition, successful application of current vaccine technologies toward immunotherapy against cancer has been an elusive goal. These major challenges facing the vaccine research field are currently being addressed in many laboratories around the world. Importantly, new exciting discoveries that have advanced our understanding of molecular and cellular interactions between pathogens and our immune system are paving ways for innovative approaches to achieve strong immune activation while avoiding tissue damage and toxicities [1].

This chapter focuses on one class of technology that is revolutionizing the field of vaccine research, namely application of nanotechnology toward vaccine development. Nanotechnology, which has transformed the landscape of drug

development, has recently been the subject of intense investigations and has made significant strides for vaccine applications [2-5]. Nanoparticle-based vaccination strategy offers key distinct advantages over conventional vaccines. Specifically, (1) nanoparticles can effectively protect their cargo from degradation in physiological conditions characterized by high salt and enzyme concentrations. (2) Size and shape of nanoparticles can be precisely tuned to mimic characteristics of pathogens, allowing effective draining through the lymphatic system to lymphoid tissues and subsequent internalization in antigen-presenting cells (APCs). (3) Moreover, particle size and charge strongly affect biodistribution and retention of particles in lymph nodes and spleens, thus offering additional means to modulate vaccine delivery to APCs and promote effector and memory immune responses [6-9]. (4) Surface modification of nanoparticles also provides additional opportunities for enhancing targeted delivery to specific cells by conjugation of receptor ligands or antibodies [10-12]. (5) Another major benefit includes nanoparticle-based co-delivery of antigens and adjuvants targeted to the same APCs, which leads to optimal immune activation and antigen presentation [13-17]. (6) Additionally, cytosolic delivery of antigens within APCs can be achieved by "smart" nanoparticle systems designed to promote endosomal escape, thus allowing for effective antigen cross-presentation and induction of cytotoxic CD8+ T lymphocyte (CTL) responses, which are notoriously difficult to induce via traditional vaccination methods [18-22]. (7) Also, vaccine nanoparticles composed of biocompatible materials with low immunogenicity can minimize induction of anti-vector immune responses [23]. This approach permits multiple administrations of the same formulation without loss of potency, which is a major advantage over viral vectors. (8) Furthermore, their potential as off-the-shelf vaccine nano-formulations offers an alternative solution to labor-intensive and expensive dendritic cell (DC)-based vaccine strategies.

2 Activation of Innate and Adaptive Immunity

The immune system, which has developed to protect the host from a variety of pathogens such as bacteria and viruses, can be divided into two branches: innate and adaptive immunity. Innate immunity plays an important role in eradication and/or containment of infectious pathogens at an early stage and relies on common and evolutionarily preserved elements of viruses and bacteria known as pathogen-associated molecular patterns (PAMPs) for recognition using pathogen recognition receptors (PRRs) [24]. This early innate immune response acts as the first line of defense against infection and provides time for the adaptive immunity to form. Adaptive immune responses are orchestrated by APCs that sample the environment for immunogenic antigens and present them to effector T cells. Complementary to this, B cells directly recognize cognate antigens and secrete antibodies with help from CD4+ helper T cells.

APCs bridge innate and adaptive immunity by capturing pathogens and delivering antigens to the effector T and B cells. Among subtypes of APCs, DCs

are the major class of professional APCs proficient at capturing wide range of antigens and processing them for presentation to effector T cells. DCs function by preferentially recognizing pathogens through the action of PRRs in a similar way to macrophages [25]. However, rather than upregulating phagocytic behavior and trafficking pathogens to lysosomes for degradation as in macrophages, DCs process and conserve antigens and begin their migration to the local lymph node [26]. Once there, antigens are presented on the DC surface along with an array of stimulatory transmembrane proteins in order to activate antigen-specific naïve T cells. In particular, certain DC subsets possess a unique ability for cross-presentation, a process involving presentation of extracellular antigens on major histocompatibility complex class I (MHC-I), strictly necessary for CTL responses [27].

MHC-I is expressed by every cell in the body and in a traditional antigen-presentation pathway functions as a surveillance mechanism for viral and intracellular infections [28]. Cells continuously turn over cytosolic proteins through the action of proteasome, which cleaves them into short amino acid chains. These peptides are then transported into the endoplasmic reticulum (ER) via TAP1/2, where they bind to MHC-I producing unique peptide-MHC-I complexes, which are then trafficked to the plasma membrane, allowing healthy cells to present self-antigens on their surface [29]. Infected cells contain bacterial or viral proteins, which are processed in the exact same way, leading to foreign antigen display on the plasma membrane. These cells are then readily recognized and killed by antigen-specific CTLs.

Normal cells fail to express immunostimulatory molecules such as CD80/86 to activate and expand the antigen-specific CTL clone. Thus, for the traditional antigen-presentation pathway to work, APCs would have to be infected directly, which may be a rare and unreliable event. Because of this, a process of cross-presentation has evolved, where APCs can take up antigens from the extracellular space and present them on MHC-I [27]. This complex and still not fully understood process involves antigen capture and trafficking into endosomes, which can briefly fuse with the ER and extract the necessary machinery for cross-presentation. Proteins can then be degraded directly by endosomal proteases and loaded onto MHC-I, which is known as the vacuolar pathway, or they can be translocated into the cytoplasm with the assistance of Sec61 protein, degraded by the proteasome, and trafficked back via TAP1/2 complex to be loaded onto MHC-I [27].

Cross-presentation often occurs due to naturally occurring cues, such as Toll-like receptor (TLR) signaling [13]. However, cross-presentation is very difficult to induce for subunit antigens as extracellular antigens phagocytosed into APCs are usually trapped in endosomes, eventually leading to degradation of antigens in lysosomes and presentation in the context of MHC-II [30]. Hence, cross-presentation in APCs has been recognized as the major bottleneck for achieving strong CTL responses in response to protein vaccination [30]. Recent studies have shown that nanoparticle-based vaccine delivery systems can address these challenges by disrupting endosomal membrane and thus allowing antigens to enter

the cytosol. One of these approaches involves conjugation of fusogenic or cell-penetrating peptides to the surface of nanoparticles [19]. These peptides are generally composed of positively charged amino acids and can adsorb and disrupt the negatively charged lipid bilayer. When chicken ovalbumin (OVA)-loaded liposomes were modified with octaarganine (R8), this resulted in 5- and 55-fold increase in OVA presentation on MHC-I compared to cationic liposomes and soluble OVA, respectively [19]. Another strategy employed conjugation of pH-dependent fusogenic polymer (linear 3-methylglutarylated poly(glycidol)) to the liposomal surface, which promoted efficient OVA delivery to the cytosol. This strategy led to increased CTL responses, providing protective immunity against challenge with OVA-expressing lymphoma and delaying growth of established tumors in mice [31]. Another complementary approach involves biomimetic incorporation of listeriolysin O, a pathogenic element that induces endosomal lysis in response to acidic endolysosomal pH, leading to increased cytosolic delivery of antigens and efficient induction of antigen cross-presentation [18].

3 Lipid-Based Nanoparticles for Vaccine Delivery

Vaccine nanoparticles can be composed of variety of materials ranging from metals and synthetic polymers to natural polymers and lipid-based systems [5]. Their characteristics can be finely tuned although risks and benefits must be carefully weighed. In particular, lipid-based nanoparticles offer advantages of material biocompatibility, biodegradability, and safety, which are ideal characteristics for a vaccine delivery system [32]. Despite significant progress made over the last three decades with liposomal vaccines [32-37], their clinical development has been challenging. This is partially attributed to the fact that lipid vesicles are prone to degradation and premature release of encapsulated biomacromolecules [38,39]. In this section, we discuss two prime examples of lipid-based nanoparticle systems that are addressing these limitations of conventional lipid-based vehicles and showing early signs of promise in pre-clinical stages of vaccine development.

3.1 Interbilayer-Crosslinked Multilamellar Vesicles (ICMVs)

Toward the goal of producing a stable vaccine delivery vehicle that can promote cross-presentation, a new class of vaccine delivery system, termed ICMV, has been developed [23]. ICMVs are formed by stabilizing multilamellar vesicles with short covalent crosslinks linking lipid headgroups across the opposing faces of adjacent tightly-stacked bilayers within the vesicle walls (Fig. 1a) [23]. As ICMVs have aqueous core surrounded by multilayers of lipid wall, they can serve as a delivery system for co-delivery of both hydrophilic and hydrophobic antigens and adjuvants. In addition, their thick lipid wall renders the particles highly resistant to serum-mediated degradation. Importantly, this feature enhances their in vivo

stability and allows stable delivery of vaccine components to lymphoid tissues, thus generating potent CTL and antibody responses as presented below.

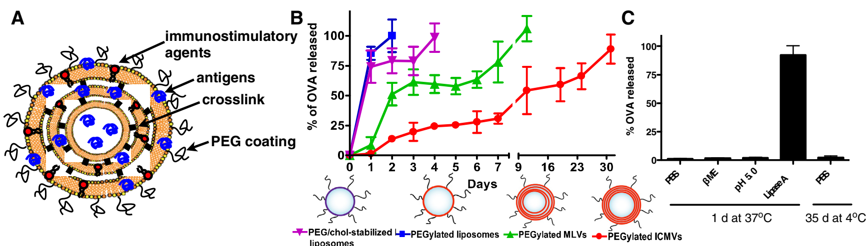

Fig. 1 (A) Schematic illustration of interbilayer-crosslinked multilamellar vesicles (ICMVs) co-loaded with antigens and immunostimulatory agents. (B) ICMVs carrying fluorophore-tagged OVA showed steady release of protein antigen over 30 days at 37ºC in 10% FBS RPMI. In contrast, other lipid vesicles showed rapid release of OVA, thus demonstrating serum-stability of ICMVs. (C) ICMVs were incubated with endolysosomal conditions and examined for OVA release. Lipase A, an enzyme found at high concentration in APCs, was able to degrade the particles within 1 day, showing lipase-sensitivity of ICMVs. Figure modified from [23].

3.1.1 ICMVs for Parenteral Vaccination

ICMV synthesis involves preparation of lipid films consisting of maleimide-functionalized lipids, followed by fusion of liposomes into multilamellar structures and crosslinking of lipid multilayers. Briefly, simple liposomes are formed by hydrating lipid films with an aqueous buffer containing water-soluble antigens and adjuvants. Antigen/adjuvant-loaded liposomes are then fused using divalent cations to form multilamellar structures, which are then crosslinked using dithiol linker covalently binding to maleimide-functionalized lipids on apposing lipid layers within multilamellar vesicles. Notably, crosslinked multilayers of lipid wall significantly increased stability of ICMVs, compared with conventional lipid vesicles, leading to stable and prolonged protein release over 30 days in serum-containing media at 37°C (Fig. 1b) [23]. Remarkably, in the presence of lipase A, a common enzyme found in endolysosomes, ICMVs were rapidly degraded and cargo was released within 24 hours (Fig. 1c). These results suggested that ICMVs would undergo rapid degradation upon cellular internalization, leading to delivery of antigens to cytosol [23]. The final step of ICMV synthesis includes conjugation of polyethylene glycol (PEG) to the particle surface in order to decrease aggregation, prevent deposition *in vivo*, and increase *in vivo* trafficking. Aside from superior stability in physiological conditions, ICMVs exhibit a sustained and prolonged draining profile from the site of the injection and accumulated within the local lymph node for over 14 days after vaccine administration [40].

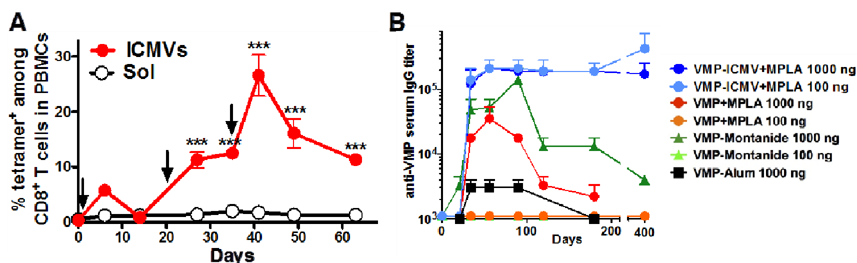

Fig. 2 (A) C57Bl/6 mice were immunized subcutaneously on days 0, 21, and 35 with 10 μg OVA and 0.3 μg MPLA either in ICMVs or soluble form. Assessment of the frequency of OVA-specific T-cells among peripheral blood mononuclear cells indicated strong induction of antigen-specific CTLs in response to ICMV vaccination. (B) C57Bl/6 mice were immunized on days 0 and 21 with vivax malaria sporozoites protein (VMP) in indicated doses formulated in ICMVs or in soluble form admixed with MPLA, Montanide, or Alum. ICMVs elicited long-sustained, high antibody titers against VMP, surpassing those induced by conventional adjuvants. Panel (A) and (B) modified from [23] and [40], respectively.

Subcutaneous vaccination with ICMVs co-loaded with OVA and monophosphoryl lipid A (MPLA, a FDA-approved TLR4 agonist) led to extensive expansion of OVA-specific $CD8^+$ T cells reaching nearly 30% of total CTLs following administration of primary and two booster doses (Fig. 2a) [23]. In addition, CTLs from ICMV-immunized mice expressed significantly higher levels of CD44 and CD62L, indicative of robust memory responses. Finally, ICMV vaccination also promoted high levels of IFNγ secretion by splenocytes after *ex vivo* restimulation [23]. In another study, particles were co-loaded with malaria antigen, VMP001, and MPLA and administered as primary and booster immunizations three weeks apart [40]. This regimen led to high and durable antibody responses lasting over a year with a very low dose of 100 ng of protein and 100 ng of MPLA, with significantly enhanced antibody titers compared with vaccination with currently approved adjuvants, such as Alum and Montanide (Fig. 2b).

3.1.2 ICMVs for Mucosal Vaccination

Intratracheal immunizations with OVA-ICMVs also dramatically expanded OVA-specific $CD8^+$ T cells and reached approximately 12% of systemic CTLs following one booster immunization on day 28 [41]. Formulations were co-loaded with the MPLA adjuvant, but the vaccine was administered along with soluble PolyI:C (TLR3 agonist), which has shown superior responses to MPLA-only particles. These results implicate the possibility of enhancing responses from ICMV-based vaccinations with the use of other adjuvants as well as adjuvant combinations, which need to be explored further in future studies. In response to the pulmonary route of administration, CTLs expressed high levels of integrin $\alpha_4\beta_7$ known as mucosal homing phenotype, and had an effector memory phenotype

capable of rapid killing of target cells in the early stages of the infection [41]. In addition to pulmonary route of vaccine delivery, transcutaneous delivery of ICMVs was recently examined via microneedle-mediated vaccination (Fig. 3a) [42]. Microneedle arrays composed of poly(lactide-co-glycolide) acid (PLGA) polymer were coated with alternating layers of negatively charged ICMVs and a biodegradable cationic poly(β-amino ester) (PBAE) via layer-by-layer assembly. After 5 min of transcutaneous administration, PBAE/ICMV films were rapidly transferred from microneedle surfaces into the tissue, targeting antigen-loaded ICMVs to skin-resident DCs. Microneedle-mediated vaccination with ICMVs elicited humoral immune responses that are characterized by a more balanced Th1/Th2 responses with 10-fold higher antibody titers, compared by bolus delivery of soluble or intradermal injection of ICMVs (Fig. 3b). Overall, these studies provide strong evidence that ICMVs designed to co-deliver protein antigens and adjuvant molecules elicit potent CTL and antibody responses when administered via mucosal surfaces, suggesting that non-invasive route of ICMV delivery may provide an alternative method of vaccine administration to facilitate vaccine deployment without the aid of trained healthcare professionals.

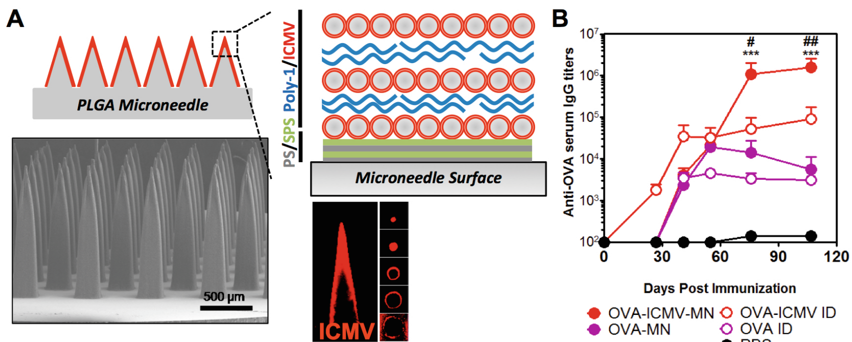

Fig. 3 (A) PLGA microneedle (MN) arrays were coated with ICMVs via layer-by-layer assembly and deposition of ICMVs was confirmed by confocal microscopy. (B) C57Bl/6 mice were immunized on days 0 and 21 with microneedles coated with either OVA-containing ICMVs or soluble OVA. Control groups included OVA-containing ICMVs or soluble OVA given via intradermal injection. Microneedle-mediated transcutaneous vaccination with ICMVs elicited > 10-fold higher anti-OVA serum IgG titers, compared with other groups. Figure reproduced from [42].

In summary, ICMVs are an attractive platform for vaccine delivery due to their potential for efficient co-delivery of hydrophilic and hydrophobic antigens and adjuvants along with their superior stability and persistent draining to local lymph nodes. ICMV vaccination elicits robust effector and effector memory immune responses, characterized by high frequency of CTLs and Th1/Th2 balanced antibody responses, thus highlighting the promise of ICMV-based vaccination strategy against intracellular pathogens and cancers.

3.2 Lipoproteins

Lipoproteins are complexes of lipids and proteins [43]. They play important roles in the transport and metabolism of lipids such as triglycerides, free cholesterol, cholesterol esters and phospholipids [44]. There are four major types of lipoproteins, including chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) [45]. Different types of lipoproteins vary in sizes, lipid, and protein compositions and functions (Table 1) [46].

In addition to the transport of lipids, lipoproteins have also been reported to transport various proteins, hormones, vitamins, and endogenous microRNA and target them to recipient cells, indicating they are multi-functional, endogenous in vivo drug delivery vehicles [47]. Indeed, development of lipoproteins as drug delivery systems aims to utilize their endogenous functions to transport various molecules with the ability to target them to specific cell types. In particular, lipoproteins have major advantages as vaccine delivery systems; (1) lipoproteins are endogenous nanoparticles with minimal safety concerns; (2) lipoproteins have multiple sites for cargo loading, including hydrophobic core and hydrophilic surface that can facilitate delivery of a broad range of molecules; (3) in vivo properties of lipoproteins can be readily tailored by changing their compositions (Table 1), thus offering the capability to effectively modulate their in vivo pharmacokinetic profiles and biodistribution. Recently there has been significant progress in development of lipoproteins as vaccine delivery carriers. In the following section, we will highlight key advances in this field of vaccine research.

Table 1 Compositions of major types of lipoproteins

Lipoprotein	CM	VLDL	LDL	HDL
Density (g/mL)	<0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (nm)	100-1000	30-80	18-28	5-15
Apoprotein	ApoA4, ApoB48	ApoB100, ApoC1, ApoC2, ApoC3, ApoE	ApoB100	ApoA1, ApoA2, ApoAV (5), ApoD, ApoE, ApoM
Total lipids* (wt%)	99	91	80	44
Triacylglycerols	85	55	10	6
Cholesterol esters	3	18	50	40
Cholesterol	2	7	11	7
Phospholpid	8	20	29	46

*The rest are mainly composed of apolipoproteins. Data adapted from [44].

3.2.1 Delivery of Adjuvants by Nanolipoproteins

TLR agonists have been used in pre-clinical settings as immunotherapy for infectious diseases and cancer [48-50]. However, TLR agonists used as monotherapy mediate marginal and short-lived protection, thus requiring frequent administrations of high doses of TLR agonists that often lead to systemic toxicity [51]. To improve the therapeutic efficacy and reduce systemic toxicity, Dina R. et al. used phospholipids and recombinant apolipoproteins (apoE422k) to prepare discoidal nanolipoprotein particles (NLPs) for the delivery of MPLA and CpG (oligonucleotides with unmethylated CpG motifs, a potent TLR9 agonist) [52]. MPLA can naturally be incorporated into NLPs due to its lipophilic nature, while CpG requires modification with lipophilic cholesterol to facilitate particle partitioning. MPLA and CpG were loaded into NLPs at molar ratios of less than 3:1 and 8:1 of TLR agonist to NLP, respectively. By fluorescently labeling apoE422k moiety of NLPs with AF488, the authors have confirmed nanoparticle uptake by mouse primary peritoneal macrophages and J774 cells as well as their localization within lysosomes.

Compared with free CpG and MPLA, CpG:NLPs and MPLA:NLPs increased IL-6, TNF-α, and RANTES cytokines release by both J774 cells and primary peritoneal macrophages in vitro [52]. It is speculated that efficient cellular uptake of NLPs led to trafficking of CpG:NLPs to endosomes where TLR9 is located, thus enhancing TLR9 activation by CpG:NLPs, compared with free CpG. In addition, the authors have reported that empty NLPs without CpG or MPLA also exhibited dose-dependent cytokine release profiles, which might be due to residual endotoxin in NLPs or the intrinsic immunostimulatory properties of structural components of NLPs. The exact mechanisms of immune activation by empty NLPs remain to be investigated.

In vivo administration of CpG:NLPs and MPLA:NLPs also increased levels of serum IL-6, TNF-α, MIP-1α, and RANTES, compared with administration of free CpG and MPLA. At the cellular level, CpG:NLPs and MPLA:NLPs activated macrophages to up-regulate their expression of MHC class II and CD40 as well as DCs to up-regulate co-stimulatory markers, such as CD40, CD80, and CD86.

When CpG molecules in CpG:NLPs were fluorescently labeled by Quasar 670 (CpG-Quasar:NLPs) and intraperitoneally injected into mice, a significant increase in the percent of splenocytes positive for Quasar 670 was observed for CpG-Quasar:NLPs, compared with free CpG-Quasar over 72 h time window post-injection (Fig. 4). These data indicate that complexation of CpG to NLPs can increase their cellular uptake by macrophages and DCs in spleen, leading to up-regulation of activation markers and increased cytokine secretion.

Importantly, CpG:NLPs provided superior protective immunity against infection, compared with free CpG. To demonstrate this, authors treated mice intranasally with 10 μg of CpG:NLPs, CpG, or PBS 24 hours before intranasal infection with a lethal dose of A/PR8/34 strain of influenza. Mice receiving CpG:NLPs survived the challenge until day 14, whereas mice receiving either free CpG or PBS succumbed to viral infection by day 7. These results showed that innate immune responses induced by CpG:NLPs can protect animals against viral infection.

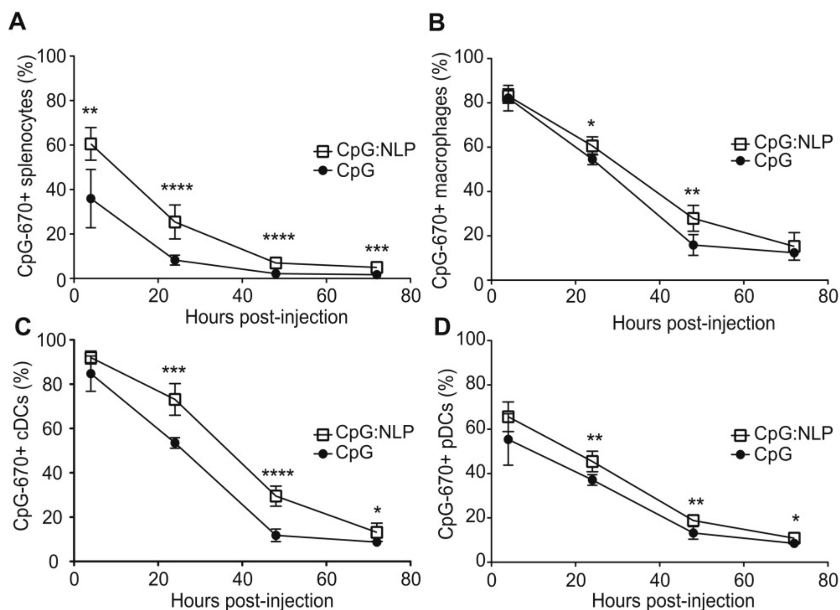

Fig. 4 Uptake of free CpG or CpG:NLP by different cell types at different time points after intraperitoneal injection analyzed by flow cytometry. Conjugation of CpG to NLP promoted the uptake by (A) splenocytes, (B) macrophages (defined as $CD11b^{+}CD11c^{-}$), (C) conventional dendritic cells (cDCs, defined as $CD11c^{+}CD45R^{-}$), and (D) plasmacytoid dendritic cells (pDCs, defined as $CD11c^{+}$ $CD45R^{+}$). CpG:NLP enhanced delivery of CpG to APCs, compared with free CpG. Figure reproduced from [52].

3.2.2 Delivery of Peptide and Protein Antigens by Nanolipoproteins

Peptide and protein antigens are attractive surrogates of live attenuated or inactivated vaccines due to their good safety profiles and relatively simple manufacturing processes. Moreover, each component of peptide and protein antigens can be purified and precisely analyzed, facilitating industrial scale-up and quality control [53] . However, subunit vaccines solely based on peptide and protein antigens typically lack sufficient immunogenicity, thus requiring the use of vaccine adjuvants [54]. Nicholas O. Fischer et al. addressed these issues by applying the same NLP platform technology for delivery of protein antigens [55]. Specifically, phospholipids, recombinant apoE protein, and nickel-chelating lipid 1,2-dioleoyl-sn-glycero-3-(nickel salt) were used to prepare the Nickel-Chelating Nanolipoprotein Particles (NiNLPs). To conjugate West Nile virus (WNV) envelope protein (trE) to NiNLP, trE was tagged by polyhistidine (trE-His) and incubated with NiNLPs for 30 min [56]. Excess, unconjugated antigen was removed, and the presence of surface bound trE-His on NiNLPs was confirmed by SDS-PAGE assay and also by detection of released trE-His upon treatment with EDTA that chelates nickel and disrupts interaction between polyhistidine and nickel containing lipid.

To confirm if conjugation of trE-His to NiNLPs can improve its immunogenicity, 6-week-old mice were immunized once with formulations (free trE-His or NiNLPs-trE-His). Three weeks after vaccination, only NiNLP-trE-His group induced detectable IgG responses to trE whereas free trE-His group failed to achieve sero-conversion. As expected, blank NiNLP did not induce detectable antibodies either, clearly indicating that conjugation of trE-His to NiNLP improves its immunogenicity.

To learn if the vaccination could protect animals from infection, vaccinated animals were challenged with 1000 ffu of live WNV NY99 on week 5 post vaccination. All mice vaccinated with NiNLP-trE-His survived the viral challenge with a minimal weight loss (Fig. 5). In contrast, the majority of animals vaccinated with trE-His, blank NiNLP, or diluent control groups succumbed to the viral challenge. These results provided strong evidence that conjugation of protein antigen to NiNLP could enhance its immunogenicity in vivo and elicit protective immune responses against viral infection.

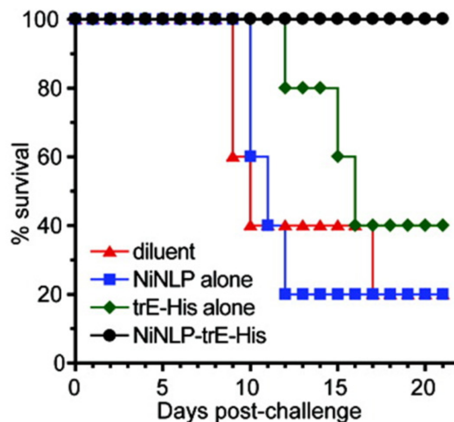

Fig. 5 Mice vaccinated with different formulations (diluent, NiNLP alone, trE-His alone, or NINLP-trE-His) were challenged with 1000 ffu of live WNV NY99 and observed daily for 21 days. Kaplan-Meier survival curves indicate that conjugation of trE-His to NiNLP improved its efficacy and better protects mice from the virus challenge. Figure reproduced from [55].

3.2.3 Co-delivery of Adjuvants and Peptide/Protein Antigens by Lipoproteins

Although conjugation of antigens to nanolipoproteins can improve their immunogenicity, NLPs themselves are not strong adjuvants. Therefore, incorporation of molecular adjuvants together with antigens into nanolipoproteins is a strategy that may further improve immunogenicity of the conjugates. To test this approach, Fischer et al. have used the NPL platform for co-delivery of protein antigens and adjuvants (MPLA or CpG) [58]. Specifically, His-tagged recombinant

viral and bacterial antigens, such as influenza hemagglutinin 5 (H5) and Yersinia pestis LcrV, were conjugated to NiNLPs. Furthermore, MPLA or cholesterol-modified CpG were incorporated into antigen-loaded NiNLPs. Quantitative analysis showed that each nanolipoprotein contained ~21 molecules of protein antigens and 3 ± 1 MPLA or 6 ± 1 CpG molecules [58].

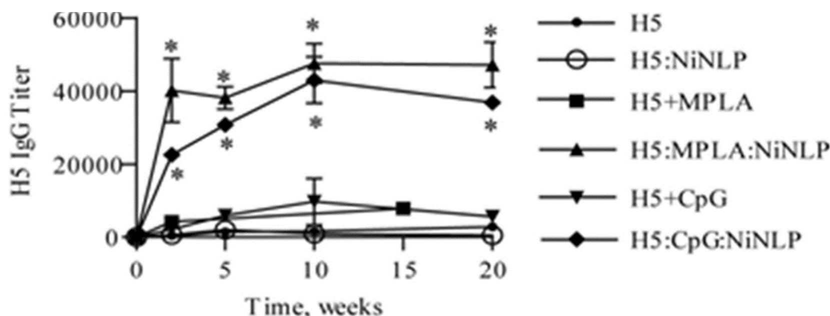

Fig. 6 Mice were vaccinated with different formulations and the kinetics of antibody titers were measured by ELISA at different time points after vaccination. * indicates $p<0.001$ compared to the physical mixture of free proteins and adjuvants. Figure reproduced from [57].

To learn if co-delivery of antigens and adjuvants by NiNLPs can increase antibody production, mice were immunized with various formulations of antigen and adjuvants, including H5, H5+MPLA (physical mixture), H5+CpG (physical mixture), H5:NiNLPs, H5:MPLA:NiNLPs, and H5:CpG:NiNLPs. As expected, H5:MPLA:NiNLPs and H5:CpG:NiNLPs induced 5-10 times higher H5 IgG titer than H5:NiNLPs, H5+MPLA (physical mixture) or H5+CpG (physical mixture) (Fig. 6). Importantly, antibody responses elicited by H5:MPLA:NiNLPs and H5:CpG:NiNLPs were maintained for at least 20 weeks without additional boost immunization. These results indicate that co-delivery of antigens and adjuvants via NiNLPs can dramatically improve immunogenicity of protein antigens and achieve strong humoral immune responses.

3.2.4 Delivery of Lipid Antigens by Lipoproteins

Unlike peptide antigens that are presented in the context of MHC class I or MHC class II complex, lipid antigens such as α-galactosyl ceramide (α-GC) are presented in the context of CD1 to activate natural killer T (NKT) cells [58,59] that can kill target cells such as infected or cancer cells [60]. Exogenous lipid antigens are trafficked to endocytic compartments, where they can bind to CD1, and presented to the surface of APCs as lipid antigen-CD1 complex. Since lipoproteins are major transporters of lipids, they may be natural, endogenous delivery vehicles for lipid antigens. In order to understand the contribution of lipoproteins in the transport of lipid antigens, Peter van den Elzen et al. used a model CD1d-presented glycolipid, galactosyl(α 1-2)galactosyl ceramide (GGC) to

investigate its trafficking patterns in vitro and in vivo [61]. GGC requires uptake and delivery to lysosomes and conversion to the active form α-GC before being presented in the context of CD1d on cell surface.

In order to understand which lipoproteins are involved in GGC trafficking and NKT cell activation, GGC was incubated with human serum in vitro, and various serum fractions, including the VLDL, LDL, and HDL fractions, were separated by fast protein liquid chromatography (FPLC). These fractions were then incubated with DCs to determine if they could activate NKT cells by measuring IFNγ production from NKT cells. Interestingly, only the fraction corresponding to VLDL led to significant NKT cell activation whereas LDL and HDL fractions exhibited little or no NKT cell activation [61] (Fig. 7a,b).

VLDL contains both apolipoprotein B (apoB) and apolipoprotein E (apoE) while LDL and HDL mainly contain apoB and apoA, respectively. Experiments performed with antagonistic antibodies revealed that the presence of antibodies against apoE led to dramatic decrease in NKT cell activation, while the presence of antibodies against apoB and apoA did not significantly affect NKT cell activation (Fig. 7b). Contribution of apoE to NKT cell activation was further confirmed by incubating GGC with apoE-depleted serum, resulting in significantly decreased NKT cell activation (Fig. 7c). NKT activation was restored by adding apoE back to the serum or incubating GGC directly with eluted apoE, further providing the evidence of apoE-mediated delivery of GGC in vitro.

To confirm the importance of apoE in vivo, GGC was intravenously injected into apoE knockout (apoE$^{-/-}$) mice or wild-type (WT) mice and NKT cell activation was analyzed by measuring intracellular IFN-γ production among CD1d-tetramer positive cells. NKT cell activation in apoE$^{-/-}$ mice was dramatically decreased, compared with that in WT mice (Fig. 7d). It is noteworthy that high doses of GGC could still activate NKT cells in apoE$^{-/-}$ mice, suggesting the possibility of alternative pathways other than apoE-mediated delivery of GGC in vivo.

Now that apoE has been confirmed to be the main player in trafficking GGC in vitro and in vivo, the next question is how apoE-bound GGC is recognized by DCs. It is already known that apoE can be taken up into the endocytic system by receptor-mediated endocytosis, which is more efficient than macropinocytosis of free small molecules such as GGC. To confirm this point, DCs were pulsed with either free GGC or apoE-bound GGC and co-cultured with NKT cells. ApoE-bound GGC led to faster and stronger NKT cell activation, compared with free GGC, suggesting that receptor-mediated endocytosis of apoE-bound GGC is more efficient than macropinocytosis of free GGC. ApoE is recognized by several cell-surface receptors, including LDL receptor (LDLR) and LDLR-like protein (LRP). Flow cytometry analysis has confirmed expression of both LDLR and LRP on DCs. Notably, LDLR is significantly up-regulated as monocytes differentiate into DCs, indicating that LDLR may be the main receptor mediating DC uptake of apoE-bound GGC. To confirm this point, apoE3 (the most common human variant with high binding affinity to LDLR) or apoE2 (a variant with low binding affinity to LDLR) was bound to GGC and used to pulse DCs, which were then co-cultured with NKT cells. Compared with apoE3-bound GGC, apoE2-bound GGC only had

40%-47% of the ability to activate NKT cells. In addition, when apoB, the primary component of LDL that can bind to LDLR but not to LRP, was added together with apoE-bound GGC to pulse DCs, NKT cell activation was decreased, compared with control groups. These results clearly showed that LDLR is the main receptor mediating cellular uptake of apoE-bound GGC.

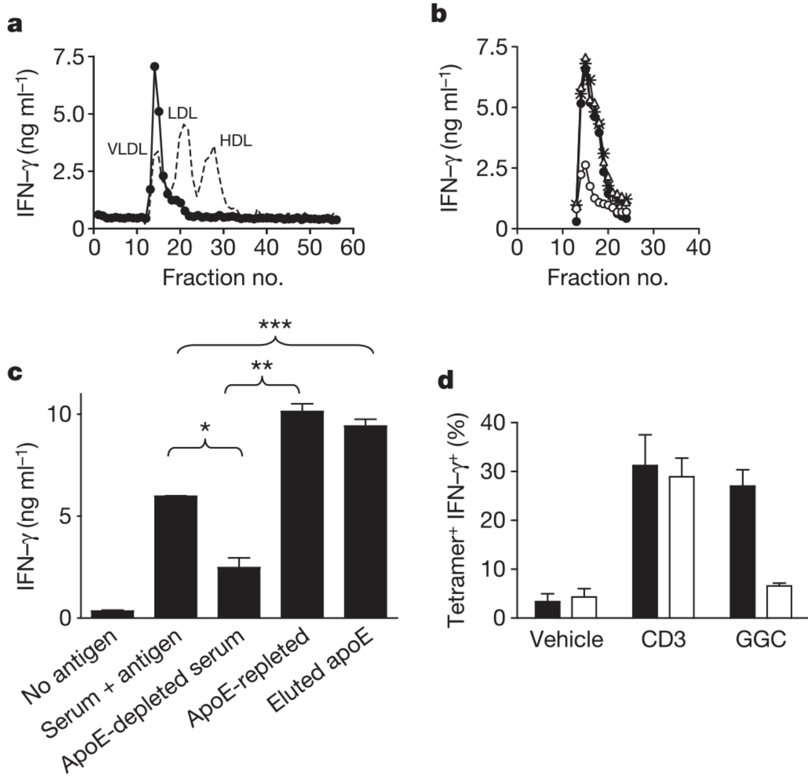

Fig. 7 (A) The lipid antigen GGC was incubated with human serum and different lipoprotein fractions (dashed line) were separated by FPLC. The NKT cell reactivity (solid line) was determined by incubating different lipoprotein fractions with dendritic cells to activate NKT cells. (B) NKT cell reactivity was measured in the absence of anti-ApoE antibodies (filled circles) or presence of anti-apoE (open circles) versus antibodies against apoB (triangles) and apoA-II (asterisks). (C) Incubation of GGC with apoE-depleted serum led to less NKT cell activation, while incubation of GGC with apoE-repleted serum or with eluted apoE directly led to more NKT cell activation. (D) Mice were intravenously injected with GGC, anti-CD3 or vehicle alone. GGC led to less NKT cell activation in $apoE^{-/-}$ mice (open bars) than in wild type mice (filled bars), while CD3 can equally activated NKT cells in $apoE^{-/-}$ mice and wild type mice. Figure reproduced from [61].

To investigate if LDLR is also important for lipid antigen presentation and NKT cell activation in vivo, GGC was intravenously injected into LDLR knockout ($LDLR^{-/-}$) mice or WT mice. NKT cell activation in $LDLR^{-/-}$ mice was

significantly decreased, compared with that in WT mice, while non-specific stimulation with anti-CD3 led to equal activation of NKT cells in LDLR$^{-/-}$ mice and WT mice. These results indicate that LDLR plays a major role in trafficking and presentation of lipid antigens in vivo.

3.3 Other Lipoproteins

In addition to being found in vertebrates and insects, lipoproteins are also found in bacteria [62]. Bacterial lipoproteins are a set of membrane proteins with distinct functions, such as adhesion to host cells, modulation of inflammatory processes, and translocation of virulence factors into host cells. These properties of bacterial lipoproteins may be utilized for development of strong vaccine adjuvants.

Chih-Hsiang Leng et al. used patented *E. coli* strains to produce recombinant lipoproteins (rlipo), which were found to be strong stimulators of TLR2 on DCs [63]. In their study, lipoprotein expressed in *E. coli* was conjugated to recombinant tumor protein antigen E7 mutant (rE7m), a viral antigen found in human papilloma virus (HPV)-induced tumors [64]. To test their immunogenicity, mice were prophylactically vaccinated twice with rlipo-E7m or free rE7m with 1 week interval and challenged with 2×10^5 TC-1 tumor cells expressing E7 antigen on day 7 post-vaccination. Mice that were vaccinated with rlipo-E7m remained free of tumor for 60 days, whereas mice administered with either rE7m alone or PBS developed tumors (Fig. 8a). The vaccines were next studied in a more rigorous therapeutic setting. Mice were inoculated with 2×10^5 TC-1 tumor cells and vaccinated on day 7. Mice vaccinated with rlipo-E7m had tumors less than 0.01 cm^3 on day 40, whereas the rE7m or PBS control group had tumors over 2 cm^3 (Fig. 8b). These results indicated fusion of rE7m to recombinant lipoproteins produced in *E. coli* can dramatically improve immunogenicity and efficacy of subunit vaccines.

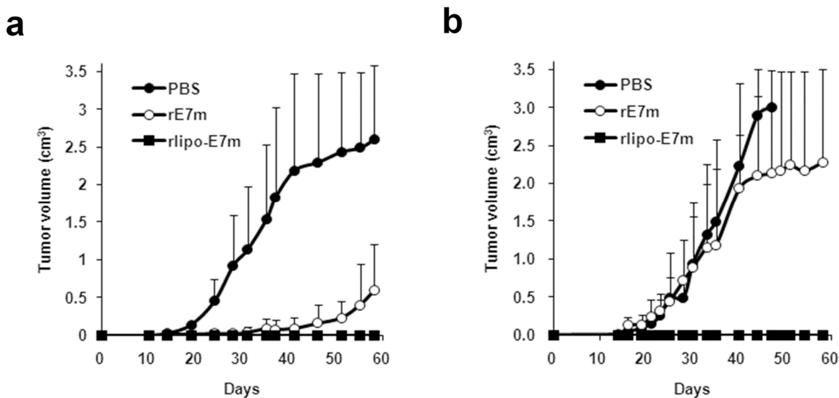

Fig. 8 Prophylactic effect of different formulations: mice were prophylactically vaccinated with different formulations twice with one week interval and inoculated with TC-1 tumor cells 7 days after the last vaccination. (b) Therapeutic effect of different formulations: mice were inoculated with TC-1 tumor cells on day 0 and vaccinated once with different formulations on day 7. Figure reproduced from [64].

4 Conclusions

Lipid-based nanoparticles are promising vaccine delivery systems. As highlighted in this chapter, their inherent biocompatibility, low toxicity and immunogenicity, combined with ease of scale-up and industrial precedence of liposome-based nanomedicines are major attributes that make lipid-based nanoparticles an ideal platform for vaccine applications. With advances in nanomaterial design and our understanding of molecular adjuvants, we can employ a reductionist approach for rational vaccine design and synthesize nanostructures that closely mimic features of pathogens without toxicities associated with them. Overall, nanoparticle-based vaccine strategies that use biocompatible materials to deliver vaccine components, target immune cells, and elicit desired immune responses will have a tremendous impact on the current status of vaccine design and development.

References

1. Pulendran, B., Ahmed, R.: Immunological mechanisms of vaccination. Nat. Immunol. **12**, 509–517 (2011)
2. Swartz, M.A., Hirosue, S., Hubbell, J.A.: Engineering approaches to immunotherapy. Sci. Transl. Med. **4**, 148–149 (2012)
3. Moon, J.J., Huang, B., Irvine, D.J.: Engineering nano- and microparticles to tune immunity. Adv. Mater. **24**, 3724–3746 (2012)
4. Irvine, D.J., Swartz, M.A., Szeto, G.L.: Engineering synthetic vaccines using cues from natural immunity. Nat. Mater. **12**, 978–990 (2013)
5. Sahdev, P., Ochyl, L.J., Moon, J.J.: Biomaterials for nanoparticle vaccine delivery systems. Pharm. Res. **31**, 2563–2582 (2014)
6. Reddy, S.T., van der Vlies, A.J., Simeoni, E., Angeli, V., Randolph, G.J., O'Neil, C.P., Lee, L.K., Swartz, M.A., Hubbell, J.A.: Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat. Biotechnol. **25**, 1159–1164 (2007)
7. Zhuang, Y., Ma, Y., Wang, C., Hai, L., Yan, C., Zhang, Y., Liu, F., Cai, L.: PEGylated cationic liposomes robustly augment vaccine-induced immune responses: Role of lymphatic trafficking and biodistribution. Journal of Controlled Release **159**, 135–142 (2012)
8. Badiee, A., Khamesipour, A., Samiei, A., Soroush, D., Shargh, V.H., Kheiri, M.T., Barkhordari, F., McMaster, W.R., Mahboudi, F., Jaafari, M.R.: The role of liposome size on the type of immune response induced in BALB/c mice against leishmaniasis: rgp63 as a model antigen. Experimental Parasitology **132**, 403–409 (2012)
9. Nakamura, T., Yamazaki, D., Yamauchi, J., Harashima, H.: The nanoparticulation by octaarginine-modified liposome improves alpha-galactosylceramide-mediated antitumor therapy via systemic administration. Journal of Controlled Release **171**, 216–224 (2013)
10. Kwon, Y.J., James, E., Shastri, N., Frechet, J.M.: In vivo targeting of dendritic cells for activation of cellular immunity using vaccine carriers based on pH-responsive microparticles. Proc. Natl. Acad. Sci. U. S. A. **102**, 18264–18268 (2005)

11. Cui, L., Cohen, J.A., Broaders, K.E., Beaudette, T.T., Frechet, J.M.: Mannosylated dextran nanoparticles: a pH-sensitive system engineered for immunomodulation through mannose targeting. Bioconjug. Chem. **22**, 949–957 (2011)
12. Trumpfheller, C., Longhi, M.P., Caskey, M., Idoyaga, J., Bozzacco, L., Keler, T., Schlesinger, S.J., Steinman, R.M.: Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. Journal of Internal Medicine **271**, 183–192 (2012)
13. Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., Kurts, C.: Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat. Immunol. **9**, 558–566 (2008)
14. Demento, S.L., Cui, W., Criscione, J.M., Stern, E., Tulipan, J., Kaech, S.M., Fahmy, T.M.: Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials **33**, 4957–4964 (2012)
15. Moon, J.J., Suh, H., Polhemus, M.E., Ockenhouse, C.F., Yadava, A., Irvine, D.J.: Antigen-displaying lipid-enveloped PLGA nanoparticles as delivery agents for a Plasmodium vivax malaria vaccine. PLoS One **7**, e31472 (2012)
16. Bershteyn, A., Hanson, M.C., Crespo, M.P., Moon, J.J., Li, A.V., Suh, H., Irvine, D.J.: Robust IgG responses to nanograms of antigen using a biomimetic lipid-coated particle vaccine. J. Control. Release **157**, 354–365 (2012)
17. Kamath, A.T., Mastelic, B., Christensen, D., Rochat, A.F., Agger, E.M., Pinschewer, D.D., Andersen, P., Lambert, P.H., Siegrist, C.A.: Synchronization of dendritic cell activation and antigen exposure is required for the induction of Th1/Th17 responses. Journal of Immunology **188**, 4828–4837 (2012)
18. Andrews, C.D., Huh, M.S., Patton, K., Higgins, D., Van Nest, G., Ott, G., Lee, K.D.: Encapsulating immunostimulatory CpG oligonucleotides in listeriolysin O-liposomes promotes a Th1-type response and CTL activity. Molecular Pharmaceutics **9**, 1118–1125 (2012)
19. Nakamura, T., Moriguchi, R., Kogure, K., Shastri, N., Harashima, H.: Efficient MHC class I presentation by controlled intracellular trafficking of antigens in octaarginine-modified liposomes. Molecular Therapy **16**, 1507–1514 (2008)
20. Hu, Y., Litwin, T., Nagaraja, A.R., Kwong, B., Katz, J., Watson, N., Irvine, D.J.: Cytosolic delivery of membrane-impermeable molecules in dendritic cells using pH-responsive core-shell nanoparticles. Nano Lett. **7**, 3056–3064 (2007)
21. Hu, Y., Atukorale, P.U., Lu, J.J., Moon, J.J., Um, S.H., Cho, E.C., Wang, Y., Chen, J., Irvine, D.J.: Cytosolic delivery mediated via electrostatic surface binding of protein, virus, or siRNA cargos to pH-responsive core-shell gel particles. Biomacromolecules **10**, 756–765 (2009)
22. Wilson, J.T., Keller, S., Manganiello, M.J., Cheng, C., Lee, C.C., Opara, C., Convertine, A., Stayton, P.S.: pH-Responsive nanoparticle vaccines for dual-delivery of antigens and immunostimulatory oligonucleotides. ACS Nano **7**, 3912–3925 (2013)
23. Moon, J.J., Suh, H., Bershteyn, A., Stephan, M.T., Liu, H., Huang, B., Sohail, M., Luo, S., Um, S.H., Khant, H., Goodwin, J.T., Ramos, J., Chiu, W., Irvine, D.J.: Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nature Materials **10**, 243–251 (2011)
24. Kumar, H., Kawai, T., Akira, S.: Pathogen Recognition by the Innate Immune System. International Reviews of Immunology **30**, 16–34 (2011)
25. Banchereau, J., Steinman, R.M.: Dendritic cells and the control of immunity. Nature **392**, 245–252 (1998)

26. Palm, N.W., Medzhitov, R.: Pattern recognition receptors and control of adaptive immunity. Immunological Reviews **227**, 221–233 (2009)
27. Joffre, O.P., Segura, E., Savina, A., Amigorena, S.: Cross-presentation by dendritic cells. Nature Reviews Immunology **12**, 557–569 (2012)
28. Barry, M., Bleackley, R.C.: Cytotoxic T lymphocytes: All roads lead to death. Nature Reviews Immunology **2**, 401–409 (2002)
29. Villadangos, J.A., Schnorrer, P.: Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nature Reviews Immunology **7**, 543–555 (2007)
30. Heath, W.R., Belz, G.T., Behrens, G.M., Smith, C.M., Forehan, S.P., Parish, I.A., Davey, G.M., Wilson, N.S., Carbone, F.R., Villadangos, J.A.: Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol. Rev. **199**, 9–26 (2004)
31. Yuba, E., Harada, A., Sakanishi, Y., Watarai, S., Kono, K.: A liposome-based antigen delivery system using pH-sensitive fusogenic polymers for cancer immunotherapy. Biomaterials **34**, 3042–3052 (2013)
32. Torchilin, V.P.: Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. **4**, 145–160 (2005)
33. Gregoriadis, G., Gursel, I., Gursel, M., McCormack, B.: Liposomes as immunological adjuvants and vaccine carriers. Journal of Controlled Release **41**, 49–56 (1996)
34. Vangala, A., Bramwell, V.W., McNeil, S., Christensen, D., Agger, E.M., Perrie, Y.: Comparison of vesicle based antigen delivery systems for delivery of hepatitis B surface antigen. J. Control. Release **119**, 102–110 (2007)
35. Steers, N.J., Peachman, K.K., McClain, S., Alving, C.R., Rao, M.: Liposome-encapsulated HIV-1 Gag p24 containing lipid A induces effector CD4 + T-cells, memory CD8 + T-cells, and pro-inflammatory cytokines. Vaccine **27**, 6939–6949 (2009)
36. Bhowmick, S., Mazumdar, T., Sinha, R., Ali, N.: Comparison of liposome based antigen delivery systems for protection against Leishmania donovani. J. Control. Release **141**, 199–207 (2010)
37. Kim, M., Song, L., Moon, J., Sun, Z.Y., Bershteyn, A., Hanson, M., Cain, D., Goka, S., Kelsoe, G., Wagner, G., Irvine, D., Reinherz, E.L.: Immunogenicity of membrane-bound HIV-1 gp41 MPER segments is dominated by residue accessibility and modulated by stereochemistry. J. Biol. Chem. (2013)
38. Allen, T.M., Mumbengegwi, D.R., Charrois, G.J.: Anti-CD19-targeted liposomal doxorubicin improves the therapeutic efficacy in murine B-cell lymphoma and ameliorates the toxicity of liposomes with varying drug release rates. Clin. Cancer Res. **11**, 3567–3573 (2005)
39. Cashion, M.P., Long, T.E.: Biomimetic Design and Performance of Polymerizable Lipids. Accounts of Chemical Research **42**, 1016–1025 (2009)
40. Moon, J.J., Suh, H., Li, A.V., Ockenhouse, C.F., Yadava, A., Irvine, D.J.: Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand T-fh cells and promote germinal center induction. Proceedings of the National Academy of Sciences of the United States of America **109**, 1080–1085 (2012)
41. Li, A.V., Moon, J.J., Abraham, W., Suh, H.Y., Elkhader, J., Seidman, M.A., Yen, M.M., Im, E.J., Foley, M.H., Barouch, D.H., Irvine, D.J.: Generation of Effector Memory T Cell-Based Mucosal and Systemic Immunity with Pulmonary Nanoparticle Vaccination. Science Translational Medicine **5** (2013)

42. DeMuth, P.C., Moon, J.J., Suh, H., Hammond, P.T., Irvine, D.J.: Releasable layer-by-layer assembly of stabilized lipid nanocapsules on microneedles for enhanced transcutaneous vaccine delivery. ACS Nano **6**, 8041–8051 (2012)
43. Biochemistry of Lipids, Lipoproteins and Membranes, *5th Edition*, pp. 1–640 (2008)
44. Plasma lipoproteins: composition, structure and biochemistry
45. Rensen, P.C.N., de Vrueh, R.L.A., Kuiper, J., Bijsterbosch, M.K., Biessen, E.A.L., van Berkel, T.J.C.: Recombinant lipoproteins: lipoprotein-like lipid particles for drug targeting. Advanced Drug Delivery Reviews **47**, 251–276 (2001)
46. Ng, K.K., Lovell, J.F., Zheng, G.: Lipoprotein-Inspired Nanoparticles for Cancer Theranostics. Accounts of Chemical Research **44**, 1105–1113 (2011)
47. Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D., Remaley, A.T.: MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nature Cell Biology **13**, 423–433 (2011)
48. Krieg, A.M.: Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene **27**, 161–167 (2008)
49. Goldstein, M.J., Varghese, B., Brody, J.D., Rajapaksa, R., Kohrt, H., Czerwinski, D.K., Levy, S., Levy, R.: A CpG-loaded tumor cell vaccine induces antitumor CD4 + T cells that are effective in adoptive therapy for large and established tumors. Blood **117**, 118–127 (2011)
50. Houot, R., Levy, R.: T-cell modulation combined with intratumoral CpG cures lymphoma in a mouse model without the need for chemotherapy. Blood **113**, 3546–3552 (2009)
51. Fischer, N.O., Blanchette, C., Rasley, A.: Enhancing the efficacy of innate immune agonists: could nanolipoprotein particles hold the key? Nanomedicine **9**, 369–372 (2014)
52. Weilhammer, D.R., Blanchette, C.D., Fischer, N.O., Alam, S., Loots, G.G., Corzett, M., Thomas, C., Lychak, C., Dunkle, A.D., Ruitenberg, J.J., Ghanekar, S.A., Sant, A.J., Rasley, A.: The use of nanolipoprotein particles to enhance the immunostimulatory properties of innate immune agonists against lethal influenza challenge. Biomaterials **34**, 10305–10318 (2013)
53. Xu, Z.H., Ramishetti, S., Tseng, Y.C., Guo, S.T., Wang, Y.H., Huang, L.: Multifunctional nanoparticles co-delivering Trp2 peptide and CpG adjuvant induce potent cytotoxic T-lymphocyte response against melanoma and its lung metastasis. Journal of Controlled Release **172**, 259–265 (2013)
54. Aguilar, J.C., Rodriguez, E.G.: Vaccine adjuvants revisited. Vaccine **25**, 3752–3762 (2007)
55. Fischer, N.O., Infante, E., Ishikawa, T., Blanchette, C.A., Bourne, N., Hoeprich, P.D., Mason, P.W.: Conjugation to Nickel-Chelating Nanolipoprotein Particles Increases the Potency and Efficacy of Subunit Vaccines to Prevent West Nile Encephalitis. Bioconjugate Chemistry **21**, 1018–1022 (2010)
56. Blanchette, C.D., Fischer, N.O., Corzett, M., Bench, G., Hoeprich, P.D.: Kinetic Analysis of His-Tagged Protein Binding to Nickel-Chelating Nanolipoprotein Particles. Bioconjugate Chemistry **21**, 1321–1330 (2010)
57. Fischer, N.O., Rasley, A., Corzett, M., Hwang, M.H., Hoeprich, P.D., Blanchette, C.D.: Colocalized Delivery of Adjuvant and Antigen Using Nanolipoprotein Particles Enhances the Immune Response to Recombinant Antigens. Journal of the American Chemical Society **135**, 2044–2047 (2013)
58. Barral, D.C., Brenner, M.B.: CD1 antigen presentation: how it works. Nature Reviews Immunology **7**, 929–941 (2007)

59. Brennan, P.J., Brigl, M., Brenner, M.B.: Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nature Reviews Immunology **13**, 101–117 (2013)
60. Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., Brossay, L.: Targeting natural killer cells and natural killer T cells in cancer. Nature Reviews Immunology **12**, 239–252 (2012)
61. van den Elzen, P., Garg, S., Leon, L., Brigl, M., Leadbetter, E.A., Gumperz, J.E., Dascher, C.C., Cheng, T.Y., Sacks, F.M., Illarionov, P.A., Besra, G.S., Kent, S.C., Moody, D.B., Brenner, M.B.: Apolipoprotein-mediated pathways of lipid antigen presentation. Nature **437**, 906–910 (2005)
62. Kovacs-Simon, A., Titball, R.W., Michell, S.L.: Lipoproteins of Bacterial Pathogens. Infection and Immunity **79**, 548–561 (2011)
63. Chen, H.W., Liu, S.J., Liu, H.H., Kwok, Y., Lin, C.L., Lin, L.H., Chen, M.Y., Tsai, J.P., Chang, L.S., Chiu, F.F., Lai, L.W., Lian, W.C., Yang, C.Y., Hsieh, S.Y., Chong, P., Leng, C.H.: A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design. Vaccine **27**, 1400–1409 (2009)
64. Huang, C.Y., Chen, J.J.W., Shen, K.Y., Chang, L.S., Yeh, Y.C., Chen, I.H., Chong, P., Liu, S.J., Leng, C.H.: Recombinant Lipidated HPV E7 Induces a Th-1-Biased Immune Response and Protective Immunity against Cervical Cancer in a Mouse Model. PLoS One **7** (2012)