# MULTICOMPONENT VACCINE DELIVERY SYSTEMS FOR SUBCELLULAR TARGETING OF ANTIGEN AND MOLECULAR ADJUVANT

**by**

**Chasity Dawn Andrews**

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**Doctoral Committee:**

**Professor Gordon L. Amidon, Co-Chair**
**Professor Kyung-Dall Lee, Co-Chair**
**Associate Professor Gustavo Rosania**
**Assistant Professor Mary X. D. O'Riordan**

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| | |
|---|---|
| APC | Antigen-presenting cells |
| BMM | Bone marrow-derived macrophages |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CFSE | Carboxyfluorescein diacetate succinimidyl ester |
| CpG | Cytosine-phosphate-guanine |
| CTL | Cytotoxic T lymphocytes |
| DMF | Dimethylformamide |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISPOT | Enzyme-linked immunosorbent spot |
| FAC | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| h | Hour(s) |
| HBSE | HEPES buffered saline |
| HRP | Horse radish peroxidase |
| IFN-γ | Interferon gamma |
| IL | Interleukin |
| ISS | Immunostimulatory sequence |
| LLO | Listeriolysin O |
| MeOH | Methanol |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| Min | Minute(s) |
| NMP | N-methyl-2-pyrrolidone |
| NP | Nucleoprotein |
| ODN | Oligodeoxyribonucleotide |
| ON | Overnight |
| OVA | Ovalbumin |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with 0.05% Tween |
| PE | Phosphatidylethanolamine |
| PE-CpG-ODN | Phosphatidylethanolamine-CpG-ODN conjugate |
| PE-non-CpG-ODN | Phosphatidylethanolamine-non-CpG-ODN conjugate |
| PRR | Pattern recognition receptor |
| RBC | Red blood cell |
| RT | Room temperature |
| TBE | Tris-Borate EDTA |
| Th | T helper |
| TLR | Toll-like receptor |

## ABSTRACT

Vaccines are a clinically proven, cost-effective means of improving public health by preventing infectious disease; however, diseases remain that lack efficacious vaccines. The development of a safe and effective multicomponent carrier is a considerable challenge in the field of vaccine delivery systems. Stimulation of cellular immune responses may be particularly desirable in vaccination against cancers or intracellular infections such as viruses. To elicit robust cellular immune responses, protein antigens must be delivered to antigen-presenting cells (APCs) in the context of proper co-stimulation. One approach is to design vaccine delivery systems such that antigens and adjuvant are co-delivered to the same APCs; however, to most effectively stimulate the desired response, the antigen and adjuvant are both delivered to a targeted subcellular location. Our lab has previously demonstrated that co-encapsulating antigen and endosomolytic listeriolysin O (LLO) into pH-sensitive liposomes results in the efficient delivery of antigen to the cytosol of APCs where the antigen can follow the cytosolic pathway of antigen presentation to stimulate cytotoxic T lymphocytes (CTLs). T helper 1 (Th1)-type immune responses may further aid in the removal of cancerous or virally infected cells; therefore, to enhance the CTL response and skew towards the Th1-type, we have incorporated CpG-oligodeoxyribonucleotides (ODNs), which are recognized by Toll-like receptor (TLR9), an endosomal receptor of the innate immune pathway. First, we demonstrate the potential of CpG-ODNs, co-encapsulated in LLO-liposomes, to

enhance the CTL response and to skew a Th1-type response utilizing the model antigen ovalbumin. Next, we show that the cellular immune response can be further enhanced by conjugating CpG-ODNs to the liposome surface as a means of promoting interaction between the CpG-ODNs and TLR9, before LLO ruptures the endosomal membrane to release the antigen into the cytosol. We demonstrate that CpG-ODNs in the lipid conjugate form retain immunostimulatory capabilities after incorporation onto liposome bilayers. Lastly we evaluate both methods (co-encapsulation and lipid-conjugation) of incorporating CpG-ODN in LLO-liposomes with the clinically relevant antigen influenza A nucleoprotein. Taken together, this work indicates the utility of LLO-liposomes with CpG-ODNs, either encapsulated or conjugated, as a vaccine delivery system to stimulate a robust cellular immune response.

# CHAPTER 1

# VACCINE DELIVERY STRATEGIES FOR STIMULATING CELLULAR IMMUNITY

## INTRODUCTION

### Successes and failures of traditional vaccination

The positive effect of vaccination on public health is second only to the introduction of a clean water supply (*1*). Vaccination is a method of exposing the immune system to an antigen such that the response will be accelerated upon re-exposure. Current vaccines prevent up to three million deaths and protect greater than 750,000 children from serious disability each year (*2*). Traditionally, vaccines have consisted of injections of live attenuated pathogens, inactivated whole organisms or inactivated toxins (*3*). The potency of live attenuated pathogen vaccines is linked to the high levels of antigen that result from pathogen replication, as well as and the immune-stimulating molecular components intrinsic to the pathogen. Although proven successful, these vaccines bear risks associated with the potential for the attenuated pathogens to revert to the virulent strain. Also, live attenuated viruses may be shed from those vaccinated, posing potential risks in unvaccinated and immune-compromised individuals. Some of these risks are alleviated by using inactivated whole organism or toxin vaccines; however, these vaccines are typically less effective at providing protection against disease than live attenuated pathogen vaccines and result in reactogenicity, which is an

unwanted immune reaction with vaccine components resulting in local swelling and often severe fever-like symptoms (*4*). Overall, the safety concerns associated with these traditional vaccines compel greater control over vaccine components; therefore, vaccine development is focusing on utilizing defined recombinant or synthetic antigens or subunits in an attempt to reduce unwanted reactions (*4*). Subunit vaccines that use viral proteins as immunogens have led to licensed vaccines against hepatitis B virus, influenza virus, and papillomavirus (*5*). However, this strategy has not obtained universal success as many subunit vaccines have failed to elicit protective immune responses in the host.

**Need for vaccines stimulating cellular immunity**

The efficacy of prophylactic vaccination against viruses and toxin-mediated bacterial diseases is due to the ability of the antibodies that neutralize pathogens with high efficiency; however, antibodies may not be the proper response against many intracellular pathogens and cancerous cells (Figure 1.1) (*6*). In these instances, the immune system primarily relies on the cellular response, including cytotoxic T lymphocytes (CTLs) and T helper (Th) 1 cells to eradiate infected or cancerous cells (Figure 1.2) (*6*). Retrospective studies of many currently marketed vaccines against infections such as hepatitis A virus, hepatitis B virus, tuberculosis, influenza, varicella and typhoid fever maintain that in addition to antibody titer, which is indicated on the licensure for protection, cellular responses also play a role in protection (Table 1.1) (*7*). Many intracellular pathogens without effective vaccines, such as human immunodeficiency virus, hepatitis C virus, tuberculosis and malaria, may require the induction of both arms of the adaptive immune system (humoral and cellular) to protect the host (*8, 9*). Live attenuated pathogen vaccines effectively stimulate both arms of the

adaptive immune system (Table 1.1) (*10*), nevertheless the focus in vaccine development has moved toward protein subunit vaccines for improved safety. Proteins are poorly immunogenic and weak stimulators of the cellular immune response. Delivery systems may be necessary to enhance the cellular immune response against protein subunits; these systems should mimic immunologically desirable qualities of live attenuated pathogens while reducing the potential safety concerns.

## APPLYING IMMUNE RESPONSE AGAINST PATHOGENS TO VACCINATION

### Innate vs. adaptive immune response

The immune system is composed of two interrelated arms: the innate and adaptive responses. Initially, the innate immune system distinguishes between self, infectious (i.e. microbial) non-self, and innocuous (i.e. non-infectious and non-microbial) non-self, via pathogen recognition receptors (PRRs) that recognize unique class-specific pathogen-associated molecular pattern (PAMPS) (*10*). If non-innocuous non-self is detected, the innate immune system reacts quickly, typically within minutes, deploying highly efficient immune effectors to combat pathogens. During infections, this first line of defense is necessary to limit the early proliferation and spread of pathogens due to the relatively slow nature of the adaptive immune system. The innate immune system is effective in its capacity to neutralize pathogens, but the magnitude of the response may be limited by the intolerance of the host to potential immunopathologies that could arise (*10*).

The adaptive immune response, requiring days to weeks, results in an antigen-specific response targeted to the invading pathogen (*11*). The adaptive immune system utilizes randomly generated receptors (on B and T cells) for antigen recognition, but

relies on PRR-induced signals to distinguish the origin of the antigen (i.e. self or non-self) (*10*). The innate immune system determines with high fidelity whether an antigen is derived from self, infectious non-self or innocuous non-self, thereby influencing the magnitude, quality and duration of the adaptive immune response (Figure 1.3) (*10*).

**Innate immunity conditions adaptive immune response**

APCs, especially dendritic cells (DCs), are responsible for linking the innate and adaptive immune response (*12*). Immature, tissue-resident DCs are strategically positioned in anatomical sites to respond rapidly to pathogen invasion; they are highly phagocytic and weakly immunogenic until they encounter non-self antigen in the periphery during which time they mature into weakly phagocytic, highly immunogenic, lymph node-homing cells (*12*). DCs express an array of PRRs, which are part of the innate immune system that allow them to sense non-self.

***Pattern recognition receptors (PRRs)***

Not all PRR stimulate the adaptive immune response equally (*10*). Table 1.2 summarizes the impact of signaling through different transmembrane and cytosolic PRRs on the adaptive immune response. Here, we focus on one type of PRR, Toll-like receptors (TLRs), and how members of this family influence the adaptive immune response.

***Influence of TLR engagement on adaptive immune response***

TLRs are germ-line-encoded PRRs expressed primarily by APCs such as macrophages and DCs (*13*). Thus far, thirteen human TLRs have been identified and TLRs 1-9 are conserved between human and mice (*14*). TLR family members can be

divided into two subpopulations based on their subcellular localization. TLRs 1, 2, 4, 5, 6, and 11 are expressed on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins, while TLRs 3, 7, 8 and 9 are located in the endosomal compartments and primarily recognize microbial nucleic acids (*15*). TLRs are type I transmembrane proteins composed of three major domains: (i) the ectodomain containing leucine-rich repeats that mediates recognition of the PAMP, (ii) the transmembrane domain, and (iii) the intracellular Toll/IL-1R (TIR) domain that is required to initiate downstream signaling pathways (*13*). Activation of APCs through TLRs ultimately results in up-regulation of MHC and co-stimulatory molecules and production of cytokines and chemokines, which influence the activation of adaptive immune cells (B and T cells).

**Adaptive immune response**

***$CD4^+$ T cells: Th1 vs. Th2-type immune response***

Mature DCs present antigenic peptides in the context of MHC class II molecules to $CD4^+$ T cells. PRR-signaling results in the expression of co-stimulatory molecules on APCs for activation of $CD4^+$ T helper (Th) cells and the inflammatory microenvironment that drives the response to either Th1- or Th2-type (Figure 1.2). APCs are induced to secrete interleukin-12 (IL-12) and IFN-γ in response to intracellular bacteria and viral infections, respectively, driving Th1-type responses and promoting cellular immunity (*16*). Conversely, helminths stimulate APCs to secrete IL-4, resulting in a Th2-type immune response that primarily drives antibody production or humoral immunity (*17*). Th cell help is crucial for both priming and generation of CTL activity and long-lasting antibody responses (*18-20*).

### *Cytotoxic T lymphocytes*

Mature DCs can also present antigenic peptides (typically 8-11 residues) in the context of MHC class I molecules to $CD8^+$ T cells. These cells also require that the DCs be stimulated through PRRs to drive the expression of cell surface co-stimulatory molecules for $CD8^+$ T cell activation. Th1 cells secrete IFN-γ that promotes the activation of CTLs (*17*); in the absence of co-stimulation, the $CD8^+$ T cells become anergic or apoptotic (*12*). Activation of naïve $CD8^+$ T cells to generate effector, particularly memory, CTLs is one goal of vaccination; activated CTLs defend the host by lysing infected cells and secreting cytokines that stimulate other cells involved in cellular immunity (*21*).

### *Antibody response*

Humoral immunity is the principal defense mechanism against extracellular microbes and toxins (*16*). B cells produce antibodies that neutralize pathogens, opsonize pathogens to promote phagocytosis and activate complement (*16*). Th2 cells secret IL-4, 5, and 10 to sustain the response (*17*).

## VACCINE ADJUVANTS

Adjuvants were first described in the 1920s by Gaston Ramon as substances that, when administered in combination with an antigen, stimulate a stronger antigen-specific immune response than the antigen alone. Adjuvants have been utilized in vaccine development to enhance immunogenicity, reduce the antigen dose, reduce the total number doses required for protective immunity, enhance the speed and duration of the immune response, reduce the non-responder rate, induce cell-mediated immunity and/or

induce mucosal immunity (*2*). Adjuvants have been classified into two categories: delivery systems and immunopotentiators (*9*). Delivery-based adjuvants enhance the amount of antigen reaching the APCs, while immunopotentiators activate the immune cells by interacting with innate immune receptors. Some examples of delivery systems include aluminum salts (alum), calcium phosphate, liposomes, virosomes, microparticles, emulsions and virus-like particles (*2*). Immunopotentiators include PAMPs recognized by PRRs, for example the TLR agonists such as, lipopolysaccharide, lipopeptides, flagellin, CpG-containing DNA, etc. These methods of classification are, however, elementary. Some delivery systems exhibit immune-potentiating activity, and immunopotentiators are often formulated in delivery systems to enhance the therapeutic effect on APCs allowing for a reduced dose and improved safety.

**Current status of adjuvants in licensed vaccines**

Aluminum salts (alum) and monophosphoryl lipid A (MPL) are adjuvants that are components of currently approved preventative vaccines in the United Sates. In addition to these adjuvants, MF59 and AS03 (oil-in-water emulsions) are also in licensed vaccines in Europe (*22*).

***Aluminum salts (Alum)***

Aluminum salts (aluminum phosphate and aluminum hydroxide; alum) were empirically developed with an emphasis on enhancing antibody responses (*23*). Alum stimulates the immune response by (i) forming a depot at the site of injection, thereby allowing antigen release to persist for a longer time, (ii) promoting phagocytosis by APCs through particulate structure formation, (iii) increasing MHC class II expression, and (iv) activating the inflammasome, a multi-protein complex that is assembled after signaling

via the innate cytosolic NOD-like receptors (NLRs), that stimulates a pro-inflammatory response in monocytes and macrophages resulting in chemokine secretion (*24, 25*). Several licensed human vaccines including diphtheria-pertussis-tetanus (DPT), diphtheria-tetanus (DT), hepatitis A, hepatitis B, *Haemophilus influenzae* type B, and *Streptococcus pneumonia* contain alum (*25, 26*). These vaccines stimulate antibodies that afford protection by neutralizing the tetanus or diphtheria toxin or activating phagocytes and complement for the destruction of pneumococci (*21*). Alum-containing vaccines have been utilized for over six decades, typically demonstrating an acceptable safety profile (*26*). However, because alum increases antibodies, particularly Th2-type, without stimulating a significant T cell response, its utility is limited in vaccines against intracellular viruses and cancer.

***Monophosphoryl lipid A (MPL)***

MPL is a glycolipid derivative of the lipopolysaccharide (LPS) from *Salmonella minnesota* and is the first and currently only TLR ligand in licensed human vaccines. LPS is composed of a hydrophilic polysaccharide portion, an oligosaccharide core, and a hydrophobic lipid moiety (lipid A) (*27*). Lipid A mediates the potent adjuvant activity of LPS, which is too toxic for human use. MPL is derived from lipid A by removing a phosphate and a fatty acid group significantly ablating toxicity but retaining immunostimulatory activity (*27*). AS04 (GlaxoSmithKline), which is a combination of alum and MPL, is a component of the FDA licensed Cervarix® for human papillomavirus (*28, 29*). In Europe, MPL has been licensed with Cervarix, Fendrix® (for hepatitis B virus), as well as for allergy treatment due to its ability to down-modulate Th2-type responses. Thousands of doses of MPL have been administered, and based on the

successful results there is great interest in developing additional TLR4-agonist vaccine adjuvants (*30*).

***Oil-in-water emulsions***

MF59 (Novartis) is a squalene-based oil-in-water emulsion that forms a < 250 nm diameter droplet that increases antibody titers and stimulates a more balanced Th1/Th2 response compared with alum (*31, 32*). It is thought that MF59 promotes antigen uptake by DCs after intramuscular injection (*33*) and stimulates cytokine and chemokine production by monocytes, macrophages, and granulocytes (*34*). Formulating the antigen with MF59 does not change the bio-distribution and clearance from the injection site suggesting that MF59 does not induce depot formation (*35*). MF59 was licensed in Europe in influenza vaccines over ten years ago (*36*) and is being tested with additional vaccine candidates for herpes simplex virus and HIV (*37*).

**Experimental adjuvants**

The current clinical status of human vaccines containing various adjuvants is summarized in Table 1.3. The potent immunogenicity of live attenuated pathogens and inactivated whole pathogens has in part been attributed to the presence of adjuvants endogenous to the pathogen, particularly PAMPs (e.g. LPS, CpG-containing DNA and peptidoglycans) (*15, 38*). Due to the success of traditional vaccines, there is substantial interest in developing TLR agonists as adjuvants to manipulate the innate immune responses and hence produce more effective vaccines. The current clinical status of TLR agonist-based vaccine adjuvants is summarized in Table 1.4; the TLR5 agonist flagellin, and the TLR9 agonist CpG-ODNs, are both undergoing clinical trials (*34*).

## CYTOSINE-PHOSPHATE-GUANINE (CPG)-DNA

### Immune recognition of CpG-DNA as a PAMP

Nucleic acids are present in both eukaryotes and pathogens; therefore, the fidelity with which the innate immune system accurately determines self from innocuous non-self is required to prevent autoimmune disorders. There are three properties that help TLR9 distinguish pathogenic from eukaryotic DNA: (i) endosomal location of TLR9, (ii) suppression of CpG frequency in eukaryotic DNA or the occurrence of eukaryotic CpGs within immunosuppressive flanking sequences, and (iii) CpG methylation status (*39-42*). TLR9 is located in the endocytic compartment where bacteria or viruses are degraded after internalization, thereby releasing their nucleic acids to interact with TLR9 (*41, 43*). A chimeric protein composed of the TLR9 ectodomain and the TLR4 transmembrane and cytoplasmic domains was localized to the cell surface where it recognized mammalian DNA, suggesting that the location of TLR9 in the endocytic pathway minimizes its interaction with self-DNA, which does not readily travel this pathway (*39*).

Unmethylated bacterial DNA stimulates B cell activation *in vitro,* while methylation of the bacterial DNA reduces B cell activation (*41*). These results, in combination with CpG-suppression in vertebrate genomes, led to the paradigm that unmethylated CpG sequences were the non-self signal for the innate immune system. Unmethylated CpG dinucleotides occur at the expected frequency in bacterial and viral genomes, but they are detected at only ~25% of the expected ratio in the vertebrate genomes where the majority of cytosines are methylated and spontaneously deaminate to form thymidine, thereby reducing the frequency of CpG sequences (*40*). Recent studies have confirmed that self-DNA can activate APCs (*44, 45*), but under typical

circumstances suitable concentrations of self-DNA do not reach the endosome to activate APCs.

**TLR9 recognition of CpG-DNA**

Unmethylated CpG motifs from bacteria, viruses and their synthetic analogues are recognized by TLR9 (*39, 46-48*), which is expressed in nearly all APCs in mice and rats, including B cells, dendritic cells (DCs), and the myeloid-derived lineage comprising monocytes, macrophages, and myeloid DCs (mDCs) (*49*). In humans and primates, TLR9 distribution is limited to B cells and plasmacytoid DCs (pDCs) (*50, 51*). TLR9 resides in the endoplasmic reticulum (ER) in resting cells (*52*). Within minutes upon CpG-DNA internalization into the endocytic compartments, TLR9 moves into the early endosomes and later into the tubular lysosomal compartment (*43*). TLR9 apparently does not follow the typical anterograde trafficking from ER through Golgi, as suggested by the lack of resistance to Endo H treatment (*39, 43*). It has been hypothesized that TLR9 instead moves into the endocytic pathway through ER-mediated phagocytosis (*43, 53*). Upon reaching the CpG-DNA-containing compartment, TLR9 binds to the nucleic acids (*43, 54*). FRET studies have demonstrated that changes in the ectodomain of TLR9 homodimers upon binding to stimulatory DNA results in close apposition of the cytoplasmic signaling domains (*55*). This is possibly required for the recruitment of signaling adaptor molecules to initiate the signaling cascade.

**CpG-ODNs as a molecular adjuvant**

Synthetic phosphorothioate ODNs (typically 8 to 30 bases in length) containing unmethylated CpG motifs, often referred to as immunostimulatory sequences (ISS), mimic bacterial DNA in their ability to stimulate an immune response (*41, 56*).

Manipulations of the sequence, composition and structure of the ODN have resulted in three different classes of CpG-ODNs that differ physically, chemically, and biologically in the responses they stimulate. The structural elements and biological activity of these ODNs are summarized in Table 1.5. The primary biological difference in the different classes of CpG-ODNs is determined by activation of B cells and stimulation of IFN-α secretion. Studies have shown that the subcellular location dictates the ability of CpG-ODNs to stimulate IFN-α; when CpG-ODNs are in particle form (A-class CpGs), they are localized to the early endosome, whereas CpG-ODN monomers (B-class CpGs) are localized to the late endosome (*57*). These results are supported by the observation that CpG-ODN monomers stimulate IFN-α secretion by complexing them with polymyxin B (PMXB), a cationic antibiotic with detergent-like properties (*57*). Most published studies using CpG-ODNs as vaccine adjuvants have been performed with B-class CpG-ODNs; the immune activation with B-class CpG-ODNs will be discussed in more detail, as that class was utilized in these studies.

***TLR9 recognition of CpG-ODNs***

Synthetic phosphorothioate ODNs, not bacterial DNA, are used as vaccine adjuvants; therefore, it is necessary to understand their interactions with TLR9. Phosphorothioate ODNs are generated by changing a non-bridging oxygen in the phosphodiester backbone to sulfur, thereby enhancing the resistance of the ODN to nucleases. ODNs synthesized with the phosphorothioate backbone have a strict CpG-sequence requirement for TLR9-dependent activation of APCs; however, this sequence dependence is not observed with phosphodiester ODNs. Non-CpG-containing phosphodiester DNA activates APCs through TLR9, albeit less efficiently than CpG-

containing DNA (*58-60*). Although phosphorothioate non-CpG-ODNs cannot activate APCs, they can bind with TLR9, as shown by a reduction in cell activation when CpG-ODNs are mixed with non-CpG-ODNs that correlates with the dose of non-CpG-ODNs (*43*). Furthermore, surface plasmon resonance studies indicated similar binding between phosphorothioate non-CpG-ODNs and phosphorothioate CpG-ODNs (*54*). By contrast, when comparing phosphodiester ODNs and plasmids, CpG-containing sequences have a higher affinity for TLR9 than sequences lacking the unmethylated CpG motif, which suggests a correlation between binding affinity and activity for phosphodiester backbones (*54, 58, 61*).

***CpG-ODN activation of immune response***

CpG-ODNs activate B cells to proliferate and to express co-stimulatory molecules (CD69, CD80, CD40) as well as MHC class II molecules, and to secrete IL-6 and IL-10. CpG-ODNs inhibit B cell apoptosis resulting in a prolongation of the immune response and enhance IgG class switch. DCs and monocytes/macrophages are activated by CpG-ODNs to express co-stimulatory molecules (CD80, CD86, CD40) as well as MHC class I and II molecules, and to secrete TNF-α, IL-6, and IL-12, and to differentiate into mature APCs with enhanced antigen processing and presentation (*56, 62*). IL-6 contributes to the activation of B and T cells, while IFN-γ stimulates macrophages for enhanced elimination of intracellular and extracellular pathogens, and IL-12 regulates IFN-γ production and contributes to NK cell activation. Ultimately, the Th1-type microenvironment generated by CpG-ODNs stimulate the activation of APCs, development and persistence of CTLs, and production of opsonizing antibodies (*63, 64*).

***Pharmacokinetics (PK) of ODNs***

The PK profiles of ODNs have primarily been obtained through antisense ODN development. When administered intravenously, phosphodiester ODNs have a half-life of approximately 5 min, whereas changing the ODN backbone to phosphorothioate results in a half-life of 30-60 min (*65*). The rapid clearance of the phosphorothioate ODNs from circulation is due to a rapid tissue distribution, with a tissue half-life of at least two days (*66*). Following intravenous and subcutaneous injection, ODNs accumulate primarily in the liver and kidneys, with lower levels spread throughout the body with the exception of the brain (*66*). ODNs are primarily eliminated by 3' exonuclease metabolism. Delivery systems such as liposomes can improve the PK properties of ODNs by protecting encapsulated ODNs from nucleases (*67*). The chemical properties of ODNs prevent them from freely traversing hydrophobic membranes to enter the cell and demonstrate a therapeutic effect; therefore, delivery systems may also promote intracellular uptake.

***Current status of CpG-ODNs in vaccination***

CpG-ODNs have been used in many phase I-III clinical trials to explore their safety, efficacy and immunomodulatory properties (*68*). Dynavax's ISS 1018 CpG-ODN was combined with hepatitis B surface antigen (HBsAg) and given the trade name Heplisav™. Co-administration of CpG-ODN with HBsAg greatly enhanced the levels of hepatitis B antibodies and the rate at which they appeared. In Phase II clinical trials, nearly 80% of Heplisav recipients demonstrated seroprotection after a single immunization compared with 12% of Engerix-B® recipients (GlaxoSmithKline's licensed hepatitis B vaccine adjuvanted with alum), while one week after the second

immunization 100% of the Heplisav-immunized subjects demonstrated protective titers compared with 18% of the subjects receiving Engerix-B (*68*). When Coley's CpG-ODN CPG 7909 was administered with Fluarix® (GlaxoSmithKline) influenza vaccine, naïve patients did not exhibit an increase in antibody response compared with Fluarix alone; however, in patients with pre-existing anti-influenza antibodies, a significant enhancement in anti-hemagglutinin inhibition titer was observed when Fluarix was administered with CpG-ODN (*69*). This study also looked at cellular responses and found that when administering CpG-ODN with one-tenth of the standard Fluarix dose, the antigen-specific IFN-γ responses from peripheral blood mononuclear cells (PBMCs) could be restored to those of the normal Fluarix dose, indicating the dose-sparing capabilities of CpG-ODN.

In association with the clinical trials, good laboratory practice-compliant toxicity studies were performed in rodents and non-human primates and indicated no clinically significant toxicities when administering CpG-ODN doses up to 12.5 mg/kg (*68*). Over ten years, five thousand doses of Heplisav have been administered to 2,500 patients. One patient in a Phase III clinical trial developed Wegener's granulomatosis, an autoimmune disease characterized by inflammation of the vasculature, resulting in a temporary clinical hold on the trial (*70*). The more typical adverse events were limited to injection site reactions or short-lived flu-like symptoms that did not interfere with daily life and the frequency of adverse events were not changed between vaccine alone or CpG-ODN-adjuvanted vaccine (*68, 69*).

## ANTIGEN DELIVERY TARGETING THE ENDOGENOUS MHC CLASS I PATHWAY

CTL responses have been shown to be important mediators of anti-viral and anti-tumor immunity. While live attenuated pathogens stimulate a strong CTL response, most exogenous soluble protein antigens are weakly immunogenic and are typically degraded in the endosome to be loaded onto MHC class II molecules for presentation to $CD4^+$ T cells. Proteins do not readily access the cytosol to follow the endogenous pathway for protein processing and $CD8^+$ T cell activation. Alternative strategies for getting antigens into the cytosolic MHC class I pathway for antigen processing and presentation involve exploiting the classical endogenous pathway or directing delivery of antigen to the cytosol of APCs.

### Exploiting the classical endogenous pathway

Viral or non-viral vector DNA vaccines require that the APCs synthesize the protein so that it can then travel through the endogenous pathway to stimulate CTLs. Currently, there are many replication-defective viral-vector vaccines (such as adenovirus (AdV), modified vaccinia Ankara (MVA), and adeno-associated virus (AAV)) in development for human use due to their ability to stimulate a strong cellular immune response (*71*). Viral vectors are in the middle of the spectrum of previously approved vaccines regarding complexity, and their use may be limited by pre-existing immunity to the vectors (*71, 72*), whereas non-viral vectors may be limited by low transfection efficiency.

**Cytosolic delivery of antigens**

In order to bypass the need for the cell to make the protein, whole protein antigen can be delivered to the cytosol of APCs using delivery systems. DCs present antigens with equal efficiency regardless of whether the antigens were from live or inactivated viruses (*73, 74*). Once the antigens are in the cytosol, they can be processed through the pathway for endogenous antigen processing and presentation for CTL stimulation.

***pH-sensitive liposomes***

Liposomes are vesicles composed of lipid bilayers that are separated by an aqueous space, which can be loaded with water-soluble compounds, including antigenic proteins. The properties of the liposomes are dictated by the lipid formulation; egg phosphatidylethanolamine (PE) and cholesteryl hemisuccinate (CHEMS) may be used to form pH-sensitive liposomes. CHEMS acts as the pH sensor; at neutral pH and above, the hydroxyl group is deprotonated resulting in a negative charge that stabilizes the bilayer (*75*). However, at acidic pH the hydroxyl group is protonated and CHEMS no longer stabilizes the membrane resulting in the PE molecules forming an inverted hexagonal structure (*75*). The destabilized liposomes deliver proteins to the endosomes and some material leaks into the cytosol (*76*).

A similar approach to pH-sensitive liposomes is virosomes, which have been licensed in Europe for use with a hepatitis A vaccine. Influenza-derived virosomes lack viral genetic material, are ~150 nm in diameter, and possess the surface hemagglutinin and neuraminidase; thus the receptor-binding and membrane fusion activity of the virus is retained (*77*). Protein antigens may be encapsulated in the virosome lumen. Virosomes stimulate Th cells, and the fusion activity of the virosomes delivers encapsulated antigen

to the cytosol of APCs to stimulate CTL responses. The virosomal hepatitis A vaccine exhibited similar immunogenicity to that of the hepatitis A alum-adsorbed vaccine; however, fewer local side effects were observed with the virosome-based vaccine (*78*).

***Bacterial toxins for delivery of MHC class I antigens***

Bacteria produce protein toxins that must enter the cytosol to access their intracellular targets to be effective (summarized in Table 1.6). The toxins primarily use two pathways access to the cytosol: (i) they can be transferred from the endosome in response to low pH or (ii) they can enter the Golgi and then the ER to be transferred to the cytosol. These toxins have been used with a variety of antigens to carry $CD8^+$ T cell epitopes into the cytosol, and their success is monitored by the induction of CTL responses (*79*). These bacteria have developed schemes to deliver toxins into the cytosol to kill the cells; conversely, *Listeria monocytogenes* (*Lm*) secretes the toxin listeriolysin O (LLO), which allows *Lm's* escape from the endosome to replicate within the cytosol.

***Listeriolysin O (LLO)***

LLO is the pore-forming protein from the Gram-positive facultative intracellular bacteria, *Lm*. Once *Lm* is phagocytosed, it secrets LLO into the endosomal compartment where LLO exhibits optimal activity at pH ~5.5 allowing *Lm* to breach the endosomal membrane (*80*). LLO binds cholesterol in the endosomal membrane whereupon it oligomerizes to form pores, thus permitting escape of the bacteria into the cytosol (*80*). Once in the cytosol, the relative activity of LLO is diminished at neutral pH, and LLO is targeted for degradation (*81-83*). The mechanism adapted by *Lm* to escape the endocytic compartment can be used to deliver macromolecules, such as antigens, to the cytosol of

cells. LLO has been engineered into vectors such as *Bacillus subtilis* and *E. coli* to deliver antigens into the cytosolic space to stimulate CTL responses *(84-86)*.

LLO can also be co-encapsulated with antigens in pH-sensitive liposomes to enhance the efficiency of cytosolic delivery (*76, 87, 88*). pH-sensitive liposomes are destabilized in the endocytic compartment, thus releasing LLO and antigen. LLO forms pores in the endosomal membrane, releasing the antigen into the cytosol, and this system has demonstrated an enhanced antigen-specific CTL response compared to pH-sensitive liposomes without LLO (*87, 88*).

**STRATEGIES FOR CO-DELIVERING ANTIGEN AND CPG-ODN**

Delivery systems for CpG-ODNs could be utilized to prevent enzymatic degradation of CpG-ODN, target CpG-ODN to the endocytic pathway, and co-deliver antigen and adjuvant to the same APC. The susceptibility of ODN to enzymatic degradation and poor uptake was previously discussed. Co-administering CpG-ODN with hepatitis B surface antigen to the same site in the muscle significantly enhanced the antibody response; however, administering CpG-ODN followed by a separate administration of the antigen resulted in no enhancement in the immune response (*89*). These results illustrate the need for co-delivery of the antigen and CpG-ODN. The importance of co-delivering antigen and adjuvant to the same APC has been demonstrated using various methods of co-delivery and numerous antigen and adjuvant combinations (summarized by (*90*)).

**Antigen and CpG-ODN conjugates**

Conjugation of the antigen and CpG-ODN via the biotin-avidin interaction or sulfhydryl-maleimide reaction has been widely utilized to promote co-delivery. When OVA is conjugated to phosphorothioate CpG-ODNs, the OVA-CpG-ODN conjugates are taken up by DNA receptor mediated endocytosis, as opposed to the less efficient fluid phase pinocytosis used for OVA antigen (*91*). OVA-CpG-ODN conjugates more efficiently trigger an *in vivo* antigen-specific CTL response compared with solution mixed OVA and CpG-ODN, indicating the potential for reducing the dose of CpG-ODN to achieve a therapeutic effect (*92-94*).

Antigen-CpG-ODN conjugates stimulate a potent Th1-biased immune response and CTLs, although the conjugates do not have a mechanism to deliver the antigen to the cytosolic pathway of antigen processing and presentation. It is believed that the antigen-CpG-ODN conjugates stimulate cross-presentation, which is the ability of APCs to present peptides from exogenous proteins in the MHC class I molecules to stimulate CTLs. Although the exact mechanism of cross-presentation has not been elucidated, the DC maturation signal, provided here by CpG-ODN, is a requirement for cross-presentation. Because the antigen-CpG-ODN conjugates are capable of stimulating a potent CTL response, they have been regarded as on a par with "live" vaccines, often considered the gold standard in vaccination. When OVA-CpG-ODN conjugates were compared with *Lm* genetically engineered to produce OVA, the results showed similar levels of OVA-specific memory CTLs between groups (*95*). Although great success has been achieved with antigen-CpG-ODN conjugates using certain antigens, the procedures for chemically linking the CpG-ODN with the proteinaceous antigen may be inefficient or may alter the immunological characteristics of other antigens (*92*).

**Liposomes**

To date, five liposomal drugs have been licensed by the US FDA (*96, 97*). In the setting of CpG-ODNs, liposome systems have the potential to improve stability, enhance delivery to and uptake by APCs, and reduce toxicity of ODNs while offering a method of co-delivery (*98*). Encapsulating either CpG-ODN or antigen into liposomes and administering a mixed population of liposomes enhanced the antigen-specific IFN-γ secreting cells compared with liposomal antigen administered with free unencapsulated CpG-ODN (*99*). This effect is potentially due to a combination of increased uptake of CpG-ODNs, increased payload per uptake, directing CpG-ODNs to endosomal compartments of APCs using liposomes, and protection of CpG-ODNs from enzymatic degradation. As expected, the antigen-specific IFN-γ secretion is further enhanced when antigen and CpG-ODN are co-encapsulated in the same liposomes (*99*), demonstrating an efficient method of co-delivering the antigen and adjuvant to the same APC. Liposomal delivery of antigens and CpG-ODNs has successfully resulted in a Th1-type response and enhanced CTL response for a number of antigens, including but not limited to, hepatitis B, HER-2/neu, OVA and tumor associated-antigens (*99-102*). Most of these liposomal systems rely on cross-presentation or leakage of endosomal contents into the cytosol to stimulate CTL responses. We describe a delivery system in which we utilize LLO-containing pH-sensitive liposomes to deliver antigen into the cytosol of APCs to enhance CTL responses while co-delivering CpG-ODNs.

**CONCLUSIONS**

Developing new vaccines is a considerable challenge in terms of balancing safety and immunogenicity. There are many combinations for adjuvants and delivery systems

that are currently being investigated. An optimal system will have a tolerated safety profile while generating the appropriate type of long-lived immunity. We take a minimalist approach by adding only components to the vaccine delivery system that have well-defined mechanisms of action for stimulating strong CTL and Th1-type responses.

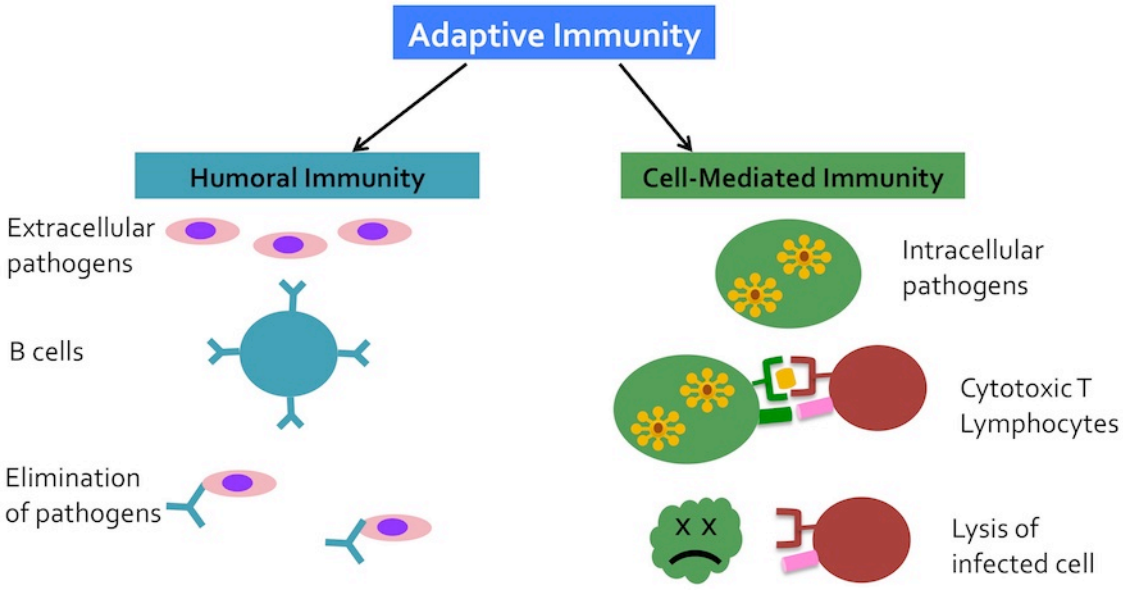


**Figure 1.1: Successful vaccination stimulates the proper arm of the adaptive immune response.**

The adaptive immune response is divided into humoral and cell-mediated immunity. Each arm of the immune response is suited for removing pathogens with different characteristics.

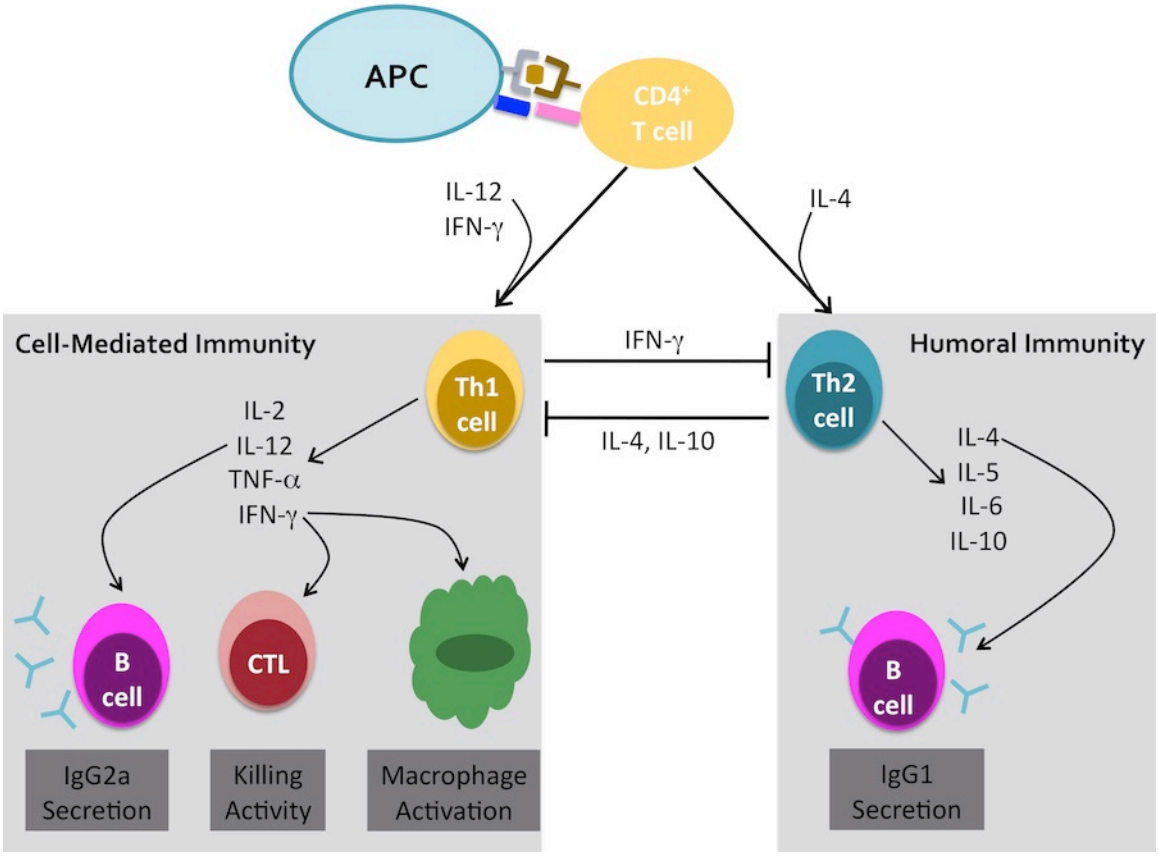


**Figure 1.2: Th1 vs. Th2 immune stimulation.**

Antigen-presenting cells (APCs) recognize pathogen-associated molecular patterns (PAMPs) resulting in the secretion of cytokines. APCs present antigen on MHC class II molecules to $CD4^+$ T cells in context of these cytokines (IL-12, IFN-γ or IL-4) driving the differentiation of $CD4^+$ T cells to Th1 or Th2-type cells. Th1-type cells then secrete cytokines that (i) drive the differentiation of B cells to secrete IgG2a, an opsonizing antibody targeting the pathogen for uptake by a phagocyte, (ii) enhance the activation of cytotoxic T lymphocytes (CTLs), resulting in the lysis of infected cells, and (iii) activate macrophages to remove pathogens. Alternatively, Th2-type cells secrete cytokines that drive B cell differentiation to secrete IgG1, a neutralizing antibody.

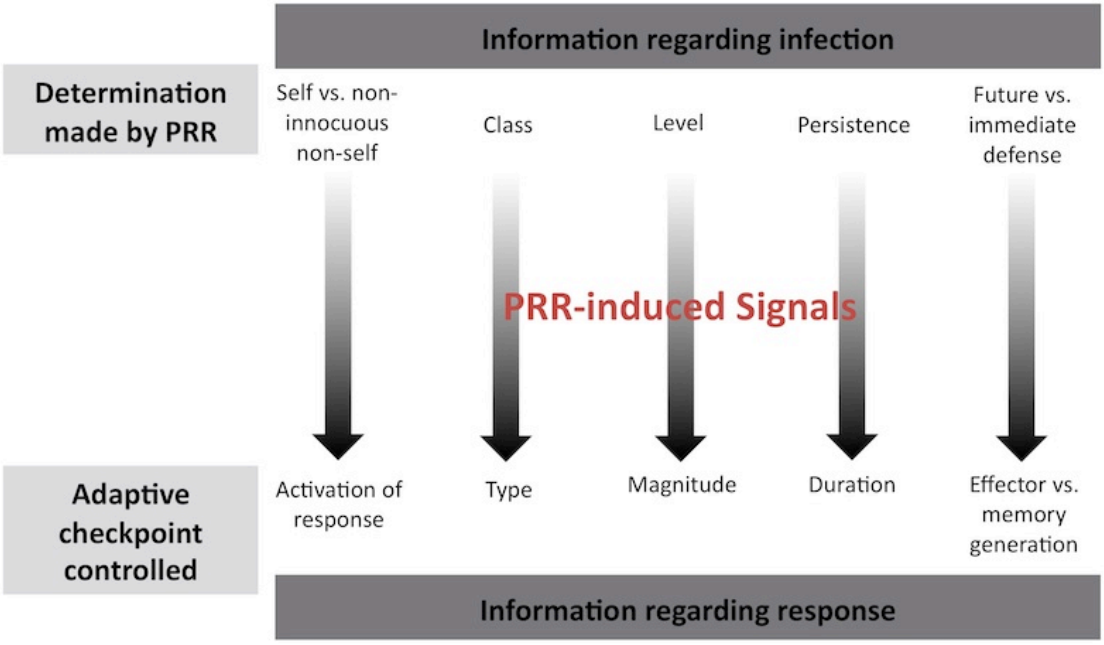


**Figure 1.3: Pattern recognition receptor (PRR)-mediated control of adaptive immunity checkpoints (adapted from (*10*)).**

Once PRRs detect the presence and characteristics of non-innocuous non-self, signals are sent to induce a productive adaptive immune response.

| Vaccine | Preparation | Year licensed | Surrogate marker of protection | |
|---|---|---|---|---|
| | | | On licensure | Based on recent studies |
| ***Preparation: Live attenuated*** | | | | |
| Bacillus Calmette-Guérin (BCG) | Live attenuated | 1950 | Ab titer | Ab titers and CMI |
| Poliovirus (OPV) | | 1962 | Ab titer | NA |
| Measles | | 1971 | Ab titer | Ab titers and CMI |
| Mumps | | 1971 | Ab titer | NA |
| Rubella | | 1971 | Ab titer | NA |
| Typhoid (Ty21a) | | 1990 | Ab titer | Ab titers and CMI |
| Influenza | | 1993 | Ab titer | Ab titers and CMI |
| Varicella | | 1995 | Ab titer | Ab titer for acute phase; CMI for chronic phase |
| Hepatitis A | | 1996 | Ab titer | Ab titer for acute phase; CMI for chronic phase |
| ***Preparation: Inactivated organism/toxin*** | | | | |
| Poliovirus (IPV) | Killed/inactivated | 1955 | Ab titer | NA |
| Diphtheria | Toxoid | 1991 | Ab titer | NA |
| Tetanus | Toxoid | 1991 | Ab titer | NA |
| Pertussis | Acellular | 1991 | Ab titer | NA |
| ***Preparation: Recombinant antigen*** | | | | |
| Hepatitis B | Recombinant antigen | 1981 | Ab titer | |
| Papillomavirus | | 2006 | Ab titer | |
| ***Preparation: Other*** | | | | |
| Meningococcal | Polysaccharide | 1981 | Ab titer | NA |
| Pneumococcal | Polysaccharide protein conjugate | 1983 | Ab titer | NA |
| Hepatitis B | Recombinant DNA vector | 1986 | Ab titer | Ab titer for acute phase; CMI for chronic phase |
| *Haemophilus influenzae* type b | Hib-conjugated polysaccharide | 1987 | Ab titer | NA |

Ab, antibody; CMI, cell-mediated immunity; IPV, inactivated poliovirus vaccine; NA, not available; OPV, oral, attenuated poliovirus vaccine.

**Table 1.1: Surrogate markers of protection for currently available vaccines (adapted from (*7*)).**

To date in the US, all of the vaccines are licensed based on the protection elicited by an antibody response. Additional studies have indicated that cell-mediated immunity (CMI) also plays a role in protection for some vaccines. Vaccines prepared from live attenuated pathogens stimulate CMI. In the case of vaccines generated from recombinant antigens, CMI may be important for controlling the disease, but is not stimulated by this type of vaccine (indicated by the shaded box).

| PRR | Ligands recognized | Microbes recognized | Adaptive response induced |
|---|---|---|---|
| TLRs | -Bacterial cell wall components<br>-Viral nucleic acids<br>-Etc. | -Gram-positive and Gram-negative bacteria<br>-DNA and RNA viruses<br>-Fungi<br>-Protozoa | -Sufficient to induce Th1, Ab (particularly IgG2) and $CD8^+$ T cell responses |
| Dectin-I | -Fungal cell wall components,<br>-β-glucan | -Fungi | -Sufficient to induce Th17 and Ab responses |
| Nod1, Nod2 | -Bacterial cell wall components<br>-Peptidoglycans | -Intracellular bacteria | -Sufficient to induce Th2 and Ab response<br>-Potentiates Th1, Th2, Th17 and Ab responses initiated by TLR<br>-May favor Th17 response |
| NALP3 | -Potassium efflux<br>-LPS plus ATP<br>-Pore-forming toxins<br>-Bacterial secretion systems | -Pathogenic bacteria | -Required for robust T cell-dependent hypersensitivity<br>-May potentiate Th2 drive Ab responses |
| ISD sensor | -Cytosolic DNA | -DNA viruses, retroviruses | -Sufficient to induce $CD4^+$ T cell and Ab responses<br>-Sufficient to induce $CD8^+$ T cell responses |
| RIG-I/ MDA5 | -Cytosolic RNA | -RNA viruses | -Sufficient to induce $CD8^+$ T cell responses<br>-Insufficient to induce $CD4^+$ T cell and Ab responses |

PRRs, pattern recognition receptors; TLR, Toll-like receptor; Nod, nucleotide-binding oligomerization domain; NALP3, Nacht domain-, LRR-, and PYD-containing protein 3; ISD, interferon stimulatory DNA; RIG-1, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; LPS, lipopolysaccharide; ATP, adenosine triphosphate; Th, T-helper, IgG2, immunoglobulin G2.

**Table 1.2: Transmembrane and cytosolic PRRs influence adaptive immunity (adapted from (*10*)).**

Signaling through different PRRs stimulates different adaptive immune responses best suited for eliminating the pathogens eliciting those signals.

| Name | Company | Class | Indications | Stage |
|---|---|---|---|---|
| | | ***Adjuvants*** | | |
| Alum | Various | Mineral Salt | Various | Licensed |
| MF59 | Novartis | O/W emulsion | Flu (Fluad)/ pandemic flu | Licensed (EU) |
| Liposomes | Crucell | Lipid vesicles | HAV, flu | Licensed (EU) |
| Montanide | Various | W/O emulsion | Malaria, cancer | Phase III |
| PLG | Novartis | Polymeric microparticle | DNA vaccine (HIV) | Phase I |
| Flagellin | Vaxinnate | Flagellin linked to antigen | Flu | Phase I |
| QS21 | Antigenics | Saponin | Various | Phase I |
| | | ***Combination adjuvants*** | | |
| AS01 | GSK | MPL + liposomes + QS21 | Malaria, TB | Phase II |
| AS02 | GSK | MPL + O/W emulsion + QS21 | Malaria | Phase II |
| AS03 | GSK | O/W emulsion + α tocopherol | Pandemic flu (Pandemrix) | Licensed (EU) |
| AS04 | GSK | MPL + Alum | HBV (Fendrix), HPV (Cervarix) | Licensed |
| RC-529 | Dynavax | Synthetic MPL + Alum | HBV | Phase II |
| Iscom | CSL, Isconova | Saponins + cholesterol + phospholipids | Various | Phase I |
| IC31 | Intercell | Peptide + oligonucleotides | TB | Phase I |
| CpG 7909 | Coley/Pfizer Novartis | Oligonucleotide +Alum, oligonucleotide + MF59 | HBV, malaria, HCV | |
| ISS | Dynavax | Oligonucleotide | HBV | Phase II |
| MF59 + MTP-PE | Chiron/ Novartis | Lipidated MDP + O/W emulsion | HIV, flu | Phase I |

AS, adjuvant series; GSK, GlaxoSmithKline; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ISCOM, immune stimulating complex; MDP, N-acetyl-muramyl-L-alanyl-D-isoglutamine; MPL, monophosphoryl lipid A; O/W, oil-in-water; PLG, poly(lactide-co-glycolide); TB, tuberculosis; W/O, water-in-oil.

**Table 1.3: Adjuvant formulations tested in humans (adapted from (*34*)).**

Delivery systems adjuvants were first used to enhance the immunogenicity of the antigen. Following the identification of synthetic PAMP analogues that stimulate the immune system, these components were added to the delivery system adjuvants, resulting in what are referred to as combination adjuvants.

| TLR | Localization | Elicitor | Stage |
|---|---|---|---|
| TLR1, TLR2 | Plasma membrane | Triacyl lipopeptides (PAM3CSK4) | Preclinical |
| TLR2, TLR6 | Plasma membrane | Diacyl lipopeptides (PAM2CSK4); MALP2 | Preclinical |
| TLR3 | Endosome | dsRNA (poly I:C) | Preclinical |
| TLR4 | Plasma membrane | LPS (MPL; synthetic MPL) | MPL licensed in US and EU |
| TLR5 | Plasma membrane | Flagellin | Clinical trial for flu |
| TLR7 | Endosome | ssRNA, imiquimod, resiquimod | Preclinical |
| TLR8 | Endosome | ssRNA, resiquimod | Preclinical |
| TLR9 | Endosome | DNA, CpG oligonucleotide, ISS | Clinical trials for various indication |
| TLR10 | Plasma membrane | Unknown | NA |

ds, double stranded; EU, Europe; ISS, Immunostimulatory sequence; MPL, monophosphoryl lipid A; NA, not applicable; ss, single stranded; TLR, Toll-like receptor.

**Table 1.4: TLRs as vaccine adjuvant targets (*34*).**

TLRs 1, 2 and 4-6 are located on the plasma membrane and recognize bacterial components, while TLRs 3 and 7-9 are located in the endosome and identify pathogenic nucleic acids. In addition to the naturally occurring pathogen components, synthetic analogues have been identified that activate TLRs.

| CpG Class | Sequences and structural elements | pDC IFN-α secretion | pDC maturation | B cell activation |
|---|---|---|---|---|
| A | PO CpG motif(s) flanked by PS poly G motif; form higher molecular weight aggregates through G tetrads | ++++ | ± | ± |
| B | CpG motifs; PS monomers | ± | +++ | +++ |
| C | 5'-TCG and > 12-nucleotide palindromic sequence; PS monomers and duplexes | +++ | +++ | +++ |

G, guanine; pDC, plasmacytoid dendritic cells; PO, phosphodiester; PS, phosphorothioate.

**Table 1.5: Characteristics of classes of CpG-ODNs (adapted from (*68*)).**

The classes of CpG sequences are based on the sequence characteristics, which determine their ability to stimulate pDCs to secrete IFN-α and/or activate B cells.

| Toxin | Bacteria | Site of translocation |
|---|---|---|
| Exotoxin A | *Pseudomonas aeruginosa* | Endoplasmic Reticulum |
| Subunit B of pertussis toxin | *Bordetella pertussis* | Endoplasmic Reticulum |
| Subunit B of Shiga toxin | *Shigella dysentariae* | Endoplasmic Reticulum |
| Heat-labile enterotoxin | *Escherichia coli* | Endoplasmic Reticulum |
| Shiga-like toxin 1 | *Escherichia coli* | Endoplasmic Reticulum |
| Diphtheria toxin | *Corynebacterium diphtheriae* | Endosomes |
| Anthrax toxin | *Bacillus anthracis* | Endosomes |

**Table 1.6: Bacterial toxins utilized for delivery of MHC class I-restricted antigens (adapted from (*79*)).**

Bacterial toxins enter the cytosol primarily through the endoplasmic reticulum or through endosomes.

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# CHAPTER 2

# ENCAPSULATING IMMUNOSTIMULATORY CPG-OLIGONUCLEOTIDES IN LISTERIOLYSIN O-LIPOSOMES PROMOTES A TH1-TYPE RESPONSE AND CTL ACTIVITY

## SUMMARY

Immunostimulatory sequences (ISS) are short DNA sequences containing unmethylated CpG dimers that have multiple effects on the host immune system, including the ability to stimulate antigen-specific cytotoxic T lymphocytes (CTLs) and drive a Th1-type immune response. Listeriolysin O (LLO)-containing pH-sensitive liposomes have been shown to efficiently deliver macromolecules to the cytosol of APCs and efficiently stimulate CTLs. We hypothesized that encapsulating ISS-oligodeoxyribonucleotides (ODNs) in this delivery system would enhance the cell-mediated immune response and skew a Th1-type response in protein antigen-based vaccination. *In vitro* studies indicated that co-encapsulation of ISS in LLO-liposomes engendered activation of the NF-κB pathway while maintaining the efficient cytosolic delivery of antigen mediated by the co-encapsulated LLO. Antigen-specific CTL responses monitored by using the model antigen ovalbumin (OVA) in mice were enhanced when mice were immunized with OVA and ISS-ODN-containing LLO-liposomes compared with those immunized with either OVA-containing LLO-liposomes or OVA-ISS conjugates. The enhanced immune responses were of the Th1-type as

monitored by the robust OVA-specific IgG2a induction and the OVA CD8 peptide-stimulated IFN-γ secretion. Our study suggests that including ISS-ODN in LLO-containing pH-sensitive liposomes yields a vaccine delivery system that enhances the cell-mediated immune response and skews this response toward the Th1-type.

## INTRODUCTION

In traditional vaccination, live attenuated viruses or inactivated pathogens have been widely utilized for the treatment and prevention of disease. Although these strategies have generated successful results for a large number of diseases, safety concerns associated with conventional vaccines have led to the development of modern vaccines such as protein antigen-based subunit vaccines (*1*). Subunit vaccines alone weakly stimulate the immune system and induce both limited antibody response and minimal cellular immunity, particularly antigen-specific cytotoxic T lymphocyte (CTL) responses, while adjuvants such as alum and oil/water emulsions have been used to enhance the antibody-based humoral immunity (*1, 2*). However, effective vaccines against tumors and viral infections need to efficiently stimulate both arms of the immune system: humoral and cell-mediated (*3, 4*). Increased efficacy may be partly achieved through the addition of molecular adjuvants, which act through an innate immune receptor to exert immunopotentiating properties that modulate the strength and type of immune response (*1, 5*). Selecting a suitable delivery system for molecular adjuvants and antigens is frequently necessary for achieving a robust immune response, and one way to accomplish this is to mimic the natural immune-stimulating properties of pathogens. Our approach tested in this study is to combine a nucleotide-based molecular adjuvant and protein antigen with LLO-containing pH-sensitive liposomes to simultaneously activate

the innate immune system, as well as deliver the antigen to the cytosol of antigen-presenting cells (APCs) to stimulate the cellular arm of immunity.

Liposomes enter APCs through the endocytic pathway; therefore, a molecular adjuvant with its main receptor located in the endosomal compartment was chosen to increase the likelihood of interaction (*6*). Immunostimulatory sequence-containing oligodeoxyribonucleotides (ISS-ODNs), are short, synthetic, single-stranded ODNs containing unmethylated cytosine phosphate guanine (CpG) sequences that mimic motifs primarily found in bacterial DNA (*7*). CpG motifs act as danger signals detected by a pattern recognition receptor (PRR), Toll-like receptor 9 (TLR9), in the endosome alerting the vertebrate innate immune system of the invasion of intracellular pathogens (*8*). Upon recognition of CpG-containing DNA by TLR9, an immunostimulatory cascade induces the differentiation, maturation and proliferation of B cells, T cells, natural killer cells and monocytes/macrophages resulting in the production of various proinflammatory cytokines such as interleukin (IL)- 1, 6, 12 and 18 and interferon (IFN)- α, β, γ (*5, 7*). The antigen-specific effects of ISS-ODNs are improved when the antigen and ISS-ODN are co-delivered to the same APC (*9, 10*).

One approach to achieve co-delivery is to conjugate protein antigens and ISS-ODNs, which results in increased antigen-specific IFN-γ secretion, IgG2a titers, and CTL activity compared with antigen and ISS-ODN in solution (*9, 10*). Although antigen-ISS-ODN conjugates stimulate antigen-specific CTL activity in some cases of model antigen (*11*), there are limitations associated with antigen-ISS-ODN conjugates as a vaccine formulation: (i) the chemical characteristics of the antigen may limit the synthesis of the conjugates, (ii) the antigenic epitopes may be altered during conjugation, and (iii) the

number antigens used in a vaccine may be restricted (*12, 13*). To overcome these problems, many types of delivery systems have been used to co-deliver antigens and ISS-ODNs, including, but not limited to, biodegradable microparticles, nanorods, and liposomes (reviewed in (*14*)). Most of these delivery systems enter the APCs via the endocytic pathway and potentiate MHC class II-dependent humoral responses, but do not possess the capacity to activate MHC class I-dependent cellular responses directly. Many systems rely on less efficient cross-presentation; that is, the ability of APCs to process and present extracellular antigens to $CD8^+$ T cells in order to engender CTL responses (*15, 16*). We previously demonstrated the utility of LLO-containing liposomes in actively potentiating CTL responses via enhanced cytosolic delivery of protein antigen: in delivering whole protein antigen to the cytosol of macrophages *in vitro* and enhancing antigen-specific CTL activity *in vivo* in a murine model (*6, 17, 18*).

LLO, the pore-forming hemolysin of a facultative intracellular bacteria, *Listeria monocytogenes (Lm),* exhibits optimal endosome-disrupting activity at pH 5.5 and promotes *Lm* escape from the phagolysosome for invasion into the cytosol (*19*). In this study, we hypothesized that incorporating ISS-ODN in the LLO-containing liposome formulations would skew the immune response to the Th1-type and further improve the CTL activity even though it may not be released until the endosome is rather acidic. We demonstrate that co-encapsulation of ISS-ODN in the LLO-containing liposomes activates the Th1-type cytokine pathway *in vitro*. Furthermore, using the model antigen ovalbumin (OVA), we show *in vivo* that the lip{LLO,OVA,ISS} (lip{} indicates liposomes encapsulating LLO, OVA and ISS-ODN) formulation enhances the number of $CD4^+$ and $CD8^+$ IFN-γ-secreting T cells, as well as the amount of bulk IFN-γ secretion.

We also show that the lip{LLO,OVA,ISS} formulation is capable of producing an increased Th1-type antibody response, while suppressing the Th2-type cytokine secretion and antibody induction, and retaining capabilities of stimulating a robust CTL response. The results from these studies indicate that the LLO- and ISS-ODN-containing liposome formulation is capable of stimulating a robust adaptive immune response.

## MATERIALS AND METHODS

### Mice

C57BL/6 (female, 8-12 weeks old; Charles River Laboratories, Portage, MI) and C57BL/10ScNJ ($Tlr4^{Lps\text{-}del}$, H-$2K^b$, female, 6-12 weeks old; Jackson Laboratories, Bar Harbor, ME) were used in this study and were handled according to Institutional Guidelines.

### Cell lines and tissue culture

All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA), and all cells were maintained and experimental incubations were conducted in a humidified incubator at 37°C and 5% $CO_2$, unless otherwise noted. B3Z cells, an OVA SIINFEKL peptide-specific $CD8^+$ T-cell hybridoma (CD8 OVA T1.3, H-$2K^b$-restricted), were maintained in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/mL streptomycin, 100 U/mL penicillin, 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 25 mM HEPES. Bone marrow was harvested from femurs and tibia of mice and differentiated into bone marrow-derived macrophages (BMM) in BMM media (DMEM supplemented with 20% HI-FBS, 30% L-cell conditioned media, 2 mM

glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 55 μM 2-mercaptoethanol) as described previously by Stier et al. (*20*). BMM were harvested on day six of culture and frozen in liquid nitrogen until the experiment. For experiments, BMM were cultured in either BMM media or complete DMEM (DMEM + 10% HI-FBS, 100 μg/mL streptomycin and 100 U/mL penicillin) as described below.

**Purification of LLO and preparation of liposomes**

The *hly* gene (which encodes for LLO) was inserted into pET29b with a polyhistidine tag. Recombinant LLO was purified from *E. coli,* analyzed for purity and monitored for hemolytic activity as previously described (*17*). ISS-ODNs used in these studies were a provided by Dynavax Technologies. ISS 1018 (5'-TGA CTG TGA ACG TTC GAG ATG A-3') unmodified and 5'-disulfide were synthesized with a nuclease-resistant phosphorothioate-modified backbone. Lipid films were made from a 2:1 (mol:mol) mixture of egg phosphatidylethanolamine: cholesteryl hemisuccinate (ePE:CHEMS; Avanti Polar Lipids, Alabaster, AL and Sigma-Aldrich, respectively) by removing chloroform and methanol using a rotary evaporator at < 10 mm Hg vacuum at RT. The lipid films were hydrated by vortexing with HBS, pH 8.4 containing LLO (100 μg), OVA (2 mg, Sigma-Aldrich, Grade VI) and/or ISS (0.625 mg). The optimal concentration of liposome-encapsulated LLO required for efficient cytosolic OVA delivery was previously determined by Mandal et al. and used in these studies (*17*). The liposomes were freeze-thawed and sonicated ten times, and unencapsulated proteins and ODNs were separated from liposomes by size-exclusion chromatography using a 1 × 25 cm Sepharose CL-4B column (GE Healthcare, Picastaway, NJ). Encapsulated OVA and LLO were analyzed by resolving the proteins in SDS-PAGE and measuring band

intensities using densitometry. Protein concentrations were determined by staining with either Coomassie Blue and digitally recorded using a KODAK Digital Sciences Electrophoresis Documentation and Analysis System, or with SYPRO Red (Invitrogen) and visualized with a Molecular Dynamics Typhoon 9200 (GE Healthcare), and calculations were based on known concentrations of the proteins run in the same gel. ODNs were resolved in a 20% Tris-Borate-EDTA (TBE) polyacrylamide gel, and the concentrations were determined by staining with SYBR Green I (Invitrogen) and calculated from known ODN concentrations.

**Preparation of conjugate{OVA-ISS}**

Conjugate{OVA-ISS} were made as described by Cho et al. with the following modifications (*9*). Briefly, OVA was activated with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Rockford, IL) at RT for 1 h, and residual sulfo-SMCC was removed by G-25 desalting column (GE Healthcare). A five molar excess of tris-(2-carboxyethyl) phosphine (TCEP, Pierce) was incubated with 5'-disulfide-linked phosphorothioate ISS-ODN at 40°C for 2 h, and residual TCEP was removed using G25-Hi Prep column. Reduced 5'-sulfhydryl-ISS ODN was mixed with modified OVA at a 5:1 molar ratio overnight at RT. Free ODN was removed from the conjugate using a Sephacryl S-200 column, and the conjugates were analyzed by SDS gel electrophoresis using NuPage 4-12% Bis-Tris gels (Invitrogen) and MOPS/SDS buffer. Conjugate{OVA-ISS} consisted of an average of 2.4 ISS molecules per OVA.

**NF-κB nuclear translocation assay**

BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were cultured on glass cover slips in 24-well plates. On the day of the experiment, cells were treated for 2 h, and then fixed with 3.7% paraformaldehyde in PBS for 15 min at RT, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 5% normal goat serum (Sigma-Aldrich) in PBS. NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was applied for 2 h followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min. The nucleus was stained using propidium iodide (Invitrogen) as per the manufacturer's instructions. Cells were visualized with an Olympus FV500 confocal microscope, and at least 300 cells were analyzed and differentiated based on nuclear or cytosolic NF-κB p65 staining.

***In vitro* IL-12 secretion analysis by ELISA**

BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were plated in 96-well tissue culture plates in BMM media one day prior to liposome treatment. Cells were pulsed with OVA-containing LLO-liposomes diluted in complete DMEM for 2 h, washed and incubated with complete DMEM for 22 h. Cell culture supernatants were collected and stored at -80 ºC until analyzed for IL-12 secretion using IL-12/IL-23p40 ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

***In vitro* antigen presentation assay**

*In vitro* antigen presentation experiments were performed as described previously with the following modifications (*6*). BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were plated in 96-well tissue culture plates in complete DMEM one day prior to liposome

treatment. BMM were incubated in serum-free DMEM for 1 h and then pulsed with liposomes in serum-free DMEM for 2 h. Cells were washed and incubated in complete DMEM for 3 h, fixed with 1% paraformaldehyde for 15 min at RT, and residual aldehydes were quenched with 0.2 M lysine for 20 min at RT. B3Z cells ($2 \times 10^5$ cells/well) were added to the plate and incubated for 15 h. Cells were washed with PBS and the β-galactosidase substrate [0.15 mM chlorophenol red-β-D-galactopyranoside (CPRG; Calbiochem, Gibbstown, NJ), 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40 in PBS] was added to the plate and incubated for 4 h, after which the absorbance was measured at 570 nm using a Molecular Devices Emax plate reader (*21*).

**Immunization protocol**

C57BL/6 mice were primed with formulations containing 8 μg of OVA in 50 μL on week 0 and boosted with the same formulation on week 2 via the subcutaneous route at the base of the tail. Sera were collected on week 4 for antibody analyses. Mice were euthanized on week 6 and spleens were harvested for assays. The data shown here is representative data from two studies performed with similar trends.

**Antigen-specific cytokine secretion**

Splenocytes ($5 \times 10^5$/well) were added to 96-well tissue culture plates in duplicate in 200 μL mouse media (RPMI 1640 (Lonza, Walkersville, MD), 10% HI-FBS (Gemini Bioproducts, West Sacramento, CA), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Lonza), 2-mercaptoethanol (55 μM) supplemented with either OVA (25 μg/mL), MHC class I OVA peptide SIINFEKL, and MHC class II OVA peptide ISQAVHAAHAEINEAGR (1 μg/mL, Research Genetics, Huntsville, AL), or

concanavalin A (10 μg/mL, Sigma-Aldrich). Splenocyte supernatants from a given mouse were combined when harvested on day 4 and stored at -80°C until analyzed for IFN-γ or IL-5 secretion by ELISA. Samples were assayed in duplicate using ELISA plates coated with purified rat anti-mouse IFN-γ capture antibody (2 μg/mL; BD Biosciences, San Jose, CA) or purified rat anti-mouse IL-5 capture antibody (1 μg/mL; BD Biosciences) in 0.1 M sodium phosphate, pH 9.0 overnight at 4°C. Plates were washed with PBST (PBS (R&D Systems) + 0.05% Tween 20), and then blocked with 1% BSA in PBS. Dilutions of the cell culture supernatants were added, and cytokines were detected with biotinylated rat anti-mouse IFN-γ or biotinylated rat anti-mouse IL-5 (BD Biosciences). Streptavidin-horse radish peroxidase (HRP, Pierce) was detected with TMB substrate (KPL, Gaithersburg, MD) and stopped with 2 N sulfuric acid. Absorbance values were read at 450 nm with a 650 nm correction using a Molecular Devices plate reader.

**ELISPOT assay for IFN-γ**

ELISPOT was used to quantify the number of OVA-specific IFN-γ secreting cells. Nunc Maxisorb plates were coated with rat anti-murine IFN-γ (5 μg/mL, U-CyTech, Utrecht) in PBS, overnight at 4°C followed by blocking with PBS + 1% BSA. Serial dilutions of splenocytes (starting at $2 \times 10^5$/well) were added to the plates in mouse media and stimulated with OVA (20 mg/mL), OVA CD8 peptide (1 μg/mL) or OVA CD4 peptide (1 μg/mL) overnight. Biotinylated rabbit anti-murine IFN-γ (U-CyTech) was detected using murine anti-biotin HRP (Sigma-Aldrich), which was developed with TMB for membranes (Sigma-Aldrich). Spots were analyzed using the BioReader 3000 immunospot image analyzer (BioSys, Karben, Germany).

### *In vitro* cytotoxic T lymphocyte assay

CTL activity was assessed using the standard $^{51}Cr$ release assay with splenocytes isolated six weeks after priming. Briefly, the splenocytes were cultured in mouse media containing 2% Rat T-Stim (BD Biosciences) with 1 μg/mL SIINFEKL-peptide, then incubated for four days. Splenocytes were washed and re-plated in mouse media containing 2% Rat T-Stim for two additional days. On day six, the splenocytes (effector cells) were counted and plated in triplicate to the 96-well plates. Five thousand $^{51}Cr$-labeled target cells, either SIINFEKL peptide-pulsed EL-4 cells or no peptide-pulsed EL-4 cells, were added to the effector cells to achieve various effector:target ratios. MHC-mismatched $^{51}Cr$-labled, peptide pulsed SV-BALB cells were also plated as targets to confirm MHC specificity (data not shown). The effector and target cells were incubated for 4 h, and the supernatants were analyzed for $^{51}Cr$ release. The percent cytotoxicity was calculated based on the amount of $^{51}Cr$ released into the supernatant using the equation: % specific lysis = [(mean killing-spontaneous release)/(maximum release-spontaneous release)] × 100, where maximal release was achieved by complete lysis of $^{51}Cr$-labeled targets in 1% Triton X-100, spontaneous release was determined by incubating $^{51}Cr$-labeled targets in media, and mean killing was determined by the cytotoxicity of $^{51}Cr$-labeled SIINFEKL-pulsed EL-4 cells.

### Anti-OVA Ig ELISA

Sera were collected on week four and analyzed by ELISA to quantify the antibody isotypes induced by the immunization formulation. ELISA plates were coated with OVA (10 μg/mL) in 0.1 M sodium phosphate, pH 9.0. Serial dilutions of sera samples were added in duplicate, and anti-OVA isotype-specific secondary antibodies

(goat anti-mouse IgG2a-biotin conjugated or goat anti-mouse IgG1-biotin conjugated; Southern Biotechnology Associates, Birmingham, AL) were detected with streptavidin-HRP (Pierce; Rockford, IL). Color was developed using TMB substrate (KPL), stopped with 2 N sulfuric acid, and the absorbance was read at 450 nm with a 650 nm background correction using a Molecular Devices plate reader. The titer was defined as the reciprocal of the serum dilution that gave an ELISA absorbance of 0.5 OD using a 4-parameter analysis (Softmax Pro97, Molecular Devices, Sunnyvale, CA).

**Statistical analyses**

Data from liposome treated animals were compared by unpaired two-tailed t-tests and the p-value is given for significant differences. A p-value $< 0.05$ was considered significant.

## RESULTS

### ISS-ODN-containing LLO-liposomes activate the NF-κB pathway

The immunomodulatory capabilities of ISS-ODNs arise as a result of their stimulating the production of specific cytokines and the downstream physiological changes they induce. Cytokine expression is driven by transcription factors such as nuclear factor kappa beta (NF-κB) p65, which is sequestered in the cytosol until cells are stimulated, during which time it moves to the nucleus to induce gene expression. We therefore determined the effect of adding ISS-ODN to LLO-containing liposomes by monitoring NF-κB p65 nuclear translocation in BMM. To confirm that the NF-κB translocation was due to ISS-ODN activation of TLR9 and not TLR4 activation, we used BMM from C57BL/10ScNJ mice, which lack TLR4 mRNA and protein. The untreated

cells show little-to-no (2 ± 1%) translocation of NF-κB to the nucleus (Figure 2.1). LPS (10 ng/mL) alone was not capable of stimulating nuclear NF-κB translocation in BMM to levels beyond those of untreated cells; however, free ISS-ODN in the media stimulated nuclear translocation of NF-κB in 90 ± 5% of cells analyzed (data not shown). Lip{LLO,OVA} (lip{} indicates liposome encapsulating LLO and OVA) stimulated NF-κB nuclear translocation in 16 ± 12%, while the inclusion of ISS-ODN in this formulation, lip{LLO,OVA,ISS}, resulted in a significantly greater response in which 75 ± 9% of cells analyzed exhibited NF-κB translocation.

To further support these results, BMM were treated with the various liposome formulations, and secreted IL-12, a Th1-type cytokine, in culture supernatants was analyzed by ELISA. The presence of ISS-ODN encapsulated in the liposomes significantly increased the amount of IL-12 secretion from 10.7 ± 0.8 pg/mL to 296.9 ± 22.7 pg/mL for lip{LLO,OVA} and lip{LLO,OVA,ISS}, respectively (Figure 2.2). Taken together, these results confirm that the presence of ISS-ODN in LLO-liposomes significantly enhances the activation of the NF-κB pathway resulting in the nuclear translocation of NF-κB and IL-12 secretion independent of TLR4 in BMM.

**Encapsulation of ISS-ODN in LLO-liposomes does not alter the cytosolic delivery of antigen to APCs *in vitro***

We previously showed that LLO in lip{LLO,OVA} liposomes significantly enhances the efficiency of cytosolic delivery of OVA (*6, 17*). Despite the well-known advantages of liposomes as a delivery vehicle, such as carrying multiple copies of macromolecules and several types of drugs; it is critical to ensure that adding ISS-ODN to LLO-liposomes does not drastically change the delivery characteristics. To test this,

we monitored the amount of OVA delivered to the cytosol of APCs upon ISS-ODN co-encapsulation using an *in vitro* OVA antigen presentation assay. BMM were treated with LLO- and OVA-containing pH-sensitive liposomes with and without ISS-ODN, and the levels of MHC class I-binding OVA peptide (SIINFEKL; $OVA_{257-264}$) presented to B3Z cells was monitored to determine the relative amounts of OVA delivered to the cytosol. B3Z, which are a *lacZ*-inducible $CD8^+$ T cell hybridoma cell line, express β-galactosidase upon binding the CD8 SIINFEKL peptide in context of $H\text{-}2K^b$ MHC class I molecules (*21*). Hence, the amount of β-galactosidase substrate (CPRG) converted to product (chlorophenol red) is an indirect measure of the number of binding events (*21*). The amount of SIINFEKL peptide presented to B3Z cells was similar when BMM were treated with lip{LLO,OVA} or lip{LLO,OVA,ISS} (Figure 2.2); however, a slight reduction in the amount of chlorophenol red was observed when ISS-ODN was included in the liposome formulation, to which we attribute a lower relative encapsulation efficiency of OVA when ISS-ODN is also encapsulated in the same liposome. The delivery of OVA to the cytosolic pathway for antigen presentation, monitored by this assay, was dependent on the amount of LLO and OVA co-encapsulated inside the liposomes (data not shown), in agreement with our previous report (*17*). The *in vitro* studies in BMM suggest that lip{LLO,OVA,ISS} formulation is capable of eliciting adjuvant-specific cytokine secretion in APCs, while retaining the efficiency of cytosolic antigen delivery for which LLO in pH-sensitive liposomes is noted.

**ISS-ODN incorporation in lip{OVA,LLO} enhances OVA-specific Th1-type cytokine secretion**

To test whether LLO-liposomes containing ISS-ODN would stimulate a Th1-type response *in vivo*, splenocytes from immunized C57BL/6 mice were analyzed for OVA-specific Th1- or Th2-type cytokine responses (IFN-γ or IL-5 secretion, respectively) upon antigen stimulation. Splenocytes from lip{LLO,OVA,ISS}-immunized mice secrete significantly more ($p=0.03$) IFN-γ than splenocytes from those mice immunized with lip{LLO,OVA}, 17,579 ± 2,778 pg/mL and 10,052 ± 1,457 pg/mL, respectively (Figure 2.3). As a way to evaluate the LLO-liposomal ISS-ODN data in the context of other ISS-ODN formulations reported in the literature, mice were immunized with sol{OVA} (OVA in solution), sol{OVA,ISS} (OVA and ISS-ODN mixed in solution) and conjugate{OVA-ISS} (OVA-ISS-ODN conjugates). Splenocytes from the sol{OVA} immunized group lacked OVA-specific IFN-γ secretion that was above detectable background levels (843 pg/mL). Adding ISS-ODN to the OVA immunization solution moderately increased the OVA-specific IFN-γ secretion (4,784 ± 1,113 pg/mL) compared with that of conjugate{OVA-ISS}, which resulted in a dramatically enhanced IFN-γ secretion (22,510 ± 3,567 pg/mL). This enhancement of IFN-γ secretion was expected based on previously published data showing antigen-ISS-ODN conjugate formulations generate a strong IFN-γ (Th1)-promoting vaccine (*9, 10*). Our data indicate that similar levels of IFN-γ secretion can be achieved when co-delivering antigen and ISS-ODN using the conjugate{OVA-ISS} formulation or the lip{LLO,OVA,ISS} formulation.

IFN-γ secretion results showed that ISS-ODN increased the production of Th1-type cytokines, which in turn are known to decrease the production of Th2-type cytokines (*22, 23*). This hypothesis was confirmed by monitoring the OVA-specific IL-5 secretion

from the splenocytes of immunized mice. Splenocytes from mice immunized with sol{OVA} secreted the lowest amount of IFN-γ and the highest amount of IL-5 (2,665 ± 1,284 pg/mL, Figure 2.3). ISS-ODN reduced the Th2-type response when it was included in solution (230 ± 92 pg/mL) and when it was conjugated with OVA (148 ± 79 pg/mL). Splenocytes from mice immunized with lip{LLO,OVA} secreted 460 ± 117 pg/mL IL-5, compared with splenocytes from those immunized with lip{LLO,OVA,ISS}, which did not secrete IL-5 above the detectable level of the assay (< 37 pg/mL). These results confirm that formulations that stimulate the greatest IFN-γ secretion also produce the lowest level of IL-5 secretion.

**OVA-specific IFN-γ secreting cells**

To further assess the types of cells activated in the cellular immune response after immunization, splenocytes isolated from C57BL/6 mice four weeks post-immunization were stimulated *ex vivo* with either (i) OVA protein, (ii) the OVA-derived peptide specifically recognized by $CD8^+$ T cells, SIINFEKL ($OVA_{257-264}$), or (iii) the OVA-derived peptide specifically recognized by $CD4^+$ T cells, ISQAVHAAHAEINEAGR ($OVA_{323-339}$), and monitored for IFN-γ-secreting cells by ELISPOT. When splenocytes were stimulated with OVA whole protein, the trend of the ELISPOT results was similar to the ELISA results for bulk IFN-γ secretion (Figure 2.3 and Table 2.1). Vaccination with lip{LLO,OVA,ISS} significantly increased the number of functional IFN-γ secreting $CD4^+$ T cells and $CD8^+$ T cells compared with lip{LLO,OVA} (Table 2.1). Vaccination with conjugate{OVA-ISS} or lip{LLO,OVA,ISS} resulted in at least twice as many $CD4^+$ and $CD8^+$ IFN-γ secreting cells in response to all stimulants compared to sol{OVA,ISS}, consistent with reports that co-delivery of antigen and ISS-ODN to the

same APC is important for stimulating an optimal immune response (*11, 24*). Relatively low numbers of IFN-γ secreting cells were observed when the splenocytes were cultured in the absence of antigen or antigenic peptide, indicating that the induced immune responses monitored by the cytokine ELISA and ELISPOT assays are antigen-specific (data not shown). ELISPOT data indicates both $CD4^+$ and $CD8^+$ T cells were activated by lip{LLO,OVA,ISS} to secrete IFN-γ, revealing that the vaccine formulation is stimulating both types of T cells that are primarily responsible for generating cell-mediated immunity.

**Adding ISS-ODN to lip{LLO,OVA} enhances the OVA-specific CTL response *in vivo***

*In vitro* studies confirmed that lip{LLO,OVA,ISS} are capable of delivering antigen to the cytosol of BMM, and the IFN-γ ELISPOT studies indicate that the lip{LLO,OVA,ISS} activates both $CD4^+$ and $CD8^+$ T cells. In addition to Th1-enhanced antigen-specific responses in the immunized mice, we tested the critical antigen-specific CTL effector function in order to assess the efficacy of our lip{LLO,OVA,ISS} vaccine carrier. We used the $^{51}Cr$ release-based CTL assay to monitor the strength of CTL function in the context of our immunization regimen. Splenocytes from lip{LLO,OVA,ISS}-immunized mice generated the strongest CTL response of all formulations tested (Figure 2.4). The OVA-specific CTL activity was significantly enhanced when mice were immunized with lip{LLO,OVA,ISS} as compared with lip{LLO,OVA} at all effector:target ratios tested. These data support previously published studies, which show that vaccination with lip{LLO,OVA} liposomes enhances IFN-γ secretion and CTL activity as compared with sol{OVA}-immunized mice (15 ± 6

% and 1 ± 1%, respectively) (*17*). Mixing ISS-ODN in solution with OVA improves CTL activity (21 ± 9%), but as shown by others the enhancement is further exaggerated when OVA and ISS are co-delivered to the same APC as is the case for conjugate{OVA-ISS} and lip{LLO,OVA,ISS} formulations, (36 ± 9% and 53 ± 11%, respectively). The antigen-specific CTL activity is similar in conjugate{OVA-ISS}- or lip{LLO,OVA,ISS}-immunized mice; however, these data show that co-encapsulating ISS-ODN in pH-sensitive LLO-containing liposomes enhances the antigen-specific CTL activity compared with lip{LLO,OVA}-immunized mice. This is a significant finding, as antigen-ISS-ODN conjugates have heretofore been regarded as the gold standard for eliciting CTL responses that are as robust as "live" vaccines in mice (*11*). Given the difficulties associated with some antigen-ISS-ODN conjugates, co-encapsulating ISS-ODN and antigen in the LLO-containing pH-sensitive liposomes may be a suitable alterative as a vaccine delivery system.

**ISS-ODN-containing lip{OVA,LLO} drive an OVA-specific Th1-type Ig response**

To investigate whether ISS-ODN in the formulations drives antigen-specific humoral response toward Th1-type, sera from immunized mice were analyzed for OVA-specific IgG1 and IgG2a antibodies. The results show that mice immunized with lip{LLO,OVA} produced more IgG1 antibodies than any of the other formulations (Figure 2.5). Co-encapsulating ISS-ODN in the lip{LLO,OVA,ISS} resulted in a significant reduction in IgG1 antibodies ($p=0.005$) and a significant enhancement in IgG2a antibodies ($p=0.007$), while lip{LLO,OVA,ISS} generated more anti-OVA IgG2a antibodies than any other formulation. The ratio of IgG2a:IgG1 anti-OVA antibody response correlates with the result of cytokine assays; as expected, lip{LLO,OVA,ISS}

and conjugate{OVA-ISS}-immunized mice have the highest IgG2a:IgG1 anti-OVA antibody response, indicative of a stronger Th1-type response (Figure 2.5).

## DISCUSSION

For the generation of an optimal immune response, APCs must receive antigens in the context of the appropriate activating signals. During an infection, APCs are stimulated through the innate immune system, which recognizes a diverse array of pathogen-associated molecular patterns (PAMPs), such as unmethylated CpG motifs, lipopolysaccharides, flagellin, and double-stranded RNA. Recognition of one or more PAMPs results in APC maturation, which is characterized by cytokine secretion, up-regulation of co-stimulatory molecules and increased antigen presentation. However, without concomitant immune stimulation along with delivery of antigen to the appropriate subcellular compartment, as is commonly the case with most subunit vaccines, the desired immune responses are hard to achieve. Mixing adjuvants with protein antigens has been shown to improve the antigen-specific immune response (*25*); but it is not an optimal vaccine formulation as both antigen and adjuvant may not be delivered to the same APC, or the APC may take up the adjuvant first resulting in activation/maturation that prevents the APC from taking up the antigen. Antigen-ISS-ODN conjugates are a relatively efficient vaccine formulation but are practically restricted by the chemical limitations of some antigens, as well as the potential for altering the antigenic epitopes as a result of the conjugation process, both of which may be overcome by co-encapsulating antigen and ISS-ODN in liposomes. Li, et al. demonstrated that co-encapsulating CpG-containing ODNs and Her-2/neu-derived peptides in liposomes stimulate robust antigen-specific secretion of IFN-γ by $CD8^+$ T

cells compared with encapsulating CpG-containing ODNs and the peptide in separate liposomes or adding free CpG-ODN to peptide encapsulated liposomes (*26*).

We previously focused on developing a vaccine delivery system that efficiently delivers antigen to the cytosol of APCs to enhance MHC class I processing and presentation as a means to augment CTL activity (*17, 18*). In this study, we hypothesized that incorporating the molecular adjuvant ISS-ODN in an LLO-containing, pH-sensitive, liposome-based vaccine delivery system would enhance the cell-mediated immune response, and that the cellular immune response against a model antigen would be skewed toward the Th1-type. This delivery system exerts its effects after entering the endocytic pathway of APCs where the pH-sensitive liposomes are destabilized by the acidic pH and release their contents (*6*). Hence, ISS-ODN was an obvious molecular adjuvant to investigate for potential use in our LLO-containing pH-sensitive liposomal delivery platform since its target receptor, TLR9, resides in endosomes (*27*). The rationale behind this approach is that ISS-ODN would be delivered to endosomes to interact with TLR9 and stimulate an innate immune response, while LLO would breach the endosomal membranes and promote the release of antigen into the cytosol for processing and presentation through the classical MHC class I pathway. The *in vitro* and *in vivo* data presented in this report clearly demonstrate that enhanced cellular immunity and Th1-skewing are achieved and the use of ISS-ODN and antigen co-encapsulated in LLO-liposomes provides a better approach than conventional vaccines when cellular immunity is required. These liposome formulations stimulate a strong cellular immune response while stimulating the humoral immune response also. Testing of this class of formulation for protection against a significant health risk is surely justified at this point.

In conclusion, results generated during this study show that LLO-containing liposomes are capable of efficiently delivering both antigen and adjuvant to stimulate (i) IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells, (ii) a robust CTL response, and (iii) the production of Th1-type antibodies. The safety and efficacy of ISS-ODN have been demonstrated in clinical trials (*7, 28*). Because of their uptake by APCs and their proven safety in currently marketed products such as Doxil, liposomes are a promising delivery system. As demonstrated here, these components may be combined with LLO for an effective vaccine delivery system to target antigens to the cytosol.

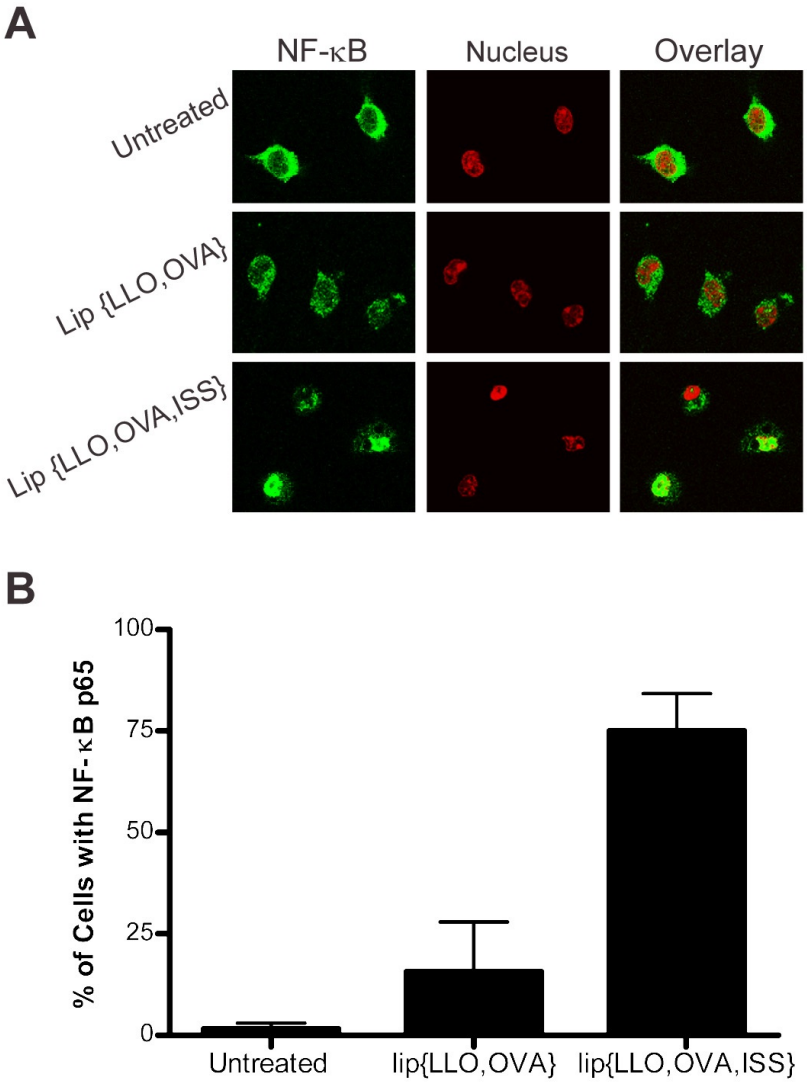


**Figure 2.1: ISS-ODN induces nuclear localization of NF-κB p65.**

BMM from C57BL/10ScNJ mice were treated with lip{LLO,OVA} (15 μg/mL OVA; 0 μg/mL ISS) or lip{LLO,OVA,ISS} (15 μg/mL OVA; 10 μg/mL ISS) for 2 h. Cells were stained with anti-NF-κB p65 antibody and the nucleus was identified by propidium iodide staining. (A) Representative images of cell staining visualized with fluorescence microscopy. (B) For each treatment, at least 300 cells were analyzed for nuclear or cytosolic NF-κB p65 localization. Results represent an average of 2-3 independent experiments ± S.D.

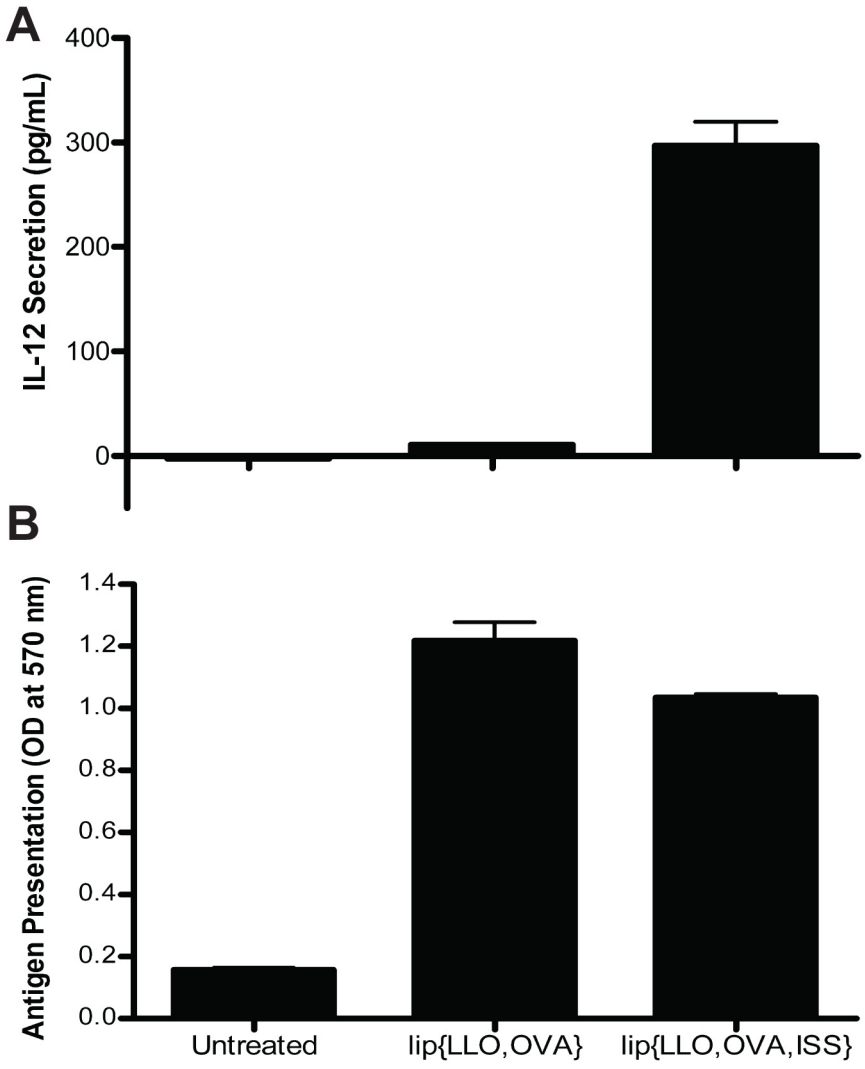


**Figure 2.2: Lip{LLO,OVA,ISS} induces IL-12 secretion and MHC class I-restricted presentation of OVA-specific peptides by BMM.**

(A) BMM were treated with lip{LLO,OVA} (10 μg/mL OVA; 0 μg/mL ISS) or lip{LLO,OVA,ISS} (10 μg/mL OVA; 4.7 μg/mL ISS) for 2 h, and then the cells were washed and further incubated for 22 h. Cell culture supernatants were analyzed for IL-12 secretion by ELISA. (B) BMM were pulsed with lip{LLO,OVA} or lip{LLO,OVA,ISS} (10 μg/mL OVA) for 2 h. Cells were washed, further incubated for 3 h and fixed; B3Z cells were added. After a 15 h incubation with B3Z cells, the level of presentation of SIINFEKL OVA-CD8 peptide by BMM to B3Z cells as monitored by the conversion of CPRG substrate to chlorophenol red in the activated B3Z cells. Cells were pulsed with SIINFEKL peptide (90 nM) as a positive control, and OD values were 1.28. Data represent the average of triplicates ± S.D.

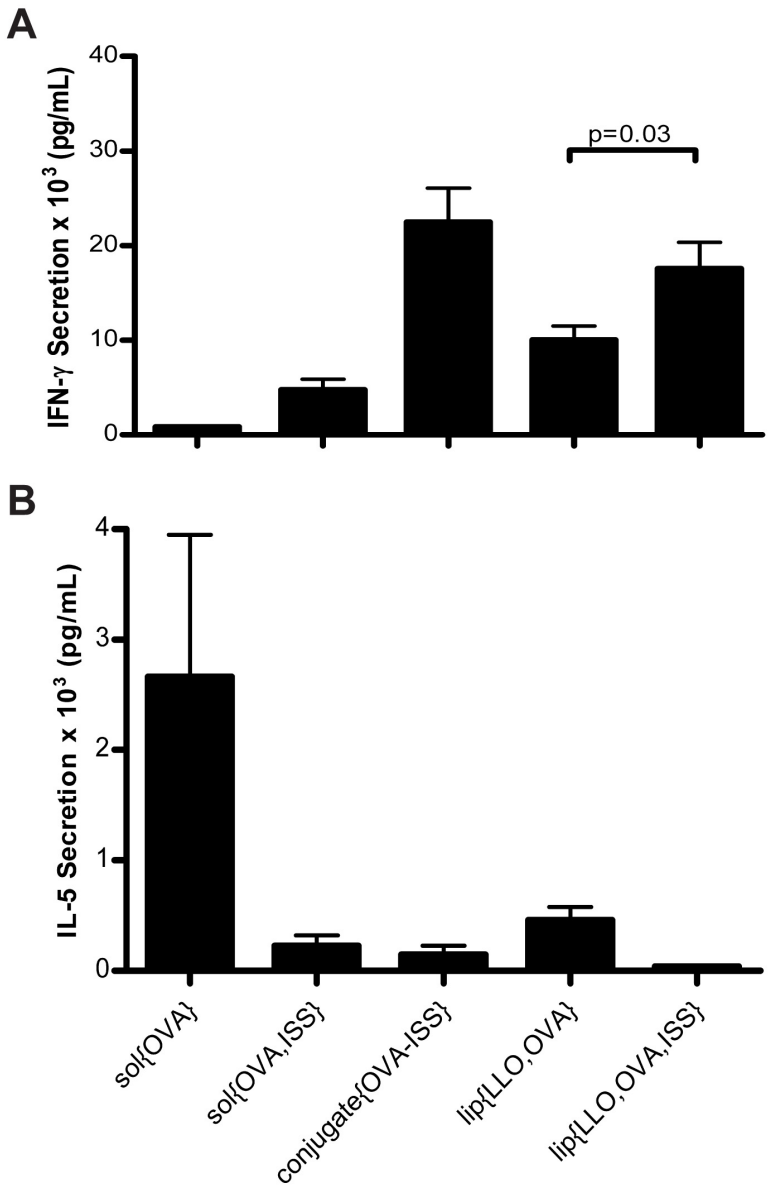


**Figure 2.3: IFN-γ and IL-5 secretion from splenocytes of immunized mice by ELISA.**

Mice were immunized subcutaneously at week 0 and boosted at week 2 with the same formulations. Mice were immunized with sol{OVA} (8 μg), sol{OVA,ISS} (8 μg OVA; 4 μg ISS) and conjugate{OVA-ISS} (8 μg OVA; 3.3 μg ISS), lip{LLO,OVA} (8 μg OVA), or lip{LLO,OVA,ISS} (8 μg OVA; 3.5 μg ISS). At week 6, splenocytes were isolated from mice and stimulated *ex vivo* with OVA (25 μg/mL). On day 4, supernatants were collected and analyzed for cytokine secretion using ELISA: (A) IFN-γ or (B) IL-5. Values below the limit of detection (LOD) were reported as the LOD × dilution factor, 843 pg/mL for IFN-γ and 37 pg/mL for IL-5. Data shown represent mean ± standard error of the mean (SEM) for each group (n=10).

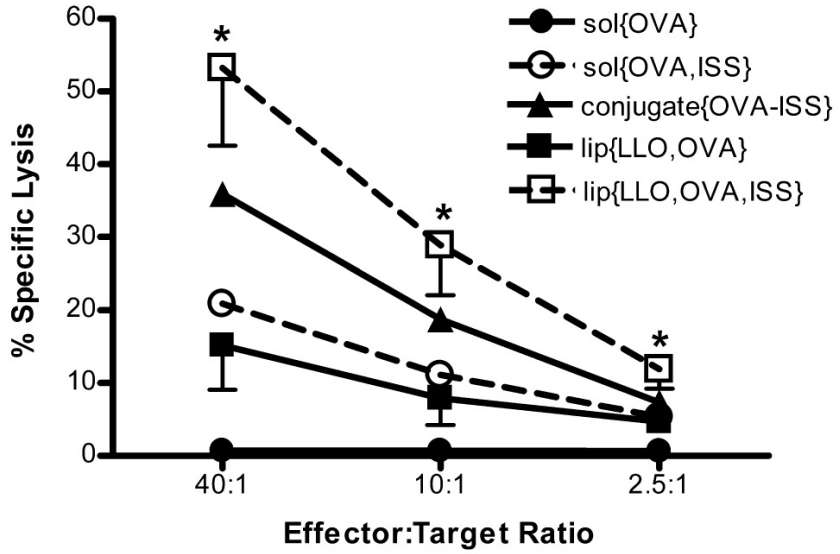


**Figure 2.4: Enhanced OVA-specific cytotoxic activity by lip{LLO,OVA,ISS}.**

Mice were immunized subcutaneously at week 0 and boosted at week 2 with the same formulations containing OVA (8 μg). OVA-specific CTL activity was monitored using $^{51}$Cr release assay. Each point represents the mean specific lysis of triplicate cultures of splenocytes for each group (n=10). SEM is shown for liposome groups. * Statistical significance was observed when comparing lip{LLO,OVA} and lip{LLO,OVA,ISS} at all effector:target ratios, p=0.006, p=0.01, p=0.03 at 40:1, 10:1 and 2.5:1, respectively.

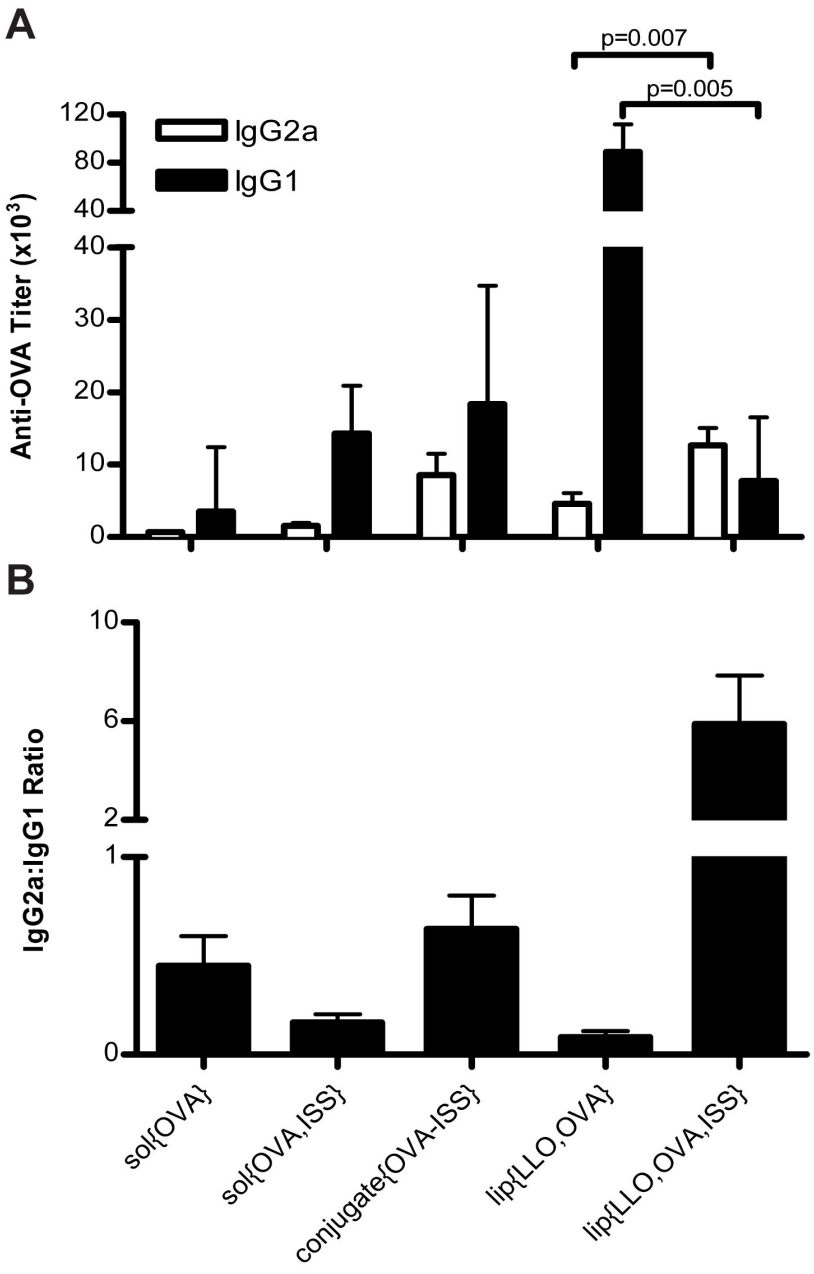


**Figure 2.5: ISS-ODN enhances the Th1-type antibody response in conjugate{OVA-ISS} or lip{LLO,OVA,ISS}-immunized mice.**

Two weeks after the boost immunization, sera were collected and analyzed for anti-OVA IgG1 or IgG2a antibodies using ELISA. (A) Each bar represents geometric mean titers (GMT) ± SEM for each group (n=10). (B) Each bar represents the average of the IgG2a:IgG1 ratio ± SEM for each group (n=10). If no titer was observed, the inverse of the lowest dilution tested, 600, was reported.

| Immunization Formulation | Stimulant | | |
|---|---|---|---|
| | **OVA** | **CD4 Peptide** | **CD8 Peptide** |
| sol{OVA} | 40 ± 1 | 41 ± 1 | 57 ± 11 |
| sol{OVA,ISS} | 49 ± 7 | 44 ± 3 | 364 ± 50 |
| conjugate{OVA-ISS} | 120 ± 15 | 92 ± 10 | 839 ± 93 |
| lip{LLO,OVA} | 84 ± 8 | 58 ± 5 | 443 ± 80 |
| lip{LLO,OVA,ISS} | 142 ± 23* | 87 ± 9* | 885 ± 115* |

**Table 2.1: Induction of OVA-specific IFN-γ-secreting cells.**

Mice were immunized subcutaneously on week 0 and week 2 with formulations containing 8 μg OVA. Splenocytes were isolated 4 weeks after the final immunization and stimulated with OVA (20 μg/mL), CD4 peptide (1 μg/mL) or CD8 peptide (1 μg/mL) for 24 h. Data represent the mean IFN-γ spot forming units (SFU) per $1 \times 10^6$ cells ± SEM for each group (n=10). Values at the limit of detection of the assay were reported as 40. * Statistical significance was observed when comparing lip{LLO,OVA} and lip{LLO,OVA,ISS} for all stimulants; p=0.03, p=0.01, p=0.005 for OVA, CD4, and CD8, respectively.

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# CHAPTER 3

# CONJUGATION OF LIPID AND CPG-CONTAINING OLIGONUCLEOTIDE YIELDS AN EFFICIENT METHOD FOR LIPOSOME INCORPORATION


## SUMMARY

For optimal stimulation of T cells, protein-based vaccines must deliver protein antigens to antigen-presenting cells and at the same time provide immunostimulatory signals. Listeriolysin O (LLO)-containing liposomes have been utilized to efficiently deliver protein antigens to the cytosolic pathway for antigen processing and major histocompatibility complex class I-dependent presentation while co-delivering immunostimulatory CpG-oligodeoxyribonucleotides (ODNs). In this report, we describe the synthesis, characterization, and biological evaluation of lipid-CpG-ODN conjugates as a means to deliver CpG-ODNs to their major receptor, Toll-like receptor 9 (TLR9), in the endosome. Maleimide-phosphatidylethanolamine (PE) lipids were conjugated to a CpG-ODN with a sulfhydryl group at the 5' terminus. We demonstrate *in vitro* in bone marrow-derived macrophages that the CpG-ODNs in their lipid conjugate form retain their immunostimulatory capabilities after incorporation onto liposome bilayers and that the conjugated CpG-ODNs interact with their receptor TLR9 as readily as liposome-encapsulated ODNs. These lipid-CpG-ODN conjugates were evaluated in mice using ovalbumin (OVA) as a model antigen, and the results indicate equally robust OVA-specific cytotoxic T lymphocyte responses between lipid-conjugated or encapsulated

CpG-ODNs. The conjugated CpG-ODNs retain Th1 immune skewing capabilities as shown by OVA-specific antibody isotypes and Th1-type cytokine secretion. Overall, this work indicates that conjugating PE lipids and CpG-ODNs results in an efficient method that allows facile incorporation of CpG-ODNs into a liposome-based delivery platform while retaining the immune-stimulating capabilities of CpG-ODNs.

## INTRODUCTION

The immune system generates humoral and cellular immune responses to viruses and bacteria by recognizing a variety of the pathogen's components. Traditional vaccination strategies have successfully exploited these responses by utilizing whole live attenuated or inactivated pathogens for the treatment and prevention of a large number of diseases; however, both safety and production concerns have led to the development of protein antigen or subunit vaccines (*1*). Compared to whole pathogens, protein-based vaccines are inherently limited in their ability to stimulate a robust immune response and their ability to deliver antigen(s) to the cytosolic pathway of antigen presentation. Hence, adjuvants have been utilized in protein vaccines as an effective means for enhancing the immunogenicity of the antigens and modulating the types of immune responses. Classical adjuvants such as aluminum hydroxide (alum) and oil/water emulsions enhance the humoral response to protein antigens but not the cellular response, thus limiting their utility (*1, 2*). A cellular immune response, particularly the T helper (Th)1-type that is associated with interferon (IFN)-γ production and cytotoxic T lymphocytes (CTLs), is desirable in a number of cases and is often needed to clear tumors or intracellular pathogens such as viruses (*3*). Traditional vaccines rely on the molecular composition of the whole pathogen to stimulate the innate immune system through an array of pathogen-

associated molecular patterns (PAMPs) such as unmethylated cytosine-phosphate-guanine (CpG)-containing DNA, lipopolysaccharides, lipoproteins, flagellin and double-stranded RNA (*4*). Recognition of one or more PAMPs induces antigen-presenting cell (APC) maturation resulting in cytokine secretion, co-stimulatory molecule expression, and enhanced antigen presentation, ultimately stimulating both the humoral and cellular arms of the immune system. The ability of some PAMPs to stimulate cellular immune responses has led to an increased interest in their utilization in vaccines as adjuvants that target innate immune receptors (*5*).

Synthetic CpG-oligodeoxyribonucleotides (ODNs) that mimic bacterial CpG-containing unmethylated DNA have been shown to activate monocytes/macrophages, dendritic cells, natural killer cells and B cells, induce the production of proinflammatory cytokines (interleukin (IL)-6, IL-12, IFNs, TNF-α) and up-regulate the expression of major histocompatibility complex (MHC) class I, MHC class II and co-stimulatory molecules (*6*). CpG-ODNs may be co-administered with antigens to stimulate a cellular immune response [reviewed in (*6*)]. When APCs receive antigen without concomitant co-stimulation, however, T cell anergy, rather than the development of a memory T cell response, may occur (*7*). To address this issue various methods of co-delivering CpG-ODNs and antigens are being explored, including, but not limited to, antigen-CpG-ODN conjugates (*8, 9*), liposomal encapsulation (*10-12*), and microparticle loading (*13, 14*).

Liposomes, which are internalized via endocytosis and disrupted inside the endosomal compartment (*15*), have tremendous potential for delivering co-encapsulated CpG-ODNs to facilitate their interaction with Toll-like receptor 9 (TLR9) in the endosome (*16*). We have previously demonstrated the ability of listeriolysin O (LLO)-

containing pH-sensitive liposomes to efficiently deliver antigen into the cytosol of APCs to enhance MHC class I processing and presentation as a means to augment CTL activity (*17-19*). LLO, the pore-forming cytolysin of the facultative intracellular bacterium *Listeria monocytogenes (Lm)*, exerts optimal activity at pH 5.5 to temporarily disrupt endosome membranes and promote *Lm*'s escape from the endolysosome uptake pathway into the cytosol (*20*). LLO can thus be exploited to mimic the cytosol-invading aspect of *Lm*'s infectivity to efficiently deliver macromolecules to the cytosol. We have also shown that co-encapsulating ovalbumin (OVA) and CpG-ODNs within LLO-containing pH-sensitive liposomes significantly enhances the CTL response, while also skewing the immune response towards the Th1-type, as compared with OVA and CpG-ODN administered in solution (*21*). Our current approach is to generate a vaccine delivery system emulating traditional whole pathogen-based vaccines to stimulate both humoral and cellular immune responses, while reducing the relative toxicity by including in the protein-based vaccine only essential well-defined components for eliciting antigen-specific immunity.

Although the previous results obtained from LLO-liposomes with co-encapsulated antigen and CpG-ODN were promising, the encapsulation efficiency of CpG-ODN was relatively low, resulting in a significant loss of material during the removal of un-encapsulated ODNs. Furthermore, because of its encapsulation within the liposomes, the access of CpG-ODN to TLR9 in the endosome may be limited both spatially and temporally, in effect reducing the overall targeting efficiency. Therefore, in order to increase the probability of CpG-ODN binding to TLR9, CpG-ODN was conjugated to lipid via an irreversible thioether bond and incorporated into the liposomal membrane.

The hypothesis tested in this report is that lipid-conjugated CpG-ODNs on the surface of the liposomes will promote the interaction between CpG-ODN and TLR9, before the endosomal contents are released into the cytosol upon LLO-mediated perforation of the endosomal membrane. Here we show that we can conjugate CpG-ODN to phosphatidylethanolamine (PE), yielding an efficient process for incorporating CpG-ODNs in liposome membranes. The lipid-CpG-ODN conjugates retain immunostimulatory activity after incorporation into liposome bilayers as tested in bone marrow-derived macrophages (BMM) and are capable of readily interacting with TLR9. In mice, we use the model antigen OVA and show that liposomes containing OVA and LLO with either encapsulated or conjugated CpG-ODN enhance CTL activity compared with liposomes containing only OVA and LLO. Both conjugated and encapsulated CpG-ODNs are capable of stimulating a Th1-type response as indicated by enhanced IFN-γ secretion and anti-OVA IgG2a antibody production.

## EXPERIMENTAL PROCEDURES

### Mice, cell culture, media and reagents

C57BL/6 (female, 8-9 weeks old) and C57BL/10ScNJ ($Tlr4^{Lps\text{-}del}$, $H\text{-}2K^{b}$, female, 6-8 weeks old; Jackson Laboratory, Bar Harbor, ME) were used in this study and were handled according to Institutional Guidelines. All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA), and all cells were maintained and experimental incubations were conducted in a humidified incubator at 37°C and 5% $CO_2$, unless otherwise noted. Bone marrow was harvested from the femurs and tibia of C57BL/10ScNJ mice and differentiated into bone marrow-derived macrophages (BMM) in BMM media (DMEM with 20% heat-inactivated FBS (HI-FBS), 30% L-cell

conditioned media, 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 55 μM 2-mercaptoethanol) as described by Stier et al. (*22*). BMM were harvested on day six and frozen in liquid nitrogen until the experiment. Unmodified and 5'-disulfide ODNs used in this study were composed of 22 bases with a phosphorothioate-modified backbone (Dynavax Technologies; Berkeley, CA). The positive control, CpG-ODN ISS 1018 (5'TGACTGTGAACGTTCGAGATGA), contains unmethylated CpG sequences, and the negative control, non-CpG-ODN ISS 1040 (5'TGACTGTGAACCTTAGAGATGA), lacks unmethylated CpG sequences.

**Lipid-ODN conjugate synthesis**

***Preparation of 5' sulfhydryl ODN***

The disulfide precursors to 5' sulfhydryl ISS 1018 CpG-ODN and 5' sulfhydryl ISS 1040 non-CpG-ODN were gifts from Dynavax Technologies (Berkeley, CA). A 700 mM tris-(2-carboxyethyl) phosphine (TCEP; Pierce, Rockford, IL) solution was made in HBSE (140 mM sodium chloride buffered with 10 mM HEPES containing 1 mM EDTA) pH 7, and used at a five molar excess to reduce 5'-disulfide-ODN at 40°C for 2 h. Residual TCEP was removed using either a PD-10 or a NAP-5 desalting column (GE Healthcare, Piscataway, NJ) and eluted in HBSE pH 6.5. Reduced 5'SH-ODN was used immediately or stored at -80°C until use.

***Conjugation procedure***

Chloroform was removed from 1,2-dioleoyol-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (*23*) (MPB-PE; Avanti Polar Lipids, Alabaster, AL) using rotary evaporation at < 10 mm Hg vacuum at RT followed by dissolving in ether, and a water-in-oil emulsion was formed by adding reduced 5'SH-ODN in HBSE at

a 2:1 organic:aqueous ratio. The optimal MPB-PE:5'SH-ODN ratios were identified as 1:10 and 1:20 for 5'SH-CpG-ODN and 5'SH-non-CpG-ODN, respectively. The emulsion was formed by brief sonication, and the MPB-PE and 5'SH-ODN reacted for 4-5 h while continuously vortexing at RT. Ether was removed using rotary evaporation, after which water was removed by lyophilization. Two chloroform washes (25 mL) were performed to remove unreacted MPB-PE from the PE-ODN, and residual chloroform was removed by evaporation. Thin-layer chromatography was performed to verify the removal of unconjugated lipids. Both the starting material of reduced 5'SH-ODN and the conjugation reaction of MPB-PE and 5'SH-ODN were resolved in a Tris-Borate-EDTA (TBE) polyacrylamide gel (15%; BioRad, Hercules, CA) with 0.5× TBE buffer (45 mM Trizma base, 45 mM boric acid, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (0.5 μg/mL in 0.5× TBE buffer). Conjugation efficiency was assessed by comparing unconjugated 5'SH-ODN from the conjugation reaction with known concentrations of ODNs resolved in the same gel. Unconjugated 5'SH-ODN was removed from PE-ODN conjugates by Sepharose CL-4B size-exclusion chromatography (1 × 25 cm; GE Healthcare). To determine which fraction was the beginning of the PE-CpG-ODN peak, in a 96-well plate a small aliquot (5 μL) of the column fraction was mixed with SYBR Green I (1:5,000; 100 μL) and the fluorescence intensity of the fractions was compared with buffer. When the fluorescence intensity increased over background, the fractions that corresponding to the first 4-5 mL containing PE-ODN were combined. A TBE polyacrylamide gel (15%) stained with ethidium bromide was used to identify all fractions containing conjugated PE-ODN but not contaminating

monomeric or homodimeric (disulfide-bonded) CpG-ODNs; PE-ODN fractions were then combined and lyophilized.

***Rehydration of PE-ODN conjugates***

The dried PE-ODN conjugates were then dissolved in either aqueous or organic solvent. The PE-ODN conjugates could be easily dissolved in HBSE pH 8.4 to form micelles, which were then added to the liposome rehydration solution. The PE-ODN conjugate was transferred into the liposome membrane through a combination of freeze/thaw and sonication. The opposing chemical characteristics of the lipid-ODN conjugate resulted in lack of solubility in the organic solvents typically used with lipids (chloroform or methanol). Various organic solvents and temperatures as listed in Table 3.1 were tested to identify a solvent in which both the hydrophobic lipid and the hydrophilic ODN are soluble. The PE-ODNs were found to dissolve in methanol:dimethylformamide (MeOH:DMF; 3.3:1), which was later replaced by MeOH:N-methyl-2-pyrrolidone (MeOH:NMP; 3.3:1) due to improved stability. Regardless of the solvent used for PE-ODNs (aqueous or organic), the amount of PE-ODN recovered from purification was determined by measuring phosphate as described by Bartlett (*24*).

**LLO expression**

The *hly* gene encoding for LLO was inserted into the bacterial expression plasmid pET29b with a polyhistidine (His6) tag. Recombinant LLO was purified from *E. coli* as described by Mandal et al. (*18*), with the following exceptions. LLO expression was induced in bacterial culture for 4-6 h with 1 mM isopropyl β-D-thiogalactopyranoside, the cell pellet was collected and resuspended in wash buffer (50 mM sodium phosphate,

300 mM sodium chloride, 20 mM imidazole, pH 8) containing 2 mM PMSF and 1 mM 2-mercaptoethanol and then lysed using a French press. Lysate supernatant was incubated with Ni-NTA agarose (Qiagen, Valencia, CA) for 2 h and the Ni-NTA extensively washed with 200 bed volumes of wash buffer. His6-tagged LLO was eluted in wash buffer containing 400 mM imidazole and extensively dialyzed against HBSE pH 8.4 at 4°C. Protein purity and activity were assessed by SDS-PAGE and hemolysis assay, respectively, as previously described (*18*). LLO was stored in single-use aliquots at -80°C.

**Liposome preparation**

***Lipid film preparation***

Lipid films containing a 2:1 (10 μmol: 5 μmol) mixture of egg phosphatidylethanolamine: cholesteryl hemisuccinate (ePE:CHEMS; Avanti Polar Lipids, Alabaster, AL and Sigma-Aldrich, respectively) were made by removing chloroform and methanol using rotary evaporation at < 10 mm Hg vacuum at RT. When indicated, PE-ODNs dissolved in MeOH:NMP were added to ePE:CHEMS (in chloroform and methanol), and solvents were removed by rotary evaporation at 40°C and < 10 mm Hg vacuum resulting in lipid films.

***Lipid film rehydration***

The films were rehydrated with HBSE pH 8.4 containing a combination of ovalbumin (OVA; 10 mg (*in vitro* studies) or 2 mg (*in vivo* studies), Sigma-Aldrich, Grade VI), LLO (0.1 mg), ODN (2 mg), and/or PE-ODN. The resulting liposomes were subjected to five freeze-thaw cycles followed by 7 × 1 min bursts of bath sonication. Un-

encapsulated materials were separated from the liposomes in Sepharose CL-4B size-exclusion columns (1 × 25 cm).

***Quantification of liposomal contents***

OVA and LLO encapsulation was determined by resolving proteins in a Tris-Glycine polyacrylamide gel (4-20%; Invitrogen) with 1X electrode buffer (250 mM Trizma base and 2 M glycine) containing 0.1% SDS. The gel was stained with Krypton (Pierce) as per the manufacturer's instructions, and band intensities were measured using a Molecular Dynamics Typhoon 9200 with ImageQuant (GE Healthcare). Calculations were based on a standard curve of known concentrations of proteins resolved in the same gel.

ODN encapsulation was determined by generating a standard curve from free ODN or PE-ODN depending on the type of ODN to be quantified. All samples and standards contained normalized lipid amounts and C12E8 (1% final; Sigma-Aldrich). SYBR Green I (Invitrogen) was added to the plate at a final dilution of 1:15,000 and the fluorescence was quantified in a Synergy HT plate reader (BioTek Instruments, Winooski, VT) using an excitation of 485 nm and emission of 528 nm.

**Ficoll density gradient separation**

A Ficoll step gradient (0, 10 and 20%) was performed; Ficoll solutions of 30% and 10% were made in HBSE pH 8.4. Liposomes or PE-ODN micelles (50 μL) were mixed with 30% Ficoll (100 μL) with a pipette and then the 20% Ficoll solution was placed in the bottom of a centrifuge tube. The 10% Ficoll solution (350 μL) was carefully layered on top of the 20% Ficoll, and HBSE pH 8.4 (100 μL) was layered on top of the 10% Ficoll. The centrifuge tubes were placed in a MLS-50 ultracentrifuge

fixed angle rotor and centrifuged at 55,000 rpm for 90 min. The interface between 0% and 10% Ficoll (300 μL) and bottom fraction (150 μL) for each sample were collected and resolved on a TBE-urea gel (15%; Invitrogen) with 0.5× TBE buffer and visualized by staining with ethidium bromide and viewing using a Typhoon 9200.

**Nuclease protection assay**

Nuclease protection assays were performed as previously described (*25*), with the following modifications. Liposomes were incubated with nuclease P1 (Sigma-Aldrich) at 60°C for 30 min. In some instances, samples were incubated with Triton X-100 (1% final) to solubilize the lipid vesicles. To degrade the nuclease, an equal volume of proteinase K solution (1 mg/mL proteinase K in 10 mM Tris pH 8, 100 mM sodium chloride, 25 mM EDTA, 0.5% SDS) was added at 50°C for 2.5 h. The reaction was quenched by adding an equal volume of stop solution (93.6% formamide, 20 mM EDTA, 0.05% bromophenol blue), and sodium hydroxide was added to the samples (final 0.1 N) to cleave the lipid-ODN bond. The samples were resolved in a TBE-urea gel (15%) and nucleic acids were stained with SYBR Green I (1:10,000 in TBE buffer) and viewed using a Typhoon 9200. ImageQuant 5.2 was used to quantify the band intensity, and the percentage of ODNs protected from nuclease degradation was calculated by comparing relative intensities.

**IL-12p40 secretion by ELISA**

BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were plated in 96-well tissue culture plates in complete DMEM (DMEM + 10% heat-inactivated (HI)-FBS, 100 μg/mL streptomycin and 100 U/mL penicillin) one day (14-18 h) prior to liposome treatment. An initial dilution of liposomes in HBSE was performed to normalize the phosphate

content of all liposomes. Liposomes were then diluted in serum-free DMEM (DMEM + 100 μg/mL streptomycin and 100 U/mL penicillin) to reduce the amount of HBSE in each sample (a minimum of a 1:10 dilution) to rule out HBSE as a potential confounding factor. These diluted liposomes were then transferred to a new 96-well plate and serially diluted in serum-free DMEM to obtain the final experimental doses (normalized for phosphate), and these samples were transferred to the plate containing BMM. BMM were incubated with the liposomes for 3 h, washed three times and incubated for 20 h in complete DMEM. Supernatants were collected and analyzed for IL-12 secretion by ELISA using IL-12p40 antibodies (R&D Systems, Minneapolis, MN) and IL-12p40 standard (Peprotech, Rocky Hill, NJ). Briefly, ELISA plates were coated with anti-mouse IL-12/IL-23p40 monoclonal antibody (MAB4991; 1 μg/mL) diluted in PBS pH 7.4, washed and then blocked with PBS containing 5% sucrose and 1% BSA prior to incubating with supernatant. Plates were then washed and incubated with biotinylated anti-mouse IL-12/IL-23p40 antibody (BAF499; 0.1 μg/mL). Plates were then washed and incubated with avidin-horse radish peroxidase (HRP; eBioscience, San Diego, CA) as per the manufacturer's instructions. The plate was washed and developed with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). The reaction was stopped using 2 N sulfuric acid, and the absorbance at 450 nm was measured using a Molecular Devices Emax plate reader.

***In vitro* antigen presentation assay**

*In vitro* antigen presentation experiments were performed as described previously (*17*), with the following modifications. BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were plated in 96-well tissue culture plates in complete DMEM one day (14-18 h) prior

to liposome treatment. Using a multi-channel pipette, BMM were washed with serum-free DMEM. An initial dilution of liposomes in HBSE followed by dilution in serum-free DMEM was performed as described above. Serial dilutions were performed in serum-free DMEM in a 96-well plate and samples were transferred to BMM using multi-channel pipette. BMM were then pulsed with liposomes in serum-free DMEM for 2 h, washed and incubated in complete DMEM for 3 h. Cells were fixed with 1% paraformaldehyde in PBS for 15 min on ice. Residual aldehydes were quenched with 0.2 M lysine in DMEM for 20 min at RT; during which time, B3Z cells were washed, counted and diluted to $1 \times 10^6$ cells/mL. B3Z cells ($2 \times 10^5$ cells/well) were added to the plate in B3Z media (RPMI-1640 media containing 10% HI-FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/mL streptomycin, 100 U/mL penicillin, 55 μM 2-mercaptoethanol and 25 mM HEPES) and incubated for 15 h. Plates were centrifuged at 1500 rpm for 5 min, media was removed and PBS was added to wash the cells. Plates were again centrifuged, PBS was removed and then the β-galactosidase substrate [0.15 mM chlorophenol red-β-D-galactopyranoside (CPRG; Calbiochem, Gibbstown, NJ), 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40 in PBS] was added to the plate and incubated for 4 h, after which the absorbance was measured at 570 nm using a Molecular Devices Emax plate reader (*26*).

**Liposome uptake by BMM**

Liposomes were made as above with 0.2 mol% rhodamine-PE (Invitrogen) dried in the lipid film to track the liposome uptake. BMM ($2.4 \times 10^6$/well) were plated in complete DMEM the day (14-18 h) before the experiment in a 24-well plate. This assay was performed in conjunction with the antigen presentation assay, so BMM were pulsed

with the highest concentration of liposomes (200 μM phosphate) in serum-free DMEM for 2 h. BMM were washed with PBS and then incubated with PBS containing 3 mM EDTA at RT for 5 min. A pipette was used to spray the buffer over the cells to assist in dislodging the cells. Cells were collected, pelleted and washed with PBS. The cells were resuspended in PBS and the untreated cells were counted with a hemacytometer. The cellular protein concentration was determined for all samples using BCA assay, and the cell concentration was normalized using the relative protein content compared with untreated cells.

**Immunization protocol**

***Prime-boost studies***

C57BL/6 mice were primed with OVA (8 μg) on day 0 and day 13 or 14 via the subcutaneous route in the hind flank. Mice were euthanized on day 27 or 28, blood was collected via cardiac puncture, and spleens were isolated for assays.

***Prime-only studies***

C57BL/6 mice were primed with OVA (8 μg) on day 0 via the subcutaneous route at the base of the tail. Mice were euthanized on day 10, blood was collected via cardiac puncture, and spleens were isolated for assays.

***In vivo* CTL assay**

Splenocytes were harvested from naïve mice and ground through a 70 μm mesh screen in a Petri dish containing mouse media (RPMI 1640, 10% HI-FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol) to form a single-cell suspension. RBCs were lysed using ACK lysis buffer (Invitrogen; 1

mL/spleen), and splenocytes ($1 \times 10^7$ cells/mL) were pulsed in mouse media with either SIINFEKL or influenza nucleoprotein $(NP)_{147-155}$ peptide (Anaspec, Fremont, CA) for 1 h at 37°C. The cells were washed and diluted to $1 \times 10^8$ cells/mL in PBS containing 0.1% FBS. The SIINFEKL peptide-pulsed and $NP_{147-155}$ peptide-pulsed populations were labeled with 4 or 0.4 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), respectively, for 10 min at RT. CFSE stock concentrations of 50 μM and 5 μM were made in PBS, and CFSE was added to the cells achieve the desired final concentration while the cells were being vortexed gently. CFSE labeling was stopped by adding an equal volume of FBS and incubating for 5 min on ice. Cells were washed thrice with PBS containing 5% FBS, counted, and diluted to $1 \times 10^8$ cells/mL. Both peptide-pulsed populations were mixed together and 200 μL ($1 \times 10^7$ cells/population) were injected via tail vein into naïve and immunized mice. Mice were euthanized 8.5-18 h post-adoptive transfer; spleens were harvested and single-cell suspensions were obtained by grinding spleens through a 70 μm mesh screen in a Petri dish containing mouse media. Splenocytes were washed, resuspended in Leibovitz's L15 media (5 mL), and placed in a 15 mL conical tube; Ficoll-Paque Premium (GE Healthcare; 5 mL) was carefully added to the bottom of the tube. A gradient was formed by centrifugation at 2200 rpm for 20 min at RT with the brake off. RBCs were pelleted and the lymphocyte layer was collected. Purified splenocytes were analyzed by FACSCalibur, and ten thousand viable CFSE-labeled cells were analyzed for each mouse. The percentage of specific lysis for each mouse was calculated by: 100 × [1-(ratio of cells recovered from naïve mice/ratio of cells recovered from immunized mice)].

**Antigen-specific cytokine secretion**

Splenocytes ($5 \times 10^5$/well) were added to 96-well tissue culture plates in duplicate in 100 μL mouse media. Stimulants were made at 2× concentration and added to splenocytes in 100 μL mouse media. Splenocytes were stimulated in media alone or media containing, $NP_{147\text{-}155}$ or MHC class I OVA peptide SIINFEKL, (5 μM final; Anaspec), or concanavalin A (10 μg/mL final; Sigma-Aldrich). After 72-85 h, splenocyte supernatants from a given mouse and treatment were combined and analyzed for IFN-γ secretion by ELISA as previously described (*21*). Briefly, samples were assayed in duplicate using ELISA plates coated with purified rat anti-mouse IFN-γ capture antibody (BD Biosciences, San Jose, CA). Cytokines were detected in dilutions of the cell culture supernatants with biotinylated rat anti-mouse IFN-γ (BD Biosciences) followed by avidin-HRP. The plate was washed and developed with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). The reaction was stopped using 2 N sulfuric acid, and the absorbance at 450 nm was measured using a Molecular Devices Emax plate reader.

**Interferon (IFN)-γ ELISPOT assay**

The frequency of OVA-specific IFN-γ secreting cells was analyzed using the IFN-γ ELISPOT Mouse Set (BD Biosciences) following manufacturer's instructions. Briefly, ELISPOT plates were coated with anti-mouse IFN-γ capture antibody (5 μg/mL) in PBS, overnight at 4°C followed by washing and blocking with mouse media for at least 2 h at RT. Splenocytes from immunized mice ($5 \times 10^5$ and $2.5 \times 10^5$/well) were added to the plates in mouse media and stimulated with either media, $OVA_{257\text{-}264}$ peptide (2.5 μg/mL) or the non-specific stimulator concanavalin A (1.25 μg/mL) for 18 h. Plates were

extensively washed with milliQ water to lyse cells. Then plates were washed six times with PBST using the ELX405 plate washer (BioTek); subsequent washes were performed with PBST and the plate washer. The plates were incubated with biotinylated anti-mouse IFN-γ detection antibody (2 μg/mL) diluted in PBS containing 10% FBS for 2 h at RT. After washing, wells were incubated with avidin-HRP (5 μg/mL) diluted in PBS containing 10% FBS for 1 h at RT. The wells were washed, and the final substrate (3-amino-9-ethylcarbazole (AEC) substrate set; BD Biosciences) was added. Color development was monitored by eye and stopped by the addition of $ddH_2O$. After drying the plates ON, the number of spot-forming units (SFU) in each well was determined using a computerized CTL ImmunoSpot Image Analyzer (Cellular Technology Limited, Shaker Heights, OH).

**Anti-OVA Ig ELISA**

Blood was collected from euthanized mice via cardiac puncture. Clotting proceeded at 37°C for at least 1 h, and samples were stored ON at 4°C. RBCs were pelleted and the post-immune sera were analyzed by ELISA as described (*21*). Briefly, ELISA plates were coated with OVA (10 μg/mL) in 0.1 M sodium phosphate pH 9.0 for a minimum of ON at 4°C. Plates were washed and blocked with PBS containing 1% BSA for a minimum of 2 h. The plates were washed, and sera was added to the plates and serially diluted down the columns in PBST containing 1% BSA. Anti-OVA isotype-specific secondary antibodies (goat anti-mouse IgG2a-biotin or goat anti-mouse IgG1-biotin; Southern Biotechnology Associates, Birmingham, AL) were detected with avidin-HRP. The plates were developed and absorbance values were determined as described above. Titer was defined as the reciprocal of the serum dilution that produced an

absorbance value of 1 using a 4-parameter analysis (Softmax Pro5.2, Molecular Devices, Sunnyvale, CA). If no titer was observed, the lowest dilution tested was reported.

**Statistical analyses**

Grubb's test was used to analyze for outliers in animal studies. Data from liposome-treated animals were compared by ANOVA and Tukey's post-test for data with homogeneous variance. In the case of non-homogeneous variance, the data were compared using the Kruskal-Wallis test followed by the Dunn's multiple comparison tests. A p-value $< 0.05$ was considered significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

**RESULTS AND DISCUSSION**

We recently described the investigation of the co-encapsulation of CpG-ODN within LLO-containing pH-sensitive liposomes as a strategy for co-delivery of a protein antigen and the adjuvant to the same APC (*21*). CpG-ODN was chosen as an adjuvant in this system because its receptor, TLR9, is located in the endocytic pathway, the primary route of uptake of liposomes by cells (*15, 27, 28*). However, the efficiency of co-encapsulation of CpG-ODNs in LLO-liposomes is relatively low (<10%). Furthermore, after the pH-sensitive LLO-liposomes are taken up by APCs and the liposomal contents are released in the acidic endosome, the perforation of the endosomal membrane by LLO leads to release of the contents, including CpG-ODN, into the cytosol where there is as yet no known CpG-ODN receptor. Therefore, we hypothesized that we could promote CpG-ODN interaction with TLR9, before endosomal rupture by LLO, using the strategy of incorporating lipid-CpG-ODN conjugates in the liposomal membrane.

**Lipid-ODN conjugate synthesis**

***Lipid:ODN conjugation ratio optimization***

CpG-ODNs with 5' sulfhydryls were conjugated to maleimide-PE (MPB-PE; sulfhydryl-reactive maleimide group conjugated to the lipid headgroup of PE) through the formation of a thioether bond. Various ratios of MPB-PE:5'SH-ODN (10:1, 20:1 and 50:1) were tested to identify the ratio that yielded the most sulfhydryl-containing ODNs conjugated without wasting MPB-PE; 10:1was found to be optimal for CpG-ODN. This procedure typically resulted in 90-95% of the CpG-ODN being conjugated, as determined by ethidium bromide staining of ODN and its lipid-conjugate after separation in TBE gels (Figure 3.1). In order to ensure that the enhanced immunopharmacological effects were not due to contaminating free CpG-ODNs, the PE-CpG-ODN conjugates were purified using size-exclusion chromatography to remove unconjugated monomeric and/or homodimeric (disulfide-bonded) ODNs. Fractions from size-exclusion chromatography were resolved using TBE-PAGE to identify the fractions that contain PE-CpG-ODN conjugate (Figure 3.2). The overall CpG-ODN recovery after pooling all fractions with conjugates was approximately 50%.

The same optimization process was performed with 5'SH-non-CpG-ODN, and >90% of the non-CpG-ODN was conjugated at the 50:1 MPB-PE:non-CpG-ODN. However, in an effort to save MPB-PE a 20:1 ratio was used and typically at least 70-75% of non-CpG-ODN was conjugated. Unconjugated non-CpG-ODN was removed by size exclusion chromatography as described above. PE-non-CpG-ODN was only used as a reagent in these studies, so optimizing the conjugation procedure was less critical.

***Solubility of PE-ODN in organic solvents***

PE-ODNs were readily soluble in aqueous buffer; therefore, the PE-ODNs could be rehydrated in HBSE and added to the liposome rehydration solution as a method to incorporate PE-ODNs on the liposomal membrane. However, this method requires that part of the rehydration solution volume consist of PE-ODNs, potentially limiting the amounts of other components. The liposomal encapsulation method employed in these studies relies on passive encapsulation, thus there is a limit to increasing the volume of the rehydration solution, as going beyond that threshold will result in decreased encapsulation efficacy. An alternative method of incorporating the PE-ODNs on the liposomal surface requires that the PE-ODNs be dissolved in an organic solvent so the PE-ODNs may be dried down as part of the lipid film. Various organic solvents and conditions were tested to identify a solvent to solubilize both the lipid and ODN moieties of the PE-ODNs (Table 3.1). A combination of methanol:DMF (3.3:1) was identified to dissolve the synthesized and purified PE-ODN; however, the conjugate was not stable in this solvent when stored under argon at -20°C for 26 days as shown (Figure 3.3). Alternative solvents with similar properties were investigated; isoproponal:DMF (3.3:1), MeOH:NMP (3.3:1), trifluoroethanol:NMP (3.3:1) and MeOH:DMF (3.3:1). The PE-ODN was found to be soluble only in MeOH:NMP or MeOH:DMF, so a stability study was performed on PE-ODN stored in these two solvents. N-methyl-2-pyrrolidone (NMP) was subsequently identified as a superior alternative to DMF for both solubility and stability of the PE-ODN when stored under argon -20°C for at least 26 days (Figure 3.3). The results indicate that the PE-ODNs stored in MeOH:DMF were no longer conjugated, as indicated by the lack of a PE-ODN band and the appearance of the ODN band,

whereas the stability of the conjugate was improved when stored in MeOH:NMP as indicated by the presence of the PE-ODN band and lack of ODN band. DMF contains a residual amine as a result of the manufacturing process that may lead to the instability of the PE-ODN conjugates in this solvent (H. Showalter; personal communication).

***PE-ODNs associate with liposome***

We then tested whether PE-CpG-ODNs were incorporated and remained associated with the liposomes. Liposomes were resolved in a Ficoll step density gradient (0, 10, 20 % Ficoll), and ODNs were identified in the gradient fractions by ethidium bromide staining of TBE-urea polyacrylamide gels. In this assay, liposomes floated to the interface of the 0 and 10% layers, whereas non-liposomal PE-CpG-ODNs remained in the 20% Ficoll fractions. All of the liposomal PE-CpG-ODNs detected were in the 0 and 10% interface, indicating that all lipid-CpG-ODN conjugates were incorporated in the liposome membrane (Figure 3.4).

***Majority of PE-ODN is Extra-Luminal***

In agreement with previous work reporting the asymmetric distribution of gangliosides in liposome bilayers (*29*), using a nuclease protection assay (*25*) we found the membrane leaflet distribution of PE-CpG-ODN to be ~80% extra-liposomal and ~20% luminal (Figure 3.5). When ODNs were encapsulated 85% were protected from nucleases; however, when the ODNs were conjugated only approximately 15-20% were protected from nucleases. It should be noted that similar results were obtained when sulfhydryl-containing CpG-ODNs were conjugated to maleimide-containing PEGylated phosphatidylethanolamine (PE-PEG-maleimide) using a combination of SYBR Green I staining and C12E8 detergent solubilization (Table A.1). When this method was used to

compare total versus extra-luminal ODNs (with C12E8 versus without C12E8) for PE-ODN conjugates (without PEG), the value with C12E8 (total ODN) was consistently lower than the value without C12E8 (extra-luminal) (assay outlined in Figure 3.6). We hypothesized that the SYBR Green I was not binding readily to the PE-ODN conjugates due to steric hindrance when PE-ODNs are in the liposome membrane, and this problem could be alleviated when the PEG group is present to move the ODNs away from the liposome membrane making them more easily to SYBR Green I.

Overall, these data indicate that PE-CpG-ODNs are efficiently synthesized with approximately 50% recovered after purification, and no loss of the conjugate was incurred during incorporation into the liposome membrane using the traditional method of liposome preparation. The majority of the loss of CpG-ODNs resulted from the size-exclusion chromatography step, which may not in fact be required as the efficiency of the conjugation process was so high (typically 90-95%). Overall, the process of lipid conjugation and incorporation into liposome bilayers is much more efficient than the more commonly employed passive encapsulation technique, which typically results in less than 10% CpG-ODN encapsulated.

**IL-12 secretion is PE-CpG-ODN dose-dependent**

Next, we asked whether CpG-ODN incorporated onto the liposomes in the form of PE-CpG-ODN conjugates retain the capacity of CpG-ODN to stimulate and modulate an immune response in similar fashion to free CpG-ODN. The liposomes were evaluated for their ability to induce the secretion of the Th1-type proinflammatory cytokine IL-12 in BMM in culture. It has been shown that the surface charge of liposomes affects their uptake by macrophages (*30*). Therefore, to eliminate uptake differences the surface

charge of the liposomes was held constant by maintaining the total percentage of conjugated ODNs in the liposome membrane (total PE-ODN kept at 0.3 mole percent of total phospholipids) while varying the percentage of PE-ODNs that contain the CpG sequence at 0, 25, 50 and 100%; the remaining PE-ODNs consisted of PE-non-CpG-ODN conjugates. Increasing the percent of PE-CpG-ODN in the liposomal membranes increased the amount of IL-12 secreted in a linear fashion (Figure 3.7). No IL-12 was detected when treated with the 0% PE-CpG-ODN sample (100% PE-non-CpG-ODN). As the PE-CpG-ODN fraction of the total PE-ODNs (PE-CpG-ODN plus PE-non-CpG-ODNs) was increased in these experiments, greater than 25% PE-CpG-ODN was required to stimulate detectable IL-12 secretion; this was shown by the lack of detectable IL-12 when cells were treated with 25% PE-CpG-ODN and by the presence of detectable IL-12 secretion at 50% PE-CpG-ODN. These data suggest that a certain threshold or minimum number of PE-CpG-ODN conjugates per liposome are required to achieve optimal BMM activation. It is noteworthy that a similar dose responsiveness phenomenon was observed when dendritic cells were treated with acid-degradable microparticles loaded with CpG-ODNs (*31*).

**Comparison of methods for PE-CpG-ODN liposomal incorporation**

Two methods were utilized to incorporate the PE-ODN into the liposome membrane: (i) drying PE-ODN conjugates as part of the lipid film or (ii) adding PE-ODN conjugates to the OVA and LLO rehydration solution. These two methods were compared *in vitro* in BMM to determine if the method of incorporating PE-CpG-ODN on the liposome membrane changes the immunostimulatory capabilities of the CpG-ODN. BMM were treated with liposomes co-encapsulating OVA and LLO that included PE-

CpG-ODNs that were either included by drying the PE-CpG-ODN (solubilized in MeOH:NMP) as part of the lipid film or by adding PE-CpG-ODNs (solubilized in HBSE) to the rehydration solution. Similar levels of IL-12 secretion were observed when of PE-CpG-ODN was incorporated on the liposome surface regardless of the method used for incorporation (Figure 3.8). Because both methods perform similarly, the method of solubilizing PE-CpG-ODN in organic solvent and including it in the lipid film was utilized in additional studies, thereby allowing the rehydration solution to contain only components to be encapsulated in the liposomes.

**Lipid-conjugated ODNs likely interact with TLR9 before encapsulated ODNs**

We hypothesized and investigated that conjugating CpG-ODN to PE lipids would promote the interaction between CpG-ODN and its presumptive receptor TLR9 in the endosomal compartment, before the pH-sensitive liposomes are destabilized by the low pH of the endosome and release their LLO. Ultimately, the effects of LLO breaching the endosomal membrane may have negative implications for the binding of CpG-ODN and signaling by TLR9. First, it is well established that protease inhibitors or inhibitors of endosomal acidification prevent the activation of TLR9 (*32, 33*), and when LLO forms pores in the endosomal membrane a transient rise in pH occurs. Secondly, LLO-mediated membrane perforation results in the release of endosomal contents (*17*), in this case CpG-ODNs, into the cytosol where there is no known receptor for single-stranded ODNs (reviewed in (*34*)). To test this hypothesis, we made PE-CpG-ODN-containing liposomes that contained either encapsulated CpG-ODN or encapsulated non-CpG-ODN; we also made PE-<u>non</u>-CpG-ODN-containing liposomes that encapsulated either CpG-ODN or non-CpG-ODN. Previous reports strongly suggest that phosphorothioate ODNs

bind the ectodomain of TLR9 regardless of sequence composition (i.e. CpG-containing or not); however, activation of TLR9 is CpG-dependent (*35, 36*), indicating that non-CpG-ODNs do not stimulate IL-12 secretion despite their binding to TLR9. Based on these data, we predicted that there would be reduced IL-12 secretion with PE-non-CpG-ODNs if these ODNs were to bind TLR9 before release of encapsulated CpG-ODN, thus reducing both the interaction and the effect of encapsulated CpG-ODNs via TLR9. The results support our hypothesis that the lipid-conjugated ODNs on the surface of the liposomes interact with TLR9 before encapsulated ODNs do, as we observed significantly attenuated IL-12 secretion with non-CpG-ODNs on the outside of liposomes encapsulating CpG-ODNs (Figure 3.9).

Importantly, antigen presentation assays confirmed that the incorporation of conjugated ODNs did not adversely affect the intracellular delivery of LLO-containing PE:CHEMS liposomes as shown by cytosolic delivery of the model antigen OVA. In this study, antigen presentation was compared when CpG-ODN was either encapsulated or incorporated in the liposomes as PE-CpG-ODN (0.1, 0.3 and 0.6 mole percent of total phospholipid). BMMs were treated with liposomes and antigen presentation results were normalized for liposome uptake and OVA and LLO content in the liposomes. The results indicate that PE-CpG-ODNs do not inhibit the cytosolic delivery of OVA (Figure 3.10); therefore, we expected co-encapsulated ODNs to also have been efficiently released from the liposomes to interact with TLR9.

**PE-non-CpG-ODNs stimulate an immune response**

***PE-ODNs do not inhibit CTL responses compared with encapsulated ODNs***

The *in vitro* studies indicated that lipid-conjugated CpG-ODNs retained immunostimulatory capabilities; to corroborate our results *in vivo*, we immunized mice to compare the two methods for including CpG-ODNs in the liposomes, incorporation of the CpG-ODN lipid conjugate onto liposome membranes versus incorporation via encapsulation. Our lab previously demonstrated that co-encapsulating OVA and CpG-ODN in LLO-containing pH-sensitive liposomes drives the immune response to a Th1-type, resulting in an increased IgG2a and reduced IgG1 antibody production, and elicits a robust CTL response (*21*). Therefore, this system was used as a positive control in all *in vivo* studies. Negative controls were performed by replacing CpG-ODNs with non-CpG-ODNs in both liposome-encapsulated and conjugated formulations. In this study, mice were primed and boosted subcutaneously in the hind flank with pH-sensitive liposomes containing LLO and OVA as well as CpG-ODN or non-CpG-ODN that was incorporated through co-encapsulation or lipid-conjugation (study outlined in Figure 3.11). The dose was normalized to OVA amount (8 μg). The generation of OVA-specific CTLs was monitored using an *in vivo* CTL assay; equal populations of $CFSE^{HIGH}$- and $CFSE^{LOW}$-labeled splenocytes were injected intravenously. The target cells, $CFSE^{HIGH}$, were pulsed with the $CD8^+$ T cell epitope SIINFEKL peptide ($OVA_{257-264}$, 10 μg/mL), while control cells, $CFSE^{LOW}$, were pulsed with a non-specific influenza NP peptide ($NP_{147-155}$, 10 μg/mL). The mice were sacrificed 18 h after intravenous injection, and CFSE profiles of splenocytes were monitored by flow cytometry. The results indicate that all formulations generate a robust OVA-specific CTL response. Mice immunized with liposomes

containing either encapsulated CpG-ODNs or PE-CpG-ODNs resulted in similar specific lysis values (66 ± 12% and 79 ± 8%, respectively) indicating that utilizing the lipid-CpG-ODN conjugate as a method of incorporating CpG-ODNs does not inhibit CTL generation (Figure 3.12). However, mice immunized with non-CpG-ODNs resulted in OVA-specific lysis values that were comparable to the values obtained when mice were immunized with liposomes containing CpG-ODNs, regardless if the non-CpG-ODNs were encapsulated or conjugated (68 ± 14% and 72 ± 12%, respectively). It was surprising to see no differences in OVA-specific lysis between mice immunized with CpG-ODNs or non-CpG-ODNs. However, these data suggest that incorporating PE-ODNs in the liposomes does not negatively alter the *in vivo* delivery of the antigen.

***Cellular immune response is not inhibited by PE-ODNs***

To further examine the effects of CpG-ODNs on the cellular immune response when either conjugated or co-encapsulated in OVA and LLO-containing liposomes, splenocytes from immunized mice were stimulated *ex vivo* with the OVA CD8 peptide and bulk IFN-γ secretion and IFN-γ spot-forming units (SFUs) were monitored (Figure 3.13). The level of bulk IFN-γ secretion measured by ELISA after splenocytes were stimulated *ex vivo* with SIINFEKL (1 μg/mL) for 85 h was similar for all formulations used in immunizations. The level of IFN-γ generated by splenocytes without the addition of peptide was not detectable in any of the groups (data not shown). Splenocytes from all groups exhibited a strong cytokine response when treated with the T cell mitogen concanavalin A (data not shown). The number of SFUs as measured by ELISPOT from splenocytes stimulated *ex vivo* with SIINFEKL (2.5 μg/mL) for 18 h was also similar for all formulations used in immunizations. The results are in agreement with the *in vivo*

CTL data (Figure 3.12), which show that there is no negative impact on the cellular immune response when including CpG-ODNs on the liposome surface by lipid-conjugation. These results also confirm that there is no CpG effect, as all mice respond similarly regardless of whether the ODN sequence contains CpG or not.

***PE-CpG-ODNs stimulate Th1-type anti-OVA antibody response***

Previous data show that adding CpG-ODNs to LLO- and OVA-liposomes enhanced the CTL response and IFN-γ secretion compared with mice immunized with liposomes containing LLO and OVA (*21*). In the studies performed here, non-CpG-ODNs were added to LLO- and OVA-containing liposomes as the negative control. The cellular immune assays did not detect a CpG-ODN effect, as there was no enhancement in OVA-specific CTLs (Figure 3.12) or IFN-γ production (Figure 3.13). Sera were collected from the mice and analyzed for OVA-specific IgG2a (Th1-type) or IgG1 (Th2-type) antibodies to determine if CpG-ODN was influencing the antibody isotype stimulated through immunization. Mice immunized with liposomes containing CpG-ODNs either encapsulated or conjugated (36,089 ± 5,201 or 23,719 ± 14,167, respectively) resulted in an increased OVA-specific IgG2a antibody titer compared with mice immunized with liposomes containing non-CpG-ODNs, either encapsulated or conjugated (5,141 ± 2,390 or 2,479 ± 3,532, respectively) (Figure 3.14). The anti-OVA IgG2a:IgG1 (Th1:Th2) ratio indicates that there is a CpG effect regarding the OVA-specific antibody isotype stimulated by immunization (Figure 3.14).

Overall, these results indicate that CpG-ODN is capable of stimulating the immune response to generate a Th1-type OVA-specific antibody response, but there is no enhancement of CTL or IFN-γ secretion mediated by CpG-ODNs. Non-CpG-ODN

seemed to be the optimal negative control; however, rarely in the literature was non-CpG-ODN used as a negative control in immunization studies. In one example, when CpG-ODN or ODN were administered with HSV antigen, the authors demonstrated no differences in CTL response (by chromium release), percent cells positive for IFN-γ (by intracellular staining) or the number of cells capable of secreting IFN-γ (by ELISPOT); although, the values were consistently higher for both groups than antigen alone (*37*). This study, however, demonstrated protection differences when the HSV antigen was administered with CpG-containing ODN compared with ODN lacking CpG (*37*). The authors hypothesized that the protection difference could be attributed to a CpG-sequence effect resulting in the shift in the anti-HSV antibody isotype from IgG1 to IgG2a (*37*).

**LLO- and OVA-containing liposomes as negative control for *in vivo* CTL assay**

Our results as well as those of others (*37*) showing that non-CpG-ODNs stimulate the cellular immune response prompted us to revert to liposomes containing only OVA and LLO as a negative control for our *in vivo* assays. However, the utility of the *in vivo* CTL assay in distinguishing differences in CTL responses stimulated by liposomal immunization had not been demonstrated. An *in vivo* study (n=3-4 mice/group) was performed in order to compare liposomes containing only OVA and LLO to liposomes containing OVA, LLO and CpG-ODN (study outlined in Figure 3.15). Mice were primed and boosted with liposome formulations based on OVA content (8 μg). The generation of OVA-specific CTLs was monitored using an *in vivo* CTL assay; equal populations of $CFSE^{HIGH}$- and $CFSE^{LOW}$-labeled splenocytes were injected intravenously. The target cells, $CFSE^{HIGH}$, were pulsed with the $CD8^+$ T cell epitope SIINFEKL ($OVA_{257-264}$ peptide, 1 μg/mL), while control cells, $CFSE^{LOW}$, were pulsed with a non-specific

influenza NP peptide ($NP_{147-155}$, 1 μg/mL). CFSE profiles were monitored 18 h after adoptive transfer. Note that the concentration of peptide used to load the splenocytes was decreased relative to that used in previous studies in an attempt to reduce the OVA-specific lysis, because in those studies some mice showed complete lysis of the OVA peptide-pulsed group. In the current study, mice immunized with OVA- and LLO-liposomes result in a specific lysis of 37 ± 11%, compared with mice immunized with OVA- and LLO-liposomes co-encapsulating CpG-ODN or non-CpG-ODN (57 ± 13% and 54 ± 18%, respectively) (Figure 3.16). The results from this study agree with previous data indicating no difference in CTL stimulation with CpG-ODN and non-CpG-ODN (Figure 3.12). Although the difference turned out to be statistically insignificant, this study is the first time that we had observed such a large difference between the mean OVA-specific lysis values (37% for mice immunized with liposomal OVA and LLO compared with 57% for mice immunized with liposomal OVA, LLO and CpG-ODN) using the *in vivo* CTL assay. These data are in good agreement with data we had previously obtained using the standard chromium release assay to determine CTL activity in which significant differences were observed (Figure 2.4). It is worth noting that the differences reported in the chromium release assay were obtained using ten mice per group as compared with the *in vivo* CTL assay in which only three to four mice per group were tested; therefore, it is likely that statistical significant differences were not observed in this study due to the relatively high variability and small sample number. The *in vivo* CTL assay is advantageous over the chromium release assay because the CTLs are not stimulated *ex vivo*; however, there may be increased variability associated with the *in vivo* CTL assay due to the requirement for tail vein injections. We therefore opted to

repeat this study using a larger sample size to test whether the experimental differences were significant.

**PE-CpG-ODNs stimulate CTL immune responses similar to encapsulated CpG-ODNs**

The above studies suggest that attaching CpG-ODN to lipid as a method of placing CpG-ODN on the liposomal membrane did not have deleterious effects on the cellular immune stimulating ability of CpG-ODN. However, through all of the cellular assays performed no significant differences had been observed between the positive control and negative controls. From the above studies and literature we had concluded that non-CpG-ODN was not the proper negative control for cellular assays and that a larger number of mice in each group should be used to determine whether any differences are statistically meaningful. We also hypothesized that the differences between liposomes containing OVA and LLO and liposomes containing OVA, LLO and CpG-ODN may be more exaggerated using a prime-only study rather than the previous prime-boost studies.

OVA and LLO were co-encapsulated in pH-sensitive liposomes with either lipid-conjugated or co-encapsulated CpG-ODNs as methods for simultaneously delivering antigen and co-stimulatory molecules to the same APC. These liposomes were tested for their capacity to stimulate OVA-specific CTL activity relative to that of OVA-containing LLO-liposomes without CpG-ODN (study outlined in Figure 3.17). Mice were vaccinated subcutaneously at the base of the tail with liposomes normalized for the amount of OVA (8 μg), and ten days later equal populations of $CFSE^{HIGH}$- and $CFSE^{LOW}$-labeled splenocytes were injected intravenously. The $CFSE^{HIGH}$ target cells were pulsed with the $CD8^+$ T cell epitope SIINFEKL ($OVA_{257-264}$ peptide, 1 μg/mL),

while the $CFSE^{LOW}$ control cells were pulsed with a non-specific influenza NP peptide (1 μg/mL). The CTL responses were comparable when mice were immunized with liposomes co-encapsulating CpG-ODN or with lipid-conjugated CpG-ODNs; specific lysis was 66 ± 22% or 69 ± 13%, respectively (Figure 3.18). The OVA-specific lysis was enhanced ($p<0.001$) when mice were immunized with either of the liposome formulations containing CpG-ODN compared with liposomes containing only OVA and LLO (specific lysis of 9 ± 6%). These results indicate that similarly to encapsulated CpG-ODNs, lipid-conjugated CpG-ODNs are capable of interacting with TLR9, providing T cells with the additional co-stimulation required for optimal activation *in vivo*.

**PE-CpG-ODNs stimulate a Th1-type cytokine response similar to encapsulated CpG-ODNs**

In addition to antigen-specific CTL induction, we evaluated the ability of $CD8^+$ T cells from the splenocytes of immunized mice to secrete IFN-γ *ex vivo* upon SIINFEKL stimulation for 76 h in culture. The supernatants were harvested and analyzed for the amount of secreted IFN-γ by ELISA. Splenocytes from mice immunized with liposomes co-encapsulating OVA and LLO secreted 2,702 ± 1,401 pg/mL IFN-γ (Figure 3.19). IFN-γ secretion was significantly enhanced ($p<0.01$ or $p<0.05$, respectively) when CpG-ODN was co-encapsulated or lipid-conjugated: 42,599 ± 9,460 and 25,848 ± 8,534 pg/mL, respectively. As negative controls, media alone or the irrelevant NP peptide generated no detectable IFN-γ in any of the groups, while the positive control mitogen concanavalin A stimulated IFN-γ secretion from splenocytes from all groups.

**PE-CpG-ODNs stimulate a Th1-type antibody response similar to encapsulated CpG-ODNs**

The induction of anti-OVA Th1- and Th2-type antibodies in the immunized mice was monitored, in addition to investigating the cytokine milieus induced by the APCs. CpG-ODNs stimulate a Th1-type immune response mediated by the cytokine IFN-γ, which drives the production of Th1-type antibodies such as IgG2a. Mice immunized with (i) liposomal OVA and LLO only, (ii) liposomes encapsulating OVA and LLO with CpG-ODN, or (iii) liposomes encapsulating OVA and LLO with lipid-conjugated CpG-ODN all produced similar levels of anti-OVA IgG1 (Figure 3.19). However, adding CpG-ODN, either encapsulated or conjugated, significantly enhanced the IgG2a antibody response (80 ± 31 or 158 ± 43, respectively), compared with mice immunized with liposomal OVA and LLO (15 ± 2). Note that these antibody responses are relatively weak which is due to the fact that prime only, and not prime-boost, immunization schedule was employed; similar studies performed with prime-boost regimens (Figure 3.14) resulted in stronger anti-OVA antibody responses, confirming the results illustrated here by the prime-only study. These results are consistent with the IFN-γ secretion data, indicating that conjugating CpG-ODN to lipid and incorporating it onto the liposomal membrane does not alter the ability of the CpG-ODN to stimulate a Th1-type response as shown by the *ex vivo* IFN-γ secretion upon CD8 peptide stimulation and the Th1-type antibody induction.

Although reduced immunostimulatory activity of CpG-ODNs has been reported for some CpG-containing sequences after conjugation at the 5'end (*31, 38*), the activity of the CpG-ODN ISS 1018 used in this study appears to be unaffected by the choice of terminus (5' or 3') used for coupling when conjugated to proteins (G. Ott personal

communication). Consistent with these findings, we observed similar CTL and Th1 responses when CpG-ODN were conjugated through the 5' end or encapsulated. It is possible that the predicted reduction in immunostimulatory activity observed with 5'-conjugated CpG-ODNs was offset here by (i) the liposomal surface position of PE-CpG-ODN more readily allowing interactions with TLR9 vis-à-vis encapsulated CpG-ODN or (ii) PE-CpG-ODNs on the liposome surface may enhance uptake by APCs, similar to that which has been observed with antigen-CpG-ODN-conjugates (*39*). Regardless, of any potential mechanistic implications, this study demonstrates that lipid-conjugated CpG-ODNs are capable of stimulating an immune response that is skewed toward the Th1-type, and future studies may include conjugation at the 3' end, which typically results in a relatively smaller reduction in immunostimulatory activity (*31, 38*). The study reported here was performed solely to compare the effects of conjugating CpG-ODNs with encapsulating CpG-ODNs; however, future studies are required to determine the optimal PE-CpG-ODN density in the liposome membrane for LLO-liposomes as well as the orientation of the conjugation.

## CONCLUSIONS

We have evaluated two methods for including CpG-ODNs in an LLO-liposome vaccine carrier. Conjugating CpG-ODN to lipid allows the design of a liposome-based delivery platform that results in greater recovery of CpG-ODNs than does passive encapsulation. Moreover, conjugated CpG-ODNs retain immunostimulatory activity both *in vitro* and *in vivo*. The results in this report strongly suggest that LLO-containing liposomes with conjugated CpG-ODN are a versatile and efficient delivery vehicle that may be used with a variety of antigens for vaccination. The strong CTL and Th1-type

immune responses indicate the potential for this vaccine delivery system for a variety of vaccines against viruses or cancer.

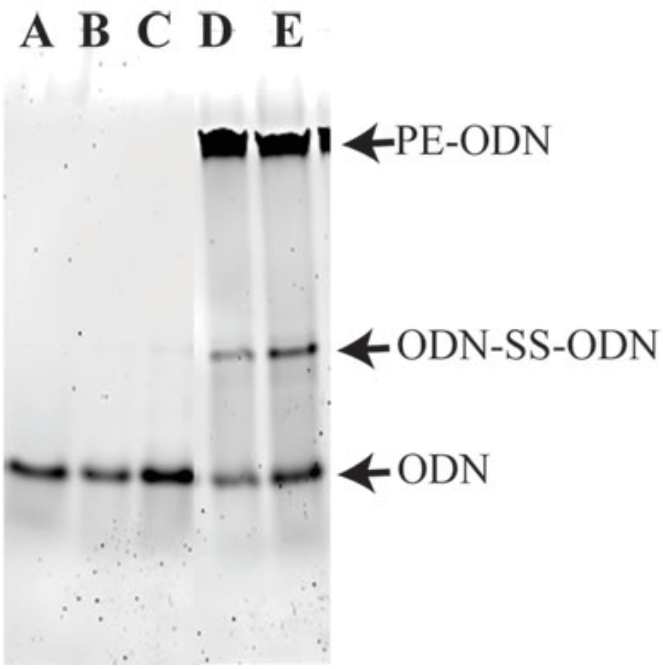


**Figure 3.1: Representative TBE gel of PE-CpG-ODN conjugation.**

Conjugation was assessed by resolving PE-CpG-ODNs from disulfide-bonded (ODN-SS-ODN) and unconjugated 5'SH-CpG-ODN in a 15% TBE polyacrylamide gel stained with ethidium bromide. The amounts of disulfide-bonded and unconjugated ODN were calculated from a standard curve of known ODN amounts. (A) 176 ng CpG-ODN standard, (B, C) reduced 5'SH-CpG-ODN added to MPB-PE (0.03, 0.06 μL, respectively), (D, E) PE-CpG-ODN conjugation reaction (5.25, 10.5 μL, respectively). These results indicate that > 95% 5'SH-CpG-ODN is conjugated.

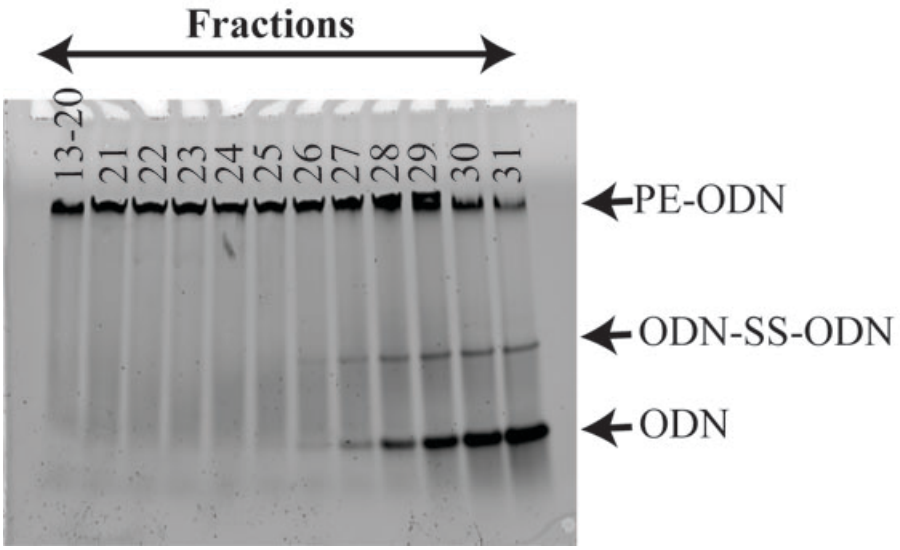


**Figure 3.2: Representative TBE gel of column fractions from PE-CpG-ODN purification by Sepharose CL-4B.**

Purification of PE-CpG-ODNs was assessed by resolving PE-CpG-ODNs from disulfide-bonded (ODN-SS-ODN) and unconjugated 5'SH-CpG-ODN in a 15% TBE polyacrylamide gel stained with ethidium bromide. Only the fractions containing PE-CpG-ODN were combined for use in studies (fractions 13-26 in this gel).

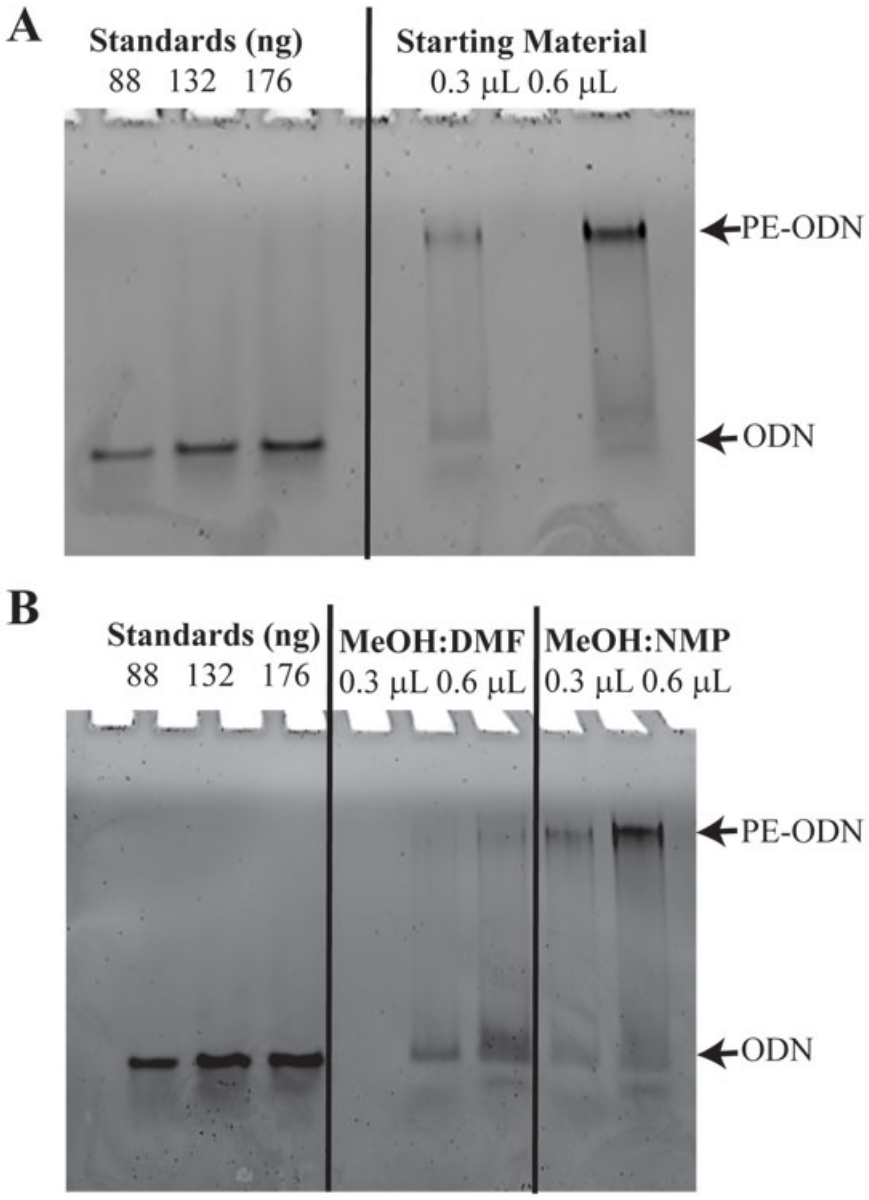


**Figure 3.3: Stability of PE-ODN in organic solvents.**

PE-ODNs were solubilized in either MeOH:DMF (3.3:1) or MeOH:NMP (3.3:1) and stored under argon at -20°C for 26 days. Solvent was removed from each sample using rotary evaporation, and each sample was rehydrated in HBSE. An aliquot of each sample was resolved on a 15% TBE gel and stained with ethidium bromide. PE-ODN on (A) day 0 and (B) day 26.

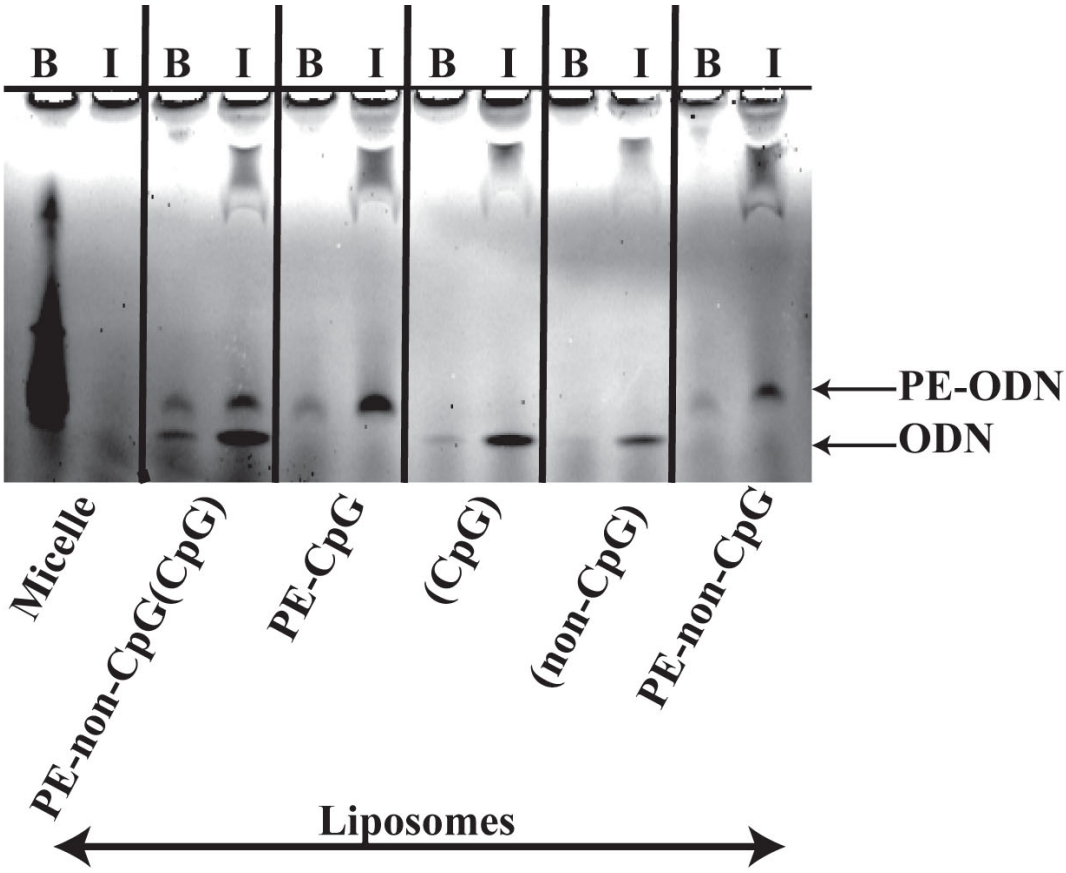


**Figure 3.4: PE-ODNs associate with liposomes.**

PE-ODN micelles or liposomes containing PE-ODNs and/or encapsulated ODNs [encapsulation denoted by the ODN type in parentheses in the figure caption] were separated in a Ficoll density gradient. A sample from each fraction was separated in a 15% TBE-urea gel and stained with ethidium bromide. Micelles that contain PE-ODNs not incorporated in liposomes were found in the bottom (B) fraction of the gradient; whereas, encapsulated or conjugated ODNs were primarily identified in the interface (I) fraction.

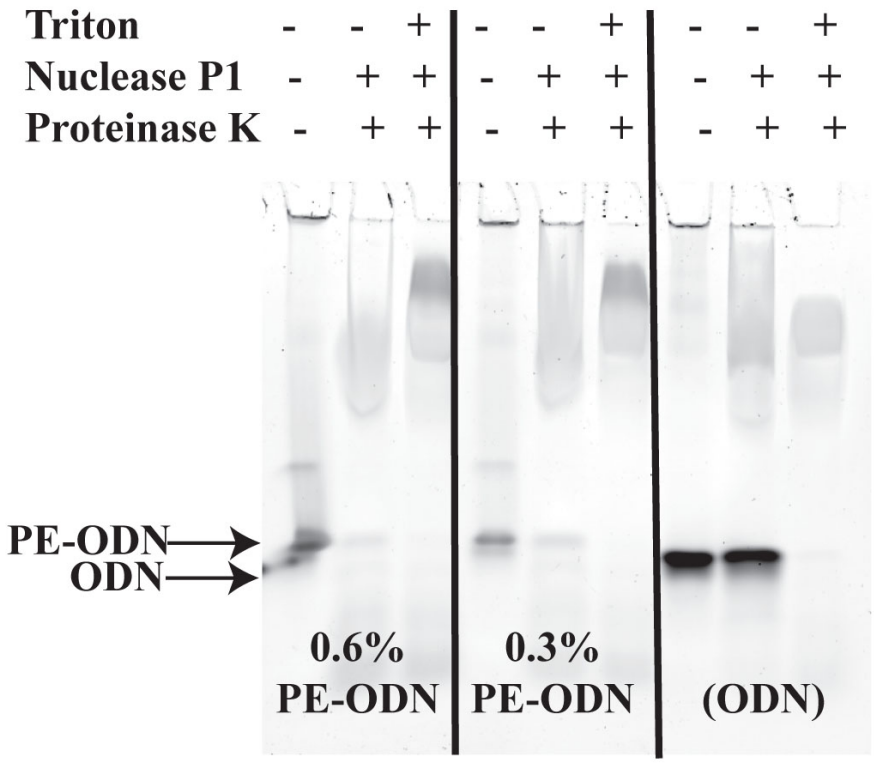


**Figure 3.5: Nuclease protection of ODN.**

Encapsulation and protection of ODN was demonstrated using a nuclease protection assay. Liposomes containing PE-ODNs or encapsulated ODN [encapsulation denoted by the parentheses in the figure caption] were incubated with nuclease P1 at 60°C for 30 min and quenched with proteinase K at 50°C for 2.5 h. Liposomes that were not treated with nuclease served as a reference for total ODN and liposomes treated with Triton X-100 (1%) served as a positive control for the nuclease.

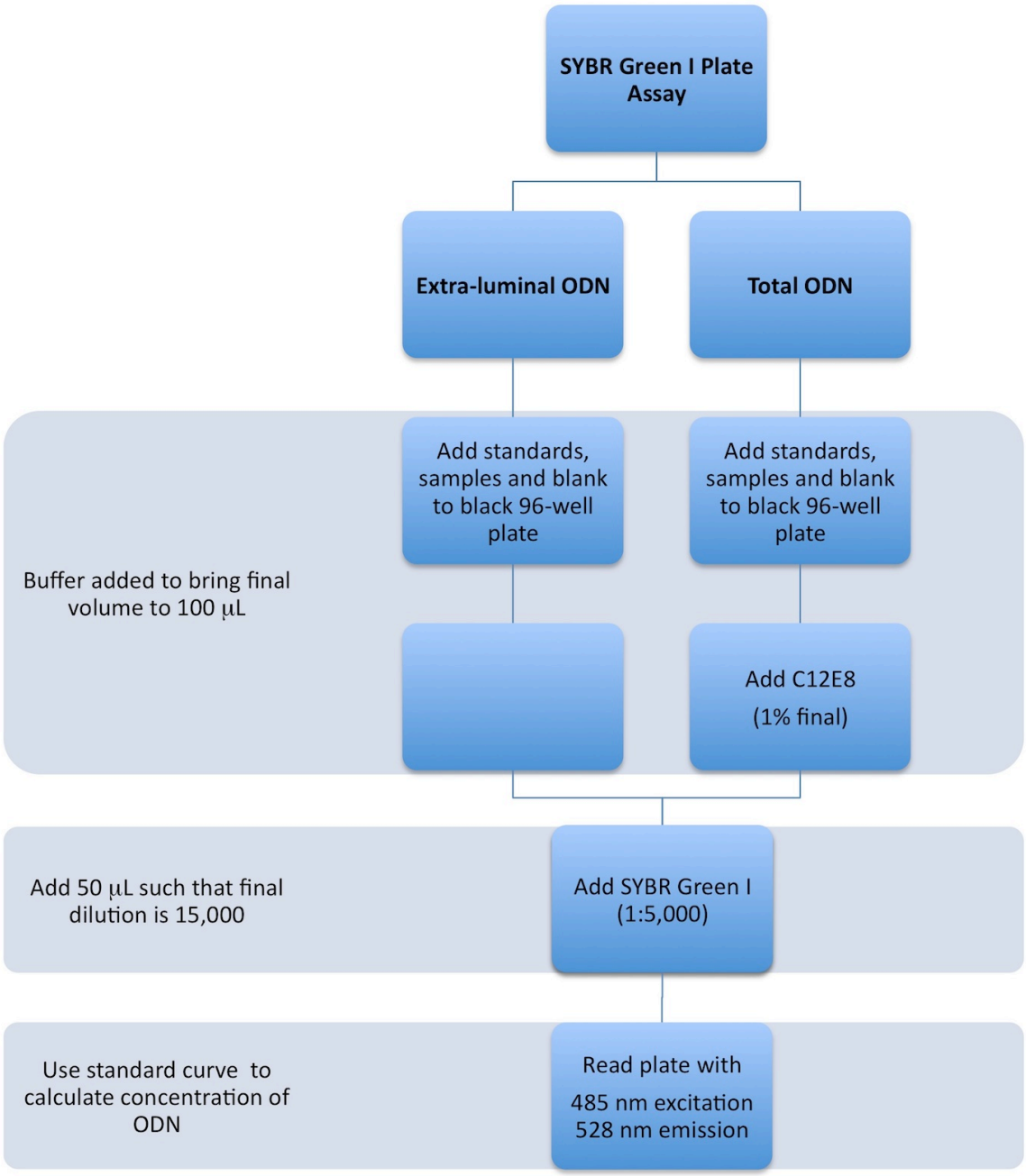


**Figure 3.6: Flowchart for SYBR Green I plate assay.**

Summary of method used to perform SYBR Green I plate assay.

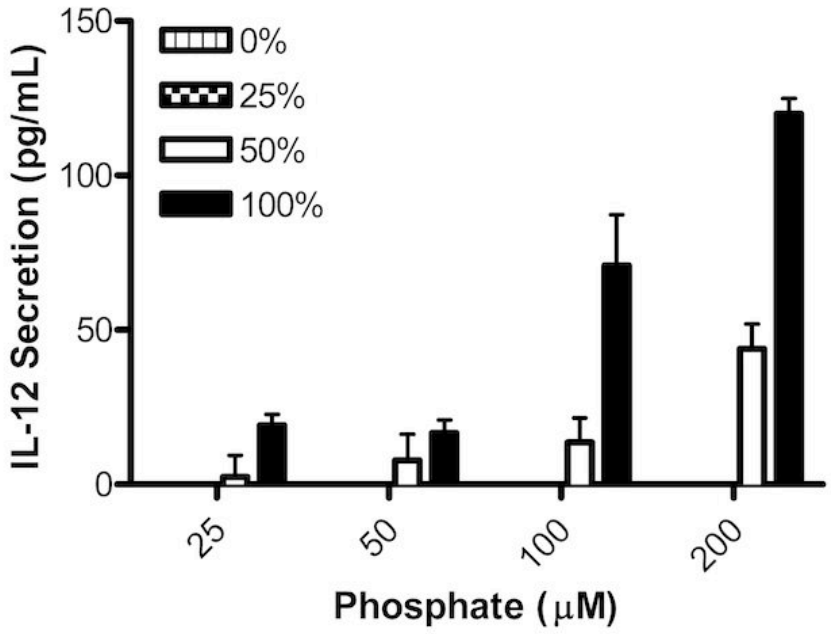


**Figure 3.7: IL-12 secretion is PE-CpG-ODN dose-dependent.**

The percentage of PE-CpG-ODN incorporated in liposomes was varied while maintaining total PE-ODN (PE-CpG-ODN plus PE-non-CpG-ODN) fixed at 0.3 mole percent of phospholipids. BMM were treated in serum-free media with serial dilutions of liposomes for 3 h, washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for secreted IL-12p40 by ELISA. Data represent the average of triplicates ± S.D.

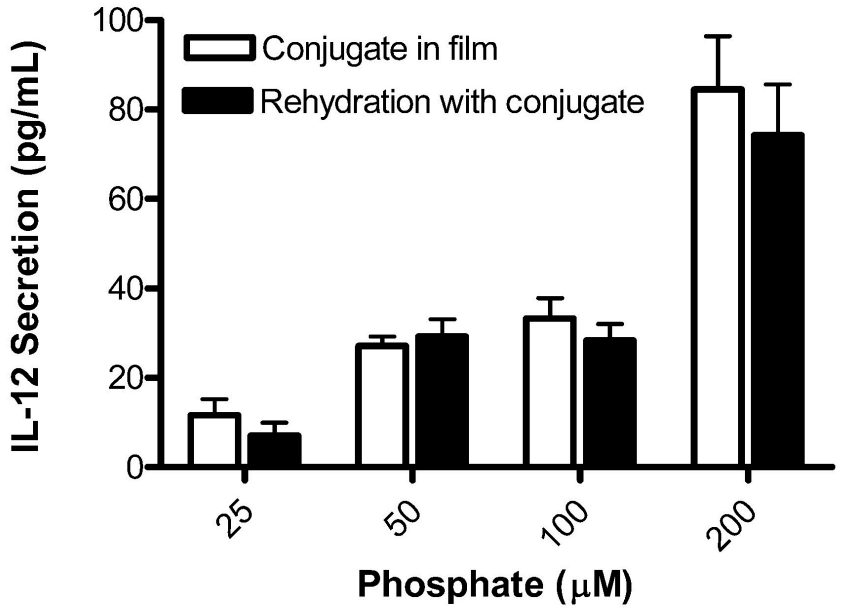


**Figure 3.8: IL-12 secretion comparing different methods for incorporating PE-CpG-ODNs in liposomes.**

BMM were treated with serial dilutions of liposomes for 3 h in serum-free media. The cells were washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for IL-12p40 secretion by ELISA. Data represent the average of triplicates ± S.D.

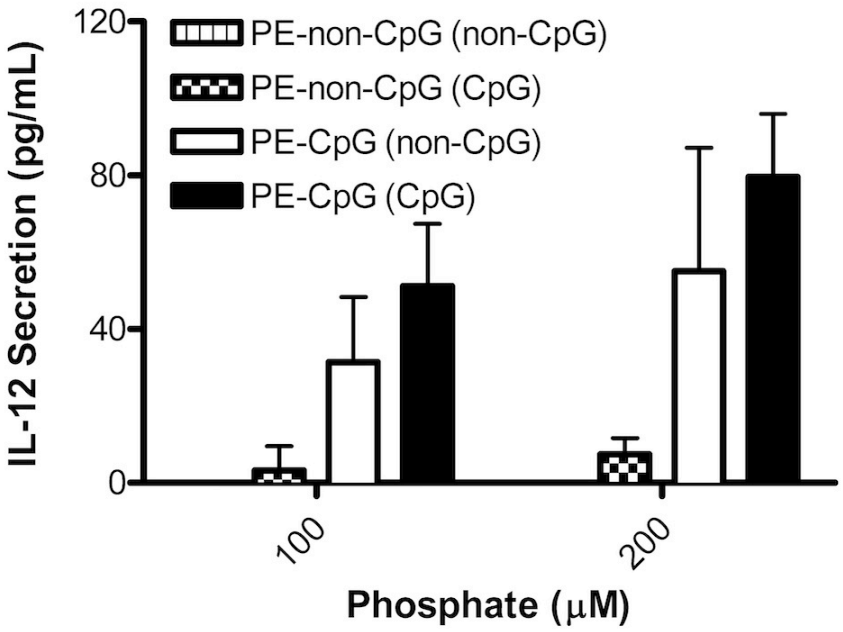


**Figure 3.9: Lipid-conjugated ODNs interact with TLR9 before encapsulated ODNs.**

Liposomes encapsulating either CpG-ODN or non-CpG-ODN [encapsulation denoted by the ODN type in parentheses in the figure caption] were made containing 0.3 mole percent PE-CpG-ODN or PE-non-CpG-ODN. BMM were treated in serum-free media with serial dilutions of liposomes for 3 h, washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for secreted IL-12p40 by ELISA. Data represent the average of triplicates ± S.D.

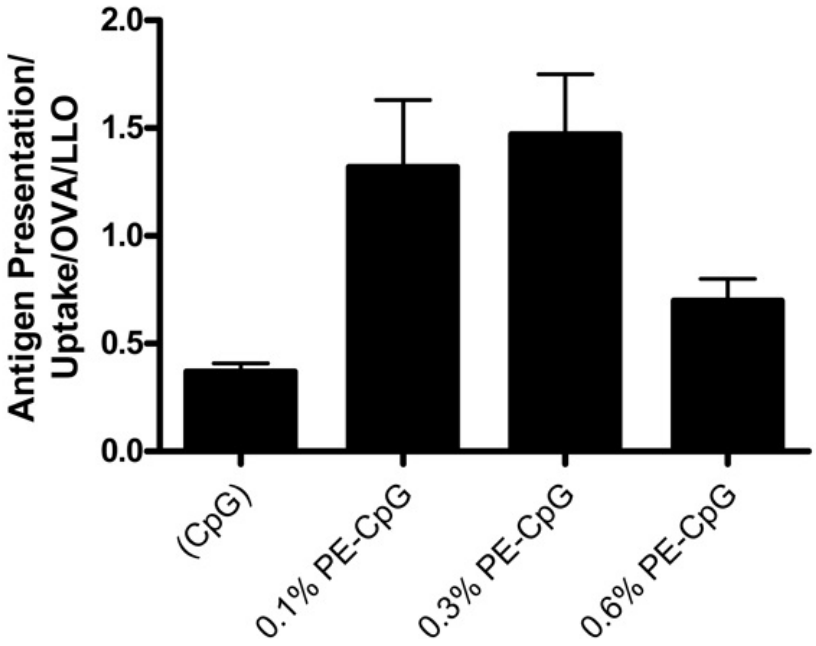


**Figure 3.10: PE-CpG does not inhibit cytosolic OVA delivery *in vitro*.**

BMM were pulsed with serial dilutions of liposomes for 2 h in serum-free media. The cells were washed and chased in complete media for 3 h. B3Z cells were added and incubated with BMM for 15 h. The level of presentation of SIINFEKL OVA-CD8 peptide by BMM to B3Z cells was monitored by the conversion of CPRG substrate to chlorophenol red in the activated B3Z cells. Antigen presentation values were monitored as absorbance at 570 nm and these values were normalized by the liposome uptake and OVA and LLO contents in the liposomes. Data represent the average of triplicates ± S.D.

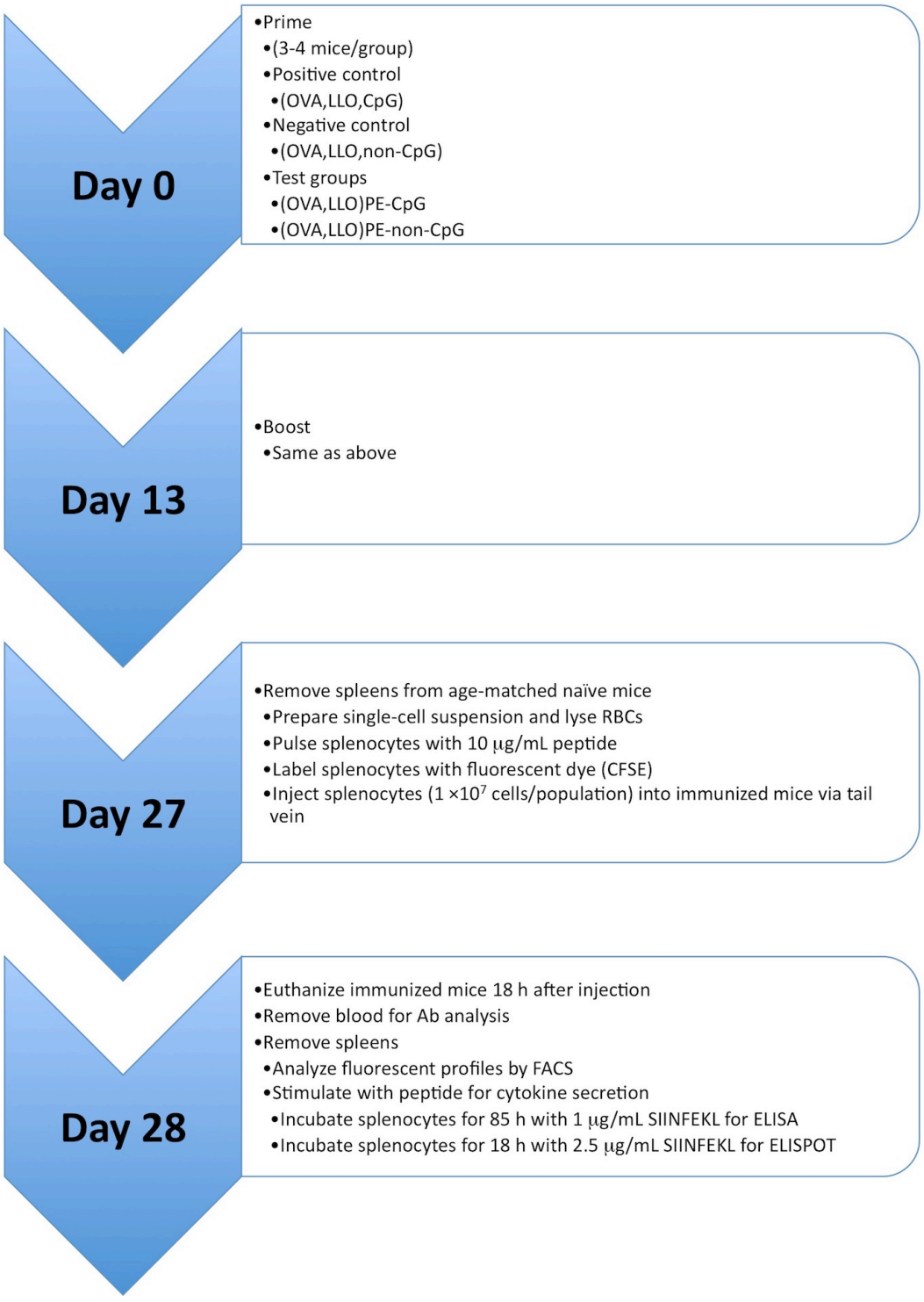


**Figure 3.11: Flowchart for immunization and assay conditions.**

For data shown in Figure 3.12 to Figure 3.14.

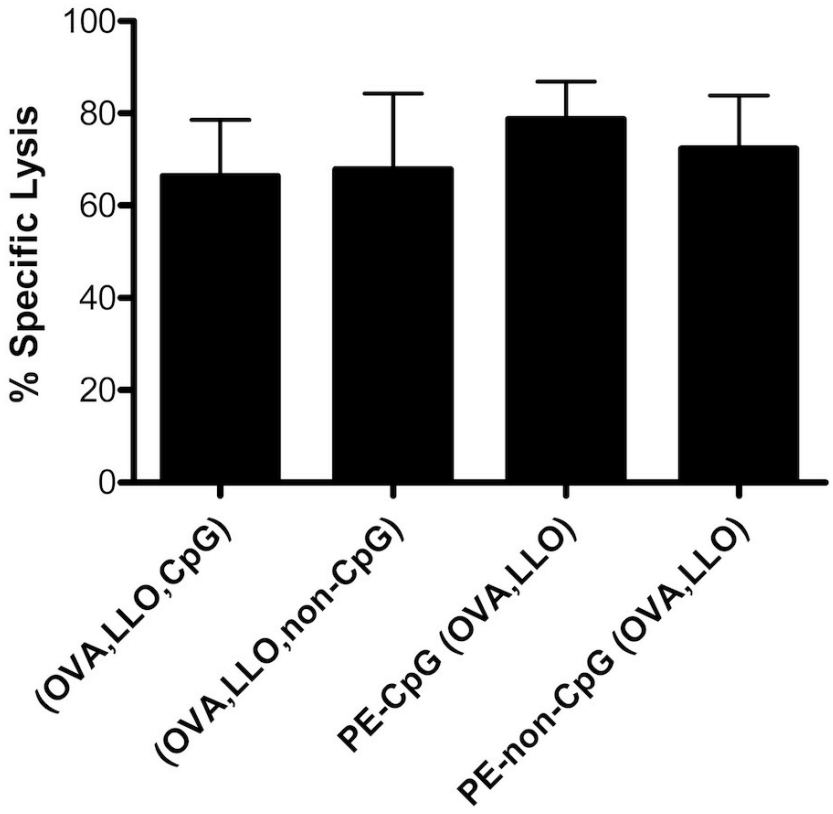


**Figure 3.12: PE-ODNs do not inhibit CTL activity compared with encapsulated ODNs.**

Mice were immunized subcutaneously on the hind flank on day 0 and day 13 with liposomes normalized to OVA content (8 μg). On day 27, target cells were prepared from naïve splenocytes. $CFSE^{HIGH}$ cells were pulsed with $OVA_{257-264}$ peptide (10 μg/mL) and $CFSE^{LOW}$ cells were pulsed with $NP_{147-155}$ peptide (10 μg/mL) as a control. The CFSE profiles were analyzed by FACS. The percentage of specific lysis was calculated by: 100 x [1-(ratio of cells recovered from naïve mouse/ratio of cells recovered from immunized mouse)]. The mean ± SEM is shown (n=3-4 mice per group).

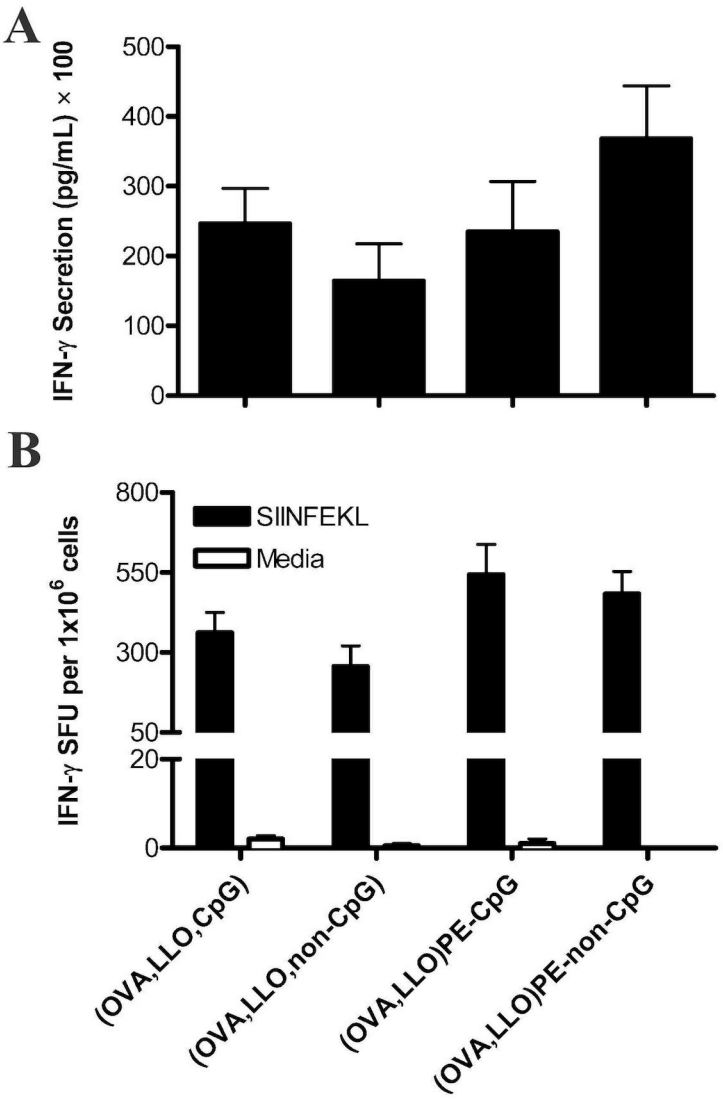


**Figure 3.13: Cellular immune response is not inhibited by PE-ODNs.**

Mice were immunized subcutaneously on day 0 and day 13 with liposomes normalized to OVA content (8 μg). (A) Splenocytes were isolated in day 28 and simulated with SIINFEKL (1 μg/mL) for 85 h and the resulting IFN-γ secretion was monitored by analyzing cell supernatant using ELISA. (B) IFN-γ spot forming units (SFU) were monitored by ELISPOT. Isolated splenocytes were stimulated with media or SIINFEKL (2.5 μg/mL) for 18 h. The mean ± SEM is shown (n=3-4 mice per group).

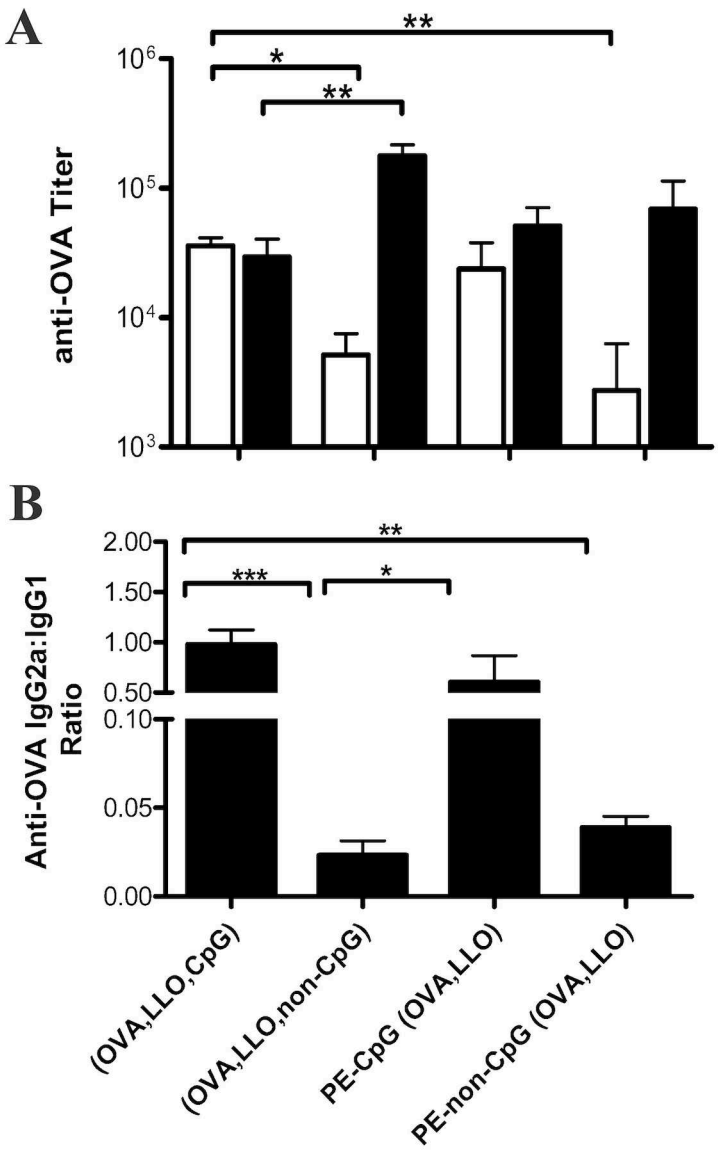


**Figure 3.14: PE-CpG-ODN elicits a Th1-type anti-OVA antibody response.**

Mice were immunized subcutaneously on day 0 and day 13 with liposomes normalized to OVA content (8 μg). Sera were obtained from blood of immunized mice harvested by cardiac puncture on day 28. (A) Anti-OVA IgG2a (open bars) and IgG1 (closed bars) were monitored using ELISA. (B) The IgG2a:IgG1 (Th1:Th2) ratio was calculated for each mouse. The mean ± SEM is shown (n=3-4 mice per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

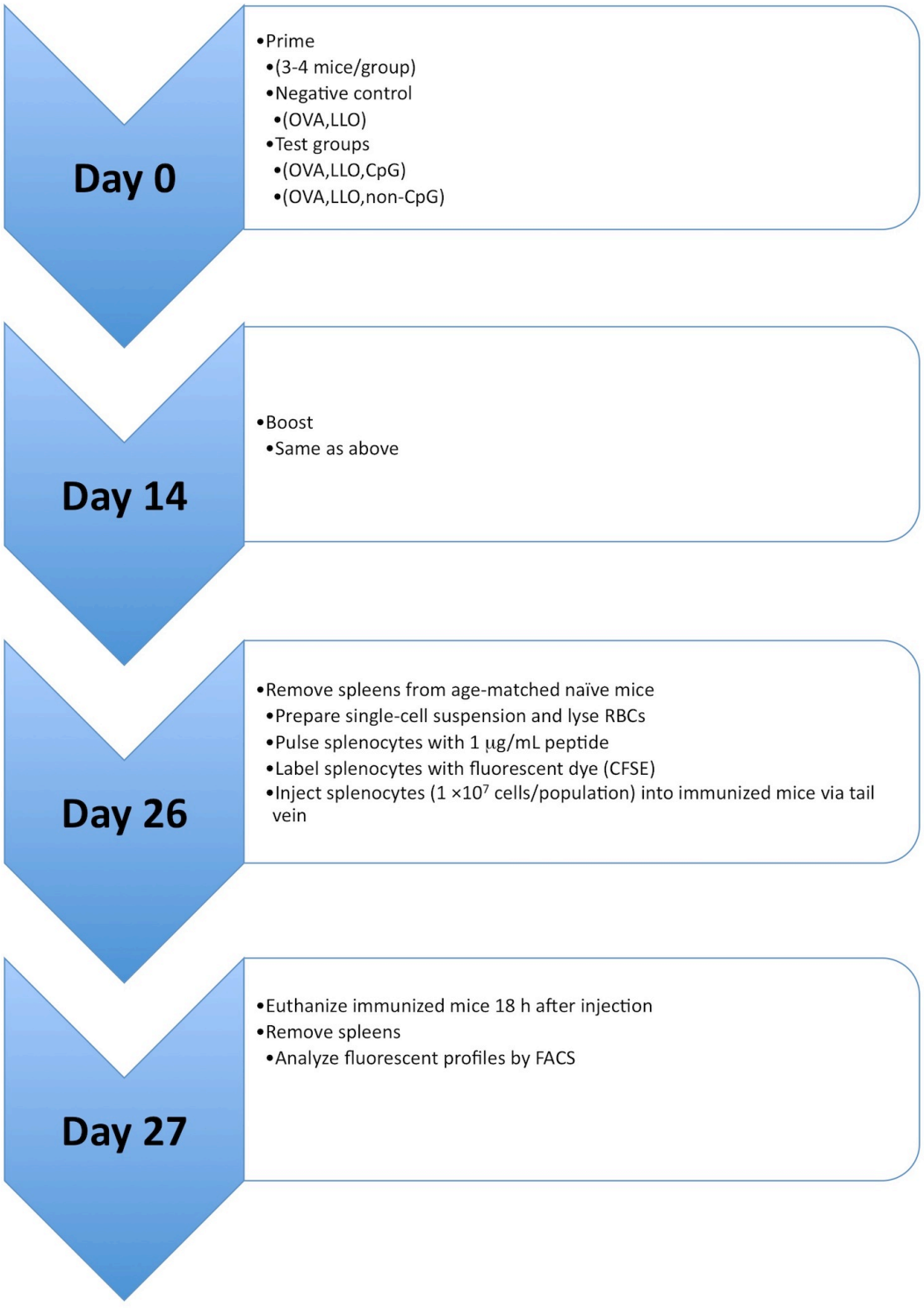


**Figure 3.15: Flowchart for immunization and assay conditions.**

For data shown in Figure 3.16.

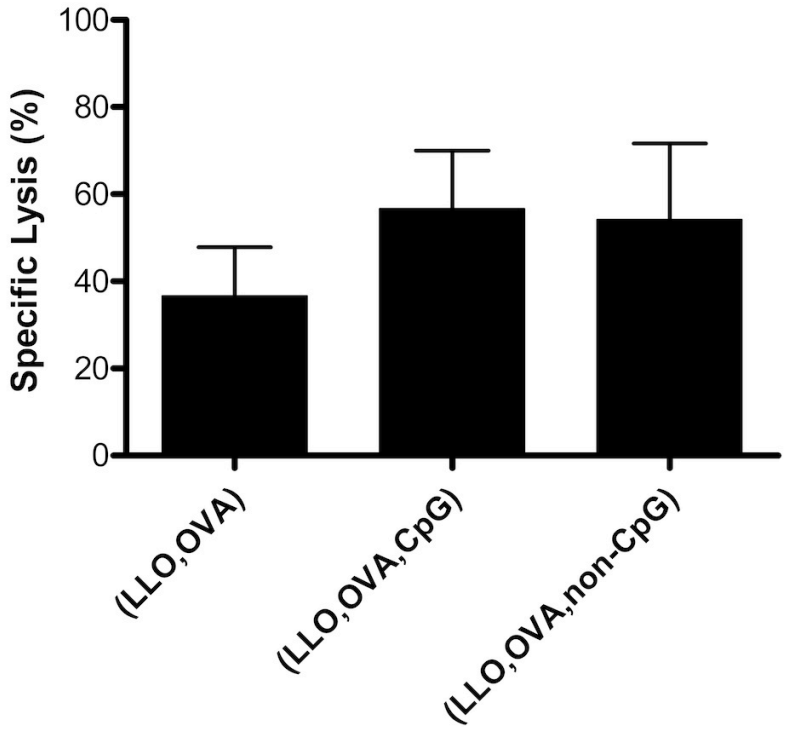


**Figure 3.16: Use of LLO- and OVA-containing liposomes as negative control in CTL assay.**

Mice were immunized subcutaneously on day 0 and day 14 with liposomes normalized to OVA content (8 μg). On day 26, target cells were prepared. $CFSE^{HIGH}$ cells were pulsed with $OVA_{257-264}$ peptide (1 μg/mL) and $CFSE^{LOW}$ cells were pulsed with $NP_{147-155}$ peptide (1 μg/mL) as a control. The CFSE profiles were analyzed by FACS 18 h after adoptive transfer. The mean ± SEM is shown (n=3-4 mice per group).

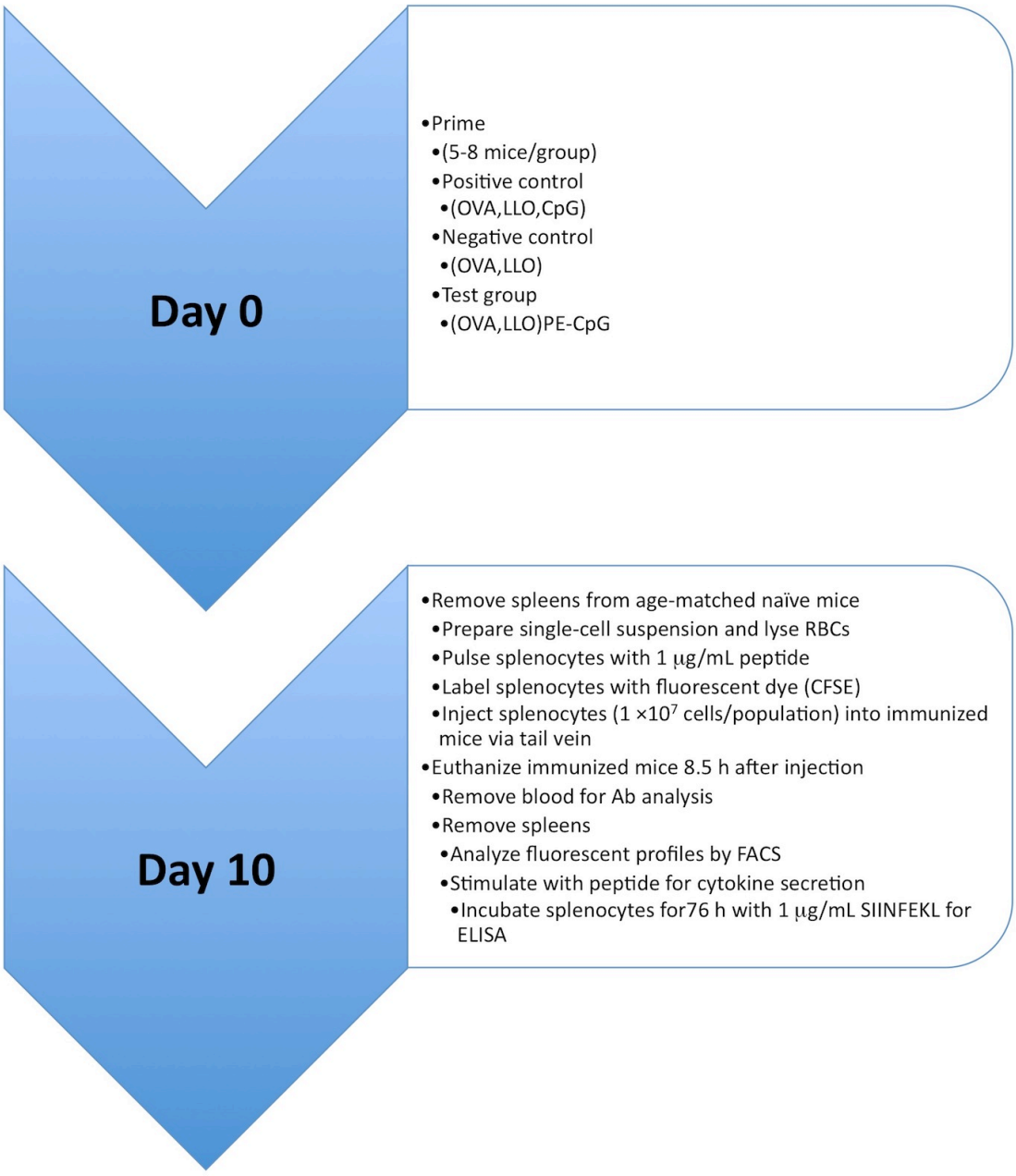


**Figure 3.17: Flowchart for immunization and assay conditions.**

For data shown in Figure 3.18 to Figure 3.19.

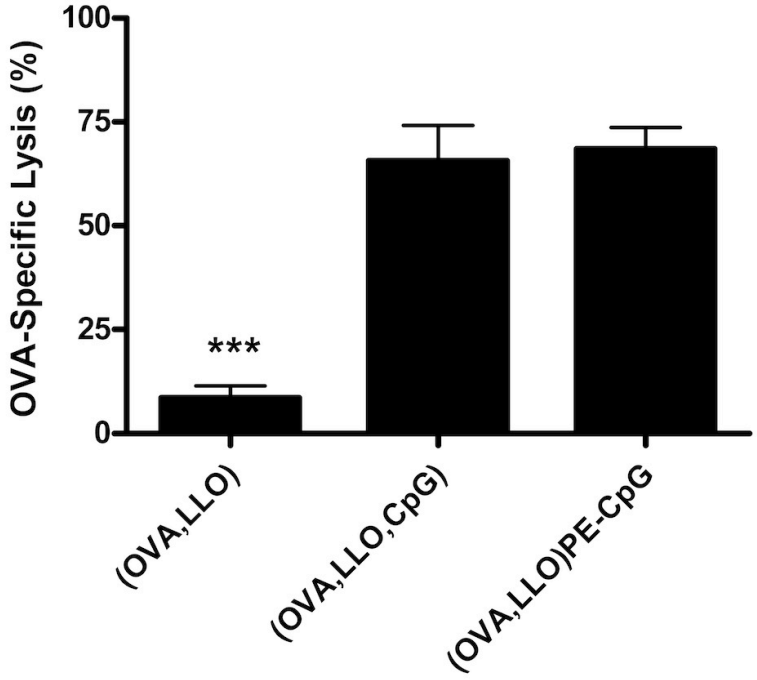


**Figure 3.18: Encapsulated and lipid-conjugated CpG-ODNs induce an OVA-specific CTL response.**

Mice were immunized subcutaneously on day 0 with liposomes containing OVA and LLO (8 μg OVA) or OVA, LLO and CpG-ODN (8 μg OVA and 3.8 μg CpG-ODN) or OVA and LLO with PE-CpG-ODN conjugates (8 μg OVA and 4.6 μg CpG-ODN). OVA-specific CTL activity was monitored *in vivo* on day 10 by lysis of intravenously injected $CFSE^{HIGH}$-labeled SIINFEKL-pulsed splenocytes in comparison to $CFSE^{LOW}$-labeled influenza NP peptide-pulsed splenocytes. The CFSE profiles were analyzed by FACS 8.5 h after adoptive transfer. The mean ± SEM is shown (n=5-7 mice per group). ***$p<0.001$

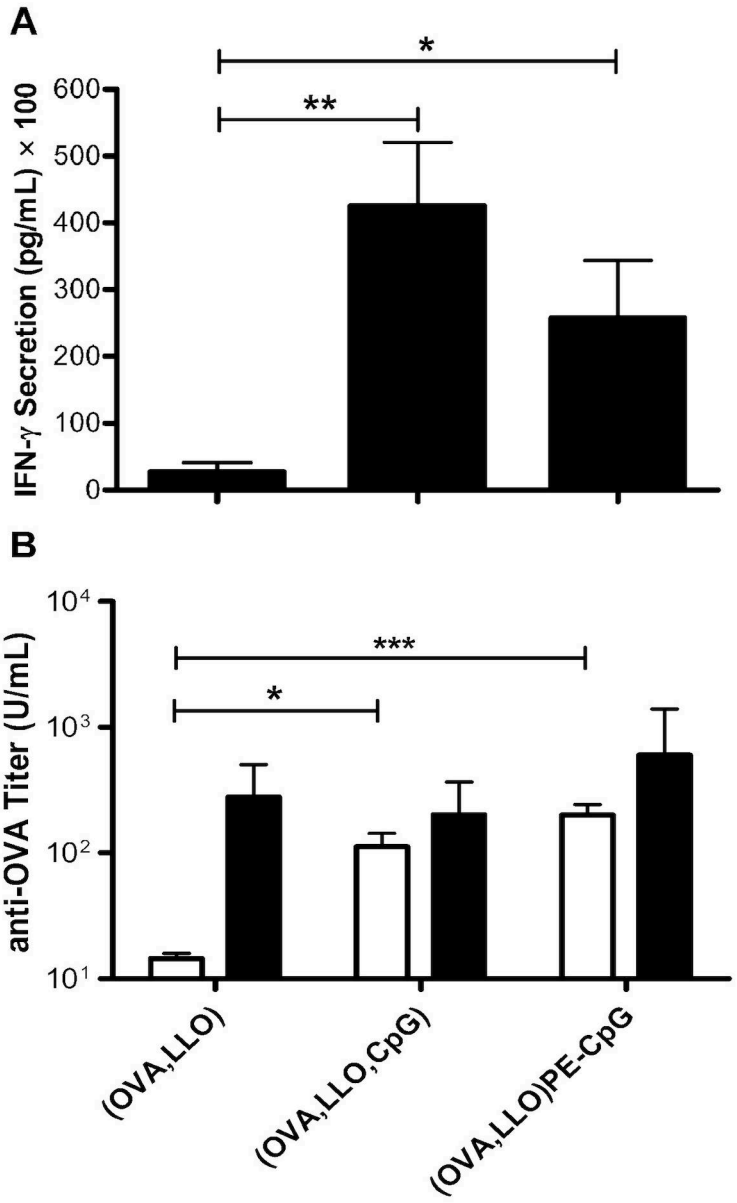


**Figure 3.19: PE-CpG-ODNs stimulate a Th1-type immune response similar to that of encapsulated CpG-ODNs.**

(A) Mice were immunized with liposome formulations on day 0, and spleens were harvested on day 10. Isolated splenocytes were stimulated with SIINFEKL for 76 h and the resulting IFN-γ secretion was monitored by ELISA. The mean ± SEM is shown. (B) Sera were obtained from blood of immunized mice harvested by cardiac puncture on day 10. Anti-OVA IgG2a (open bars) and IgG1 (closed bars) were monitored using ELISA. The geometric mean titer ± SEM is shown (n=7-8 mice per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

| Solvent | Temp | Result |
|---|---|---|
| Methanol | 70°C | Small particles floating |
| Methanol | 90°C | No visible particulates until sample cooled |
| Methanol:Tetrahydrofuran (1:1) | RT | Large amounts of white precipitate |
| Acetone | RT | Precipitate |
| Methanol:Acetone (1:1) | RT | Precipitate |
| Methanol:Acetone (1:1) | 55°C | Precipitate |
| Dimethylformamide (DMF) | RT | Precipitate |
| Methanol:DMF (3.3:1) | RT | No visible precipitate |

**Table 3.1: Solvents evaluated to solubilize PE-ODN.**

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# CHAPTER 4

# LIPOSOMES CONTAINING INFLUENZA NUCLEOPROTEIN, LISTERIOLYSIN O, AND CPG-OLIGONUCLEOTIDE STIMULATE NUCLEOPROTEIN-SPECIFIC CYTOTOXIC T LYMPHOCYTES AND TH1-TYPE RESPONSES

## SUMMARY

Current influenza vaccines are produced yearly based on predicted strain dominance to stimulate antibody production against viral envelope glycoproteins. These flu vaccines have reduced efficacy when the predicted strain antigens do not match the infectious strain. In order to generate a more broadly based influenza vaccine, our approach is to stimulate a cell-mediated immune response to the relatively conserved influenza nucleoprotein (NP) by using both a cytosolic targeting mechanism as well as a molecular adjuvant. This study evaluated listeriolysin O (LLO)-containing pH-sensitive liposomes as a delivery system for NP and monitored the adjuvant effect of unmethylated cytosine-phosphate-guanine (CpG)-containing oligodeoxyribonucleotide (ODN) incorporation. LLO-liposomes have been used previously, but never in the context of a clinically relevant antigen together with adjuvants. We hypothesized that our approach would simultaneously generate a cytotoxic T lymphocyte (CTL) response and favor a Th1-type response by delivering NP to the cytosol via LLO and stimulating Toll-like receptor 9 with CpG-ODN, respectively. Mice immunized with liposomal NP, LLO, and CpG-ODN exhibited a significantly stronger CTL response compared with liposomal NP-

immunized mice, and their splenocytes secreted the most IFN-γ and the least IL-5. This study indicates that LLO-containing pH-sensitive liposomes carrying NP and CpG-ODN can favor a Th1-type response and stimulate a CTL response to a clinically relevant antigen.

## INTRODUCTION

Influenza A is a single-stranded RNA virus that causes respiratory viral infections by gaining entry through the airway leading to an estimated 148,000 hospitalizations and 36,000 deaths in the United States each year (*1*). To minimize the severity of epidemics, two types of vaccines are currently used: (i) non-infectious preparations of detergent-disrupted virus particles or purified viral glycoproteins (hemagglutinin (HA) and neuraminidase (NA)) and (ii) live attenuated, cold-adapted vaccines. Both vaccines engender strong antibody responses to HA and NA. HA antibodies protect cells against virus infection, whereas NA antibodies decrease the amount of virus released from infected cells. These vaccines are highly effective (70-90%) in children and young adults when the glycoproteins of the influenza vaccine strain are closely related to those of the epidemic circulating strain (*2*). However, the low fidelity of the influenza virus polymerase permits the introduction of mutations in the distal HA epitopes of the circulating influenza strain, thus limiting vaccine effectiveness (*3*). Also, many influenza A virus strains co-circulate during a single season giving rise to the possibility for multiple viruses to infect a single cell, rearrange, and produce a virus subtype with new surface glycoproteins, resulting in the potential for a pandemic (*3*). This was last observed in the 2009 H1N1 pandemic, in which the H1N1 virus was genetically dissimilar to seasonal H1N1 strains but akin to viruses that circulate in pigs (*4*). The

2009 seasonal influenza vaccine was ineffective at protecting against the 2009 H1N1 pandemic due to the lack of cross-protective immunity, demonstrating the need for new strategies to supplement the current vaccines in order to limit morbidity in the absence of cross-reactive neutralizing antibodies.

One strategy for providing broader protection against the ever-changing influenza strain is to generate an influenza vaccine that elicits immunity to a more conserved viral protein instead of the continuously mutating epitopes of surface glycoproteins. Many of the conserved influenza proteins, however, reside within the virus, hence the need for stimulating cellular immunity rather than relying solely on antibodies like the current seasonal flu vaccine does. Our strategy is to generate immunity to nucleoprotein (NP), a highly conserved and internal viral protein that encapsidates the viral genome for purposes of viral transcription and packaging (*5*). This would more closely mimic natural influenza infection in which the humoral response is assisted by cytotoxic T lymphocytes (CTLs) that facilitate viral clearance by killing infected respiratory epithelia cells that present internal viral antigen peptides (*6*).

Evidence of CTL-mediated protection was demonstrated in animals following heterologous prime/challenge studies (*7*). CTLs generated in B cell-deficient mice upon immunization with sublethal doses of influenza virus promote recovery from a lethal secondary influenza infection, and cloned influenza-specific CTLs passively transfer protection (*8*). This protection was ablated upon depleting mice of $CD4^+$ and $CD8^+$ T cells during the challenge period, thus illustrating the potential of T cells to control infection (*9*). The literature strongly supports the utility of CTLs to prevent influenza-related mortality in mice, and there is epidemiological evidence that $CD8^+$ T cell-

mediated immunity is important in protection against influenza in humans. A retrospective study of adults with confirmed H1N1 influenza contracted between 1950-1957 were three times less likely to become infected with H2N2 influenza in 1957, indicating the involvement of the cellular immune response in preventing infection (*10*).

For the most efficient stimulation of CTL responses, the antigen must be present in the cytosol in order to gain access to the eukaryotic antigen presentation machinery. Thus many of the previous animal studies stimulating NP-specific CTLs have been performed using DNA due to its ability to produce cytosolic proteins (*11*). Previous studies have shown that DNA prime/recombinant adenoviral boost vaccinations using NP results in heterosubtypic immunity, reducing morbidity and mortality that is T cell-dependent, and is more potent than a DNA prime alone (*12*). Due to the low transfection efficiency associated with DNA vaccines and the problems coupled with using viral vectors in vaccination, we are using an alternate approach that relies on listeriolysin (LLO)-containing liposomes to deliver the NP antigen to the cytosol of antigen-presenting cells (APCs). LLO is the pore-forming hemolysin of the facultative intracellular bacterium *Listeria monocytogenes* (*Lm*) and exhibits optimal endosome-disrupting activity at pH 5.5 to promote *Lm's* phagolysosomal escape for cytosol invasion (*13*).

Cytosine-phosphate-guanine-containing oligodeoxyribonucleotides (CpG-ODN) are vaccine adjuvants that interact with Toll-like receptor 9 in the endosome to stimulate the immune system. CpG-ODNs have been tested in clinical trials as an adjuvant for the trivalent killed split influenza vaccine, Fluarix® (*14*). In patients with pre-existing anti-influenza antibodies, a significant enhancement in anti-hemagglutinin inhibition titer was

observed when Fluarix was administered with CpG-ODN, and when administering CpG-ODN with one-tenth of the standard Fluarix dose the antigen-specific IFN-γ responses from peripheral blood mononuclear cells (PBMCs) could be restored to those of the normal Fluarix dose (*14*). We hypothesized that incorporating CpG-ODNs in the liposomes would further stimulate the NP-specific cellular immune responses.

In this study, we demonstrate that mice immunized with NP-, LLO- and CpG-ODN-containing liposomes stimulate a more robust NP-specific CTL response than mice immunized with NP-liposomes. Similarly, splenocytes from mice immunized with NP-, LLO- and CpG-ODN-containing liposomes secrete the most IFN-γ when stimulated *ex vivo* and generate NP-specific IgG responses that are skewed to the Th1-type. Lastly, an alternative method of incorporating CpG-ODNs into the liposome delivery system via lipid-conjugation was evaluated. We demonstrate that CpG-ODNs on the liposome membrane in the lipid-conjugated form retain similar biological activities to those of encapsulated CpG-ODNs when studied with NP as the antigen.

## MATERIALS AND METHODS

### Reagents

Dynavax Technologies (Berkeley, CA) provided the CpG-ODN immunostimulatory sequence (ISS) 1018, influenza nucleoprotein (NP) used for immunizations and anti-NP monoclonal antibody (#10E1G7). CpG-ODN, ISS 1018 (5'-TGA CTG TGA ACG TTC GAG ATG A-3'), was synthesized with a nuclease-resistant phosphorothioate-modified backbone.

**NP expression for immunization**

NP used for immunization and splenocyte stimulation was cloned from A/Puerto Rico/8/34/Mount Sinai (H1N1, ATCC Catalog # VR-95) with the sequence optimized for *E. coli* expression by Coda Genomics (Laguna Hills, CA). NP was ligated into the *Nde*I and *Bam*HI cloning sites in the pET9a expression vector. *E. coli* strain BL21(λDE3) was transformed with the plasmid and induced with isopropyl β-D-thiogalactoside (IPTG). Following high-pressure cell disruption, the cell lysate was centrifuged and the supernatant was purified by cation exchange chromatography. A final buffer exchange was performed by ultrafiltration using a 30 kDa PES cut off membrane. The protein was stored at -80°C in 100 mM sodium phosphate, 150 mM sodium choride, pH 7.0.

**NP expression for ELISAs**

NP used for coating ELISA plates was cloned from A/Puerto Rico/8/34/Mount Sinai (a kind gift from Peter Palese). NP was subcloned using *Nde*I and *Xho*I restriction enzymes sites into the pET29b expression vector in frame with the polyhistidine tag. *E. coli* strain BL21(λDE3) was transformed with the plasmid and induced with IPTG. After a 6 h induction at 30°C, bacteria were pelleted for 20 min at 4000 × g at 4°C. Pellets were stored overnight at -20°C and then resuspended in 0.02× culture volume using wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 8), containing 2 mM PMSF, 2 U/mL DNase, and 50 U/mL RNase. Lysozyme was added at 1 mg/mL and solutions were rocked at RT for 1 hour. Insoluble debris was pelleted by centrifugation at 20,000 × g at 4°C for 1 h and then the soluble fraction was poured into a column containing Ni-NTA agarose (Qiagen), washed with 16× column volume (CV) of wash buffer and eluted with 4× CV wash buffer containing 400 mM imidazole. Purified

NP was dialyzed against HBSE pH 8.4 (10 mM HEPES containing 140 mM sodium chloride and 1 mM EDTA) at 4°C.

**LLO expression**

The *hly* gene encoding LLO was inserted into the bacterial expression plasmid pET29b with a polyhistidine (His6) tag. Recombinant LLO was purified from *E. coli* as described by Mandal et al., (*15*) with the following exceptions. LLO overexpression was induced in bacterial culture for 4-6 h with 1 mM IPTG, the cell pellet was collected and resuspended in wash buffer containing 2 mM PMSF and 1 mM 2-mercaptoethanol and then lysed using a French press. Lysate supernatant was adsorbed to Ni-NTA agarose and extensively washed with 200 bed volumes of wash buffer. His6-tagged LLO was eluted in wash buffer containing 400 mM imidazole and extensively dialyzed against HBSE pH 8.4 at 4°C. Protein purity and activity were assessed by SDS-PAGE and hemolysis assay, respectively, as previously described (*15*). LLO was stored in single-use aliquots at -80°C.

**PE-CpG-ODN conjugate preparation**

The disulfide precursor to 5'-sulfhydryl-CpG-ODN ISS 1018 was a gift from Dynavax Technologies. A 70 mM tris-(2-carboxyethyl) phosphine (TCEP; Pierce, Rockford, IL) solution was made in HBSE (140 mM sodium chloride buffered with 10 mM HEPES containing 1 mM EDTA) pH 7, and used at a five molar excess to reduce 5'-disulfide-CpG-ODN at 40°C for 2 h. Residual TCEP was removed using a PD-10 (GE Healthcare, Piscataway, NJ) and eluted in HBSE pH 6.5. Reduced 5'SH-CpG-ODN was used immediately or stored at -80°C until use. After chloroform was removed from 1,2-dioleoyol-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide]

(MPB-PE, Avanti Polar Lipids, Alabaster, AL) using rotary evaporation at < 10 mm Hg vacuum at RT, it was dissolved in ether. 5'SH-CpG-ODN in HBSE was added to the lipid at a 2:1 organic:aqueous ratio. An emulsion was formed by brief sonication, and the MPB-PE and 5'SH-CpG-ODN (10:1) reacted for 4-5 h while continuously vortexing at RT. Ether was removed using rotary evaporation, after which water was removed by lyophilization. Unreacted MPB-PE was removed from the PE-CpG-ODN through two chloroform washes (25 mL), and residual chloroform was removed by evaporation. Unconjugated 5'SH-CpG-ODN was removed from PE-CpG-ODN conjugates by Sepharose CL-4B size-exclusion chromatography (1 × 25 cm; GE Healthcare). Tris-Borate-EDTA (TBE) polyacrylamide (15%) gel electrophoresis (PAGE) stained with ethidium bromide was used to identify fractions containing PE-CpG-ODN, which were then combined, lyophilized, and the dried solid was dissolved in methanol:N-methyl-2-pyrrolidone (MeOH:NMP; 3.3:1). The amount of PE-CpG-ODN recovered was determined by measuring phosphate as described by Bartlett (*16*).

**Liposome preparation**

Egg phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) and cholesteryl hemisuccinate (Sigma) lipid stocks (2:1) were combined and solvent was removed using Buchi rotary evaporation at < 10 mm Hg vacuum at RT. PE-CpG-ODN dissolved in MeOH:NMP were incorporated in the lipid film at 0.1 mole percent, and solvent was removed by rotary evaporation at 45°C and < 10 mm Hg vacuum. Lipid films were rehydrated with HBSE pH 8.4 containing a combination of 475 μg NP, 100 μg LLO and/or 2 mg CpG-ODN. Liposomes were prepared as previously described (*17*). Briefly, liposomes were freeze/thawed five times followed by five one-minute sonication

bursts, and unencapsulated materials were separated from liposomes by CL-4B size exclusion chromatography.

**NP and LLO quantification**

NP was quantified by resolving standards with known amounts of NP and LLO in a 4-20% Tris-Glycine gel (Invitrogen) with 1X electrode buffer (250 mM Trizma base and 2 M glycine) with 0.1% SDS and then transferring the protein to a PVDF membrane (Immobilon-P 0.45 μm) using semi-dry transfer buffer (10 mM Trizma base, 4 mM glycine and 20% methanol) at 3.1 $mA/cm^2$. The membranes were blocked with 1% casein in PBS, washed with PBST (PBS + 0.05% Tween 20) and incubated with mouse anti-NP antibody (3.5 μg/mL) or rabbit anti-LLO polyclonal antibody in PBST containing 2% BSA. Blots were washed thrice with PBST and incubated with goat anti-mouse alkaline phosphatase (AP)-conjugated antibody (Bio-Rad, 1:2,000) or goat anti-rabbit AP-conjugated antibody (Sigma, 1:20,000) in PBST containing 2% BSA. Blots were washed and developed with ECF western blotting reagent (GE Healthcare). Alternatively, NP and LLO were resolved using a 4-12% Bis-Tris gel (Invitrogen) using MES running buffer (Invitrogen) and protein concentrations were determined by staining with Krypton (Pierce). For western blot or fluorescent protein staining, the bands were visualized with a Typhoon 9200 (GE Healthcare). Densitometry was performed using ImageQuant 5.2, and calculations were based on known concentrations of proteins run in the same gel to determine NP and LLO concentrations in the liposome formulations.

**ODN quantification**

ODN encapsulation efficiency was determined by generating a standard curve of free ODN or PE-CpG-ODN depending on the type of ODN to be quantified. All samples

and standards contained normalized lipid amounts and C12E8 (1% final, Sigma-Aldrich). SYBR Green I (Invitrogen) was added to the plate at a final dilution of 1:15,000 and the fluorescence quantified in a Synergy HT plate reader (BioTek Instruments, Winooski, VT) using an excitation of 485 nm and emission of 528 nm.

**Ectodomain of matrix protein 2 (M2e)**

M2e is the 23 amino acid ectodomain of the influenza A matrix protein 2. In these studies, we used an M2e peptide consisting of amino acids 2-17 (SLLTEVETPIRNEWGC). This peptide was a kind gift from Dynavax Technologies, and was dissolved in PBS (1.43 mg/mL) and stored in aliquots at -80°C until use. Liposomes were made as described above containing NP, LLO, CpG-ODN and M2e (15.1 or 100 μg).

***M2e quantification using PAGE***

In an attempt to quantify M2e, known amounts were resolved in a 4-12% Bis-Tris gel (Invitrogen) using MES Running buffer (Invitrogen) and visualized using a Typhoon 9200 after staining gels with either Krypton (Pierce) or SYPRO Ruby (Invitrogen) as per the manufacturer's instructions. More stringent fixing methods consisting of either 50% MeOH/10% acetic acid or 40% MeOH/10% trichloroacetic acid (TCA) for 30 min were attempted followed by either Krypton staining or Coomassie staining (0.25g Coomassie G-250 in 50% MeOH/10% acetic acid) for 1 h at RT, followed by destaining with 5% MeOH/7% acetic acid. To reduce the stain reaction time, a microwave procedure using the 50%MeOH/10% acetic acid fix for 2-3 min, followed by Coomassie staining for 2-3 min and destaining as above was also performed. Gels poured in-house consisting of a 10% running gel (10% 29:1 acrylamide:bisacrylamide, 10.5% glycerol, 0.0167%

ammonium persulfate, 0.033% TEMED in 1 M Tris-HCl, 0.1% SDS, pH 8.45) and 3.9% stacking gel (3.9% 29:1 acrylamide:bisacrylamide, 0.06% ammonium persulfate, 0.28% TEMED in 0.74 M Tris-HCl, 0.074% SDS, pH 8.45) were run with 1X Tris/Tricine/SDS Buffer (Bio-Rad), fixed with 40% MeOH/10% TCA for 30 min, stained with Krypton for 1 h, and destained as described above.

***M2e quantification using dot blot***

PVDF membranes were pre-wet in MeOH, soaked in semi-dry transfer buffer and secured in the dot-blot apparatus. M2e-containing samples were loaded onto the blot in either sample buffer (2% SDS, 10% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue in 50 mM Tris, pH 6.8) diluted in HBSE or in HBSE only. Membranes were blocked in 1% casein/PBS, washed thrice with PBST and incubated with anti-influenza A M2 monoclonal antibody (Thermo Scientific; 1 μg/mL) for 2 h at RT with rocking. After washing thrice, the membrane was incubated with goat anti-mouse alkaline phosphatase (AP)-conjugated antibody (Bio-Rad, 1:2,000) for at least 1 h at RT with rocking. Blots were washed and developed with ECF western blotting reagent and visualized using a Typhoon 9200.

**Immunization**

Female BALB/c mice (8-9 weeks old, Harlan; Indianapolis, IN) used in this study were handled according to Institutional Guidelines. Mice were primed on week 0 and boosted on week 2 subcutaneously at the base of the tail with liposome formulations normalized to NP (10-24 μg per mouse, held constant within experiment). Mice were euthanized on week 5, sera were collected via cardiac puncture and spleens were isolated

for assays. The data shown here is representative data from two studies performed with similar trends.

***In vivo* CTL assay**

The *in vivo* CTL assay was performed as previously described with the following modifications (*18*). Briefly, red blood cell (RBC)-lysed, single-cell spleen suspensions from naïve mice were pulsed at $2 \times 10^7$ cells/mL in mouse media (RPMI 1640 containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol) with either influenza $NP_{147-155}$ or SIINFEKL peptide (Anaspec) for 1 h at 37°C, 5% $CO_2$. The cells were washed and diluted to $1 \times 10^8$ cells/mL in PBS containing 0.1% FBS. The $NP_{147-155}$ peptide-pulsed and SIINFEKL peptide-pulsed populations were labeled with 4 and 0.4 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 10 min at RT. CFSE labeling was stopped by adding an equal volume of FBS and incubating on ice for 5 min. Cells were washed thrice with PBS containing 5% FBS, and an equal number of $NP_{147-155}$-pulsed target cells ($CFSE^{HIGH}$) and SIINFEKL-pulsed cells ($CFSE^{LOW}$) were mixed together and $1 \times 10^6$ cells of each peptide-pulsed population were injected intravenously via tail vein into naïve or immunized mice. Mice were euthanized 18 h post-adoptive transfer, spleens of recipient mice were harvested, and single cell suspensions were obtained by grinding spleens through a 70 μm mesh screen in a Petri dish containing mouse media. Splenocytes were washed, purified using Ficoll-Paque Premium (GE Healthcare) and analyzed by FACSCalibur. At least four thousand CFSE-labeled cells were analyzed for each mouse. The percentage of specific lysis for each mouse was calculated by: 100 ×

[1-(ratio of cells recovered from naïve mice/ratio of cells recovered from immunized mice)].

**Cytokine secretion assays**

After splenocytes from the CTL assay were purified by the Ficoll density gradient as described above, additional splenocytes were contaminating the pelleted RBC fraction. Therefore, this fraction was washed with mouse media, and RBCs were lysed with ACK lysis solution (Invitrogen) for 2-3 min. Splenocytes were washed, counted and $5 \times 10^5$ cells were stimulated with either $NP_{147-155}$ peptide (2.5 μg/mL) or NP protein (25 μg/mL) in duplicate for 72 h at 37°C, 5% $CO_2$. Supernatants were combined, and analysis for IFN-γ or IL-5 secretion was performed by ELISA as previously described (*17*). Maxisorp plates (Nunc) were coated at 4°C with IFN-γ or IL-5 (BD Bioscience; 2 μg/mL or 1 μg/mL, respectively) in coating buffer (0.1 M sodium phosphate pH 9.0). Plates were blocked (1% BSA in PBS), and the supernatants were diluted in dilution buffer (1% BSA in PBST) and added to the plates in duplicate. Biotin IFN-γ or biotin IL-5 (BD Bioscience, 1 μg/mL) was added to the appropriate plates in dilution buffer, followed by avidin-horse radish peroxidase (HRP, eBioscience) and detected with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). Sulfuric acid (2 N) was added to stop color development, and absorbance values were detected at 450 nm using Emax plate reader (Molecular Devices). IFN-γ secretion values were determined from a standard curve, and all IL-5 plates were normalized to a control absorbance value.

**Anti-NP Ig ELISA**

After mice were euthanized, cardiac puncture was performed to obtain blood, which was allowed to clot at 37°C for at least 1 h before it was placed at 4°C ON. Sera

were separated from RBCs by centrifugation at 1200 rcf for 15 min. Sera were collected and added to ELISA plates that were coated with NP (10 μg/mL) in coating buffer and blocked. Biotin goat anti-mouse IgG2a, biotin goat anti-mouse IgG1 (Santa Cruz Biotechnology, 0.1 μg/mL or 0.033 μg/mL, respectively) or biotin anti-mouse IgG whole molecule $F(ab)_2$' fragment (1:20,000; Sigma) was used as a secondary antibody and detected by avidin-HRP (eBioscience). Plates were developed, stopped and read as described above. The data were fit using the 4-parameter curve available in the Softmax Pro software, and anti-NP titers were calculated by solving the curve for the inverse dilution factor resulting in an absorbance value of 1.

**Anti-M2e Ig ELISA**

Serial dilutions of sera were added to ELISA plates that were coated with M2e (3-10 μg/mL) in coating buffer and blocked with PBS containing 1% BSA. Biotin anti-mouse IgG whole molecule $F(ab)_2$' fragment (1:20,000; Sigma) was detected with avidin-HRP (eBioscience) and developed as described above. The anti-influenza A M2 monoclonal antibody (Thermo Scientific; highest concentration 10 μg/mL) served as a positive control for this assay. The absorbance values were monitored at the lowest dilution, and an M2e-response was reported if the absorbance value was greater than two-times background.

***In vitro* NP antigen presentation assay**

BALB/c mice were immunized with NP (35 μg) emulsified in Complete Freud's Adjuvant (CFA) on day 0 and boosted with NP (50 μg) emulsified in Incomplete Freud's Adjuvant (IFA) on day 14. On day 20, BALB/c macrophages ($2 \times 10^5$ cells/well) were plated in a 96-well tissue culture treated plate in complete DMEM. On day 21, BMM

were washed in serum-free DMEM and then pulsed with liposomes containing NP and LLO in serum-free DMEM for 2 h. Cells were washed and chased in complete DMEM for 3 h, during which time spleens were removed from immunized mice (n=10). Spleens were ground into a single-cell suspension through a 70 μm screen in mouse media. RBCs were lysed using ACK lysis solution (1.5 mL/mouse), and splenocytes were counted. Following the BMM chase, BMM were fixed with 1% paraformaldehyde in PBS for 15 min on ice, and residual aldehydes were quenched with 0.2 M lysine for 20 min at RT. Splenocytes ($2 \times 10^5$ cells/well) were added to the plate and incubated for 72 h. Cell supernatant was harvested from the plate and analyzed for IFN-γ secretion by ELISA as described above.

**Statistical analyses**

Grubb's test was used to analyze the animal study data for outliers. Data from liposome-treated animals were compared by ANOVA and Tukey's post-test for data with homogeneous variance. In the case of non-homogeneous variance, the data were compared using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. A p-value < 0.05 was considered significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

## RESULTS

### NP-containing liposomes induce a CTL response

To evaluate the immunogenicity of the liposome formulations, BALB/c mice were subcutaneously primed on week 0 and boosted on week 2 with three liposomal vaccine formulations: (i) NP, (ii) NP with LLO, and (iii) NP with LLO and CpG-ODN (study outlined in Figure 4.1). On week 5, NP-specific CTL activity was monitored

using an *in vivo* CTL assay. CFSE-labeled target cells pulsed with $NP_{147-155}$ were mixed in equal ratios with control cells pulsed with $OVA_{257-264}$ and injected intravenously. Viability of the target cells in the spleen was examined 18 h after injection. $NP_{147-155}$-pulsed ($CFSE^{HIGH}$) and $OVA_{257-264}$-pulsed ($CFSE^{LOW}$) spleen cells were present at similar levels in the non-immunized mice (Figure 4.2). Mice immunized with liposomes containing NP, LLO and CpG-ODN induced an enhanced ($p<0.05$) NP-specific lysis compared with mice immunized with NP-containing liposomes, 39 ± 5% and 22 ± 3%, respectively (Figure 4.2). No significant difference in NP-specific lysis was observed between mice immunized with NP- and LLO-liposomes (25 ± 5%) and the other groups. The variability of the assay was 5 ± 2%, which corresponds to the NP-specific lysis in naïve mice (n=5).

**NP-liposomes containing LLO and CpG-ODN modulate a Th1-type cytokine response**

To evaluate NP-specific T cell immune responses, splenocytes were isolated from the immunized mice three weeks after the boost and stimulated *ex vivo* with $NP_{147-155}$ CD8 peptide. NP-specific IFN-γ secretion is an additional measure of cellular immunity, and the same trend was observed between the *in vivo* CTL data and the NP-specific IFN-γ secretion. Splenocytes from mice immunized with NP-liposomes containing LLO and CpG-ODN secreted higher ($p<0.01$) levels of IFN-γ than those of mice immunized with NP-containing liposomes (18,776 ± 4,903 and 2,500 ± 518 pg/mL, respectively) (Figure 4.3). In agreement with the *in vivo* CTL results, there was not a significant enhancement of IFN-γ secretion when LLO was added to the NP-liposome formulation (11,611 ± 2,926 pg/mL). IFN-γ secretion was not detectable in any group from splenocytes without

peptide stimulation (data not shown). Splenocytes from all groups exhibited a strong cytokine response when stimulated with concanavalin A (data not shown).

To monitor the Th2-type cytokine profile, splenocytes were also stimulated *ex vivo* with NP protein. As expected, the results for IL-5 secretion demonstrated an inverse trend compared with IFN-γ secretion (Figure 4.3). Adding CpG-ODN to the NP- and LLO-liposome formulation reduced ($p<0.05$) the amount of *ex vivo* IL-5 secretion compared with splenocytes from mice immunized with NP- or NP- and LLO-containing liposomes. Co-encapsulating CpG-ODN in the NP- and LLO-containing liposome formulation enhances the Th1-type cellular immune response and decreases the Th2-type response, as shown by IFN-γ and IL-5 secretion, respectively.

**Adding LLO to NP-liposomes does not reduce the total NP-specific IgG**

Adding LLO to the liposome formulation directs the NP antigen to the cytosol for proteasome processing, and to ensure that re-routing the NP does not abolish the NP-specific antibody response, we looked at the total NP-specific IgG. Serum samples were collected on week 5 and titrated on ELISA plates coated with recombinant NP. Detectable anti-NP antibodies were not found in the sera of the naïve mice (data not shown). Adding LLO to the NP-containing liposome formulation did not reduce the mean anti-NP IgG titer compared with NP-liposomes (Figure 4.4). However, adding CpG-ODN to the NP- and LLO-containing liposomes enhances the total NP-specific serum IgG compared to mice immunized with NP-liposomes ($p<0.01$). This is in agreement with previously published data; when adding CpG-ODN to formalin-inactivated influenza virus the influenza-specific IgG response is increased (*19*). Using

LLO to direct NP into the cytosol of APCs does not reduce the total anti-NP IgG response generated.

**NP- and LLO-containing liposomes co-encapsulating CpG-ODN stimulates a Th1-type antibody response**

The cytokine secretion results indicate that the NP-, LLO- and CpG-ODN-containing liposome formulation stimulates a Th1-type immune response. To further verify this response, sera from immunized mice were titrated for IgG1 (Th2-type) and IgG2a (Th1-type) NP-specific responses. The data show that comparing NP-liposomes with NP- and LLO-containing liposomes results in no difference in the geometric mean IgG1 and IgG2a NP titers (Figure 4.5). Adding CpG-ODN to the NP- and LLO-containing liposomes formulation resulted in a decreased IgG1 titer and an increased IgG2a titer. The IgG2a:IgG1 (Th1:Th2) ratios were compared between groups immunized with different formulations. Adding CpG-ODN to the NP- and LLO-containing liposome formulations enhanced ($p<0.001$ and $p<0.05$, respectively) the IgG2a:IgG1 ratio compared with the ratio from liposomal NP-immunized mice and liposomal NP- and LLO-immunized mice (Figure 4.5). These results are in good agreement with the cytokine secretion results that show that NP, LLO, and CpG-ODN formulation stimulated a strong Th1-type response, while suppressing the Th2-type response.

**Encapsulated or conjugated CpG-ODN stimulate similar NP-specific immune responses**

We have previously developed a method for incorporating CpG-ODNs onto the liposome membrane by conjugating a sulfhydryl-containing CpG-ODN to a maleimide-containing PE lipid (*20*). This method is efficient, resulting in increased recovery of

CpG-ODNs compared with the traditional passive encapsulation technique, and the PE-CpG-ODN conjugates demonstrated comparable biological properties compared with encapsulated CpG-ODN when using the model antigen ovalbumin (OVA) (*20*). In this study, we compare mice immunized with NP- and LLO-containing liposomes as a control to NP- and LLO-containing liposomes with CpG-ODN either encapsulated or conjugated (study outlined in Figure 4.6). The results indicate that mice immunized with PE-CpG-ODN stimulate a stronger ($p<0.01$) NP-specific CTL response than mice immunized with encapsulated CpG-ODN (67 ± 6% and 50 ± 3%, respectively) (Figure 4.7). Splenocytes from each mouse were also stimulated with NP protein to determine the amount of IFN-γ secretion *ex vivo*, and the results indicate that there were similar levels of IFN-γ secretion from both groups of mice, regardless of the method of CpG-ODN incorporation, encapsulation or conjugation ($1.4 \times 10^6 \pm 2 \times 10^5$ and $1.7 \times 10^6 \pm 1.1 \times 10^5$ pg/mL, respectively) (Figure 4.7). In both assays the response generated in mice immunized with liposomes containing NP and LLO with CpG-ODN either conjugated or encapsulated was enhanced over the response generated in mice immunized with liposomes containing only NP and LLO.

Enhancement ($p<0.001$ or $p<0.05$, respectively) in the anti-NP IgG2a response was observed when CpG-ODN was encapsulated or conjugated ($6.9 \times 10^6 \pm 1.5 \times 10^6$ and $3.3 \times 10^6 \pm 6.2 \times 10^5$, respectively) compared with mice immunized with liposomes containing only NP and LLO ($8.6 \times 10^4 \pm 9.2 \times 10^4$). The IgG1 responses were reduced ($p<0.01$) when mice were immunized with encapsulated CpG-ODN ($3.5 \times 10^5 \pm 6.8 \times 10^4$) compared with mice immunized with liposomes containing only NP and LLO ($2.7 \times 10^6 \pm 6.6 \times 10^5$). No differences in the anti-NP IgG1 titer were observed when CpG-

ODN was conjugated ($1.2 \times 10^6 \pm 1.9 \times 10^5$). Overall, these results support the previous data obtained when using PE-CpG-ODNs with LLO-liposomes and the model antigen OVA (*20*). Conjugating CpG-ODNs to PE lipid provides an efficient, alternative method for incorporating CpG-ODNs onto liposomes that retain biological activity as demonstrated with both OVA and influenza NP.

**Mutating $LLO_{91-99}$ epitope enhances NP presentation *in vitro***

Previous results from our lab indicate that adding LLO to pH-sensitive liposomes enhanced the CTL response to both the model antigen OVA as shown with a standard chromium assay (*15, 17*), and to LCMV NP, as shown by tetramer staining (*21*); however, in the current studies with influenza NP we have not demonstrated an LLO-mediated enhancement of CTL responses using the *in vivo* CTL assay. We hypothesized that the presentation of the $LLO_{91-99}$ epitope by the MHC class I $K^d$ subtype is competing with the presentation of $NP_{147-155}$ peptide on MHC class I molecules, effectively reducing the NP-specific CTL response when LLO is added to the vaccine delivery system. In order to address this possibility, we mutated the immunodominant epitope $LLO_{91-99}$ with the point mutation I99Y that was shown to increase the $k_{off}/k_{on}$ rate of the peptide and soluble MHC class I (*22*). Two other LLO mutants, K103G and RK89GG, which are outside the immunodominant epitope, were studied based on previous data that suggested mutating these amino acids could change the protease cleavage of the protein, resulting in peptides that are less efficiently loaded on the MHC class I molecule (*23*). Wild type (WT) LLO or LLO mutants were co-encapsulated in pH-sensitive liposomes with NP, and BMM were pulsed with these liposomes. NP-specific peptide presentation was quantified by monitoring IFN-γ secretion induced by the NP-specific splenocytes upon

recognition of NP peptides presented by BMM. The results were normalized to the amount of NP and LLO encapsulated in each liposome formulation, and the results indicate that the I99Y mutant enhances ($p<0.05$) the NP-specific peptide presentation *in vitro* by the BMM, indicating that the $LLO_{91-99}$ immunodominant epitope may be competing with the $NP_{147-155}$ epitope for MHC class I loading (Figure 4.8). There was no significant enhancement of NP-specific presentation when using either the K103G or RK89GG mutant compared with WT LLO. These results provide a potential explanation as to why LLO did not significantly enhance NP-specific CTL responses, which were observed when LLO was added to liposomes containing either OVA or LCMV NP.

**Co-encapsulating M2e in NP-, LLO-, and CpG-ODN-containing liposomes does not stimulate a robust anti-M2e humoral response**

Initially, M2e (15.1 μg) and influenza NP were co-encapsulated in the liposomes at an equimolar ratio. Because this amount of M2e was below the limit of detection, it was increased to 100 μg for all experiments described here. Various combinations of gel types and fixing/staining methods were attempted to quantify M2e as outlined in the Materials and Methods; however, these assays lacked adequate sensitivity for detecting the peptide. Using a dot blot, we determined that adding the peptide to the membrane in the presence of lipid increased the sensitivity of the assay; however, the lack of linearity of the standard curve in this case prohibited its use in quantification.

Without quantifying M2e, we proceeded with immunizing one group of mice with liposomes co-encapsulating NP, LLO, CpG-ODN and M2e (24 μg NP). The anti-NP immune response generated by these mice, as demonstrated by the *in vivo* CTL, cytokine secretion and anti-NP IgG isotype responses assays, were all similar between mice immunized with liposomes containing NP, LLO, and CpG-ODN with or without M2e.

However, when analyzing the sera from these mice for anti-M2e total IgG response, only two of the five mice from the group immunized with NP-, LLO-, CpG-ODN-, and M2e-liposomes generated a weak anti-M2e antibody response, barely above background. These data indicated that co-encapsulating M2e in the NP-, LLO-, and CpG-ODN-liposomes at the amounts described above is not sufficient for stimulating a humoral response to M2e.

**DISCUSSION**

The recent emergence of a pandemic influenza virus has highlighted the need for influenza vaccines that provide broad coverage across subtypes. Currently, protection is afforded against influenza infection by a vaccine that stimulates the immune response to generate antibodies to the constantly mutating surface glycoproteins. We explored a vaccination strategy that closely mimics influenza infection by delivering NP to the cytosol of cells and priming the immune response toward a Th1-type profile. In this study, we monitored the ability of LLO-liposomes to deliver NP antigen and CpG-ODN and generate antigen-specific cellular and humoral immunity in mice. The results demonstrate that vaccinating mice with liposomes containing NP, LLO and CpG-ODN successfully induce NP-specific CTL responses (Figure 4.2), IFN-γ producing cells (Figure 4.3) and skews the NP-specific immune response to the Th1-type (Figure 4.5).

Conventional vaccines that consist of either inactivated viruses or soluble proteins are inefficient at inducing CTL responses. Alternatively, DNA vaccines have been investigated for their ability stimulate cellular immunity when CTL responses are desired for protection. NP DNA vaccination can protect mice against low-dose influenza challenges (*24*), while boosting with a recombinant adenovirus expressing NP results in

more effective protection (*12*). We previously demonstrated the utility of co-encapsulating antigen in LLO-liposomes in stimulating strong cellular immune responses as compared with DNA-based vaccination (*18*). In this case, a non-viral vector, liposome-entrapped polycation-condensed DNA delivery system (LPDII), was utilized to enhance DNA transfection efficiency. In a prime-boost regimen, mice primed with either protein antigen encapsulated in LLO-liposomes or LPDII encapsulating LLO stimulate comparable OVA-specific cellular immune responses, which are enhanced compared to mice immunized with the LPDII formulation (*18*).

The protection afforded by NP DNA vaccines was attributed to the CTL response as well as the Th1-type cytokine secretion (*25*). Specialization of the immune response (Th1 or Th2) influences how the immune system combats a pathogen. Th1-type responses are associated with high levels of IFN-γ-producing CTLs, complement-dependent antibodies, and activation of phagocytic cells, which are desired responses for fighting intracellular pathogens such as influenza. Antiviral cytokines such as IL-12, IFN-γ and TNF-α act early in infection to inhibit viral growth and spread, while ultimately aiding in viral killing. Synthetic CpG-ODNs can mimic bacterial and plasmid CpG-DNAs in their capacity for Th1-type immune skewing. Adding CpG-ODNs to influenza vaccines has been explored experimentally and clinically as a means to enhance the antibody response for stronger hemagglutinin inhibition activity (*14, 19*). CpG-ODNs have been used with a variety of viral antigens to stimulate cellular immunity (*26, 27*). We have previously demonstrated that co-encapsulating CpG-ODNs in LLO-liposomes skew the antigen-specific immune response to that of the Th1-type for the model antigen OVA (*17*), and here we observe similar results using the clinically relevant

antigen influenza NP. We also developed an alternative, more efficient method of incorporating CpG-ODNs in the delivery system that relies on conjugating sulfhydryl-containing CpG-ODNs to maleimide-containing lipids. We demonstrated that this method of adding CpG-ODNs into LLO-liposomes stimulates a more robust NP-specific CTL response and similar anti-NP IgG isotype titers compared with encapsulated CpG-ODNs. These data indicate the potential of CpG-ODN conjugation as an alternative method of CpG-ODN inclusion in this vaccine delivery system that has advantages over co-encapsulation.

In the face of an emerging influenza pandemic, cross-reactive CTLs have the potential to promote an enhanced recovery, thereby ameliorating disease. However, the propensity for the virus to undergo mutational escape resulting in a loss of T cell recognition is possibly a substantial issues for vaccine design. Nevertheless, some viral variants generate cross-reactive T cell responses, as demonstrated by a recent study that indicates that between the 1918 "Spanish Flu", the 2009 H1N1 "swine flu" and the seasonal isolates for the past nine decades, there are ~20 different immunodominant NP peptide sequences, but only two distinct T cell subsets are required for cross-reactivity to all of these sequences (*28*). This is a promising finding for vaccine design, indicating that a limited number of influenza NP T cell epitopes may provide cross-reactivity between the majority of influenza isolates (seasonal and pandemic) dating back to 1918.

Recent experiments have demonstrated an enhanced protection when generating immune responses to numerous relatively conserved influenza antigens (*29, 30*). Our data indicate that we can stimulate both a strong cellular response as well as anti-NP antibodies; therefore, we co-encapsulated M2e in the NP-, LLO-, and CpG-ODN-

containing liposomes to try to stimulate a similarly strong anti-M2e antibody response. However, the results here indicate that the majority of the mice were not capable of stimulating an M2e-specific humoral immune response above background levels. The inherently weak immunogenic characteristics of M2e have been improved by conjugating the peptide to carrier proteins (*31, 32*). Vaxinnate has also been successful in stimulating protective immune responses by conjugating four tandem copies of the M2e peptide to TLR agonists (*33*). Future work aiming to stimulate an anti-M2e antibody response using the LLO-liposome system should consider conjugating M2e to the liposome surface, generating an NP and M2e recombinant protein (potential with tandem repeats of M2e), and/or conjugating tandem repeats of M2e to CpG-ODNs.

Using a vaccine delivery system with recombinantly produced antigens is advantageous from a manufacturing standpoint. To date, the majority of inactivated influenza vaccines are prepared by propagating the influenza viruses in the allantoic cavity of embryonated chicken eggs (*34*). The egg-based system has inherent weaknesses that may limit the vaccine supply, including (i) the potential for disease in layer flocks interrupting the egg supply chain, (ii) the limitations associated with manufacturing expansion, and (iii) the prospect for poor growth of some reassorted vaccine strains. Furthermore, the processing of virally infected fluids possesses many potential safety concerns (*34*). These manufacturing problems become exacerbated during pandemics because the egg-based seasonal influenza vaccines require nearly year-round processing; pandemic preparedness therefore requires the reallocation of seasonal influenza vaccine manufacturing resources (*35*). The 2009 H1N1 pandemic demonstrated that the production and distribution of an antigenically matched vaccine is

possible, but the availability of a sufficient supply was delayed for several months before the vaccine was publicly available, and based on production capacity, several years were needed to produce enough pandemic vaccine to immunize the world's population (*4*). A vaccine that conveys cross-strain protection has the potential to reduce mortality during the vaccine manufacturing period. Furthermore, cross-strain protection could reduce mortality when the seasonal vaccine does not closely match the circulating seasonal strain. Vaccine production is also limited by the requirement of one to two eggs for each dose; therefore, mammalian tissue culture expression of influenza antigens was originally explored as an attractive alternative that also retains protein glycosylation (*36*). However, recently it was demonstrated that the glycosylation of HA was not required to stimulate antibody responses, allowing these proteins to be generated using bacterial expression which was estimated to produce 1000-fold protein than the egg-based system (*35*).

NP-, LLO- and CpG-ODN-containing liposomes stimulate a robust NP-specific immune response, but no enhancement in antigen-specific CTL or IFN-γ secretion was observed upon the addition of LLO to NP-containing liposomes, which was surprising based on previous data (*15, 17, 21*). The absence of an LLO response may be attributed to using influenza NP as the antigen or utilizing different test methods to monitor CTL response. We hypothesized that competition between the LLO and influenza NP immunodominant peptides for binding to the MHC class I $H2K^d$ molecules may prevent an enhancement of NP-specific CTLs when LLO is added to the formulation. In keeping with this hypothesis, we mutated LLO in an attempt to reduce LLO peptide binding to the MHC class I molecule. The LLO mutants retained hemolytic activity (data not shown),

and when co-encapsulated with NP in liposomes one LLO mutant, I99Y, results in enhanced NP-peptide presentation compared with WT LLO. Future work should be performed to (i) evaluate this NP- and LLO-liposome delivery system in crossbred mice that are more representative of the human population and (ii) evaluate the I99Y mutant LLO *in vivo*.

## CONCLUSIONS

We have demonstrated the utility of our pH-sensitive LLO-containing liposomes as a vaccine carrier for influenza NP and CpG-ODNs. These liposomes package vaccine antigens and deliver them to the cytosolic pathway of antigen presentation while providing the proper co-stimulation to stimulate a robust CTL response. We demonstrate the ability to stimulate a strong NP-specific CTL and Th1-type immune response while retaining antibody-stimulating abilities indicating the potential for this vaccine delivery system to be utilized with a combination of influenza antigens.

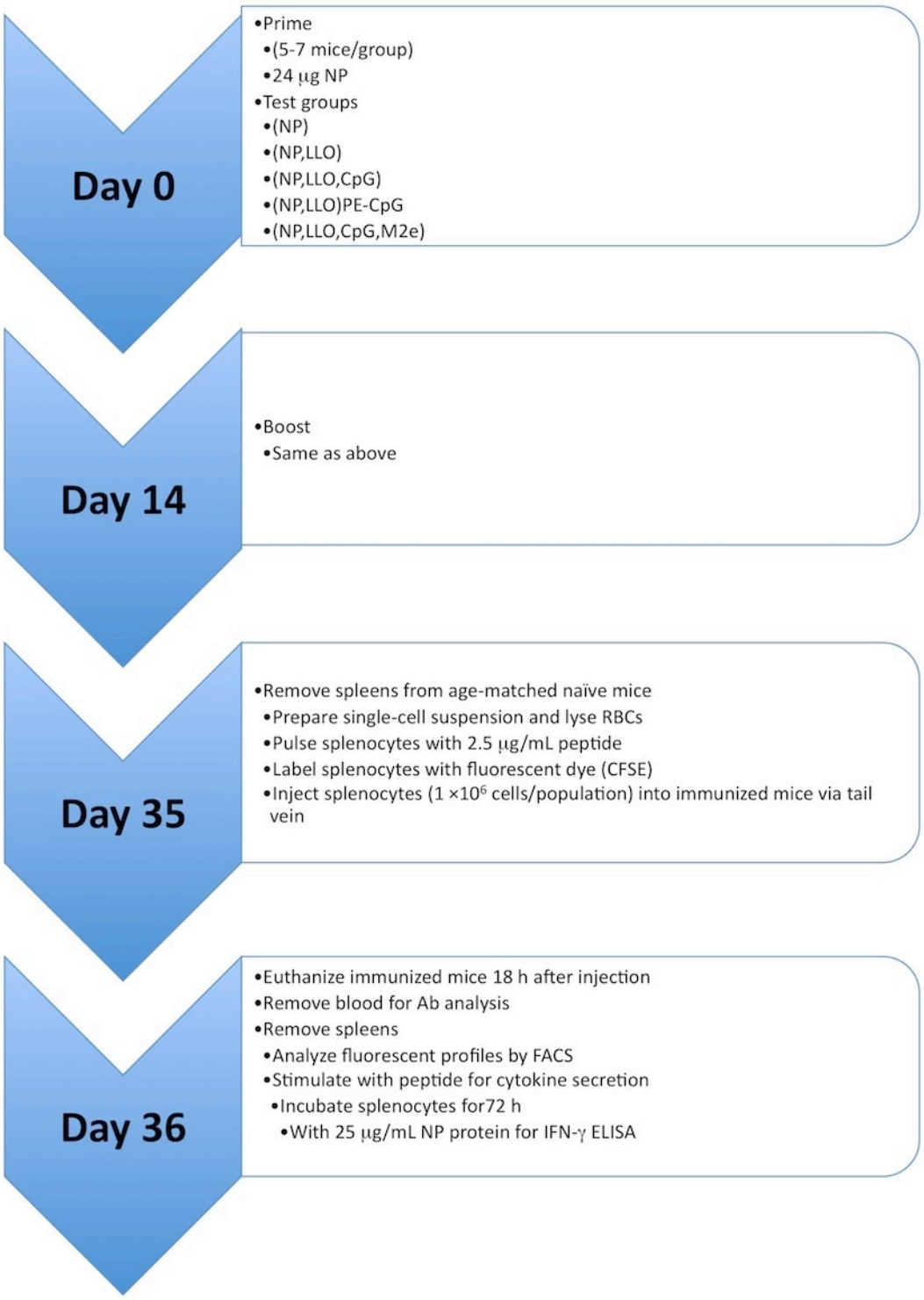


**Figure 4.1: Flowchart for immunization and assay conditions.**

For data shown in Figure 4.2 to Figure 4.5.

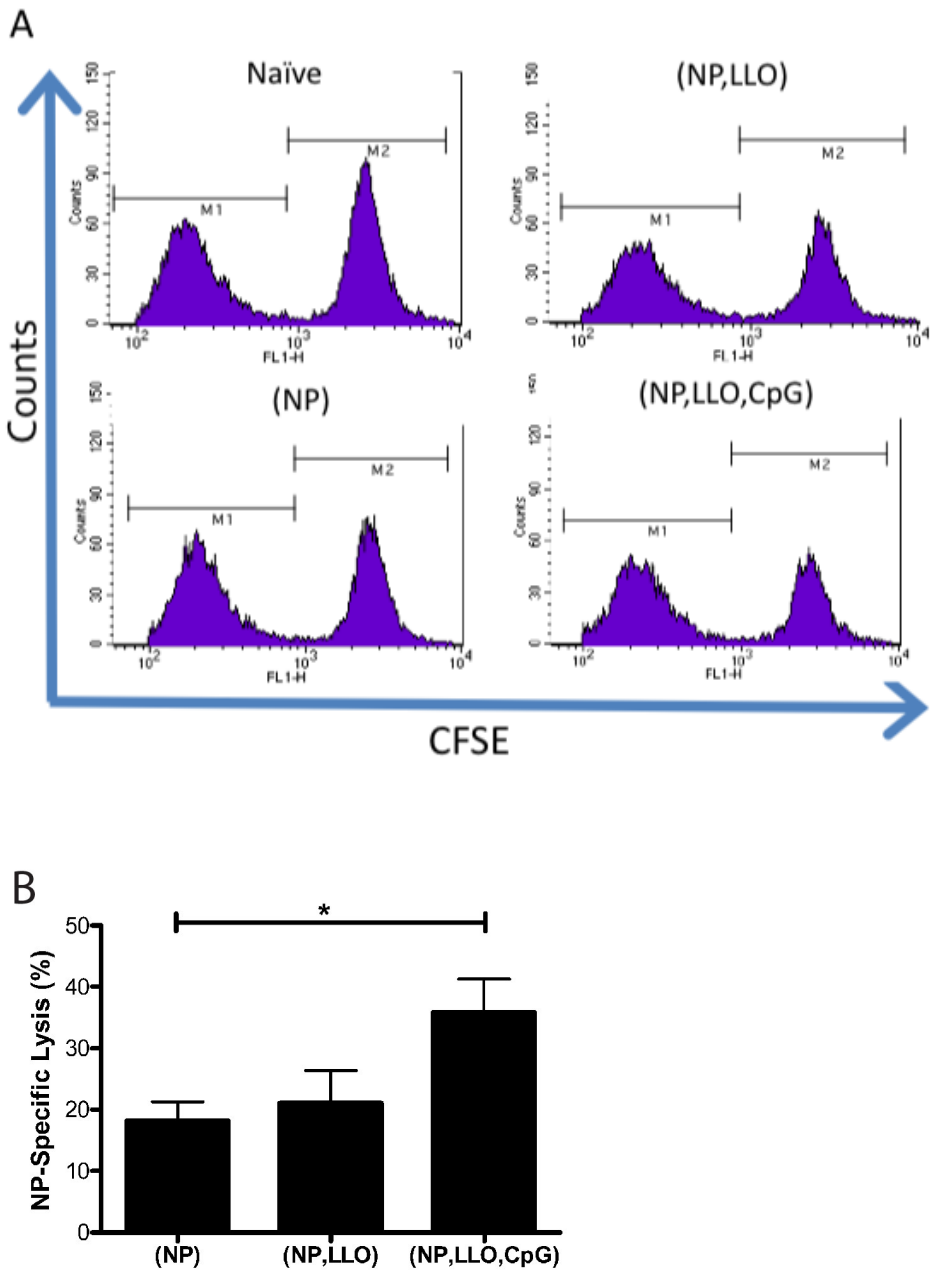


**Figure 4.2: CTL responses in mice immunized with NP-liposomes.**

Mice (n=5-6) were primed on day 0 and boosted on day 14 subcutaneously at the base of the tail with liposome formulations normalized to NP amount (10 μg per mouse). On day 35, target cells were prepared. $CFSE^{HIGH}$ cells (M2) were pulsed with influenza $NP_{147-155}$ peptide (1 μg/mL) and $CFSE^{LOW}$ cells (M1) were pulsed with $OVA_{257-264}$ peptide (1 μg/mL) as a control. (A) Representative results of flow cytometric analysis for splenocytes from each group. (B) Data shown represent mean ± SEM for each group. * $p<0.05$

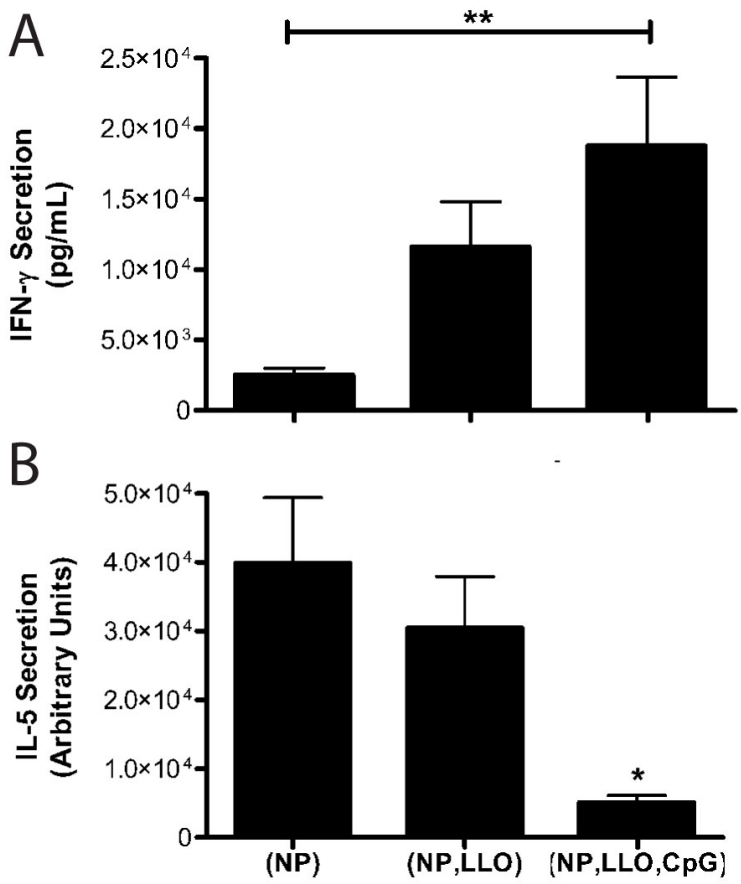


**Figure 4.3: CpG-ODN modulates a NP-specific Th1-type cytokine response.**

Splenocytes from immunized mice (n=5-7) were stimulated with either (A) $NP_{147-155}$ peptide (2.5 μg/mL) for IFN-γ analysis or (B) NP protein (25 μg/mL) for IL-5 analysis in duplicate for 72 h at 37°C. IFN-γ or IL-5 secretion was determined by ELISA. IFN-γ secretion values were determined from a standard curve, and all IL-5 plates were normalized to a control absorbance value. The mean ± SEM for each group is shown. * $p<0.05$, ** $p<0.01$

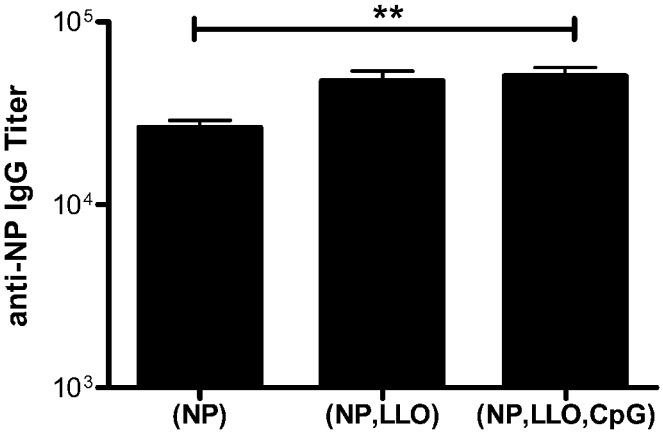


**Figure 4.4: Anti-NP IgG responses are not diminished in LLO-containing formulations.**

Total anti-NP IgG titers were determined by ELISA of sera collected from immunized mice on day 36. Titer data are represented as geometric mean titer ± SEM for each group (n=6-7). ** $p<0.01$

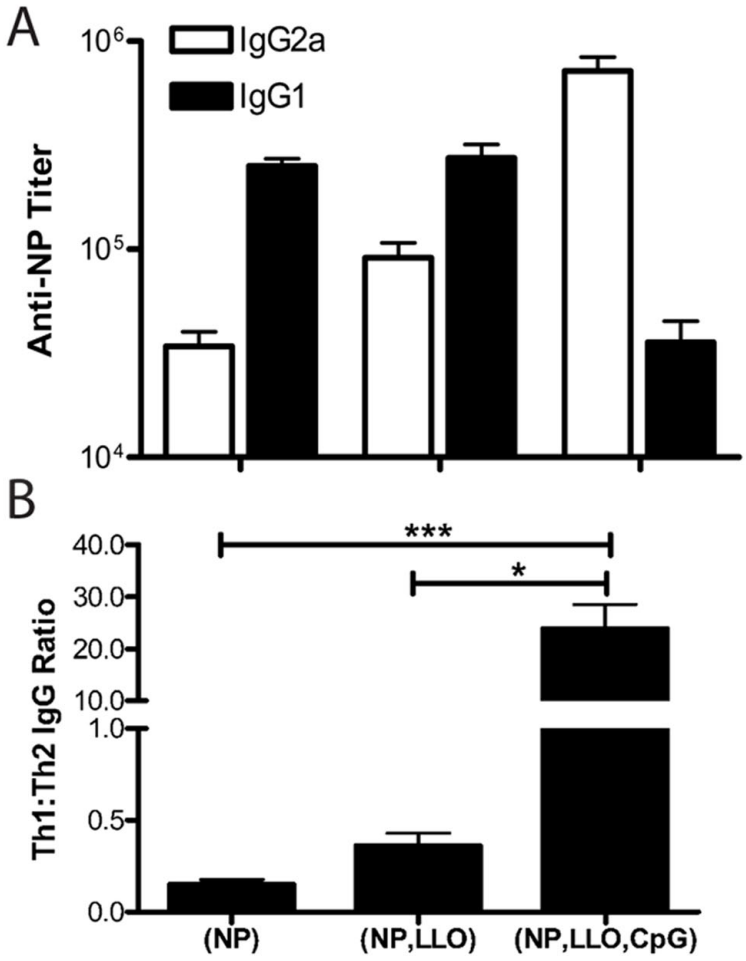


**Figure 4.5: CpG-ODN stimulates a NP-specific Th1-type antibody response.**

Sera were collected on day 36 and analyzed for anti-NP IgG1 and IgG2a antibodies using ELISA. (A) Each bar represents geometric mean titers ± SEM for each group (n=6-7). (B) Each bar represents the average of the Th1:Th2 (IgG2a:IgG1) ratio ± SD for each group (n=6-7). * $p<0.05$ , *** $p<0.0001$

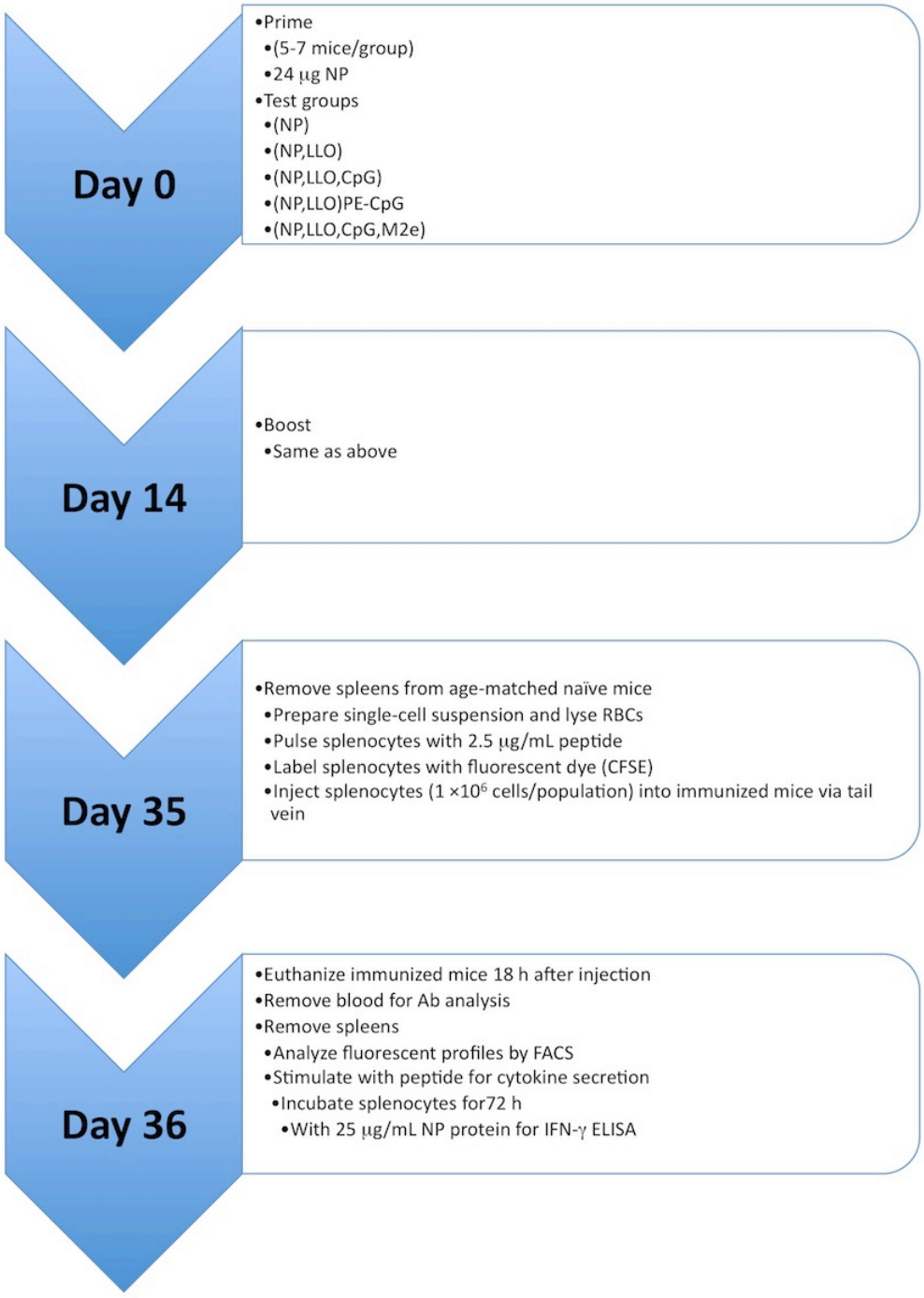


**Figure 4.6: Flowchart for immunization and assay conditions.**

For data shown in Figure 4.7. Note that data from (NP)- and (NP,LLO,CpG,M2e)-immunized groups are not shown for simplicity; however, the statistical analyses were performed comparing all five groups. The data from the (NP)-immunized mice were in good agreement with what was shown in previous figures.

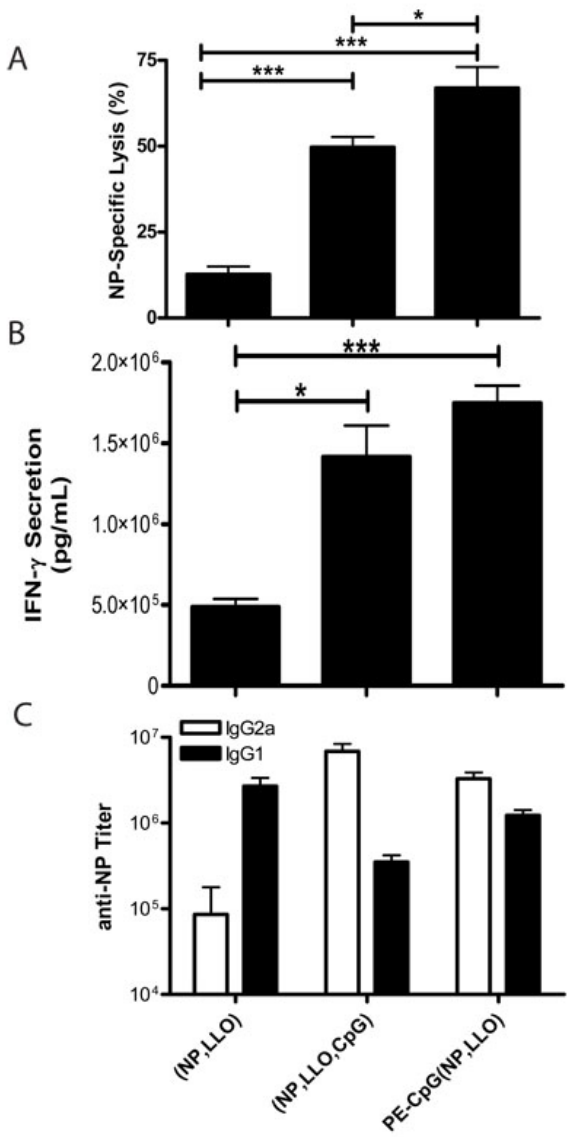


**Figure 4.7: Lipid-conjugated CpG-ODNs stimulate a similar NP-specific CTL and Th1-type immune response compared with co-encapsulated CpG-ODNs.**

(A) Mice (n=5-6) were primed on day 0 and boosted on day 14 subcutaneously at the base of the tail with liposome formulations normalized to NP amount (24 μg per mouse). Cytotoxic activity was demonstrated *in vivo* on day 35 by intravenously injected $CFSE^{HIGH}$-labeled $NP_{147-155}$ peptide (2.5 μg/mL)-pulsed splenocytes in comparison to $CFSE^{LOW}$-labeled $OVA_{257-264}$ peptide (2.5 μg/mL)-pulsed splenocytes. The CFSE profiles were analyzed by FACS 18 h post adoptive transfer. Data shown represent mean ± SEM for each group. (B) Splenocytes from immunized mice (n=5-7) were stimulated with NP protein (25 μg/mL) in triplicate for 72 h at 37°C. IFN-γ secretion was determined by ELISA. The mean ± SEM for each group is shown. (C) Sera were collected on day 36 and analyzed for anti-NP IgG1 and IgG2a antibodies using ELISA. Each bar represents geometric mean titers ± SEM for each group (n=6-7). * $p<0.05$; ***$p<0.001$.

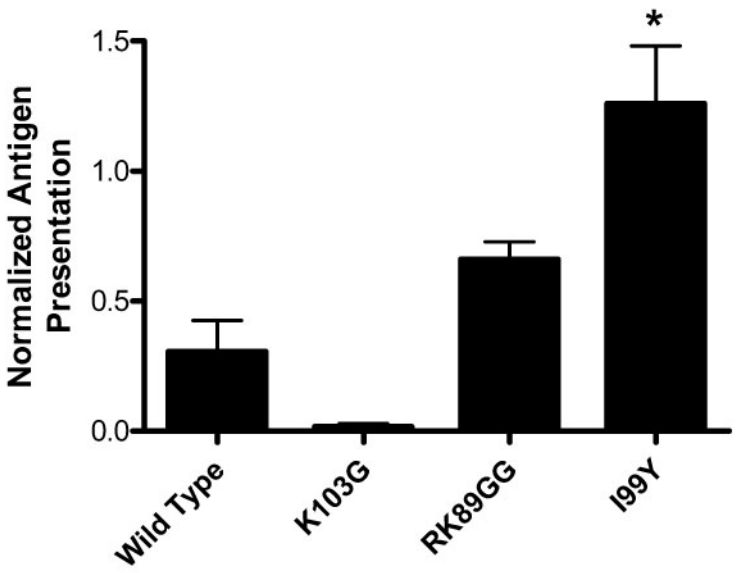


**Figure 4.8: Mutating $LLO_{91-99}$ enhances NP-specific antigen presentation *in vitro.***

BMM were pulsed in serum-free media with liposomes for 2 h, washed and chased in complete media for 3 h. Splenocytes from mice immunized with NP were added to the BMM for 72 h. The level of NP-peptide presentation by BMM to splenocytes was monitored by IFN-γ secretion in cell supernatant using ELISA. Antigen presentation values were normalized by NP and LLO contents in the liposomes. Data represent the average of quadruplicates ± S.D.

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# CHAPTER 5

# SIGNIFICANCE AND FUTURE DIRECTIONS

## SIGNIFICANCE

This is the first time that LLO-liposomes have been utilized in conjunction with CpG-ODN adjuvants. Liposomes are advantageous for delivering CpG-ODN as they can target CpG-ODNs to their receptor in the endocytic pathway, deliver a relatively large payload and co-deliver CpG-ODN and antigen to the same APC. LLO-liposomes enhance the delivery of antigen to the cytosol of APCs to augment antigen processing and presentation in the MHC class I pathway. Utilizing LLO-liposomes for co-delivering antigen and CpG-ODN demonstrated similar antigen-specific CTL responses compared with a more traditional strategy that utilized antigen-CpG-ODN conjugation. Antigen-CpG-ODNs have been successful in many cases, but conjugation has the potential to adversely affect the immunogenicity of antigens and the bioactivity of CpG-ODN. Also, some vaccinations (e.g. papillomavirus) require generating immune responses to numerous antigens for protection, and in these cases it may be advantageous to utilize a delivery system such as liposomes to simultaneously co-deliver antigens and adjuvants.

In keeping with the idea of engineering systems to deliver the components to their subcellular targets, CpG-ODNs were targeted to their receptor, TLR9, by inserting lipid-conjugated CpG-ODNs onto the liposomal membrane. Pharmaceutically, developing methods that minimize the loss of components without sacrificing their biological activity

is essential in reducing costs and time associated with vaccine production. The conjugation procedure developed here results in ~95% of the CpG-ODNs conjugated and ~50% recovered from the purification procedure. Virtually all of the PE-CpG-ODNs are retained in the liposome membrane, compared with less than 10% when utilizing passive encapsulation for CpG-ODNs.

Lastly, we demonstrated that these LLO-liposomes with CpG-ODN encapsulated or conjugated are also capable of enhancing antigen-specific CTL and Th1-type responses to a clinically relevant protein, influenza A NP. Cellular immune responses may be advantageous in providing protection against influenza when cross-reactive neutralizing antibodies are not present. This system provides an alternative to DNA vaccination to stimulate influenza-specific cellular responses, which has demonstrated efficacy in the pre-clinical setting, but has been limited in clinical testing due to safety concerns.

**FUTURE DIRECTIONS**

In the studies described in this thesis, the dose of CpG-ODN was matched to a previously established sample group that has been shown to stimulate a response. For example, in Chapter 2 the dose of CpG-ODN encapsulated in liposomes was closely matched to that mixed in solution with OVA or conjugated to OVA. In Chapter 3, the dose of PE-CpG-ODN closely matched the encapsulated CpG-ODNs, allowing us to fairly compare the immune responses. The relatively larger payload that is possible with liposomes may allow for a reduced CpG-ODN dose; therefore, a dose optimization study should be performed. Utilizing the lowest dose of CpG-ODNs while maintaining a

therapeutic effect has the potential for positively correlating with a tolerated safety profile.

LLO-containing liposomes can be used to investigate the potential existence of a cytosolic receptor for CpG-ODNs. Currently, cytosolic receptors have been identified for RNA and double-stranded DNA (*1*). Positively charged DOTAP lipids were utilized to enhance DNA transfection efficiency, and in these studies double-stranded DNA in the form of PCR products or adenovirus DNA activated the inflammasome, resulting in IL-1β secretion; however, this was not observed when cells were treated with CpG-ODNs (*1*). This work suggested a minimum length requirement of 250 base pairs to be recognized by cytosolic receptors (*1*). Our system provides an alternative method of delivering CpG-ODNs to the cytosol. Utilizing TLR9 knockout macrophages, we can compare LLO-liposomes with and without CpG-ODN and monitor for cellular activation using a microarray. Alternatively, based on the signaling pathways stimulated via other cytosolic receptors, preliminary evaluation may be performed by monitoring nuclear NF-κB, IRFs 3, 5, and 7 and secreted IL-1β. Also, both phosphorothioate and phosphodiester CpG-ODNs should be monitored to ensure that any negative responses are not due to the phosphorothioate ODN backbone.

Although LLO-liposomes have been shown to be a suitable vehicle for co-delivering antigen and CpG-ODN, those results were demonstrated by monitoring only cellular assays (e.g. antigen-specific CTL and IFN-γ secretion). In order to demonstrate a more widespread utility of this delivery system, influenza protection assays must be performed. Intranasal infection of fully anesthetized mice with influenza A causes viral replication in the lung and death at 6 to 8 days post infection (*2*). Survival of mice

challenged with influenza A virus reflects the ability of CTLs to limit the severity of infection. In order to further characterize the potency of the NP-specific CTL response and Th1-type response generated by the NP-, LLO- and CpG-ODN-containing liposome formulation, mice should be monitored for protection against influenza virus. In the studies performed in Chapter 4, mice were vaccinated with NP from the H1N1 viral strain. Ideally, mice should be monitored for protection against H1N1 viruses to demonstrate homologous protection and against heterologous influenza strains such as the avian influenza H5N1 or H3N2. Throughout the course of the experiment the mass of the mice should be monitored; effective vaccinations typically lead to a recovery in mass at around day 7 or 8 (*2*). Determining the viral titers in the lungs of immunized mice after sublethal challenge is another method to monitor the effectiveness of the immune response generated through immunization to verify that any protection observed is due to a reduction in viral load.

The ideal influenza vaccine would mimic the natural immune response generated for clearing an influenza infection in terms of the breadth, robustness and *in vivo* sites at which the T cell responses are generated. Recent work has demonstrated the importance and relevance of the physiological location of the CTLs (*3*), and the antiviral capacity of influenza-specific $CD8^+$ T cells is strongly reliant on their ability to migrate and localize to the lungs. Future experiments should exploit nasal delivery of the liposomes in an attempt to stimulate CTLs in the lungs.

In continuing with the idea of mimicking natural infection, vaccines containing multiple influenza virus antigens are being explored for their ability to confer enhanced protection. Recently published studies suggest that the protection afforded by

immunization with NP in mice diminishes markedly as the dose and virulence of the challenge virus increases, indicating that there may be a need to stimulate both arms of the immune system for more robust protection (*4, 5*). Anti-NP antibodies have not been demonstrated to afford protection; therefore, different conserved antigens must be identified as humoral immune targets. In addition to stimulating a cellular response to NP, antigens such as matrix protein 2 (M2) and a conserved region of HA are potential vaccine targets. M2 is a transmembrane viral protein with the N-terminus on the surface of the virus (ectodomain) and the C-terminus exposed to the viral lumen. M2 forms a proton-selective channel that plays a pivotal role in pH-dependent uncoating of the endocytosed virus, ultimately leading to the fusion of hemagglutinin with the endosomal membrane to promote viral escape into the cytosol. M2, which is also found in the plasma membrane of infected cells, has been widely targeted as a possible broadly protective vaccine candidate. A recent study using adenovirus encoding three M2e (M2 ectodomain) proteins from diverse influenza A strains and H1N1 NP demonstrated an increase in protective efficacy when both arms of the immune system are stimulated by vaccination (*6*). This study was supported by one from another group that immunized BALB/c mice with recombinant adenovirus expressing M2 and NP, and protection was subsequently demonstrated against highly virulent H1N1, H3N2 and H5N1 (*7*). HA is a surface glycoprotein that is composed of a stalk-like structure with a globular head. The seasonal vaccine generates antibodies to the ever-changing globular head, while the stalk domain is relatively well conserved. A recent study demonstrated the utility of immunizing mice with the stalk domain of HA in generating antibodies with broader reactivity, yielding protection against mortality following lethal viral challenge (*8*).

One of the motivations for this work was to generate a vaccine delivery system that mimics the desirable potent immune-stimulating properties of the live attenuated pathogen-based vaccines while reducing the toxicity. LLO-liposomes mimic certain pathogens in their ability to deliver macromolecules to the cytosol, while the use of purified recombinant proteins reduces potential toxicity. While we tested this vaccine delivery system with only one antigen, influenza NP, we envision that a combination of influenza antigens, as discussed above, may be added to the LLO-liposomes based on their protective merits. In addition to the delivery of multiple antigens, pathogen-based vaccines generate potent immune response due to stimulation of the innate immune system through PRRs. Toxicity results from signaling by certain PAMPs such as lipopolysaccharide (LPS), while some synthetic analogues such as monophosphoryl lipid A (MPL) are better tolerated. The studies presented here only explored CpG-ODN as an adjuvant; however, the potential exits to include numerous molecular adjuvants, with acceptable toxicity profiles and known cellular receptors, in the LLO-liposome vaccine delivery system. We have demonstrated that LLO-liposomes provide a platform for stimulating robust cellular and humoral immune responses, and by tailoring it with the proper combination of antigens and adjuvants this vaccine delivery system has the potential to mimic the protective immune responses of live attenuated pathogens while avoiding potential toxicities.

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# APPENDIX A

# USE OF PEGYLATED LIPIDS FOR CONJUGATION OF CPG-CONTAINING OLIGONUCLEOTIDE

## SUMMARY

In order to efficiently stimulate a robust antigen-specific response, antigen needs to be co-delivered in the context of proper co-stimulatory signals; cytosine-phosphate-guanine-containing oligodeoxyribonucleotides (CpG-ODNs) can provide these co-stimulatory signals. Here, we describe the synthesis and characterization of phosphatidylethanolamine-polyethylene glycol-CpG-ODN (PE-PEG-CpG-ODN) and *in vitro* and *in vivo* biological characterization of PE-PEG-CpG-ODN-containing liposomes.

## INTRODUCTION

The purpose of these studies was to evaluate polyethylene glycol (PEG)-containing functionalized lipids compatible with sulfhydryl-containing oligodeoxyribonucleotides (ODNs) to generate an efficient method of conjugation. Phosphatidylethanolamine (PE) lipids are very hydrophobic, whereas PEG-containing lipids were evaluated because the hydrophilic properties of PEG give the PE-PEG lipid properties more similar to those of the ODNs, which may have advantages when establishing a conjugation method. Here, we established a method of conjugating either maleimide-containing PE-PEG lipids or sulfhydryl-containing PE-PEG lipids with 5'

sulfhydryl-containing CpG-ODNs, and the PE-PEG-CpG-ODN micelles were evaluated for immunostimulatory capabilities *in vitro*. PE-PEG-CpG-ODNs were incorporating into liposomes using sonication and freeze-thaw, and the liposomes were evaluated for IL-12 secretion in BMM and as vaccine delivery systems *in vivo*.

## MATERIALS AND METHODS

### Mice, cell culture, media and reagents

C57BL/6 (female, 7-9 weeks old; Jackson Laboratory, Bar Harbor, ME) and C57BL/10ScNJ ($Tlr4^{Lps\text{-}del}$, $H\text{-}2K^{b}$, female, 6-8 weeks old; Jackson Laboratory) were used in this study and were handled according to Institutional Guidelines. All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Bone marrow was harvested from the femurs and tibia of C57BL/10ScNJ mice and differentiated into bone marrow-derived macrophages (BMM) as described (*1*). BMM were harvested on day six and frozen in liquid nitrogen until the experiment. Unmodified and 5'-disulfide-CpG-ODN 1018 used in this study were composed of 22 bases with unmethylated CpG-sequences (5'TGACTGTGAACGTTCGAGATGA) in a phosphorothioate-modified backbone (Dynavax Technologies; Berkeley, CA).

### Lipid-ODN conjugate synthesis

#### *Reduction of 5'-disulfide-ODN*

A 700 mM tris-(2-carboxyethyl) phosphine (TCEP; Pierce, Rockford, IL) solution was made in HBSE (140 mM sodium chloride buffered with 10 mM HEPES containing 1 mM EDTA) pH 7, and used to reduce 5'-disulfide-CpG-ODN at 40°C for 2 h. A 500 mM dithiothreitol (DTT) solution was made MilliQ water and used to reduce 5'-

disulfide-CpG-ODN (100 mM final concentration) at RT for 2 h. Residual reducing agent was removed using a NAP-5 desalting column (GE Healthcare) equilibrated with degassed HBSE pH 6.5 or degassed HBSE pH 8.0 when the resulting 5'SH-CpG-ODN was added to maleimide- or sulfhydryl-containing lipids, respectively (*2*).

***PE-PEG-maleimide and 5'SH-CpG-ODN conjugation***

Chloroform was removed from 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG(2000) maleimide; Avanti Polar Lipids, Alabaster, AL) using rotary evaporation at < 10 mm Hg vacuum at RT. The lipids were then rehydrated with the freshly reduced 5'-SH-CpG-ODNs by vortexing the lipid film at 10:1 PE-PEG-maleimide:ODN. The solution was sealed under argon, and the reaction proceeded while rocking overnight until quenched by adding cysteine (1 mM final).

***PE-PEG-PDP and 5'SH-CpG-ODN conjugation***

The same procedure as above was performed using 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000] (DSPE-PEG(2000) PDP).

***PE-PEG-CpG-ODN conjugation efficiency***

Tris-Borate-EDTA (TBE) polyacrylamide gels (15%; BioRad) stained with SYBR Green I (Invitrogen) were used monitor conjugation efficiency. The amount of unconjugated 5'-SH-CpG-ODNs were determined by using densitometry comparing the values with known concentrations of CpG-ODNs resolved in the same gel. Band intensities were measured using a Molecular Dynamics Typhoon 9200 with ImageQuant (GE Healthcare). The freshly reduced 5'SH-CpG-ODN was added directly to the lipids

after NAP-5 purification; therefore, the reactions were set up as 10:1 lipid:ODN; although, the actual ratio was established later, and was typically 15:1 to 30:1.

**Liposome preparation**

Lipid films containing egg phosphatidylethanolamine:cholesteryl hemisuccinate (ePE:CHEMS; Avanti Polar Lipids, Alabaster, AL and Sigma-Aldrich, respectively) (2:1) were made by removing chloroform and methanol using rotary evaporation at < 10 mm Hg vacuum at RT. When indicated, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000) was included in the lipid film. The molar ratio of DSPE-PEG2000 in each experiment was the same as the molar ratio of PE-PEG-CpG-ODN to be used in the rehydration solution. Lipid films were rehydrated with 0.5 mL HBSE pH 8.4 containing a combination of CpG-ODNs (4 mg/mL) and/or LLO (200 μg/mL) and ovalbumin (OVA; 20 mg/mL). The resulting liposomes were subjected to five freeze-thaw cycles followed by 5 × 1 min bursts of bath sonication. Unencapsulated materials were separated from the liposomes using Sepharose CL-4B size-exclusion chromatography (1 × 25 cm). OVA and LLO encapsulation was determined by staining proteins resolved in SDS-PAGE with SyproRed (Invitrogen) and measuring band intensities using a Molecular Dynamics Typhoon 9200 with ImageQuant (GE Healthcare). Calculations were based on known concentrations of proteins resolved in the same gel. ODN concentrations were determined by either SYBR Green I plate assay or by using TBE-PAGE stained with SYBR Green I or ethidium bromide.

**SYBR Green I plate assay**

Using phosphate concentration values (*3*) from purified liposomes, samples were diluted to known concentration of phospholipid. Blank (unloaded) liposomes were

spiked into the standards and the blank to achieve the same concentration of phospholipid in the samples. To ensure that the liposome-encapsulated materials were released, C12E8 (1% final) was added to all samples, standards, and blank. Samples, standards, and blanks were added to a black 96-well plate (100 μL) in duplicate and SYBR Green I diluted 1:5,000 in HBSE pH 8.4 was added to the wells (50 μL/well). The fluorescence was quantified in a Synergy HT plate reader (BioTek Instruments, Winooski, VT) using an excitation of 485 nm and emission of 528 nm. SYBR Green I cannot permeate the liposome membrane; therefore, extra-liposomal CpG-ODNs were monitored by performing the assay without detergent solubilization.

**IL-12p40 secretion by ELISA**

BMM ($2 \times 10^5$/well) from C57BL/10ScNJ mice were plated in 96-well tissue culture plates in complete DMEM (DMEM + 10% heat-inactivated (HI)-FBS, 100 μg/mL streptomycin and 100 U/mL penicillin) one day prior to treatment and incubated at 37°C, 5% $CO_2$. BMM were pulsed in serum-free DMEM (DMEM + 100 μg/mL streptomycin and 100 U/mL penicillin) with serial dilutions of sample for 3 h. BMM were washed three times and incubated for 20 h in complete DMEM. Cell supernatants were analyzed using mouse IL-12/IL-23 p40 (eBioscience) as per the manufacturer's instructions.

**Immunization protocol**

C57BL/6 mice were primed with OVA (8 μg) on day 0 and day 12 via the subcutaneous route in the hind flank. Mice were euthanized on day 24-26; sera were collected via cardiac puncture.

**Anti-OVA Ig ELISA**

Post-immune sera were analyzed by ELISA as described (*1*). Briefly, Maxisorp ELISA plates (Nunc) were coated with OVA (10 μg/mL), blocked, and incubated with serial dilutions of sera. Anti-OVA isotype-specific secondary antibodies (goat anti-mouse IgG2a-biotin or goat anti-mouse IgG1-biotin; Southern Biotechnology Associates, Birmingham, AL) were detected with avidin-HRP (eBioscience). The plate was developed using SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL) and stopped with 2N sulfuric acid. The absorbance values were monitored using a Molecular Devices Emax plate reader at 450 nm. The titer was defined as the reciprocal of the serum dilution that produced an absorbance value of 1 using a 4-parameter analysis (Softmax Pro5.2, Molecular Devices, Sunnyvale, CA). If no titer was observed, the inverse of the lowest dilution tested was reported.

**Statistical analyses**

Grubb's test was used to analyze for outliers in animal studies. Data from liposome-treated animals were compared by ANOVA and Tukey's post-test for data with homogeneous variance. In the case of non-homogeneous variance, the data were compared using the Kruskal-Wallis test followed by the Dunn's multiple comparison tests. A p-value < 0.05 was considered significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

## RESULTS AND DISCUSSION

### *PE-PEG-CpG-ODN conjugation*

Following the reduction method utilized by others (*4, 5*), TCEP was employed to reduce the phosphorothioate ODNs. Initially, TCEP at a final concentration of 50 mM

was used to reduce the 5'disulfide-CpG-ODN; however, the conjugation efficiency was very low. We were concerned that the ODNs were not being reduced efficiently; therefore, we attempted to use DTT to reduce the 5'disulfide-CpG-ODN. The resulting PE-PEG-maleimide and 5'SH-CpG-ODN reaction resulted in seven bands when the reaction was resolved in a TBE polyacrylamide gel and stained with SYBR Green I. These results were surprising since at most, three bands were expected, one corresponding to PE-PEG-CpG-ODN conjugate, CpG-ODN dimer, and unconjugated CpG-ODN. The number of bands in the gel was not reduced to two (conjugated and unconjugated CpG-ODN) by treating the samples with 2-mercaptoethanol and boiling before running the gel. The poor conjugation efficiency between PE-PEG-maleimide and 5'-SH-CpG-ODN regardless of the reducing agent used, either TCEP or DTT, indicated a different problem. While performing these reductions, a constant ratio of reducing agent:ODN was not well maintained, and a vast molar excess (up to 100×) of reducing agent was used. Reducing agent was removed from the ODN using a NAP-5 desalting column directly prior to adding the reduced ODN to PE-PEG-maleimide; however, this column typically results in a minimum of 3% of the reducing agent remaining in the sample to compete with the 5'-SH-CpG-ODN for binding to the PE-PEG-maleimide. Ellman's assay was performed to monitor the reduction of the 5'disulfide-CpG-ODN, and the results typically indicated that there were more free sulfhydryls present after NAP-5 purification than 5'disulfide-CpG-ODNs added to the reduction reaction, suggesting that reducing agent was co-purifying with the ODN. Therefore, we lowered the amount of reducing agent to make it comparable to the amount of 5'disulfide-CpG-ODN. Because of the problems with the additional bands when DTT was used as the reducing agent, we

reverted to TCEP and used a five-fold excess over 5'disulfide-CpG-ODN. Using these reducing conditions, adding the freshly reduced 5'SH-CpG-ODN to the PE-PEG-maleimide film resulted in greater than 95% of the ODN conjugated (Figure A.1).

PE-PEG-CpG-ODNs were evaluated in micellar form for their ability to induce the secretion of the Th1-type cytokine, IL-12. PE-PEG-CpG-ODNs were compared with free CpG-ODNs in media for BMM-stimulating capabilities (Figure A.2). The results indicate that micelles stimulate IL-12 secretion to a lesser extent than free CpG-ODNs, but there are likely uptake differences between free CpG-ODNs and micellar PE-PEG-CpG-ODN. These results confirm that CpG-ODNs maintain immunostimulatory activity once conjugated to PE-PEG lipids.

**IL-12 secretion from BMM treated with PE-PEG-CpG-ODN**

Next, we asked whether CpG-ODNs incorporated onto the liposomes in the form of PE-PEG-CpG-ODN conjugates retain the capacity of CpG-ODNs to stimulate and modulate the immune response similarly to encapsulated CpG-ODNs. We compared liposomes with either 0.5, 1, 2.5, 5, or 10% PE-PEG-CpG-ODN with PE:CHEMS liposomes or PE:CHEMS:PE-PEG (10% mole percent PEG) co-encapsulating CpG-ODN. PE-PEG-CpG-ODNs are capable of stimulating IL-12 secretion from BMM and increasing the dose of CpG-ODNs results in increased IL-12 secretion (Figure A.3). A minimum of 2.5% PE-PEG-CpG-ODN was required for detectable IL-12 secretion using this system. CpG-ODNs co-encapsulated in pH-sensitive liposomes were the positive control for the assay, and BMM treated with this formulation resulted in the highest IL-12 secretion. PE:CHEMS:PE-PEG liposomes encapsulating CpG-ODNs stimulated less IL-12 secretion than 10% PE-PEG-CpG-ODN. It was expected that the PE:CHEMS

liposomes would stimulate the most IL-12 secretion, as the presence of PEG on the liposome surface is known to decrease liposome uptake (*6*). These results indicate that incorporating CpG-ODNs on the surface of the liposome by conjugating to PE-PEG-maleimide retains immunostimulatory capabilities of the CpG-ODNs.

For PE-PEG lipids, unconjugated lipids were not removed from PE-PEG-CpG-ODNs; therefore, the liposomes were discussed regarding the mole percent of PE-PEG. The conjugation efficiency for the data reported here was 1/20 PE-PEG-maleimide conjugated to CpG-ODNs corresponding to 0.025, 0.05, 0.125, 0.25 and 0.5 mole percent of total phospholipids containing CpG-ODNs. These data are in good agreement with the data reported in Chapter 3, in which *in vitro* studies monitoring BMM stimulation with PE-CpG-ODNs were typically performed at 0.3 mole percent CpG-ODN.

**Incorporation of CpG-ODNs in liposomes is proportional to mole percent of PE-PEG-CpG-ODN used in rehydration solution**

We also varied the amount of PE-PEG-CpG-ODN used in rehydration to determine if we could control the number of CpG-ODNs incorporated in the liposome. We added 0.5, 1, 2.5, 5 and 10 mole percent of total phospholipid to the lipid film rehydration solution and removed unencapsulated materials as described above. The results indicate that the number of CpG-ODNs incorporated in the liposome is proportional to the mole percent of PE-PEG-CpG-ODN used in the rehydration solution (Table A.1). We also show that above 1% there is an asymmetrical distribution of PE-PEG-CpG-ODNs, in which approximately 60-80% of the CpG-ODNs are on the extra-liposomal side of the liposome, which is in agreement with previously published data (*7*).

**Utilizing a(n) reversible or irreversible bond for lipid-CpG-ODN conjugation does not influence immune stimulation**

Conjugated CpG-ODNs were inserted on the liposomal membrane as a method to promote interaction between CpG-ODN and its receptor before LLO perforates the endosomal membrane and promotes the release of the endosomal contents into the cytosol. Next we wanted to determine if there was any difference in immunostimulatory capabilities when 5'SH-CpG-ODN was conjugated through a reversible or irreversible bond. We hypothesized that conjugating CpG-ODNs through an irreversible thioether bond may negatively impact the interaction between CpG-ODNs and TLR9, and this effect may be overcome by using a reversible disulfide bond that would be reduced in the endosome allowing the release CpG-ODNs to interact with TLR9, before LLO causes the release of endosomal contents into the cytosol. We compared micelles composed of PE-PEG-CpG-ODN conjugates that were conjugated through the irreversible thioether bond using PE-PEG-maleimide or reversible disulfide bond using PE-PEG-PDP. The results indicate that when the lipids are in the micelle form, there is no difference in the immune-stimulating capabilities of the CpG-ODNs regardless of the bond used in conjugation, irreversible or reversible (Figure A.4). The PE-PEG-maleimide conjugation efficiency was slightly higher, and thus future studies were performed using the thioether bond formed between 5'-SH-CpG-ODN and PE-PEG-maleimide.

***In vivo* evaluation of liposomes containing PE-PEG-CpG-ODNs**

The *in vitro* studies indicated that lipid-conjugated CpG-ODNs retained their immunostimulatory capabilities. To corroborate our results *in vivo*, we immunized mice to compare the methods for including CpG-ODNs in the liposome: incorporation of the CpG-ODN lipid-conjugate onto liposome membranes versus incorporation via

encapsulation. We compared PE:CHEMS and PE:CHEMS:PE-PEG liposomes co-encapsulating OVA, LLO and CpG-ODN as a control for the effect of PEG on the liposome formulation. These formulations were compared with PE:CHEMS:PE-PEG-CpG-ODN liposomes co-encapsulating OVA and LLO. The results from two different experiments are shown here (Figure A.5). In the first experiment, no significant differences were observed in the IgG2a titers regardless of the formulation used for immunization. However, mice immunized with CpG-ODN encapsulated in PE:CHEMS liposomes resulted in a significantly reduced ($p<0.01$ or $p<0.05$, respectively) IgG1 response compared with mice immunized with CpG-ODN encapsulated in PE:CHEMS:PE-PEG liposomes or PE-PEG-CpG-ODNs on the surface of liposomes. When this experiment was repeated, there was a reduced ($p<0.05$) IgG2a response when mice were immunized with PE-PEG-CpG-ODN compared with PE:CHEMS liposomes encapsulating CpG-ODN. In both experiments, the IgG2a:IgG1 response is decreased upon the addition of PEG, although not to a significant extent. It seems that adding PEG to the liposomes may impair the Th1-skewing abilities of CpG-ODNs, and future experiments should be performed to determine how increased PEG concentrations affect the immune response. As part of the second experiment, an *in vivo* CTL assay was performed as described before (Chapter 3); however, the data were inconclusive since complete lysis of the OVA-peptide pulsed population was observed (10 μg/mL OVA peptide loading, $4.6 \times 10^6$ cells/ population injected intravaneously).

**CONCLUSION**

These experiments show that we can develop an efficient method for conjugating 5'sulfhydryl-containing CpG-ODNs using PE-PEG-maleimide lipids. The PE-PEG-

CpG-ODNs retain immunostimulatory capabilities when included on the liposome membrane when tested *in vitro* in BMM. PE-PEG-CpG-ODNs were evaluated in the micellar form and the bond used to conjugate CpG-ODNs was compared; the results indicate no differences in immunostimulatory activity when CpG-ODN is conjugated through a irreversible thioether bond or a reversible disulfide bond. The *in vivo* studies performed here were inconclusive regarding the effect of PEG on the Th1-type immune stimulating activity; therefore, additional animal studies should be performed to determine the PEG effect on the Th1-type immune skewing activity of CpG-ODNs *in vivo*.

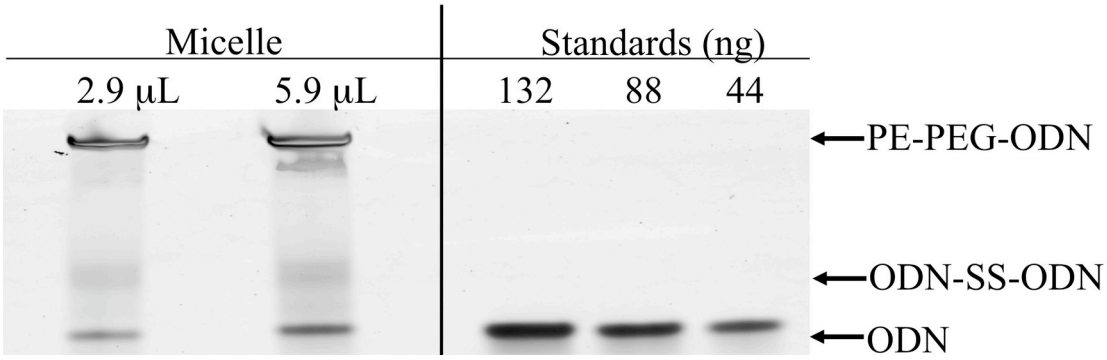


**Figure A.1: Representative TBE gel of PE-PEG-CpG-ODN conjugation.**

Conjugation was assessed by resolving PE-PEG-ODNs from disulfide-bonded (ODN-SS-ODN) and unconjugated ODN in a 15% TBE polyacrylamide gel stained with SYBR Green I. The amounts of disulfide-bonded and unconjugated ODN were calculated from a standard curve of known ODN amounts. These results indicate that >95% 5'SH-CpG-ODN is conjugated.

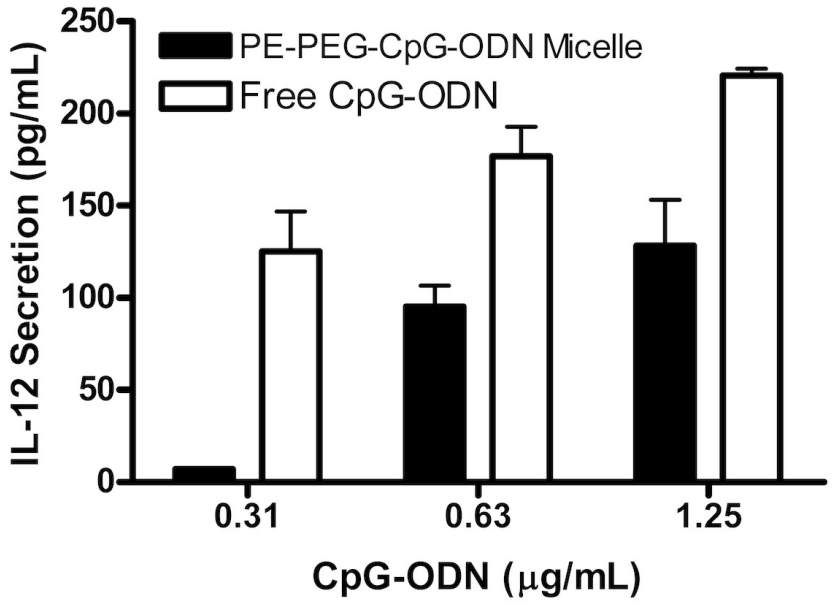


**Figure A.2: PE-PEG-CpG-ODN micelles stimulate IL-12 secretion.**

Micelles formed from PE-PEG-CpG-ODNs were compared with free CpG-ODNs in solution in their capabilities to stimulate BMM to secrete IL-12. BMM were treated in serum-free media with serial dilutions CpG-ODN for 3 h, washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for secreted IL-12p40 by ELISA. Data represent the average of triplicates ± S.D.

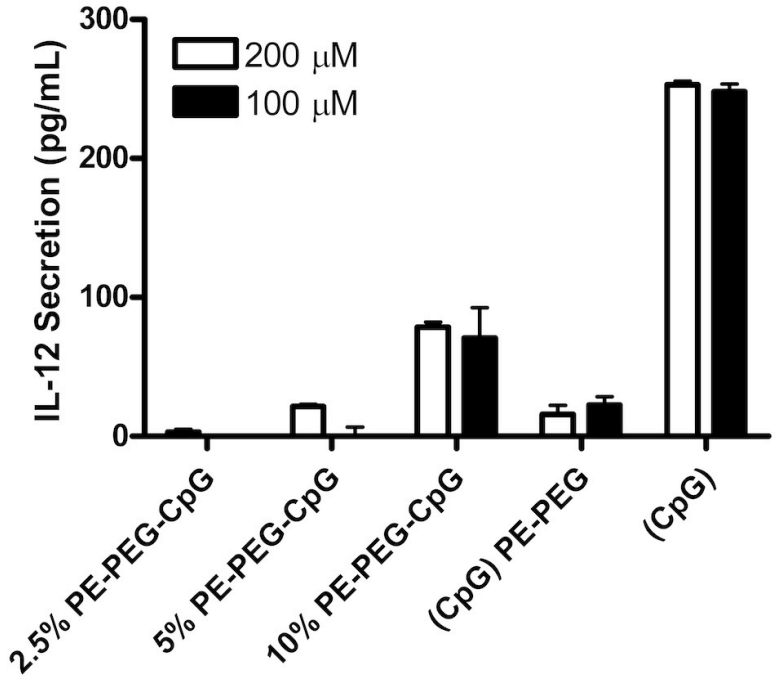


**Figure A.3: Liposomes containing PE-PEG-CpG-ODNs stimulate IL-12 secretion.**

PE-PEG-CpG-ODNs were included in liposomes at the final mole percent of total phospholipid as indicated in the graph. For liposomes encapsulating CpG-ODN containing PE-PEG, 10 mole percent was included [encapsulation denoted by the parentheses in the x-axis label]. BMM were treated in serum-free media with serial dilutions of liposomes (200 and 100 μM phosphate) for 3 h, washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for secreted IL-12p40 by ELISA. Data represent the average of triplicates ± S.D.

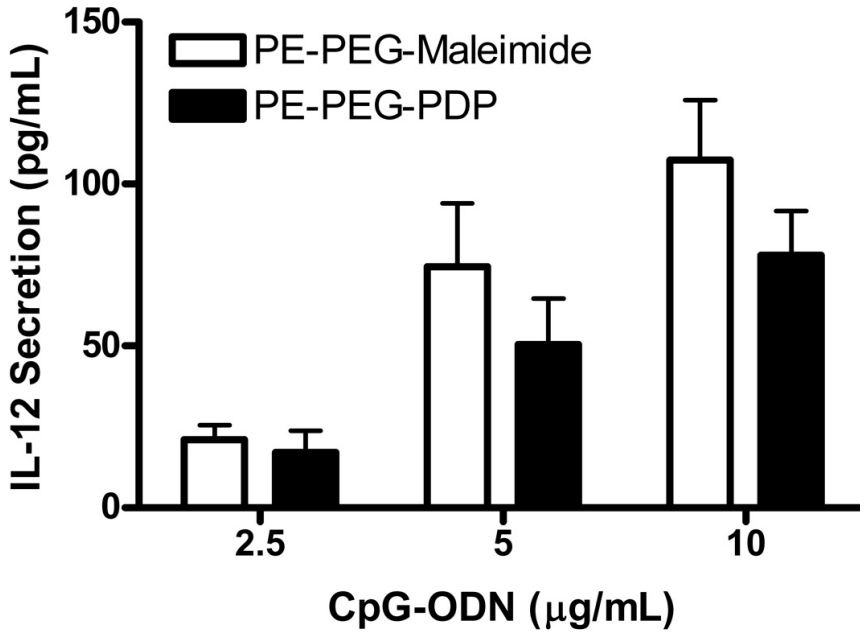


**Figure A.4: Utilizing either reversible or irreversible bonds for CpG-ODN and PE-PEG conjugation results in similar IL-12 secretion.**

5'SH-CpG-ODNs were conjugated to either PE-PEG-maleimide forming a reversible thioether bond or PE-PEG-PDP forming an irreversible disulfide bonds. Micelles formed from these lipids were used to compare the effect of the bond on the ability of the CpG-ODNs to stimulate IL-12 secretion. BMM were treated in serum-free media with serial dilutions of CpG-ODN for 3 h, washed and incubated in complete media for 20 h. The supernatant was collected and analyzed for secreted IL-12p40 by ELISA. Data represent the average of triplicates ± S.D.

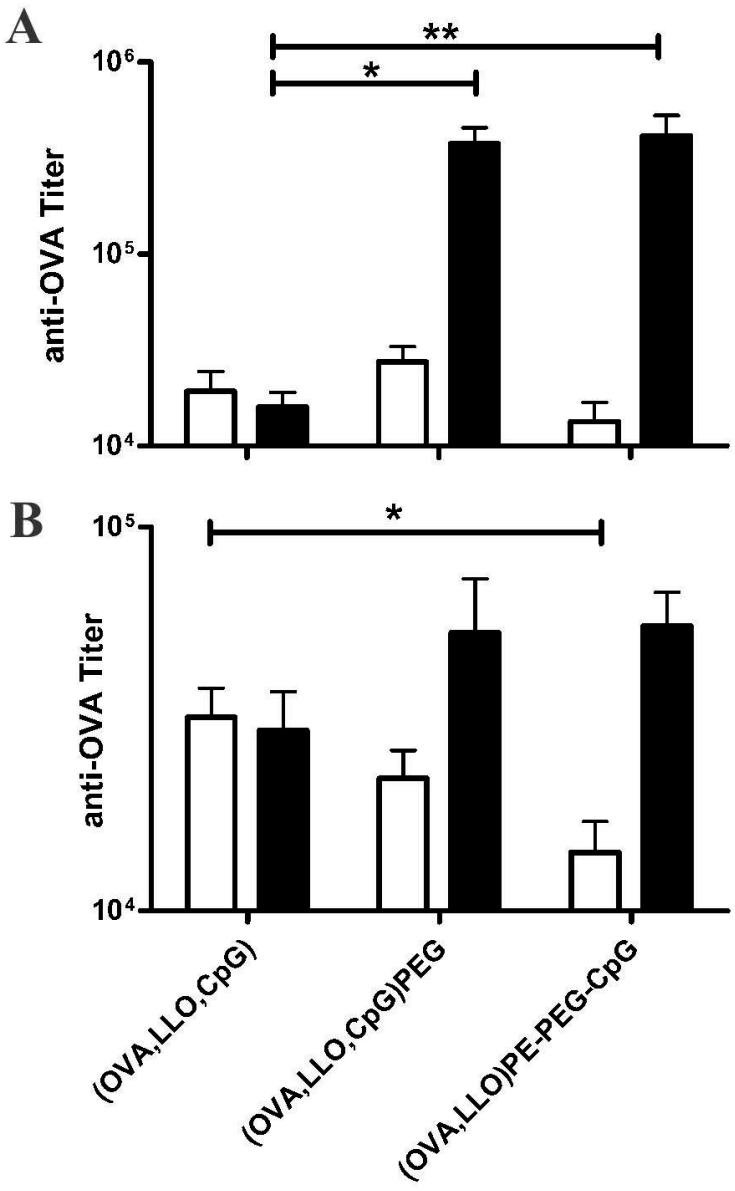


**Figure A.5: PE-PEG-CpG-ODNs effect the OVA-specific IgG isotype response.**

Mice were immunized subcutaneously on day 0 and day 12 with liposomes normalized to OVA content (8 μg). Sera were obtained from blood of immunized mice harvested by cardiac puncture on day 24-26. Anti-OVA IgG2a (open bars) and IgG1 (closed bars) were monitored using ELISA. The mean ± SEM is shown. (A) Data from experiment one (n=3-4 mice per group). (B) Data from experiment two (n=7-8 mice per group). *$p<0.05$, ** $p<0.01$

| Micelle used in rehydration (mole percent) | CpG-ODN molecules per liposome | Molecules extra-liposomal CpG-ODN | Extra-liposomal CpG-ODN (%) |
|---|---|---|---|
| 0.5 | 189 | 189 | 100 |
| 1 | 221 | 221 | 100 |
| 2.5 | 480 | 391 | 81 |
| 5 | 855 | 541 | 63 |
| 10 | 1387 | 962 | 70 |

**Table A.1: Percent of micelles used in rehydration is proportional to extra-liposomal CpG-ODNs.**

This procedure provides a controllable method to incorporate CpG-ODNs onto the surface of liposomes. Note that the percent micelle is the total amount of PEG incorporated in the liposome surface; the conjugation reaction was performed at 10:1 PE-PEG-maleimide:5'SH-CpG-ODN resulting in one tenth of the micelle containing CpG-ODN.

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# APPENDIX B

# EFFECT OF PRE-EXISTING ANTI-LLO IMMUNITY ON GENERATION OF IMMUNITY TO SUBSEQUENT ANTIGENS DELIVERED IN LLO-LIPOSOMES

## SUMMARY

The effectiveness of vaccines can be limited by either pre-existing or vaccine-induced vector specific immunity. Here, we establish pre-existing LLO immunity using LLO-liposomes, and monitor its effects on the immune response to influenza NP antigen upon immunization with NP in LLO-containing liposomes.

## INTRODUCTION

We have demonstrated the utility of LLO-liposomes in delivering antigens to the cytosol for endogenous MHC class I processing and presentation (*1-3*). In order to use the LLO-liposomes as a broadly based vaccine vector that spans many platforms, it must be demonstrated that pre-existing immunity to LLO does not inhibit the functionality of the vaccine. This has been observed in the case of adenovirus-based vaccines in which pre-existing immunity prevents effective vaccination with an adenoviral vector (*4, 5*). We hypothesized that because LLO is encapsulated within the luminal space of the liposome any pre-existing immune response generated against LLO will not be detrimental to the vaccine delivery system, as antibodies could not access LLO to bind and change the properties of the delivery system. In order to test our hypothesis, we

established LLO pre-immunity by immunizing mice with liposomes containing ovalbumin (OVA) and LLO. The control group without LLO pre-existing immunity was immunized with liposomes containing only OVA. Both groups were then immunized with liposomes containing influenza NP, LLO and CpG-ODN. We monitored the NP-specific immune cytotoxic T lymphocytes (CTLs), IFN-γ secretion, and antibodies in the groups with and without the pre-existing LLO immunity.

## EXPERIMENTAL PROCEDURES

### NP expression

NP was cloned from influenza strain A/Puerto Rico/8/34/Mount Sinai (a kind gift from Peter Palese). NP was subcloned using *Nde*I and *Xho*I restriction enzymes sites into the pET29b expression vector in frame with the polyhistidine (His6) tag. *E. coli* strain BL21(λDE3) was transformed with the plasmid and induced with 1 mM IPTG. After a 6 h induction at 30°C, bacteria were pelleted for 20 min at 4000 × g at 4°C. Pellets were stored overnight at -20°C and then resuspended in 0.02× culture volume using wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 8), containing 2 mM PMSF, 2 U/mL DNase, and 50 U/mL RNase. Lysozyme was added at 1 mg/mL and solutions were rocked at RT for 1 hour. Insoluble debris was pelleted by centrifugation at 20,000 × g at 4°C for 1 h and then the soluble fraction was poured into a column containing Ni-NTA agarose (Qiagen), washed with 16× column volume (CV) of wash buffer and eluted with 4× CV wash buffer containing 400 mM imidazole. Purified NP was dialyzed against HBSE pH 8.4 (10 mM HEPES containing 140 mM sodium chloride and 1 mM EDTA) at 4°C.

**LLO expression**

The *hly* gene encoding LLO was inserted into the bacterial expression plasmid pET29b with a polyhistidine (His6) tag. Recombinant LLO was purified from *E. coli* as described by Mandal et al., (*3*) with the following exceptions. LLO overexpression was induced in bacterial culture for 4-6 h with 1 mM IPTG, the cell pellet was collected and resuspended in wash buffer containing 2 mM PMSF and 1 mM 2-mercaptoethanol and then lysed using a French press. Lysate supernatant was adsorbed to Ni-NTA agarose and extensively washed with 200 bed volumes of wash buffer. His6-tagged LLO was eluted in wash buffer containing 400 mM imidazole and extensively dialyzed against HBSE pH 8.4 at 4°C. Protein purity and activity were assessed by SDS-PAGE and hemolysis assay, respectively, as previously described (*3*). Heat-inactivated LLO was prepared by heating at 70°C for 10 min (*6*).

**Liposome preparation**

Egg phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) and cholesteryl hemisuccinate (Sigma) lipid stocks (10 μmol:5 μmol) were combined and solvent was removed using Buchi rotary evaporation at < 10 mm Hg vacuum at RT. For establishing pre-immunity, lipid films were rehydrated with HBSE pH 8.4 containing a combination of OVA (2 mg) with or without LLO (100 μg). For NP-containing liposomes, lipid films were rehydrated with HBSE pH 8.4 containing NP (600 μg), LLO (100 μg) and CpG-ODN (2 mg). Note that the CpG-ODN was included in the liposomes to enhance then NP-specific immune response such that it was detectable by the established assays. Liposomes were freeze/thawed five times followed by five one-

minute sonication bursts, and unencapsulated materials were separated from liposomes by CL-4B size exclusion chromatography.

**Protein quantification**

OVA and LLO were resolved with standards containing known amounts of OVA and LLO in a 4-20% Tris-Glycine gel (Invitrogen) with 1X electrode buffer (250 mM Trizma base and 2 M glycine) with 0.1% SDS. Gels were stained with Krypton (Pierce) as per the manufacturer's instructions, and proteins were quantified by generating a standard curve using densitometry using a Typhoon 9200 (GE Healthcare) with ImageQuant 5.2 software.

NP-liposome samples were resolved with standards with known amounts of NP and LLO in a 4-20% Tris-Glycine gel as above and then transferred to a PVDF membrane using semi-dry transfer buffer (10 mM Trizma base, 4 mM glycine and 20% methanol) at 3.1 mA/cm$^2$. The membranes were blocked with 1% casein in PBS, washed with PBST (PBS + 0.05% Tween 20) and incubated with mouse anti-NP antibody (3.5 μg/mL, Dynavax) or rabbit anti-LLO polyclonal antibody (1:1,000) in PBST containing 2% BSA. Blots were washed thrice with PBST and incubated with goat anti-mouse alkaline phosphatase (AP)-conjugated antibody (Bio-Rad, 1:2,000) or goat anti-rabbit AP-conjugated antibody (Sigma, 1:20,000) in PBST containing 2% BSA. Blots were washed and developed with ECF western blotting reagent, and the bands were visualized with the Typhoon 9200. Densitometry was performed using ImageQuant 5.2, and calculations were based on known concentrations of proteins run in the same gel to determine NP and LLO concentrations in the liposome formulations.

**Immunization**

Female BALB/c mice (7-8 weeks old, Harlan; Indianapolis, IN) used in this study were handled according to Institutional Guidelines. Pre-existing immunity was established by immunizing mice subcutaneously at the base of the tail on day 0 and 7 with liposomes containing either OVA (12.7 μg) or liposomes containing OVA and LLO (12.7 μg and 1.3 μg). On day 14, three mice from each group were randomly selected and euthanized, blood was collected via cardiac puncture and spleens were isolated for cellular assays to verify that anti-LLO immunity was established. The remaining mice were immunized with liposomes containing NP, LLO and CpG-ODN on day 14 and day 27 (10 μg NP and 2 μg LLO). Mice were euthanized on day 37, blood was collected via cardiac puncture and spleens were isolated for assays.

**Cytokine secretion assays**

RBCs were lysed with ACK lysis solution (Invitrogen) for 2-3 min. Splenocytes were washed, counted and $5 \times 10^5$ cells were stimulated with either $NP_{147-155}$ peptide (Anaspec; 2.5 μg/mL), $OVA_{257-264}$ peptide (Anaspec; 2.5 μg/mL), $LLO_{91-99}$ peptide (Elim Biopharmaceuticals, Hayward, CA; 2.5 μg/mL), NP protein, OVA protein, heat-inactivated LLO (25 μg/mL) in duplicate for 72 h at 37°C, 5% $CO_2$. Supernatants were combined, and analysis for IFN-γ secretion was performed by ELISA as previously described (*7*). Briefly, Maxisorp plates (Nunc) were coated (for at least 3 d) at 4°C with IFN-γ (BD Bioscience; 2 μg/mL) in coating buffer (0.1 M sodium phosphate pH 9.0). Plates were blocked (1% BSA in PBS), and the supernatants were diluted in dilution buffer (1% BSA in PBST) and added to the plates in duplicate. Biotin IFN-γ (BD Bioscience, 1 μg/mL) was added in dilution buffer followed by avidin-horse radish

peroxidase (HRP, eBioscience) and detected with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). Color change was stopped with 2 N sulfuric acid, and absorbance values were detected at 450 nm using Emax plate reader (Molecular Devices). IFN-γ secretion values were determined from a standard curve.

**Ig ELISAs**

After mice were euthanized, cardiac puncture was performed to obtain blood, which was allowed to clot at 37°C for at least 1 h before it was placed at 4°C ON. Sera were separated from RBCs by centrifugation at 400-500 × g for 15 min, and added to ELISA plates coated with antigen (10 μg/mL in coating buffer) as indicated in results tables and blocked. Biotin anti-mouse IgG whole molecule $F(ab)_2$' fragment (1:20,000; Sigma) was used as a secondary antibody and detected by avidin-HRP (eBioscience). Plates were developed, stopped and read as described above. The data were fit using the 4-parameter curve from Softmax Pro software, and anti-antigen titers were calculated by solving the curve for the inverse dilution factor resulting an absorbance value at least two-fold greater than background.

***In vivo* CTL assay**

During the *in vivo* CTL optimization, in addition to determining the number of cells to adoptively transfer, a protocol for labeling the transferred cells with three serial dilutions of CFSE was established. This allows us to monitor CTLs generated to two different peptides, while using one population as the control. The *in vivo* CTL assay was performed as previously described with the following modifications (*6*). Briefly, RBC-lysed, single-cell spleen suspensions from naïve mice were separated into three populations that were pulsed at $2 \times 10^7$ cells/mL with either influenza $NP_{147-155}$ peptide (1

μM), $LLO_{91-99}$ peptide (1 μM), or media without peptide for 1 h at 37°C, 5% $CO_2$. The cells were washed and diluted to $1 \times 10^8$ cells/mL in PBS containing 0.1% FBS. The $NP_{147-155}$ peptide-pulsed, un-pulsed, and $LLO_{91-99}$ peptide-pulsed populations were labeled with 5, 1.25 and 0.31 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 8 min at RT. CFSE labeling was stopped by adding an equal volume of FBS and incubating for 5 min on ice. Cells were washed thrice with PBS containing 5% FBS, and an equal number of $NP_{147-155}$-pulsed ($CFSE^{HIGH}$), un-pulsed ($CFSE^{MED}$) and $LLO_{91-99}$-pulsed ($CFSE^{LOW}$) cells were mixed together. Either $1 \times 10^6$, $5 \times 10^6$ or $1 \times 10^7$ cells per population were injected intravenously via tail vein into naïve or immunized mice. Mice were euthanized 6 or 18 h post-adoptive transfer, spleens of recipient mice were harvested, and single cell suspensions were obtained by grinding spleens through a 70 μm mesh screen containing mouse media (RPMI 1640 containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol). Splenocytes were washed, purified using Ficoll-Paque Premium (GE Healthcare) and analyzed by FACSCalibur. At least four thousand CFSE-labeled cells were analyzed for each mouse. The percentage of peptide-specific lysis for each mouse was calculated by: 100 × [1-(ratio of cells recovered from naïve mice/ratio of cells recovered from immunized mice)]. Ratio of cells recovered: percent gated in peptide-pulsed (NP or LLO) cells/percent gated in un-pulsed cells.

**Statistical analyses**

Statistical differences were analyzed by two-tail Student's *t*-test, and a p-value < 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Verifying anti-LLO pre-immunity was established

Mice were primed and boosted as outlined in Figure B.1 to establish anti-LLO pre-immunity. To mimic a situation in which LLO-containing liposomes would be used as a vaccine carrier for one antigen, and then subsequent immunizations would be performed with LLO-containing liposomes as a carrier for an alternative antigen, we included the model antigen ovalbumin (OVA) in the liposomes when the LLO pre-immunity was established. Mice were immunized with liposomes containing only OVA or liposomes containing OVA and LLO; on day 14 three mice from each group were euthanized and assays were performed to ensure that LLO-specific cellular and humoral immune responses were stimulated.

#### *Pre-immunity: anti-LLO cellular response*

Splenocytes were stimulated *ex vivo* and IFN-γ secretion was monitored to verify activation of cellular immune responses. When splenocytes were stimulated with either OVA CD8 peptide SIINFEKL or OVA protein, there was no IFN-γ secretion from mice immunized with liposomes containing OVA or liposomes containing OVA and LLO (Table B.1). This was surprising based on previous studies (*3, 7*), which showed IFN-γ secretion from mice immunized with OVA-containing LLO-liposome formulations; however, in the previous studies the immunization schedule allowed two weeks between the prime and boost, and IFN-γ secretion was monitored two to four weeks after the boost. The earlier time point used in this study may not be sufficient for generation of detectable OVA-specific IFN-γ cells. The splenocytes from mice immunized with liposomes containing OVA and LLO secreted IFN-γ when stimulated *ex vivo* with the

LLO CD8 peptide ($3.6 \times 10^5 \pm 2.4 \times 10^5$ pg/mL) compared with undetectable levels of IFN-γ from splenocytes of liposomal OVA immunized mice (Table B.1). Lastly, splenocytes were stimulated with NP CD8 peptide and NP protein as a control to verify that the mice were not previously exposed to NP. There was no detectable IFN-γ secretion from splenocytes from mice immunized with either formulation when stimulated with NP CD8 peptide, indicating that the mice had not generated NP-specific $CD8^+$ T cells that could be activated with the immunodominant peptide. Unexpectedly, IFN-γ secretion was apparent when splenocytes from both groups were stimulated with NP protein, and there was significantly ($p=0.004$) more IFN-γ secretion from mice immunized with liposomes containing OVA and LLO ($1.1 \times 10^5 \pm 2.9 \times 10^4$ pg/mL) compared with mice immunized with liposomes containing OVA ($5.2 \times 10^3 \pm 6.5 \times 10^2$ pg/mL). These mice were not previously immunized with influenza NP, and the immunodominant NP CD8 peptides did not stimulate the splenocytes to secrete detectable IFN-γ. These data may indicate that there is an immunogenic component of LLO that is also found in the NP protein formulation (but not NP CD8 peptide), thereby activating the splenocytes from mice vaccinated with LLO to secrete IFN-γ upon NP stimulation.

***Pre-immunity: anti-LLO humoral response***

To verify the humoral immune responses, serial dilutions of mouse sera were added to ELISA plates coated with antigens. Both groups of mice generated similar anti-OVA antibody response regardless of the presence of LLO (Table B.2). As expected, the mice immunized with LLO-containing liposomes stimulated an enhanced ($p=0.016$) anti-LLO antibody response compared with mice immunized with liposomes only containing

OVA. Mice were also monitored for anti-NP antibody titers, and the results indicate that there were no differences in anti-NP antibody responses if the mice were immunized with liposomes containing OVA and LLO compared with mice immunized with liposomes containing OVA (Table B.2).

The ability of NP to stimulate splenocyte secretion of IFN-γ was surprising; therefore, to determine if we could narrow down the immunogenic component of NP, we looked for cross-reactive antibodies to other proteins with various similarities to NP (e.g. His-tag, bacterial expression). ELISA plates were coated with various proteins, and ELISA titers were determined by adding serial dilutions of sera from immunized mice. First, we determined that there was an enhanced anti-yellow fluorescent protein (YFP) titer when mice were immunized with liposomes containing OVA and LLO compared with mice immunized with liposomes containing OVA (Table B.2). This indicates that the immune response may be to a contaminant from the bacterial expression or from the His-tag on the protein. To test this hypothesis, we looked at the anti-NP titer using NP purified from Dynavax, which lacks a His-tag and was purified using a different method (see Chapter 4) than the NP made in the Lee lab; very low titer values that were not different between the groups were observed (Table B.2). In order to try to determine if the response was to a bacterial protein or the His-tag, we coated ELISA plates with either GST-tagged LCMV NP (*2*) or His-tagged apolipoprotein B (apoB)-YFP purified from Sf9 insect cells. There was a stronger response from sera from mice immunized with LLO and OVA compared with mice immunized with OVA when the plate was coated with GST-tagged LCMV NP ($1.2 \times 10^3 \pm 3.0 \times 10^2$ and $1.5 \times 10^2 \pm 1.2 \times 10^2$, respectively) or with His-tagged apoB-YFP ($1.6 \times 10^3 \pm 1.5 \times 10^2$ and $1.5 \times 10^1 \pm 1.6 \times$

$10^1$, respectively). However, statistical tests were not performed on these data because there were only enough sera from two mice from the LLO and OVA liposome immunized group. Overall, these data are not conclusive as to what is in the NP protein preparation that is activating splenocytes from mice immunized with LLO. It is clear that the immune response is different when comparing proteins purified in-house using His-tag with Dynavax's purification scheme. More sera would be required to compare responses between insect cell purified and GST-tagged proteins to better narrow down the potential activating substance.

**NP-specific immune responses in mice with pre-existing anti-LLO immunity**

***CTL response is diminished in mice with pre-existing anti-LLO immunity***

While performing these studies, extra mice were included in the group immunized with LLO- and OVA-containing liposomes, and these mice were used to determine the optimal number of cells to inject in order to be able to observe NP-specific lysis. In addition to monitoring NP-specific lysis, we wanted to monitor LLO-specific lysis to determine if any differences observed between the groups could be attributed to changes in LLO-specific lysis between the mice pre-immunized with OVA-containing liposomes or OVA- and LLO-containing liposomes. In order to do this, we established a method to label cells with three concentrations of CFSE in order to differentiate NP peptide-pulsed, LLO peptide-pulsed, and un-pulsed cell populations. Then, equal ratios of these cells were combined, and three different concentrations of cells were injected into mice that were euthanized at two different time points (6 h and 18 h) post-adoptive transfer. The results indicate that there was no NP-specific lysis in any of the mice that were euthanized 6 h post-adoptive transfer. In the mice that were euthanized 18 h post-

adoptive transfer, there was NP-specific lysis above background in the mice injected with $1 \times 10^6$ cells per population (Figure B.2), but not in the mice injected with $5 \times 10^6$ or $1 \times 10^7$ cells per population. These results indicate that for monitoring NP-specific lysis injecting $1 \times 10^6$ cells per population and allowing the killing to proceed for 18 h is optimal.

When monitoring LLO-specific lysis, there was a relatively high specific lysis of ~40% regardless of the number of cells per population that were injected. It was expected that there would be a trend of increasing lysis as the number of cells injected decreased, but this was not observed, and is potentially due to the fact that the 6 h time point only represents one mouse. The optimal LLO-specific lysis was observed when $1 \times 10^7$ cells per population were injected for 18 h (Figure B.2). Complete lysis was observed with $1 \times 10^6$ cells per population and one of the mice showed complete lysis in the $5 \times 10^6$ cells per population group. These results indicate that ten-fold more LLO-labeled cells are required to determine an LLO-specific lysis value compared with NP-labeled cells.

The optimization experiment was performed by injecting the same ratio of three populations of cells; it was unclear if injecting ten-fold more LLO-labeled cells would change the kinetics of killing. Therefore, we used the number of cells deemed optimal for NP-specific lysis ($1 \times 10^6$ cells per population). It was also hypothesized that since we used the group that was pre-immunized with OVA- and LLO-containing liposomes in the optimization that the group pre-immunized with OVA-containing liposomes may have a reduced LLO-specific lysis. Therefore, the experiment was performed as outlined in the optimization by injecting three populations of cells, un-pulsed or pulsed with either

NP peptide or LLO peptide, at $1 \times 10^6$ cells per population. The results indicate that there was a significant reduction ($p=0.0037$) in the NP-specific lysis when the mice were pre-immunized with liposomal OVA and LLO compared with liposomal OVA (45% ± 16 and 72% ± 14, respectively). Based on previous results performed in the lab, it was unexpected to see the reduction in NP-specific lysis in the group of mice with pre-immunity established against LLO. The LLO-specific lysis indicated that mice pre-immunized with liposomal OVA or liposomal OVA and LLO showed complete lysis of the LLO-pulsed peak (87% ± 3 and 84% ± 4). To use this method of determining LLO-specific lysis, future studies should be performed to ensure that injecting more LLO-pulsed cells does not change the NP-specific killing, and if so, extra mice would be required to separately monitor LLO-specific lysis.

***Reduced NP CD8 peptide-stimulated IFN-γ secretion in mice with pre-existing anti-LLO immunity***

Antigen-specific cytokine responses were determined by culturing the splenocytes from the immunized mice with either whole protein or the MHC class I immunodominant epitope from OVA, LLO, or NP for 72 h, and splenocyte supernatants were monitored for IFN-γ secretion using ELISA. No differences in IFN-γ secretion from the splenocytes of mice with or without anti-LLO pre-immunity were observed upon stimulation with whole OVA protein or $OVA_{257-264}$ peptide. It is, however, worth noting that four weeks after the OVA boost an immune response was observed, but this was not the case one-week after the OVA boost. When the splenocytes were stimulated with the $LLO_{91-99}$ peptide, there was also no significant difference observed; however, the splenocytes from the mice with anti-LLO pre-existing immunity secreted significantly less IFN-γ when stimulated with

heat-inactivated LLO (p=0.0309). In good agreement with the *in vivo* CTL results, the NP-specific secretion when stimulated with $NP_{147-155}$ peptide or NP protein was significantly reduced from mice pre-immunized with OVA- and LLO-containing liposomes compared with the mice immunized with only OVA-containing liposomes. We postulate that there was an immune response generated to an as yet unidentified component in the LLO-liposomes that resulted in the reduction in the NP-specific cellular immune response as shown by a reduction in CTLs and IFN-γ secretion.

***Anti-NP antibody response is diminished in mice with pre-existing anti-LLO immunity***

At the conclusion of the study, sera from the mice were monitored for antibodies to antigens used throughout the study by coating ELISA plates with various antigens and adding serial dilutions of sera to the ELISA plates. The anti-OVA antibodies were significantly enhanced (p=0.0025) in the mice pre-immunized with liposomes containing OVA and LLO compared with the mice pre-immunized with liposomes containing only OVA (Table B.4). The anti-LLO antibodies were significantly enhanced (p=0.0006) in the mice pre-immunized with liposomes containing OVA and LLO compared with the mice pre-immunized with liposomes containing OVA (Table B.4). This was not surprising since the mice in the pre-existing LLO immunity group had received four doses of LLO compared with the other group that had only received two doses of LLO. Lastly, in agreement with all of the cellular data indicating a reduction in the NP-specific cellular response in the mice with pre-existing anti-LLO immunity, there was also a significant (p=0.0112) reduction in the anti-NP antibody response (Table B.4).

These data do not agree with one previous study (M. Mandal; unpublished observations) performed in the Lee lab in which pre-existing LLO immunity was

generated using liposomes containing only LLO and the control was unloaded liposomes. In that study, the pre-existing anti-LLO immunity was monitored for its effect on generating an immune response to OVA when delivered in LLO-liposomes. The immunization timetable used in the current study very closely mimicked the previous study (day 0, 7, 16, 29, 40; see Figure B.1). Using OVA as the subsequent antigen may be advantageous as it is neither purified from bacteria nor contains a His-tag. Lastly, influenza NP is positively charged at pH used for liposome encapsulation, and we have demonstrated that its encapsulation efficiency is much greater than the theoretical maximum expected for passive encapsulation. This leads us to hypothesize that the NP may be electrostatically interacting with the negative charge of the liposomal membrane, thereby placing NP on the surface of the liposome to be recognized by the immune system.

**CONCLUSION**

We have shown that when establishing pre-immunity to LLO, an immune response is generated against either the His-tag or unidentified bacterial contaminants, as indicated by the NP protein-stimulated IFN-γ secretion from splenocytes of mice immunized with LLO and OVA. This non-LLO-specific pre-immunity complicated the interpretation of the results because it is currently unknown whether the reduction in the NP-specific immune responses is due to the anti-LLO pre-immunity or the non-LLO-specific pre-immunity.

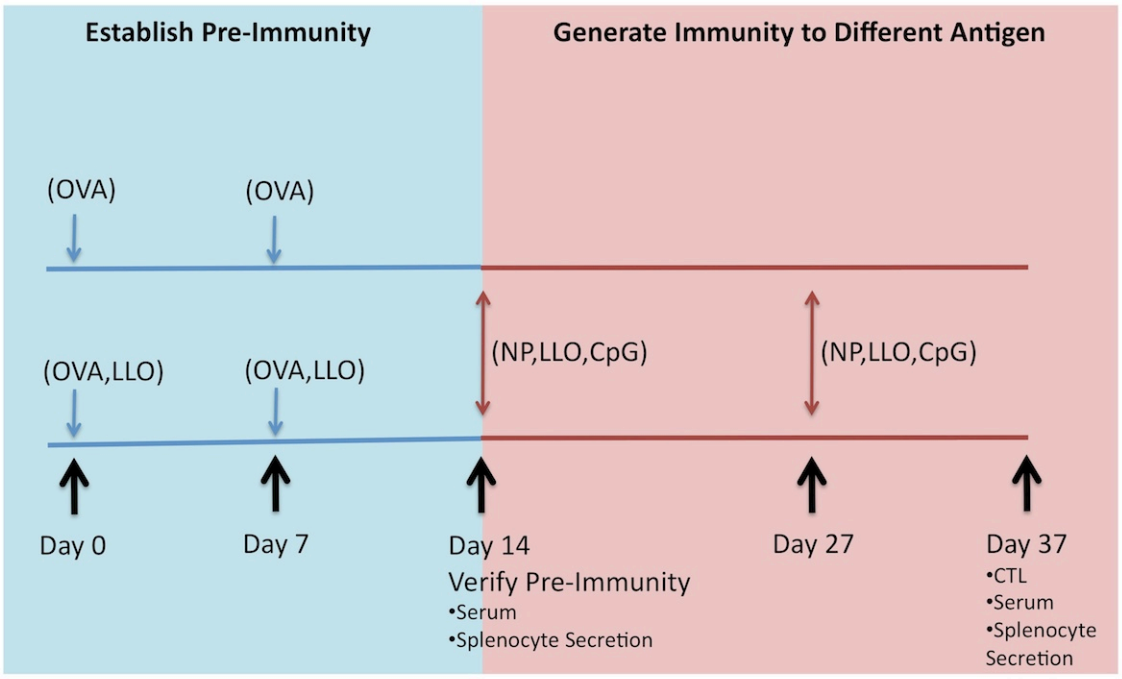


**Figure B.1: Schematic of immunization schedule.**

Anti-LLO pre-existing immunity was established by immunizing mice (BALB/c) subcutaneously at the base of the tail on day 0 and 7 with liposomes containing either OVA (12.7 μg) or liposomes containing OVA and LLO (12.7 μg and 1.3 μg). On day 14, three mice from each group were randomly selected and euthanized, blood was collected via cardiac puncture and spleens were isolated for assays to verify anti-LLO pre-immunity. The remaining mice were immunized with liposomes containing NP, LLO and CpG-ODN on day 14 and day 27 (10 μg NP and 2 μg LLO). Mice were euthanized on day 37, blood was collected via cardiac puncture and spleens were isolated for assays.

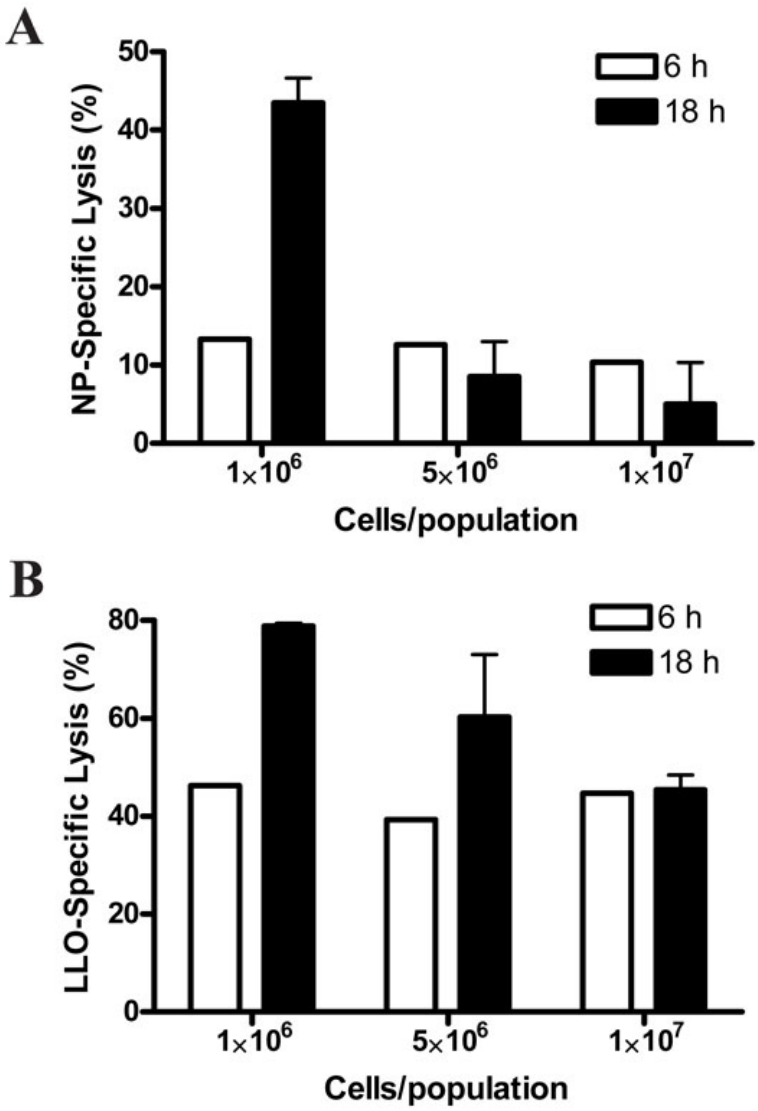


**Figure B.2: Optimization of *in vivo* CTL assay.**

Mice (n=9) were primed with OVA and LLO-containing liposomes as outlined in Figure B.1. On day 34, target cells were prepared. $CFSE^{HIGH}$ cells were pulsed with influenza $NP_{147-155}$ peptide (1 μM), $CFSE^{MED}$ cells were un-pulsed, and $CFSE^{LOW}$ cells were pulsed with $LLO_{91-99}$ peptide (1 μM). Equal numbers of each cell population were combined, and cells were adoptively transferred into mice (3 mice per group). One mouse from each group was euthanized 6 h after adoptive transfer, and two mice were euthanized 18 h after adoptive transfer. (A) NP-specific lysis and (B) LLO-specific lysis are shown. The mean lysis value for one mouse at 6 h is shown, and the mean ± SEM for two mice is shown for 18 h.

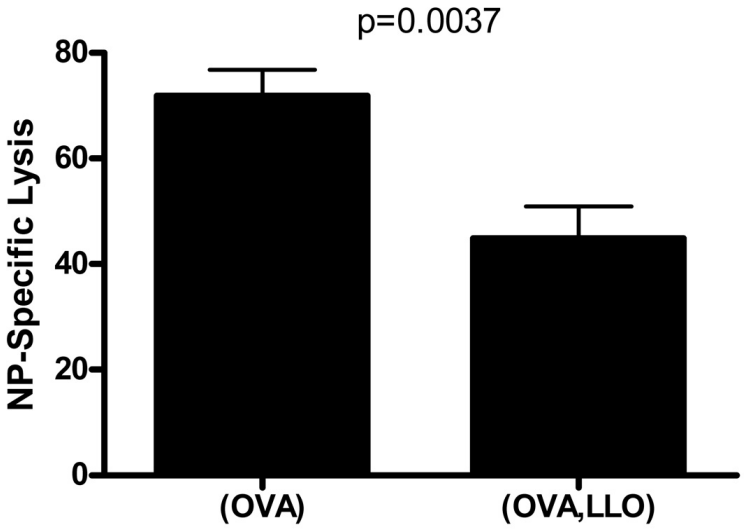


**Figure B.3: Anti-LLO pre-immunity diminishes the NP-specific CTL response.**

Mice were immunized as outlined in Figure B.1. On day 36, target cells were prepared. $CFSE^{HIGH}$ cells were pulsed with influenza $NP_{147-155}$ peptide (1 μM), $CFSE^{MED}$ cells were un-pulsed, and $CFSE^{LOW}$ cells were pulsed with $LLO_{91-99}$ peptide (1 μM). Equal numbers of each cell population were combined, and cells ($1 \times 10^6$ cells/population) were adoptively transferred into mice, and mice were euthanized 18 h post adoptive transfer. The mean ± SEM is shown (n=7-8 mice per group).

| Stimulant | Pre-Immunity Immunization (OVA) | Pre-Immunity Immunization (OVA,LLO) | p-value |
|---|---|---|---|
| $OVA_{257-264}$ CD8 peptide | ND | ND | |
| OVA protein | ND | ND | |
| | | | |
| $LLO_{91-99}$ CD8 peptide | ND | $3.6 \times 10^5 \pm 2.4 \times 10^5$ | |
| | | | |
| $NP_{147-155}$ CD8 peptide | ND | ND | |
| NP protein | $5.2 \times 10^3 \pm 6.7 \times 10^2$ | $1.1 \times 10^5 \pm 3.1 \times 10^4$ | 0.0309 |

**Table B.1: Verification of pre-existing anti-LLO cellular immunity, IFN-γ secretion (pg/mL).**

Three mice from each group were euthanized on day 14 (before NP immunization), and splenocytes were stimulated with peptide (2.5 μg/mL) or protein (25 μg/mL) for 72 h at 37°C, 5% $CO_2$. IFN-γ secretion (pg/mL) was determined by ELISA; the mean ± SEM is shown. ND, not determined.

| Coating antigen | His-tag | Bacterial Expression | Pre-Immunity (OVA) | Immunization (OVA,LLO) | p-value |
|---|---|---|---|---|---|
| **OVA** | **N** | **N** | $1.1 \times 10^4 \pm 1.2 \times 10^4$ | $2.4 \times 10^4 \pm 9.0 \times 10^3$ | NS |
| **LLO** | **Y** | **Y** | $6.0 \times 10^1 \pm 1.9 \times 10^1$ | $7.8 \times 10^3 \pm 3.3 \times 10^3$ | 0.016 |
| **NP (Lee Lab)** | **Y** | **Y** | $5.2 \times 10^1 \pm 2.2 \times 10^1$ | $5.3 \times 10^2 \pm 5.4 \times 10^2$ | NS |
| **NP (Dynavax)** | **N** | **Y** | $2.8 \times 10^1 \pm 1.7 \times 10^1$ | $7.5 \times 10^1 \pm 5.3 \times 10^1$ | NS |
| **YFP** | **Y** | **Y** | $3.8 \times 10^1 \pm 3.0 \times 10^1$ | $3.6 \times 10^2 \pm 1.6 \times 10^2$ | 0.039 |
| | | | | | |
| **GST LCMV NP** | **N** | **Y** | $1.5 \times 10^2 \pm 1.2 \times 10^2$ | $1.2 \times 10^3 \pm 3.0 \times 10^2$ | |
| **ApoB-YFP** | **Y** | **N** | $1.5 \times 10^1 \pm 1.6 \times 10^1$ | $1.6 \times 10^2 \pm 1.5 \times 10^2$ | |

**Table B.2: Verification of pre-existing anti-LLO humoral immunity, anti-antigen titer.**

Three mice from each group were euthanized on day 14 (before NP immunization), and blood was obtained by cardiac puncture. Plates were coated with antigens of interest (first column), and sera were added to plates in serial dilutions. The total anti-antigen antibody titer was determined by ELISA. Titer was defined as the reciprocal dilution resulting in an absorbance value of at least two-fold greater than background. If no titer was observed, the reciprocal of the lowest dilution tested was reported as the titer. Data shown are mean ± S.D. Statistics were not performed for anti-GST LCMV NP and anti-apoB YFP titers because there were only two values for the (OVA,LLO)-immunized group. N, no; NS, not significant; Y, yes.

| Stimulant | Pre-Immunity Immunization (OVA) | (OVA,LLO) | p-value |
|---|---|---|---|
| $OVA_{257-264}$ CD8 peptide | $3.2 \times 10^3 \pm 3.0 \times 10^3$ | $1.9 \times 10^3 \pm 2.4 \times 10^3$ | NS |
| OVA protein | $1.3 \times 10^4 \pm 9.9 \times 10^3$ | $2.0 \times 10^4 \pm 1.4 \times 10^4$ | NS |
| | | | |
| $LLO_{91-99}$ CD8 peptide | $2.6 \times 10^4 \pm 3.3 \times 10^4$ | $1.6 \times 10^4 \pm 1.8 \times 10^4$ | NS |
| Heat-inactivated LLO | $6.5 \times 10^5 \pm 2.4 \times 10^5$ | $4.2 \times 10^5 \pm 1.1 \times 10^5$ | 0.0309 |
| | | | |
| $NP_{147-155}$ CD8 peptide | $3.6 \times 10^4 \pm 3.0 \times 10^4$ | $6.8 \times 10^3 \pm 5.2 \times 10^3$ | 0.0326 |
| NP | $1.5 \times 10^6 \pm 1.2 \times 10^5$ | $1.3 \times 10^6 \pm 3.0 \times 10^5$ | 0.0437 |

**Table B.3: NP CD8 peptide-specific IFN-γ-secretion is inhibited by pre-existing anti-LLO immunity.**

Splenocytes from immunized mice (n=7-8) were stimulated with peptide (2.5 μg/mL) or protein (25 μg/mL) for 72 h at 37°C, 5% $CO_2$. IFN-γ secretion was determined by ELISA and all results are reported as mean (pg/mL) ± SEM. NS, not significant.

| Coating antigen | Pre-Immunity Immunization (OVA) | Pre-Immunity Immunization (OVA,LLO) | p-value |
|---|---|---|---|
| **OVA** | $1.4 \times 10^3 \pm 5.6 \times 10^2$ | $3.9 \times 10^3 \pm 1.5 \times 10^3$ | 0.0025 |
| **LLO** | $1.2 \times 10^4 \pm 8.1 \times 10^3$ | $9.7 \times 10^4 \pm 5.3 \times 10^4$ | 0.0006 |
| **NP** | $2.5 \times 10^5 \pm 1.2 \times 10^5$ | $1.1 \times 10^5 \pm 4.2 \times 10^4$ | 0.0112 |

**Table B.4: Humoral immune responses following NP immunizations.**

On day 37, mice (7-8) from each group were euthanized and sera were obtained by cardiac puncture. Plates were coated with antigens of interest (first column), and sera were added to plates in serial dilutions. The total anti-antigen antibody titer was determined by ELISA. Titer was defined as the reciprocal dilution resulting in an absorbance value of 1. Data shown are mean ± S.D.

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