EFFECT OF GLYCOLIPID INCORPORATION ON LIPOSOME UPTAKE BY ANTIGEN-PRESENTING CELLS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen-presenting cell
BMM	Bone marrow-derived macrophage
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHEMS	Cholesteryl hemisuccinate
Chol	Cholesterol
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DOPC	Dioleoyl phosphatidylcholine
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ePC	Egg phosphatidylcholine
ePE	Egg phosphatidylethanolamine
GM1	Monosialotetrahexosylganglioside
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HI-FBS	Heat-inactivated fetal bovine serum
HBS	HEPES buffered saline

HBSE	HEPES buffered saline with EDTA
iDC	Immature dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
LLO	Listeriolysin-O
LN	Lymph node
mDC	Mature dendritic cell
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute
mol%	mole percentage
MPS	Mononuclear phagocyte system
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine

PEG	Poly(ethylene glycol)
PI	Phosphatidylinositol
PG	Phosphatidylglycerol
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RT	Room temperature
s.c.	Subcutaneous
Th	T helper
TLR	Toll-like receptor

ABSTRACT

Robust cell-mediated immune responses are considered vital for successful vaccination against intracellular pathogens and cancer. Enhancing antigen uptake by dendritic cells (DCs) is a proposed way to stimulate cell-mediated immune responses when using typically low immunogenic protein antigens. Formulating antigen into a nanoparticle carrier can increase antigen uptake by two major antigen-presenting cells (APCs), DCs and macrophages. However, macrophage uptake is generally accepted as the main route of nanoparticle clearance due to macrophages' high phagocytic capability. To skew the antigen uptake by DC, previous approaches have relied on attaching complex targeting moieties to antigen itself or onto nanoparticle antigen carriers such as liposomes. We hypothesized that by retarding high macrophage uptake of liposomes, we could facilitate enhanced uptake of liposomes by DCs. Simple liposome formulations containing phosphatidylinositol (PI) or monosialotetrahexosylganglioside (GM1) are well documented to deter rapid uptake by macrophages. *In vitro* uptake studies showed that incorporating 10mol% PI promoted uptake by DCs, while having minimal effect on uptake by macrophages. This trend was not observed with 10mol% GM1 incorporation. *In vivo* uptake studies upon subcutaneous injection confirmed PI-liposomes are indeed internalized more by DCs than GM1-liposomes, however, this result was also observed in macrophages. These liposomal formulations are of interest as vaccine carriers to stimulate enhanced cell-

mediated immune responses and the generation of $CD8^+$ T cells via enhanced uptake by DCs. Extending on our previous work using liposomes co-encapsulating the model antigen (OVA) and a hemolysin, listeriolysin-O (LLO), we show, herein, that PI- and GM1-liposomes encapsulating OVA and LLO can deliver OVA to the cytosol of DCs and macrophages in cell culture, resulting in efficient antigen presentation to $CD8^+$ T cells. Mice immunized with PI-liposomes or GM1-liposomes co-encapsulating OVA and LLO generated similar $CD8^+$ T cell-mediated immune responses as determined by MHC I tetramer staining and IFN–gamma ELISPOT analysis. Vaccination with either liposome formulation resulted in enhanced antigen-specific serum IgG2a titers indicative of better Th1 helper T cell activation. Both PI-liposomes and GM1-liposomes represent simple, inexpensive vaccine carriers for effectively stimulating cell-mediated immune responses utilizing subunit protein antigens.

CHAPTER 1

Directing Vaccine Delivery to Dendritic Cells

INTRODUCTION

Vaccination approaches in history

One of the most important medical discoveries, the act of vaccination, remains the most powerful and cost-effective tool for preventing disease [1]. As a result of widespread vaccination, the incidence and mortality of life-threatening diseases such a diphtheria, meningitis, measles, polio, and smallpox has been reduced by >97-99% [2, 3]. Most vaccines in use today consist of live attenuated pathogen, inactivated pathogen, or protein subunit preparations. Live attenuated pathogens are the most successful vaccines and have been administered to billions of people worldwide, conferring protective immunity that often lasts for decades with just one vaccination [1, 4]. Live attenuated vaccines are effective at preventing infection because the vaccine elicits strong cellular and antibody immune responses, mimicking the protective immunity a person would develop in response to a natural infection [4]. However, the use of live attenuated vaccines is limited to invariant pathogens and infections that can be reduced as a result of prior exposure(s). Even live attenuated vaccines would not be effective at controlling some of the world's most concerning diseases resulting from the variable pathogen human immunodeficiency virus (HIV), or malaria, where prior

infections do not confer lasting immunity [1]. Furthermore, live vaccination is contraindicated for immunocompromised individuals, which includes patients with HIV/AIDS, young children, and the elderly population. Safety issues are also of concern considering that live attenuated pathogens persistently replicate inside the host, leaving the potential to revert back to a pathogen with partial or full virulence, as was the case with the oral polio vaccine [5]. Safety concerns stemming from live vaccination together with increased knowledge on recombinant expression of proteins shifted vaccine development towards protein subunit vaccine preparations [3, 4]. Subunit protein vaccines utilize antigenic protein(s) to initiate host immune responses. The drawback of this type of vaccine is that poorly immunogenic protein antigen(s) are primarily responsible for conferring immunity in a host. The success of protein subunit vaccines has so far been limited as many fail to produce protective, memory immune responses [1, 6].

Intracellular pathogens require cellular immune responses

All currently licensed vaccines (killed or inactivated, whole-cell, recombinant protein, or live attenuated) generate humoral [antibody] immune responses (Table 1.1) [7-9]. Antibody production as a result of vaccination is effective against many viral and bacterial infections [3, 10]. However, for intracellular viral infections or cancer-causing cells, antibody production alone is not the proper immune response to clear the infection or disease. Many intracellular pathogens such as HIV, *Plasmodium* (causing malaria), hepatitis C, and *Mycobacterium tuberculosis*, which require cell-mediated immune

responses in addition to humoral responses in order to protect the host, remain without effective vaccines [1, 10-13]. Cell-mediated immune responses involve eliminating infected or cancerous cells via the cytotoxic T lymphocytes (CTLs) with support from T helper (Th) 1 cells. This type of response is primarily directed at intracellular pathogens, which persist inside host cells protected from antibody recognition and opsonization. Many of the marketed live attenuated vaccines including those against hepatitis A, hepatitis B, influenza, varicella, typhoid fever and measles have been shown to induce cell-mediated immune responses (Table 1.1). Those cellular immune responses generated are believed to be in part responsible for the long-lasting protective immunity generated against these pathogens [9]. However, as mentioned previously, safety concerns of live vaccines have limited their future development. New vaccine strategies focus on enhancing the immunogenicity of protein subunit vaccines. To increase the effectiveness of protein subunit vaccines often requires the use of high antigen doses, booster immunizations, the addition of an adjuvant(s), or a combination of these measures [9].

Types of adjuvants

Adjuvants are a heterogenous group of substances that are capable of increasing or modulating humoral and/or cellular immune responses to the delivered antigen [14]. When utilized in vaccine formulations, adjuvants can facilitate a decrease in antigen amounts required in the formulation, less frequent vaccine dosing, improved responses among former non-responders, and stronger, swifter immune responses [15]. Adjuvants

can be broadly separated into two main classes, immunostimulants and delivery systems. Adjuvants found in either class can be used as the only adjuvant component in vaccine formulations but a combination of immunostimulant and a delivery system has been shown to further enhance immune responses and even provide a synergistic effect [16].

Immunostimulants

Adjuvants classified as immunostimulants activate the innate immune response. Many of these adjuvants are pathogen-associated molecular patterns (PAMPs), conserved components of microbes (e.g. viral nucleic acids, components of bacterial and fungal cell walls and flagellar protein) that are not found in eukaryotic life. Receptors that recognize these PAMPs, called pattern recognition receptors (PRRs), provide the innate immune system with a way to distinguish self and non-self derived molecules [17]. There are many receptor families defined as PRRs, including toll-like receptors (TLRs), C-type lectin receptors (CLRs) and RIG-I-like receptors (RLRs).

Delivery systems

This first generation of adjuvants includes the aluminum salts (collectively termed as alum), oil-in-water emulsions, polymeric particles and liposomes. Alum and emulsions induce local inflammation which recruits immune cells to the site of injection resulting in increased antigen exposure [18]. Polymeric particles and liposomes act as adjuvants by protecting the antigen against degradation and enhancing antigen delivery to antigen-presenting cells of the immune system [17]. Both approaches extend antigen duration in the host and additionally increase uptake of the antigen by immune cells,

providing an adjuvant effect.

Immune system responses

The immune system is comprised of two response types, the innate and the adaptive. The innate immune response is an immediate, non-pathogen specific response to infection. Using a variety of PRRs the innate immune system can sense self from non-self by PAMPs [19]. PAMPs are conserved patterns commonly expressed by microbes including viral nucleic acids, components of bacterial and fungal cell walls and flagellar proteins [20]. Innate immune responses are essential for early inhibition of infection and viral/bacterial proliferation. The innate immune system functions to produce molecules that stimulate the immune system (e.g., chemokines, cytokines and interferons (IFNs)) and activate leukocytes (e.g., macrophages) in response to infection [21].

The other branch of the immune system, the adaptive immune response, takes anywhere from days to weeks to develop and is an antigen-specific response to infection. The adaptive immune response relies on the innate immune response to recognize whether a detected antigen is self or non-self. Based on the determination of non-self, the adaptive immune response develops to aid in long-term protective immunity towards the antigen [19]. Memory cells are activated by the adaptive immune response to provide an immunological memory of encountered pathogens. Upon future encounters with a memorized pathogen, the immune system is able to react faster and stronger to clear the infection [22].

Tailoring adaptive immune responses

The adaptive immune response is further divided into the cell-mediated and humoral responses. While the cell-mediated immune response is essential for directly killing infected cells, the antibody response provided by humoral immunity is important for lasting protective immunity [23]. The branch of adaptive immunity that is activated is dependent on where the antigen is detected in the cell. The major histocompatibility complex (MHC) class I and class II pathways deliver antigen found inside the cell to the cell surface for immune recognition. The purpose of the MHC class I pathway is to present antigens found in the cytosol while MHC class II molecules present antigens found in the endocytic pathway [24]. The MHC molecules that perform the actions of binding and presenting antigen have distinct immune activating properties. MHC class I molecules brought to the surface with an exogenous antigen activate $CD8^+$ T cells (CTLS). Activation of CTLs promotes the specific lysis of infected cells necessary for clearing intracellular infections. MHC class II molecules brought to the surface with exogenous antigen recognized in the endocytic pathway will activate $CD4^+$ T cells [24, 25].

$CD4^+$ T cells can polarize into Th1 or Th2 cells that can either enhance or inhibit cell-mediated immunity, respectively [26]. Skewing towards Th1 promotes cell mediated-immunity with the secretion of Th1-type cytokines such as IL-12, TNF-α and IFN-γ. These molecules enhance the activation of CTLs and macrophages and promote the differentiation of B cells [23, 26]. B cells that are activated by Th1-type cytokines secrete IgG2a, an opsonizing antibody associated with viral clearance. On the other

hand, skewing T cell differentiation towards Th2 results in IL-4 and IL-5 production and the proliferation of B cells that secrete IgG1, a neutralizing antibody [26-28]. A Th2 type response promotes the other side of adaptive immunity, the humoral response [23]. All currently approved vaccines evoke humoral immune responses. However, humoral immunity alone is not able to clear some intracellular infections. Additional components are needed to skew Th1 polarization in response to vaccination [11].

Antigen-presenting cells

Antigen-presenting cells (APCs) are defined by their expression of MHC class II molecules. Propagation of the immune response is dependent upon the ability of APCs to capture, internalize, process and present antigen on the cell surface via MHC molecules (I or II). Many cell types express MHC class II molecules including endothelial cells, epithelial cells, tumor cells, B lymphocytes, macrophages and dendritic cells (DCs). All cell types expressing MHC class II molecules are able to engage T cells in an antigen-specific manner. However, antigen-presenting cells have different efficiencies of antigen-presentation, leading to the term 'professional' APCs, which includes B cells, macrophages and DCs [29].

B lymphocytes

A B cell's primary function is to produce antibodies. B cells are not particularly efficient in antigen capture due to poor phagocytic capability [30, 31]. Rather they are able to internalize antigen that binds via their immunoglobulin (Ig) surface receptors [32, 33]. B cells express high levels of MHC class II molecules as well as abundant

costimulatory and adhesion molecules needed for T cell interactions. They are efficient at generating antigen-MHC complexes and MHC class II presentation to T cells, which in turn promotes B cell expansion and antibody production [29, 34].

Macrophages

Macrophages have an extraordinary capacity for internalizing antigen of many forms including soluble, whole-cell, and receptor- or ligand-bound [29, 35]. Macrophages express MHC-I and MHC-II as well as the costimulatory molecules needed for cell-cell interactions. However, their expression of MHC-II molecules is lower than that of B cells and DCs, which leads to a lower capacity to stimulate T cells [36, 37]. In addition, the endocytic pathway of macrophages is more tailored for degradation than loading of antigen for subsequent MHC presentation to T cells [38, 39].

Dendritic cells

Dendritic cells' main function appears to be antigen-presentation to T cells [39]. DCs have a higher capacity to stimulate T cell interactions due to their high expression of MHC-II and costimulatory molecules [24, 29, 40, 41]. Lysosomes of immature DCs sequester large amounts of MHC-II molecules which antigen is ultimately directed to after internalization; however, only upon DC maturation can antigen-MHC complexes be efficiently presented to T cells. In response, DCs sequester antigen-MHC complexes in lysosomes until the immature DC has matured [42]. Thereafter, DCs have a unique ability to facilitate the movement of MHC II molecules from lysosomes to the cell surface [43, 44]. Further supporting their role in antigen-presentation, DC's characteristic shape enables them to interact with multiple T cells simultaneously [29]. Due to these unique

properties, DCs' ability to stimulate T cells can surpass macrophages' by orders of magnitude [45, 46].

Dendritic cell identification and plasticity

Ralph Steinman first identified dendritic cells in the peripheral lymphoid organs of mice in 1973. These cells were weakly and transiently adherent while displaying a distinctly different morphology from that of macrophages or lymphocytes. Dendritic cells are so named because of their stellate appearance, displaying dendrites from the body of the cell outwards [47]. Since initial discovery, research efforts on dendritic cells has grown expansively and several subsets of DCs have been identified, all of which have specialized functions in immune responses (Figure 1.1).

The maturity of a given dendritic cell shapes that cell's function. Normally, DCs in the peripheral tissues and spleen, as well as approximately 50% of DC in the lymph nodes are immature [48, 49]. Immature dendritic cells (iDCs) are highly efficient at antigen capture and therefore act as early-warning systems for the presence of non-self antigens via numerous uptake routes such as phagocytosis, macropinocytosis and receptor-mediated endocytosis [25]. Macropinocytosis and receptor-mediated endocytosis, in particular, facilitate efficient antigen uptake. Antigen internalization by iDCs via these routes allow for antigen presentation even at nanomolar antigen concentrations, a distinct improvement from the usual micromolar values required by other APCs [24]. Antigen-capture and proper inflammatory maturation stimuli direct iDCs to mature. However, if DCs do not receive maturation signals (present in an

inflammatory environment), these cells will remain immature and antigen presentation will lead to immune regulation and/or suppression. Maturation of DCs is associated with the down-regulation of antigen-capture activity, the increased expression of surface MHC class II molecules and co-stimulatory molecules (CD86, CD80 and CD40), the ability to secrete cytokines, as well as the acquisition of CCR7, which allows migration of the DC into the draining lymph node. Mature DCs (mDCs) can initiate differentiation of antigen-specific T cells into effector T cells with unique functions and cytokine profiles and also interact with B cells for humoral responses [24, 49, 50].

The dendritic cell's unique role in immunity

From a vaccination standpoint, dendritic cells represent an important target for stimulating robust cellular immune responses towards delivered antigens. DCs are present in nearly all tissues with enriched cell numbers where antigen exposure is more frequent, such as in lymphoid organs, at body surfaces and at mucosal surfaces [51]. DCs are potent stimulators of T cells where *in vitro* it has been estimated that one mDC can activate 100-3,000 T cells, underlining their integral role in the development of cell-mediated immune responses [24].

Antigen cross-presentation

While both macrophages and dendritic cells are capable of the traditional MHC Class I and II types of antigen presentation, it is DCs that are unique in their ability to "cross-present" antigens to T cells and induce T cell activation *in vivo* [43, 52, 53]. Cross-presentation allows exogenously delivered antigen to be loaded on MHC Class I

molecules resulting in presentation to $CD8^+$ T cells, which are critical in cell-mediated immune responses [54, 55]. DCs, unlike macrophages, are able to prime naïve CTLs and helper $CD4^+$ T cells using cross-presented antigens [52, 53, 56]. Presumably this is because DCs are the only cells that possess the necessary costimulatory signals, such as CD80 and CD86, required for T cell activation. Activation of naïve $CD4^+$ and $CD8^+$ T cells, leads to T cell proliferation and their transition into effector cells [49, 57].

Interactions with other antigen-presenting cells

DCs, highly acknowledged for their T cell interactions, are also fundamental in directing B cell growth, differentiation and immunoglobulin production [24]. B cells can be influenced by DCs indirectly through the release of cytokines from DCs and T cells, as well as directly though cell-cell interactions [58]. There may also be a role for macrophages in enhancing DC antigen presentation. Dendritic cells have been shown to develop antigen-specific T cell responses from antigenic peptides generated by macrophages [59, 60].

Dendritic cell vaccines-successes/failures

Ex vivo DC-based vaccines

Given their central role in controlling cellular and humoral immunity, dendritic cells represent a logical target for antigen delivery and therapeutic vaccines against intracellular infections and cancer. DC targeting has historically been dominated by the *ex vivo* approach. This approach involves isolating dendritic cells present in the patient's blood, stimulating the DCs with selected antigen(s) and/or maturation stimuli *ex vivo,*

and then reinjecting those modified DCs into the patient with the intention of inducing antigen-specific B and T cell responses. Thousands of reports have published clinical findings based on the *in vitro* priming of DCs with antigen, however most of these DC-based vaccines did not progress past Phase II clinical trial testing [51]. The only vaccine that proved the *ex vivo* approach to be a success came in 2010 by FDA approval of Sipuleucel-T, a DC-based vaccine for prostate cancer [61]. Several issues may explain the lack of success using this vaccination approach, including uncertainty in the route of cell administration and numbers of DCs to inject, shortage of TLR agonists (to induce DC maturation), choice of antigen and clinical testing primarily on late-stage cancer patients [62, 63]**.** Addition concerns relate to the high costs and labor-intensive aspect of the personalized vaccine therapy. Overall, what these DC-based vaccine trials did show is that they were safe, well tolerated by patients and capable of inducing potent immune responses in some cases [49] (Table 1.2).

In vivo DC-based vaccines

Exploiting DCs for vaccination has largely turned to *in vivo* targeted approaches. Intensive research on DC biology has identified cell surface receptors that are more or less DC-specific, allowing for an opportunity to target these APCs directly (Table 1.3). Options for DC targeting include the use of: antibodies for DC-specific surface molecules, ligands to bind to DC-specific surface molecules, and DC-specific liposome or virosome preparations. Antibodies for receptors have the potential to be highly specific targeting moieties. In mice, antibody-fused antigen is hundreds of times more efficient than untargeted antigens and offers options for antigen presentation on both

MHCI and MHCII molecules to $CD8^+$ and $CD4^+$ T cells, respectively [64, 65]. Particles bound to anti-DEC205 or anti-CD11c also show enhanced uptake by DCs compared to control particles *in vivo* [66], resulting in protection against tumor growth in mice [67, 68]. However, antibody-based targeting faces numerous drawbacks, including their higher cost to develop and the potential to negatively affect antibody affinity by addition of a conjugate. Even if these challenges could be overcome, the antibody-based targeting method still relies on the addition of another molecule (cytokine, interleukin, PAMP) to the formulation in order to promote immunity rather than tolerance [65, 69, 70]. Other studies have shown that conjugating a ligand for a DC-specific cell surface receptor results in enhanced antigen-specific responses [71-73]. Although ligands for the DC-SIGN receptor on DCs are known, they are rather complex, which would ultimately place a burden on the development of a vaccine. To conclude, a few approaches to target DCs have proved promising in harnessing DCs' immunogenicity, yet there still is room for improvement in terms of simpler preparation and cost.

Multicomponent vaccine delivery carriers

Delivery systems themselves can be classified as adjuvants due to their abilities to target material to APCs, target antigen to the cytosol, and protect antigen from degradation. The combination of these abilities can improve the immune response while reducing the amount of antigen needed.

Co-delivery of antigen and adjuvant

Traditional vaccines comprised of soluble antigen formulated with alum or in an emulsion form have not been practical for combatting infections that require robust cellular immune responses. Soluble antigen is typically weakly stable and has low bioavailability [74]. By formulating soluble antigen and adjuvant into a particulate carrier, antigen can be protected from degradation. In addition, previous work has shown that delivering antigen and adjuvant to the same APC via a nanoparticle carrier can enhance both humoral and cellular immune responses [75-77]. Further, delivery of antigen and adjuvant to the cytosol of cells generates enhanced cellular immunity and Th1 responses [78]. An inherent advantage of using a carrier to deliver antigen is the versatility of the delivery system. Multiple antigens can be encapsulated to enhance overall specific immune responses towards the target pathogen and additional incorporation of adjuvant(s) such as TLR or CLR ligands into the carrier would allow for selective tailoring of immune responses.

Mediating endosomal escape via Listeriolysin O (LLO)

Proteins ingested by cells are typically degraded in the endosome and loaded onto MHC Class II molecules for presentation to $CD4^+$ T cells. Proteins cannot readily access the cytosol for loading onto MHC Class I molecules and presentation to $CD8^+$ T cells. To enhance antigen delivery to the cytosol, a bacterial hemolysin from *Listeria monocytogenes (Lm)*, listeriolysin-O (LLO), can be co-encapsulated with antigen in liposomes [79-81]. When *Lm* is phagocytosed, LLO protein is secreted into the host's endosomal compartment. At pH 5.5, conditions present in the acidifying endocytic

pathway, LLO is most active and forms pores in the endosomal membrane by binding membrane cholesterol and oligomerizing, thus facilitating the bacteria's escape into the cytosol [82]. How LLO secretion forms pores in the endosomal membrane is not completely elucidated but the presence of two bacterial phospholipases PI-PLC and PC-PLC may be additionally required to enhance release of the endosomal components [83, 84]. Nevertheless, it has been shown that using purified LLO, co-encapsulated with antigen and adjuvant, delivers antigen to the cytosol of APCs [78, 85].

Liposome uptake and the generation of long-circulating liposomes

Liposome carriers

Liposomes are particularly appealing as a vaccine carrier because a liposome's size and shape resembles that of viral particles, which are naturally targeted for uptake by APCs. Liposomes are also useful platforms for delivery of materials, with their hydrophobic bilayer and aqueous center enabling these carriers to encapsulate both hydrophilic and hydrophobic cargo. The liposomal membrane additionally provides protection of encapsulated cargo from recognition by neutralizing antibodies and degradation by blood serum proteins. However, the attractiveness of liposomes as a carrier diminishes greatly when delivery of material requires bypassing macrophage uptake in the MPS (mononuclear phagocyte system) (i.e., delivery of antigen to peripheral tissue including lymph nodes). Conventional liposome formulations have circulation half-lives that can vary from a few minutes to multiple hours (depending on the dosage, particle diameter, surface charge, and bilayer fluidity) as a result of their

rapid uptake into the cells of the MPS [86]. Thus, conventional liposomes' therapeutic use has been limited to applications involving delivery of drugs to this system (i.e., spleen and liver). The use of glycolipids and hydrophilic polymers in the liposome formulation has been proven to decrease uptake of liposomes by the mononuclear phagocyte system [86, 87].

Long circulating liposomes

Regardless of the administration route, antigens and vaccine carriers, particularly nanosized carriers, are avidly taken up by local macrophages and by macrophages of the MPS when introduced into systemic circulation. It was discovered that some anionic lipids incorporated into conventional liposome formulations could alter circulation half-life by reducing macrophage uptake of liposomes in the spleen and liver [88]. All anionic lipids are not capable of reducing macrophage uptake; while phosphatidylserine (PS) or phosphatidylglycerol (PG) reduce liposome half-life *in vivo* to minutes, the glycolipids phosphatidylinositol (PI) or monosialotetrahexosylganglioside (GM1) results in prolonged half-lives [88-91]. Liposomes with anionic lipids that markedly reduce macrophage uptake by the MPS were termed long circulating liposomes. Compared to neutral liposomes, negatively charged liposomes had once been strictly associated with decreased circulation times and increased uptake by cells [92, 93]. However, the realization that incorporating negatively charged GM1 and PI lipids could reduce liposomal uptake by macrophage cells offered up a different theory to explain anionic liposomes and their interactions with cells. To explain this discrepancy, anionic lipids can be divided into two categories. For example, PS and PG

lipids, which reduce half-life *in vivo*, have negatively charged groups that are exposed to the environment on the outer surface of the liposome. This may allow for direct interaction between the negatively charged group on the liposome surface and either plasma proteins or cell-surface proteins, thus accelerating liposome clearance. In the case of both GM1 and PI, a bulky hydrophilic group is thought to shield the negative charge and reduce liposome interaction with opsonizing proteins [88]. The affinity of divalent calcium ions in the blood to exposed negatively charged lipid head groups may also play a role in liposome aggregation and instability [94, 95]. A shielded negative charge in that case would prevent vesicle aggregates and deter rapid clearance [88].

The main purpose of long-circulating liposomes, including PI- and GM1-containing liposomes, at the time of their development was to deter the macrophage uptake (by the MPS) of liposomes after intravenous injection. This allowed for increased numbers of liposomes to travel through the MPS, ultimately reaching peripheral tissues, especially tumor [86]. Delivery to tumor via lipid nanoparticles was a challenge before long-circulating liposomes, however with their development, encapsulated chemotherapeutics could now be delivered directly to tumor tissues [96, 97]. The unique ability of long-circulating liposomes to deter rapid macrophage uptake *in vivo* has additional potential in the context of vaccine design. Directing antigen-containing liposomes away from dominant macrophage uptake may facilitate potential interaction and uptake by DCs in the body. When long circulating liposomes were developed, there was scarce knowledge of dendritic cells and their functions. In the past 40 years, DCs have become a well-characterized cell type known for its distinct and fundamental role

in the immune response. Yet, interactions between long circulating liposome formulations and DCs have not been studied in detail. The behavior of PI- and GM1-containing liposomes has also not been previously studied after subcutaneous injection, which would be a preferable route of administration for vaccines.

Conclusion

Directing more antigen to dendritic cells, away from macrophages, has the potential to improve immune responses to subunit protein vaccines. Directly targeting antigen (and adjuvant(s)) to dendritic cells using DC-specific antibodies or ligands specific for DC surface receptors are expensive, labor-intensive targeting approaches. We embraced the idea that long-circulating liposome formulations, capable of reducing initial rapid macrophage uptake, may be useful for passively targeting DCs in peripheral tissues such as the lymph nodes, where DCs are in high numbers and close proximity to effector immune cells. Increasing the antigen uptake by DCs ever slightly could have a profound effect on T and B cell activation, proliferation and immune stimulating properties, which may enhance the immunogenicity of a liposomal subunit protein vaccine preparation.

Pathogen	Type of vaccine	Vaccine-induced protective immunity	Mechanisms of immune control during infection
Virus			
Smallpox	Live	Antibodies, CTL	CTL
Rabies	Killed virus	Antibodies	Antibodies, CD4, CTL
Polio	Live or killed virus	Antibodies	Antibodies
Measles	Live	Antibodies, CTL	Antibodies, CD4, CTL
Mumps	Live	Antibodies	Antibodies
Rubella	Live	Antibodies	Antibodies
Varicella zoster	Live	Antibodies, CTL	Antibodies, CTL
Haemophilus influenza type B	Hib-conjugated polysaccharide	Antibodies	NA
Influenza	Protein	Antibodies	Antibodies, CD4, CTL
Hepatitis A	Killed virus	Antibodies	Antibodies, CD4, CTL
Hepatitis B	Protein	Antibodies	Antibodies, CD4, CTL
Human papillomavirus	VLP or protein	Antibodies	CD4, CTL
Bacteria/Toxoid			
Diphtheria	Toxoid	Antibodies	NA
Tetanus	Toxoid	Antibodies	NA
Pertussis	Acellular	Antibodies	NA
Bacillus Calmette-Guérin (BCG)	Live	Antibodies, CTL	Antibodies, CD4, CTL
Typhoid (Ty21a)	Live	Antibodies, CTL	Antibodies, CD4, CTL
CD4, $CD4^+$ T cell; CTL, cytotoxic T lymphocyte; NA, not available			

Table 1.1 Correlates of immune protection (adapted from (9) and (10))

All currently licensed vaccines in the United States established protective immunity based on antibody responses following immunization. Further studies have indicated that $CD4^+$ and $CD8^+$ (CTL) T cell responses play a role in protection for several vaccines.

Vaccine and antigen	Indication	Key observations
GM-CSF–IL-4 DCs with or without HLA-A*0201-restricted peptides or peptides alone	Metastatic prostate cancer	Immunogenicity of DCs
GM-CSF–IL-4 DCs with peptides, tumor lysates or autologous tumor-eluted peptides	Stage IV melanoma, renal cell carcinoma and malignant glioma	Loading DCs with complex antigen preparations
Blood DCs and idiotype antigens	Multiple myeloma	• Immunogenicity of DCs • Tumor regression
Mature GM-CSF–IL-4 DCs and peptides	Stage IV melanoma	• Well-controlled and validated vaccine manufacture process • Testing mature DCs • Immunogenicity
$CD34^+$ HPC-derived DCs and peptides	Stage IV melanoma	• Loading vaccines with a mixture of well-defined peptides • Durable immune responses in long-term survivors
FLT3 ligand-expanded blood DCs and altered peptides	Advanced CEA^+ cancer	Immunogenicity
Immature GM-CSF–IL-4 DCs	Healthy volunteers	Antigen-specific inhibition of effector T cell function after injection of immature DCs
GM-CSF–IL-4 DCs and tumor lysates	Refractory paediatric solid tumors	Immunogenicity
Mature cryopreserved GM-CSF–IL-4 DCs	Stage IV melanoma	Immunogenicity
DCs loaded with autologous tumor RNA	Colon cancer	• Feasibility • Immunogenicity
DCs loaded with killed allogeneic tumor cells	Stage IV melanoma	• Immunogenicity • Durable objective clinical responses • Long-term survival
Monocyte-derived DCs loaded with the NK T cell ligand α-galactosylceramide	Advanced cancer	Adjuvant effect of NK cell activation on $CD8^+$ T cell-mediated immune response
Monocyte-derived DCs	Melanoma	*In vivo* identification of antigen-specific immune response by PET imaging in patients
		Route of DC administration affects T cell activation

Continued on next page

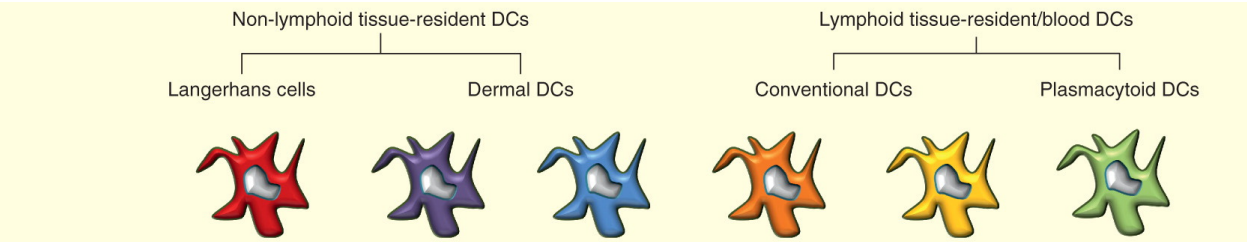
Vaccine and antigen	Indication	Key observations
Comparative study of $CD34^+$ HPC-derived LCs versus monocyte-derived DCs	Melanoma	LC-based vaccines stimulated significantly greater tyrosinase-HLA-A*0201 tetramer reactivity than the monocyte-derived DC vaccines
Type 1-polarized monocyte-derived DCs	Glioma	Combination of DC vaccination with polyICLC to trigger systemic inflammation driven by type I interferon family members
CEA, carcinoembryonic antigen; DC, dendritic cell; IL-4, interleukin-4; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA, human leukocyte antigen; HPC, haematopoietic progenitor cell; LC, Langerhans cell; NK cell, natural killer cell; PET, positron emission tomography; polyICLC, polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose		

Table 1.2 Examples of clinical trials testing vaccination with *ex vivo* DCs (adapted from (49))

Studies using *ex vivo* generated DCs proved that dendritic cell therapies were safe, well tolerated and generally effective.

Targeted receptor	Receptor family	Expression by human cells
Mannose receptor	CLR	iDCs (low on mDCs), monocytes, macrophages, subsets of endothelial cells, retinal pigment epithelium, kidney mesangial cells, tracheal smooth muscle cells
CD205 (DEC-205)	CLR	mDCs (low on iDCs), thymic epithelial cells, monocytes, B cells, NK cells, T cells
DC-SIGN	CLR	iDCs (low on mDCs), macrophages, megakaryocytes
LOX1	CLR	iDCs, macrophages, fibroblasts, smooth muscle cells, endothelial cells
Dectin-1	CLR	iDCs (low on mDCs), monocytes, macrophages, neutrophils, eosinophils, B cells, subpopulation of T cells
FcϒRI	FcR	DCs, monocytes, macrophages, activated neutrophils
FcϒRIIa	FcR	DCs, monocytes, macrophages, neutrophils, eosinophils, platelets
FcϒRIII	FcR	DCs, NK cells, macrophages, neutrophils, stimulated eosinophils
FcϒR	FcR	mDCs (low on iDCs), monocytes, macrophages, neutrophils, eosinophils
CD11c–CD18	Integrin	DCs, monocytes, macrophages, granulocytes, NK cells, activated B cells, certain CTLs
MACI	Integrin	DCs, monocytes, macrophages, granulocytes, NK cells, subsets of T and B cells
CD40	TNF-receptor superfamily	DCs, B cells, macrophages, endothelial cells, keratinocytes, fibroblasts, $CD34^{+}$haematopoietic cell progenitors, thymic epithelial cells
Siglec-H	Siglec	No human orthologue identified
CLR, C type lectin receptor; CTLs, cytotoxic T lymphocytes; DC, dendritic cell; FcR, Fc receptor; iDC, immature dendritic cell; mDC, mature dendritic cell; NK cell, natural killer cell; TNF, tumor necrosis factor;		

Table 1.3: Dendritic cell surface receptors used for targeting of antigen to DCs (adapted from (63))



C-type lectin expression	Langerin, DEC -205, Dectin-1, DCIR	DC-SIGN, DEC-205, Dectin-1 LOX-1, DCIR	DCIR	CLEC12A, DCIR, Dectin-1, DEC-205	CLEC9A, DEC-205	DEC-205, DCIR
TLR expression	TLR-1, -2, -3, -6, -10	TLR-2, -4, -5, -6, -8, -10	TLR-4	TLR-1, -2, -3, -4, -5, -6, -7, -8, -10	TLR-3, -8	TLR-7, -9
Cytokine production	IL-6, IL-8, IL-15	IL-1β, IL-6, IL-8, IL-10, IL-12, GMCSF, MCP, TGF-β	IL-8, IL-15	TNF-α, IL-6, IL-12	IL-12	Type I IFNs, IL-6, IL-8, TNF-α
Function	CTL responses, cross-priming	Humoral responses	CTL responses, humoral responses	CTL induction, cross-priming, Th1 polarization	CTL induction, cross-priming, necrotic cell engulfment	CTL responses, humoral responses, tolerance induction
Localization	Epidermis, mucosal epithelia, skin-draining LNs	Dermis	Dermis, skin-draining LNs	Blood, lymphoid organs	Blood, lymphoid organs	Lymphoid organs, blood, liver

Figure 1.1 Dendritic cell subsets: their phenotypes and functions (adapted from (51))

Dendritic cells are a heterogeneous cell type with multiple roles in the immune response.

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CHAPTER 2

Uptake Behavior of PI- and GM1-Containing Liposomes in Macrophage and Dendritic Cell Types

SUMMARY

Targeting dendritic cells (DCs) with nanoparticles *in vivo* is not an easy feat. Macrophage uptake is generally accepted as the main route of nanoparticle clearance in the body due to macrophage's high phagocytic capability. We hypothesized that incorporating either phosphatidylinositol (PI) or monosialotetrahexosylganglioside (GM1) glycolipids into a liposome formulation would lead to enhanced DC uptake, especially in the peripheral lymph nodes. This presumed enhanced DC update would result from decreased initial macrophage uptake previously characterized in these two long-circulating liposome formulations. Using simple liposome formulations consisting of dioleoylphosphatidylcholine (DOPC), cholesterol (Chol), and either PI or GM1 glycolipids, we examined the *in vitro* and *in vivo* uptake of these liposome formulations by DCs and macrophages. *In vitro* studies of liposome formulations using cultured cell lines or primary cells indicate liposomes containing PI at 10 mole percentage (mol%) (PI-liposomes; PI-lip) have increased uptake in DCs compared to liposomes containing 10mol% GM1 (GM1-liposomes; GM1-lip), whereas macrophage uptake was comparable. *In vivo* uptake by mouse lymph node (LN) DCs and macrophages was

determined 4 hours and 24 hours after subcutaneous (s.c.) injection of liposomes. Supporting *in vitro* results, DCs in the lymph node internalized more PI-lip than GM1-lip in a 4-hour time period. In contrast to uptake trends described *in vitro*, PI-lip also exhibited higher uptake than GM1-lip by LN macrophages after 4 hours. LN uptake of PI-lip was diminished after 24 hours; however, there was no significant change in uptake of GM1-lip by LN DCs between 4 and 24 hours post injection. In addition, macrophage uptake of GM1-lip was higher than that for PI-lip 24 hours post injection, suggesting GM1-liposomes persist in the lymphatic system over this time period. In summary, by slightly altering liposome composition we show increased initial DC uptake of liposomes using PI-liposomes or alternatively, GM1-liposomes can be utilized to facilitate longer exposure of liposomes with DCs and macrophages.

INTRODUCTION

Developing vaccines against viral infections and/or cancer will require potent immune responses capable of detecting and clearing infected or tumor-causing cells. Clearance of intracellular viruses and cancerous cells from the body necessitates cell-mediated immune responses, especially activation of $CD8^+$ T cells (CTL), which can directly kill targeted cells [1, 2]. Live vaccination stimulates CTL activity; however, safety issues including reversion to a virulent species outweigh the benefits of live attenuated vaccines [3, 4]. Subunit protein vaccines are inherently safer than live vaccines but their use has been limited, as many do not confer protective immunity against the pathogen. This decreased effectiveness is partially due to the short half-lives of soluble protein *in*

vivo, as well as a general lack of activation of antigen-presenting cells (APCs) of the immune system, which is important for promoting immunity rather than antigen tolerance [5]. Further absent in subunit protein vaccines is the generation of $CD4^+$ or $CD8^+$ T cell-mediated immune responses [6]. There are a few approaches to improve upon this drawback of subunit protein vaccines, including the use of molecular adjuvant(s) to enhance and/or tailor immune responses, incorporating a delivery vehicle (e.g. emulsions, nanoparticles), or targeting of antigen to specific areas or cells in the body in order to enhance the immune response [7-9]. All of these approaches rely on APCs to enhance the immune response.

APCs are special cells of the immune system that recognize, internalize, process, and present antigen to T cells, including CTLs. Dendritic cells and macrophages are both APCs that have interrelated roles in the immune response. Dendritic cells and macrophages are situated at high numbers in the most common areas where foreign entities may be introduced (e.g. skin, and mucosal surfaces) and also in the lymph nodes, where they propagate immune responses to T and B cells [10]. Macrophages are highly active in phagocytosis yet seem more suitable for destruction of phagocytosed material, rather loading of antigenic peptides for presentation to T cells [11, 12]. Dendritic cells (DCs), discovered in 1973, are distinct from monocytes and macrophages phenotypically and functionally [13, 14]. Their unique and integral role in the immune response is of high interest in the field of vaccine development. DCs are well known for their efficient T cell activation, which makes them a prime target for vaccines in need of potent T cell responses. DCs can initiate T cell responses with few

numbers of cells and also small amounts of antigen [11, 12, 15]. Therefore, skewing antigen uptake even slightly towards DCs, may augment immune responses after immunization.

To harness the immune-stimulating capabilities of DCs, some antigen must be directed away from the dominant phagocytic cells in the body (i.e. macrophages). DC-specific cell surface receptors such as DEC-205, DC-SIGN, and CD11c have been utilized for DC-targeted antigen delivery. Conjugating either ligand or antibody for these receptors to antigen or antigen-delivery vehicles has demonstrated the value of DC-specific targeting. Studies have shown antigen delivery specifically to DCs can improve antigen-presentation and T cell activation, as well as provide clinically relevant decreases in tumor size after treatment [16-20]. Although these methods have shown the benefit of DC-targeting, they are ultimately too complex for a large-scale vaccination program. Ligands of DC-SIGN are complex carbohydrate structures inferring high costs; likewise, antibody production for targeting of these DC receptors is not economical.

Although liposomes are biocompatible, they are still recognized by the immune system as foreign bodies. Therefore, their main route of clearance from the body comes from phagocytes, specifically macrophages. Decades ago, the use of liposomes was limited due to the unavoidable high uptake by macrophages in the mononuclear phagocyte system (MPS), which includes the spleen and liver. Liposome cell uptake is dependent on many factors including size, lipid composition and overall charge of the particles. It was discovered that some anionic lipids incorporated into conventional liposome formulations could alter circulation half-life by reducing macrophage uptake of

liposomes in the spleen and liver [21]. All anionic lipids are not capable of reducing macrophage uptake; while phosphatidylserine (PS) or phosphatidylglycerol (PG) reduce liposome half-life *in vivo* to minutes, the glycolipids phosphatidylinositol (PI) or monosialotetrahexosylganglioside (GM1) results in prolonged half-lives averaging 16-24 hours [21-25]. Incorporation of PI at minimum 9 mol% or GM1 at minimum 7 mol% of phospholipid in a liposome formulation deterred liposome uptake by macrophages and increased half-life [23, 26]. Even at identical mole percentages (mol%) in liposomes, PS, PG, PI and GM1 lipids had remarkably different uptake characteristics by macrophages. To rationalize these data, it was postulated that because PI and GM1 have bulky hydrophilic head groups their negative charge is shielded from recognition by serum proteins whom opsonize particles [21]. Furthermore, the cell membranes of most cells in the body contain glycolipids, rendering them negatively charged and hydrophilic, a property that is thought to be in part responsible for the long-circulating properties of PI- and GM1-containing liposomes [25].

Conventional liposomes compositions not containing PI or GM1 have rapid extensive uptake by macrophages in the MPS after intravenous injection, resulting in short half-lives and limited use as delivery vehicles. The identification of long-circulating liposomes, including PI- and GM1-containing liposomes, was a significant improvement in liposome technology. PI- and GM1-containing long-circulating liposome formulations are well described for reducing the uptake by macrophages of the MPS, but their uptake was not studied in dendritic cells. PI- and GM1-containing long-circulating liposome formulations were of interest because it was hypothesized that deterring initial high

macrophage uptake would benefit liposome uptake by DCs, especially in the periphery (i.e. lymph nodes rich in DCs). The possibility of reduced initial macrophage uptake using PI- or GM1-containing liposome formulations was of interest for promoting DC uptake of liposomes. Coupled with easy preparation and inexpensive materials required, presented PI- and GM1-liposomes as promising vaccine delivery systems. However, these liposome formulations have not been studied after subcutaneous injection, which would be a preferable route of administration for vaccines.

Dendritic cells in the lymph node (LN) are strategically positioned to generate immune responses. Here, DCs in high numbers are in close proximity to T and B cells promoting cell-cell interaction and propagating the immune response [10, 27]. A majority of lymph node DCs are immature, meaning they are efficient at internalizing and processing antigen [28]. Therefore, liposomes that gain access to the lymph nodes through the lymphatic vessels could augment uptake by DCs and potentially enhance immune responses to antigen. Subcutaneously administered liposomes can be taken up via lymphatic capillaries into lymphatic vessels through which they travel to access lymph nodes. Any liposomes that are not captured by cells in the lymph nodes ultimately enter the blood stream and encounter organs such as the liver and spleen [29].

Liposome size is the most crucial factor in determining uptake into lymphatic vessels, and therefore uptake into lymph nodes [30]. Small liposomes (mean diameter <0.1 μm) can gain access to lymph nodes quicker, but they also display higher blood concentrations suggesting they pass through the lymphatic system with low uptake by DCs in the lymph nodes. Larger liposomes (>0.4 μm up to 1 μm) have been detected in

the lymphatic system; however, a significant majority of these liposomes will remain at the injection site and have low uptake efficiencies [30, 31]. Studies suggest that nanoparticles smaller than 0.2 μm can drain into the lymph nodes and be taken up by DCs in the lymph node [32]. Size is also an important factor for liposomes to retain their long-circulating properties. Liposomes containing 10mol% GM1 had low uptake by liver and spleen in the size range of 70-200 nm while those larger than 300 nm predominantly accumulated in the spleen [33]. Moreover, liposomes containing 10mol% of either PI or GM1 lipid exhibited maximum blood/MPS ratios when mean diameter was 80-120 nm [34].

We hypothesized that small liposomes (mean diameter≈100nm) composed of either PI or GM1 glycolipid at 10mol% of total phospholipid would deter initial rapid macrophage uptake, in turn, facilitating liposome interaction with DCs for uptake. We demonstrated that PI-lip and GM1-lip have differential uptake by DCs *in vitro* and *in vivo*. *In vitro* PI-liposomes promoted uptake by DCs compared GM1-lip, whereas macrophage uptake was comparable between PI- and GM1-liposome formulations. *In vivo* PI-lip also exhibited enhanced uptake by lymph node DCs within a short period after liposome injection. Contrastingly, GM1-liposomes show less initial uptake by lymph node DCs and macrophages *in vivo* but may be better suited for extended exposure of liposome and potential cargo with LN cells. These glycolipid-bearing liposomes are easy to prepare and liposome properties are reproducible, which places additional value on these liposome formulations as potential protein subunit vaccine carriers.

MATERIALS AND METHODS

Liposome preparation

Cholesterol (Chol) was obtained from Calbiochem (La Jolla, California). All non-fluorescent lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama). Oregon Green® 488 1,2-Dihexadecanoyl-*sn*-Glycero-3 Phosphoethanolamine (Oregon Green 488-DHPE) was obtained through Life Technologies (Grand Island, New York). Liposomes were prepared at a 2:1 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Δ9-cis) (DOPC) to Chol. In uptake experiments, Oregon Green 488-DHPE lipid was incorporated at 1mol% of the total phospholipid. L-α-phosphatidylinositol (Soy) (PI), monosialotetrahexosylganglioside (ovine brain) (GM1), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE) or L-α-phosphatidylserine (porcine brain) (PS) was incorporated at indicated mol% of the total phospholipid. pH-sensitive liposomes contained L-α-phosphatidylethanolamine (egg, chicken) (ePE) and cholesteryl hemisuccinate (CHEMS) (Sigma-Aldrich) at a 2:1 molar ratio. Lipid mixtures were dried into a lipid film using a Buchi Rotavapor R-200 rotary evaporator. Lipids were dried completely then rehydrated with isotonic (290 ± 10 mmol/kg osmolality) HEPES-buffered saline containing EDTA (HBSE) (10mM HEPES, 140mM NaCl, 1mM EDTA, pH 7.4). pH-sensitive liposomes composed of ePE:CHEMS were hydrated in HBSE, pH 8.4 buffer. Hydrated lipid films were vortexed, subjected to 5 freeze/thaw cycles and extruded using double-stacked polycarbonate 100 nm diameter filters (GE). The size of the liposomes and zeta potential was determined using a Malvern ZS90 ZEN 3600

zetasizer. Phospholipid content of liposomes was quantified using Bartlett's phosphate assay[35]. Determined phospholipid concentrations signified the concentration of liposomes and were used in the dosing calculations. GM1 does not have a phosphate group and thus the phospholipid assay concentrations determined do not include GM1 content of the liposomes; dosing calculations were adjusted to account for this. Prepared liposome samples were stored at 4°C and used within 10 days of preparation.

Cell culture

All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. All cells were maintained in in a humidified incubator at 37°C and 5% CO_2. J774A.1 mouse macrophages (ATCC TIB-67) and JAWSII mouse dendritic cells (ATCC CRL-11904) were purchased from ATCC (Manassas, Virginia). J774 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS (HI-FBS) and 100µg/mL streptomycin, 100U/mL penicillin. JAWSII cells were maintained in alpha minimum essential medium with ribonucleosides and deoxyribonucleosides supplemented with 20% HI-FBS, 100 µg/mL streptomycin, 100U/mL penicillin, 2mM L-glutamine and 5ng/ml murine GM-CSF (PeproTech, Inc. Rocky Hill, New Jersey).

Bone marrow was harvested from femurs and tibia of female C57BL/6 mice and differentiated into bone marrow-derived macrophages (BMMs) and bone marrow-derived dendritic cells (BMDCs). BMM media (DMEM supplemented with 20% HI-FBS, 30% L-929 cell conditioned media containing macrophage colony stimulating factor (M-

CSF), 2mM glutamine, 100µg/mL streptomycin, 100U/mL penicillin and 55µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO)) was used to maintain the BMM cultures. BMMs were derived as described previously by Stier et al [36]. In short, precursor cells were cultured for 6 days in the presence of M-CSF, a component of the L-cell media supplement. Cells were replenished with new cell media on day three of culture. BMMs were then harvested on day six of culture and stored in liquid nitrogen until needed. BMDC media (RPMI-1640 supplemented with 10% HI-FBS, 100µg/mL streptomycin, 100U/mL penicillin, and 50µM 2-mercaptoethanol) was prepared and murine GM-CSF was added fresh each time to the BMDC media at 20ng/ml immediately before use. Cells were cultured in the presence of GM-CSF for 6 days, exchanging the cell media every 2 days, after which loosely adherent and non-adherent cells were harvested and stored in liquid nitrogen until needed.

In vitro cell uptake and flow cytometry cell staining

Equal counts of cells were incubated with liposomes at 100µM [phospholipid] in serum-free DMEM for one hour at 37°C, unless otherwise noted. After treatment, any non-cell bound liposomes were removed by centrifugation and three subsequent cell washes in phosphate buffered saline (PBS) pH 7.4. Cell samples to be stained for flow cytometry were allowed to incubate in PBS containing 10% HI-FBS for 30 minutes at room temperature before addition of staining antibodies. The cell samples to be labeled with antibodies were incubated with murine anti-CD11b-PerCPCy5.5 antibody (Ab) (BD Pharmingen) and murine anti-CD11c-PE Ab (BD Pharmingen) at 4°C for 30 minutes to

one hour. Prior to flow cytometry analysis, cell samples were thoroughly washed using PBS/10% HI-FBS to remove any non-bound antibodies from the sample. Cell fluorescence was obtained using a BD FACSCalibur flow cytometer (San Jose, CA). Cell Quest software was used to plot fluorescence values and obtain the mean fluorescence intensity (MFI) of samples.

Mice

C57BL/6 (7-8 weeks old; Jackson Laboratories, Bar Harbor, ME) were used in this study and handled according the University of Michigan Institutional Animal Care guidelines. Mice were injected subcutaneously (s.c.) on each side of the hind flank near the tail base with a total of 250 nmoles phospholipid in 100 µL volume.

Preparation of lymph nodes and flow cytometry

Inguinal lymph nodes harvested from mice were sliced and squeezed through a 70-µm filter to prepare a single cell suspension in PBS containing 1% bovine serum albumin (BSA). Cell suspensions were washed with PBS/ 1%BSA then incubated with CD16/32 Fc block (eBioscience, San Diego, CA) for 10 minutes at RT and a small amount of each sample was taken for negative and single-label controls. Cells were then incubated with murine anti-CD11b-PerCPCy5.5 Ab (BD Pharmingen) and murine anti-CD11c-PE Ab (BD Pharmingen) for 1 hour on ice. Prior to flow cytometry analysis, cell samples were thoroughly washed using PBS/1% BSA to remove any non-bound antibodies from the sample. Cell fluorescence was obtained using a BD FACSCalibur

flow cytometer (San Jose, CA). Cell Quest software was used to plot fluorescence values and obtain the MFI of cell samples.

RESULTS

JAWSII dendritic cells exhibit preferential uptake of PI-liposomes

Previous investigations of liposome formulations containing a 2:1 ratio of phosphatidylcholine (PC) to cholesterol (Chol) and either PI or GM1 glycolipid focused exclusively on macrophages' role in liposome clearance. With increasing evidence of DCs' fundamental role in the immune system we sought to examine the uptake of these liposome formulations in macrophage (J774) and dendritic (JAWSII) cell lines. Liposomes containing a 2:1 ratio of dioleoylphosphatidylcholine (DOPC) to Chol with 10mol% of the total phospholipid being PI glycolipid are termed PI-liposomes (PI-lip). Liposomes containing a 2:1 ratio of DOPC:Chol with 10mol% of the total phospholipid being GM1 glycolipid are termed GM1-liposomes (GM1-lip). Liposomes containing only DOPC and Chol (DOPC:Chol liposomes) represent a neutrally charged liposome formulation that was additionally studied to assess baseline liposome uptake by both cell types. The DOPC:Chol liposomal formulation additionally containing 50mol% PS (PS-lip) was treated as a positive control, with evidence that PS displayed on nanoparticles promotes uptake by phagocytic cells [37]. Table 2.1 list the physical characteristics of the liposomes used in the following studies. Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome composition and measured using flow cytometry. Overlaid histogram plots

as seen in Figure 2.1A and 2.1B plot cell fluorescence (X) vs. cell counts (Y). The x-axis, fluorescence, is here-within always plotted on a log scale. The high cell uptake of PS-lip was comparable in J774 macrophages and JAWSII DCs, confirming both cell lines examined in the study are proficient at internalizing liposomes (Figure 2.1A and 2.1B). The observed uptake of GM1-lip was similar to DOPC:Chol neutral liposome uptake regardless of cell type (Figure 2.1A and 2.1B). DCs incubated with PI-lip displayed increased cell-associated fluorescence compared to DCs exposed to GM1-lip ($p<0.01$) or DOPC:Chol liposomes ($p<0.001$) (Figure 2.1D), whereas in macrophages the uptake of these three liposome formulations were comparable (Figure 2.1C). Moreover, we also observed this exact result when the uptake experiment was performed with cells in the presence of serum-containing media (data not shown). The presence of serum is known to have dramatic effects on liposome uptake [24, 38]; overall, liposome uptake was reduced in both JAWSII and J774 cells but the trends in liposomal uptake described previously were upheld.

Uptake trends in primary bone marrow-derived cells resemble those presented in cell lines

Uptake of PI-lip and GM1-lip was additionally examined in primary cells differentiated from progenitor cells in the bone marrow of mice. Primary cells were used for confirmation of studies performed in cell lines because, their behavior is expected to more closely resemble that of cells *in vivo*. Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome

composition and measured using flow cytometry. A majority of bone marrow-derived DCs (BMDCs) cultured (≈60%) displayed CD11c and CD11b on the cell surface, indicating DC differentiation (data not shown). Cultured bone marrow-derived macrophages (BMMs) cultured displayed no CD11c, but had high presence of CD11b (data not shown). BMMs and BMDCs had higher cell-associated fluorescence than that seen with their respective cell lines, J774 and JAWSII. BMMs exhibit enhanced uptake of all liposomes formulations compared to BMDCs, albeit with no discernable uptake trends (Figure 2.2A and 2.2C). Fluorescence histograms for BMDCs appear to show GM1-lip is internalized less than DOPC:Chol liposomes, but the difference between the two mean fluorescence intensities (MFI) (108.8 ± 26.97 and 99.6 ± 2.96 respectively) is not considered significant at $p<0.05$ (Figure 2.2B). BMDCs exhibit markedly enhanced uptake of PI-lip ($p<0.05$) compared to GM1-lip and DOPC:Chol liposome formulations (Figure 2.2D). This study repeated at a lower liposome concentration (50μM) confirmed PI-lip has enhanced uptake compared to GM1-lip in BMDCs, a trend that was not observed in BMMs (data not shown). Studies performed in cell lines and primary cells offered the same results, confirmation that indeed, PI-lip formulations present DC-specific targeting aspects *in vitro*.

Uptake of PEG-lip is comparable to GM1-lip, in BMMs and BMDCs

Liposomes bearing a PEG polymer coating represent another approach to reduce uptake by macrophages *in vitro* and *in vivo* [25]. Poly (ethylene glycol) (PEG)–phosphatidylethanolamine conjugates (PEG-PE) are incorporated into liposome

compositions to create a liposome with a hydrophilic, bulky surface polymer coating. PEG-PE was added to the DOPC:Chol lipid composition at 10mol% of total lipid (PEG-lip), matching the amount of DOPC displaced by PI or GM1 glycolipids in their respective formulations. We sought to compare the uptake behavior of glycolipid-bearing long-circulating liposomes (PI-lip and GM1-lip) to the polymer-coated long-circulating liposome formulation. The uptake of GM1-lip, PI-lip and PEG-lip were compared in BMMs and BMDCs. Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome composition and measured using flow cytometry. The uptake behavior of PEG-lip resembles that of GM1-lip in macrophages and DCs, showing decreased uptake compared to neutral DOPC:Chol liposomes (Figure 2.3A and 2.3B). BMMs display slight, yet statistically insignificant (ANOVA $p=0.0551$) differences, between liposome formulations (Figure 2.3C). However in BMDCs these uptake differences are clearly emphasized, especially when comparing PI-lip to PEG-lip (MFI=284.35 ± 9.85 and 81.04 ± 20.99 respectively, $p<0.001$) (Figure 2.3D). The data indicate that the long-circulating characteristic of PEG-liposomes holds true in bone marrow-derived macrophage and dendritic cell types.

Effect of PI density on liposome uptake by macrophages and DCs

To assess the contribution of PI lipid on the cellular uptake of liposomes we prepared four formulations with increasing PI mol%, starting with 0mol% PI (DOPC:Chol liposomes) as the lowest density and 50mol% PI as the highest density (Table 2.1). Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid

incorporated at 1mol% of the liposome composition and measured using flow cytometry. The increasing presence of PI glycolipid had dramatically different effects on liposome uptake by JAWSII DCs and J774 macrophages (Figure 2.4A and 2.4B). As described previously and shown again in Figure 2.4, liposomes containing 10mol% PI promote uptake by DCs whilst the impact on macrophage uptake is low. In the study presented, 10mol% PI is the minimum and maximum amount needed to significantly enhance DC uptake (Figure 2.4B). Increasing PI content to 25mol% or 50mol% does not change uptake by JAWSII DCs. In contrast, for J774 macrophages, increasing PI content in the liposome formulation continuously improved liposomal uptake. At 50mol% PI in the liposome formulation liposomal uptake by J774 macrophages and JAWSII dendritic cells was similar (Figure 2.4C).

ePE:CHEMS liposome compositions dissolve liposome uptake trends

Previously our lab group has used pH-sensitive liposomes composed of phosphatidylethanolamine and cholesteryl hemisuccinate (ePE:CHEMS liposomes) in combination with listeriolysin-O (LLO) protein from *Listeria monocytogenes (Lm)* to deliver encapsulated liposomal material into the cytosol of cells [36, 39, 40]. The ability to deliver antigen to the cytosol of APCs for subsequent presentation to $CD8^+$ T cells would be optimal for a protein subunit vaccine formulation. We examined the influence ePE:CHEMS composition had on the uptake of liposomes also containing 10mol% glycolipid. Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome composition and measured using flow

cytometry. Using ePE:CHEMS, rather than DOPC:Chol, increased the uptake of all liposome formulations tested regardless of cell type examined (Figure 2.5C and 2.5D). Interestingly, ePE:CHEMS liposomes containing 10mol% GM1 retained some aspect of reduced uptake in BMMs, when compared to ePE:CHEMS liposomes or ePE:CHEMS containing 10mol% PI ($p<0.01$) (Figure 2.5A). The presence of negatively charged CHEMS in the liposome formulations results in a high negatively charged liposome surface, which increased overall cell uptake of all ePE:CHEMS liposome compositions. This increase in cell association dissolved the advantage PI glycolipid had over GM1 in enhancing DC-specific uptake (Figure 2.5B and 2.5D).

PI-lip promote uptake by macrophages and DCs present in the LN

To further characterize the uptake of PI-lip and GM1-lip, their uptake by macrophages and DCs was analyzed *in vivo*. A total of 250 nmoles of the indicated liposome formulation (10mol% PI-lip, GM1-lip, or PS-lip; mean diameter≈100 nm) (Table 2.1) were subcutaneously injected into female C57BL/6 mice (n=2 mice per liposome formulation). The basis for injecting 250 nmoles of phospholipid was to mimic the amount of lipid content injected in the immunization study performed with these liposome formulations (Chapter 3). Lymph node cells were analyzed 4 hours post injection. As in *in vitro* experiments, liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome composition and measured using flow cytometry. The cell surface receptors, CD11b and CD11c were used as identifiers of macrophage and DC populations. Dendritic cells present in the

lymph nodes (LN) were selected as staining positive for both CD11c and CD11b, as determined by cellular fluorescence. Macrophages in the lymph nodes were classified as having no CD11c staining and high CD11b staining. Identified DC and macrophage populations from the same overall lymph node cell population were then plotted for their fluorescence due to liposome internalization (OregonGreen488 fluorescence) (Figure 2.6A and 2.6B).

The percentages of cells displaying any liposome-related fluorescence was similar between all liposome formulations whether LN macrophages (~60% Liposome$^+$ cells) or LN DCs (~20% Liposome$^+$ cells) were analyzed (ANOVA p=0.14 DCs; p=0.41 macrophages) (Figure 2.6D), however this result did not translate to similar uptake of liposome formulations by macrophages or DCs (Figure 2.6C). LN cells recognize and internalize liposomes with different efficiencies. To emphasize this point, the percentage of LN macrophage cells that displayed any liposomal fluorescence was approximately 60% regardless of the liposome formulation; however, the average MFI value associated with PI-lip was two times higher than the average MFI value for GM1-lip. Another observation is more LN macrophages internalize liposomes than do LN DCs ($p<0.01$) (Figure 2.6D). After only 4 hours, 60% of macrophages had internalized liposomes, suggesting that the glycolipid-containing 'long-circulating' liposomes do not largely deter macrophage uptake in the same way described of them after i.v. administration.

In contrast to cell culture experiments, evaluation of *in vivo* uptake determined PI-lip (DC MFI=51.32 ± 1.385; macrophage MFI=241.9 ± 16.5) had enhanced uptake

compared to GM1-lip (DC MFI=32.52 ± 5.705; macrophage MFI=100.9 ± 42.07) in both LN DCs and LN macrophages. Compared to *in vitro* experiments, the ability of PI-lip to promote DC-specific uptake was weakened. However, PI-lip uptake trended higher than GM1-lip in LN DCs ($p<0.05$). The uptake of GM1-lip by LN macrophages was highly variable contributing to a p-value greater than 0.05 when comparing macrophage GM1-lip to PI-lip uptake ($p=0.062$). Although we cannot statistically define PI-uptake is higher than that of GM1-lip in macrophages, uptake trends indicate GM1-lip had lower uptake by both cell types during the 4-hour exposure (Figure 2.6C). We additionally monitored the uptake of PS-liposomes by LN macrophages and DCs *in vivo* (Figure 2.6A and 2.6B). The uptake of liposomes containing PS has been studied extensively *in vitro* and *in vivo* which is why we choose to compare the biodistribution of PS-lip (10 mol% PS) to the 10mol% glycolipid liposome formulations. After 4-hour uptake, PS-lip displayed mean uptake values between those of PI-lip and GM1-lip in both cell types examined (DC MFI=46.84 ± 7.255; macrophage MFI=131.1 ± 16.22). ANOVA post-hoc multiple comparison tests determined there were no significant differences in uptake when comparing PS-lip to either glycolipid liposome formulation. These results were not expected, due to previous knowledge that PS-containing liposomes are avidly internalized by macrophages and DCs *in vivo*.

GM1-lip has prolonged uptake by cells in the LN

The same liposome batches prepared for the 4 hour *in vivo* uptake study were used for the 24 hour *in vivo* uptake. Female C57BL/6 mice (n=2 mice per liposome

formulation) were subcutaneously injected in the hind flank near the tail base with 250 nmoles of the indicated liposome formulation. Liposome uptake was monitored by fluorescence of OregonGreen488-DHPE lipid incorporated at 1mol% of the liposome composition and measured using flow cytometry. As described for the 4 hour *in vivo* uptake study, dendritic cells present in the lymph nodes were selected as staining positive for both CD11c and CD11b, as determined by cellular fluorescence; while macrophages were classified as having no CD11c staining and high CD11b staining. Identified DC and macrophage populations from the same overall LN cell population were then plotted for their fluorescence due to liposome internalization (OregonGreen488 fluorescence) (Figure 2.7A and 2.7B).

Surprisingly, uptake of GM1-lip was higher than that of PI-lip in both DC and macrophage cell types ($p<0.05$) (Figure 2.7C and 2.7 D). In comparison to 4-hour uptake values, the 24-hour uptake of PI-lip was considerably less in both LN DCs (MFI= 16.68 ± 0.775) and LN macrophages (MFI= 5.51 ± 0.17) ($p<0.01$ and $p<0.0001$ respectively). Interestingly, GM1-lip exhibited similar uptake by LN DCs at 4 and 24 hours post injection (MFI= 32.52 ± 5.705 and MFI= 25.31± 3.085, respectively). This corresponded with the percentage of LN DCs that contained GM1-lip (4 hour: 18.0% ± 3.09%; 24 hour: 16.5% ± 3.44%). Together, these results suggest GM1-lip persist in the lymphatic system for longer periods of time. Uptake of PS-lip was similar to that of PI-lip for both macrophage and DC cell types (Figure 2.7D).

DISCUSSION

In this study we sought to investigate the uptake of two glycolipid-containing liposome formulations by macrophages and dendritic cells. Liposomes containing 10mol% GM1 or PI glycolipid have been previously shown to evade rapid macrophage uptake *in vitro* and *in vivo* [24, 26, 34, 41]. We hypothesized that these long-circulating liposome formulations would reduce the liposomal uptake by macrophages, which in turn, may result in increased exposure and uptake by DCs. We have observed that 10mol% PI-liposomes (1) promote uptake by DCs *in vitro* and *in vivo* (2) lose DC-preferential uptake when ePE:CHEMS composition is used (3) are superior to GM1-lip and PEG-lip for enhancing liposomal uptake by DCs.

In literature, long-circulating liposomes are also referred to as sterically stabilized liposomes, glycolipid-bearing liposomes, or Stealth© liposomes. Their main realized function is to enhance liposomal delivery of small molecules to tumor after intravenous (i.v.) administration, a goal that was only manageable if liposomes could evade rapid uptake mediated by macrophages of the spleen and liver (i.e. MPS). Long-circulating liposomes have primarily been studied after i.v. injection, as this was the logical route of administration for the purpose of tumor-targeted delivery of small molecules. However, the applications of long-circulating liposomes may be expanded based on the studies presented in this chapter. Since i.v. injection of a vaccine formulation is not ideal for multiple reasons, subcutaneous (s.c.) administration of long-circulating liposomes was utilized. Subcutaneous administration of liposomes is advantageous for directing nanoparticles initially to the draining (local) lymph nodes. Subcutaneous administration

of PI- or GM1-containing long-circulating liposomes had not been previously examined thus it was unknown whether or not these liposomes would possess the same uptake behaviors described after i.v. administration. Since long-circulating liposomes were extensively studied in the time before DCs were well defined as potent immune cells, the interactions of these liposome formulations with DCs had also not previously been of interest to researchers. The concept of reducing rapid liposome uptake by macrophages in the MPS with long-circulating liposome formulations introduced decades ago was applied to vaccine delivery in this thesis, based on the hypothesis that this reduced rapid macrophage uptake may skew some liposome uptake towards DCs. There are actually two ideas at play that may lead to enhancing DC uptake of liposomes. One possibility is that decreased initial rapid uptake by macrophages using long-circulating liposomes may facilitate increased liposome interactions and uptake with DCs. But, another possibility is that a liposome formulation may also promote uptake by DCs by a more specific interaction with DCs, rather than simply being the result of greater liposome availability. These two approaches may stand alone, or the uptake of long-circulating liposomes may be a result of the combination of the two uptake possibilities.

In vitro studies using cell lines and primary cells, suggested the use of PI or GM1 glycolipid in the liposome formulation did not result in reduced liposome uptake compared to neutral DOPC:Chol liposomes by the macrophage cell type. However, it was realized that including PI glycolipid in the liposome formulation at 10 mol% phospholipid could improve liposome uptake by DCs, whereas in macrophages this enhancement of liposome uptake was not observed. PI- and GM1-containing liposomes,

expected to deter rapid macrophage uptake *in vivo* based on previously published research, were identified in ~60% of macrophage cells in the LN as shown in Figure 2.6D. Therefore, it appears that PI-liposomes and GM1-lipsoomes injected subcutaneously for lymph node targeting may not possess the same ability to deter macrophage uptake as described of them following i.v. administration. Although macrophage uptake is apparently still a major component of liposome uptake in the LN, there is still the possibility to promote DC uptake of liposomes. This would explain how PI-liposomes, with initially high macrophage uptake in the LN, still have higher uptake than GM1-liposomes in LN DCs. The additional novelty of PI-liposomes is they require inexpensive materials, with PI being obtained in abundance from soybean, are easy to prepare, and exhibit unique uptake behavior in DCs examined *in vitro* and *in vivo*.

Besides the well-known PEG-coated liposomes, PI-liposomes and GM1-liposomes represent the most effective formulations for deterring macrophage uptake [24, 25, 42]. One explanation for the noticeably reduced macrophage uptake of liposomes containing PEG, PI, or GM1 is the so-called shield effect, suggesting the bulky, hydrophilic lipid head groups present in these liposome formulations shield the lipid's negative charge from recognition and clearance [21]. It is interesting that the bulky hydrophilic coatings present in PEG-lip, PI-lip, and GM1-lip formulations do not equally reduce liposome uptake by DCs. PEG-lip and GM1-lip both reduced DC uptake of liposomes, while PI-lip did not. The lipid head groups of GM1 and PEG are more bulky than PI and would extend further out from the liposome membrane, possibly contributing more to the shield effect and resulting in decreased recognition of PEG-lip

and GM1-lip by both cell types. However, the less bulky PI lipid head group present in PI-lip still exhibits low macrophage uptake, but alternatively increases liposome uptake by DCs. These results imply that the shield effect cannot explain the liposome uptake differences seen in DCs.

Surface charge and size of nanoparticles are two important factors affecting recognition and uptake by macrophages and dendritic cells. The size of PI-lip and GM1-lip investigated in this report were equal (≈100nm) and comprised equal anionic glycolipid compositions (10mol%). Yet, DC uptake of these liposomes formulations was dramatically different. Apparently two of the main determinants of liposome uptake, size and surface charge, are not the dominant factors affecting DC-mediated uptake of these glycolipid-bearing liposome formulations. Rather, we propose that the lipid head groups of these liposomes also determine uptake by DCs.

The surface density of PI glycolipid plays a role in liposomal uptake by DCs and macrophages. While in DCs, 10mol% PI is sufficient to promote uptake, macrophages require 50mol% PI to exhibit significant enhancements in liposome uptake. The PI density effect on cell uptake has been previously examined in J774 cell lines, therefore we had an indication that PI-lip, with increasing PI mol% would increase liposome uptake by these cells [24]. However, 10 mol% PI-lip were internalized the same as 50mol% PI-lip in JAWSII DCs. These results emphasize the different recognition mechanism for PI-lip in the two cell types. Whereas J774 macrophages recognize PI-lip based on increasing PI surface density (and increasing negative surface charge), JAWSII DCs require only the presence of the PI lipid head group at 10mol%. This could

be a result of either different binding sites for PI on the two cell lines, or increased PI binding sites exist on JAWSII DCs. J774 macrophage uptake of 50mol% PI-lip did approach that of 10mol% PI-lip uptake in JAWSII DCs, however this high glycolipid content results in accelerated liposome clearance *in vivo* and would not be a viable liposome formulation for deterring macrophage uptake [43].

The shield effect explained above has also been used to explain the dramatic uptake differences seen between PS-liposomes and either PI- or GM1-liposomes. Unlike PI and GM1 lipid head groups, which have a bulky hydrophilic group to shield lipid charge, the PS lipid head group exposes the negative charge to the surrounding environment. Our results confirm the shield theory in macrophages, however, differential DC uptake by PI-lip and GM1-lip formulations require an additional explanation. The role of cell-surface receptors has been previously proposed as a method of uptake for anionic lipids. Currently, there is no specific PI, GM1 or PS cell-surface receptor that has been identified. However, Class B scavenger receptors such as CD36 and SR-BI have been indicated as cell-surface receptors of anionic liposomes [44, 45]. In one particular *in vitro* study, 50mol% PI-liposomes and 10-50mol% PS-liposomes competed with low-density lipoprotein (LDL) natural ligands of SR-BI and CD36, suggesting these receptors recognize anionic liposomes for uptake [44]. SR-BI and CD36 are expressed on macrophages and immature dendritic cells and have also been shown to facilitate internalization of apoptotic cells *in vitro*, likely due to binding of PS exposed on the cell's outer lipid membrane [45-47]. The presence of SR-BI and CD36 on DCs could play a role in the differential internalization of PI-lip observed in this report. If one, or both, of

these scavenger receptors is more highly expressed on the cell surface of DCs, in comparison to macrophages, this could explain the observed enhanced uptake of PI-lip by only the DCs. Further indirect support of the scavenger receptors' role in liposomal uptake by macrophages can be found in studies performed by Lee *et al*. These authors show J774 macrophage uptake of PS-liposomes is negatively affected by competition with polyinosinic acid and polycytidylic acid, both of which are poly-anionic molecules known to bind to scavenger receptors [24]. If indeed scavenger receptors are expressed by J774 macrophages, they did not appear to play a role in discriminating uptake between GM1-lip and PI-lip in these studies. Furthermore, while CD36 and SR-BI are involved in the uptake of anionic liposomes and apoptotic cells *in vitro*, there is no evidence to indicate these are the receptors mediating uptake *in vivo*. In addition, the interaction between GM1-lip and either CD36 or SR-BI to our knowledge has not been investigated. Therefore it is possible that GM1-lip, another anionic liposome formulation, may be recognized by CD36 or SR-BI as well. In conclusion, we cannot exclude the possible role that Class B scavenger receptors, CD36 and SR-BI, may have in the internalization of the anionic lipids investigated in this report. Although there is also still the possibility that other, potentially undiscovered, cell-surface receptors are additionally involved.

The *in vivo* uptake study confirmed our *in vitro* finding that PI-lip have increased DC uptake compared to GM1-lip. Although, the clear ability of PI-lip to promote DC uptake displayed *in vitro* was weakened *in vivo*. The uptake of PI-lip by LN macrophages was also higher than GM1-lip, signifying that enhanced uptake was not

unique to LN DCs. An explanation for the differing *in vivo* and *in vitro* results in macrophage type cells is that J774 macrophages and the bone marrow-derived macrophages used in the *in vitro* uptake studies are likely not representative of the macrophages in the lymph nodes. Although J774 are widely used as representatives of macrophage cells, they were originally defined only as 'macrophage-like' cells based on their many macrophage-like properties including high phagocytic capability, ability to bind antibody-coated cells and their adherence in culture [48]. Bone marrow progenitor cells exposed only to the growth factor M-CSF, generate a homogenous macrophage cell population. Therefore, it is conceivable that lymph node macrophages may phenotypically differ from J774 and BMM cells, allowing for identification and increased uptake of PI-lip in the LN. The increased recognition observed of PI-containing liposomes by LN macrophages conflicts with the many published data demonstrating PI-containing liposomes reduce uptake by macrophages in the MPS [21, 22, 24, 25, 34]. Again, this discrepancy may be a factor of the differing cell types examined. *In vivo* there are many different types of macrophages and DCs, which differ phenotypically and functionally [10, 49-53]. As shown, PI-liposomes, understood to deter uptake by macrophages located in the spleen and liver of the MPS, appear to have different uptake behavior by macrophage cells in the lymph node. The difference in PI-liposome uptake exhibited may additionally be a result of LN macrophages being in close proximity to high concentrations of liposomes in the LN, facilitating increased binding and internalization of PI-lip. Moreover, PI-containing liposomes have continually been described as having lesser ability to deter macrophage uptake (in the MPS) when

compared to GM1-containing liposomes [21, 24, 33, 34, 43], which may further explain the higher uptake of PI-liposomes by LN macrophages. Although the referenced publications intravenously administered liposomes and observed this difference between PI- and GM1-containing liposomes, the *in vivo* experiments presented here following subcutaneous administration of liposomes, agree with previous data showing PI-liposomes are internalized by macrophages more so than GM1-liposomes.

The uptake of PS-liposomes *in vivo* is more complex than anticipated. The low LN DC and macrophage uptake of PS-lip was attributed to high uptake of these liposomes at the site of injection. Oussoren *et al*. reported that approximately 40% of liposomes composed of ePC:Chol:PS (10mol% PS; mean diameter≈70nm) remain at the injection site up to 52-hours post injection in rats [30]. Across multiple liposome formulations, it was also observed that with increasing mean diameter, more of the liposome dose remains at the injection site [30, 54]. Therefore it is plausible that 40% or more of the 100nm diameter PS-liposomes used in this study were retained at the injection site and not available for uptake by the lymph nodes. Due to the well-known remarkably different cell uptake of PI- and GM1-containing liposomes compared to PS-liposomes, the same uptake behavior at the injection site should not be projected to glycolipid-containing, long-circulating liposomes formulations. To add to that point, in a separate publication, Oussoren *et al*. investigated the effect of a liposomal PEG coating on liposome uptake from the injection site. It was discovered that liposomes containing PEG could increase the amount of liposomes that drained into the lymphatic system from the injection site [54]. Since liposomes with a PEG coating, alike PI-lip and GM1-

lip, are known to have a hydrophilic bulky surface which contributes to their low uptake by phagocytic cells, we speculate that PI-lip and GM1-lip may also promote increased liposomal drainage from the injection site to the lymphatic system. This may explain why PS-lip had lower than expected uptake by the lymph nodes in our *in vivo* uptake study.

The low cell fluorescence of LN cells 24 hours after injection could be a result of high uptake by cells within a shorter time frame, as well as, the instability of the fluorescent lipid marker. Lymphatic uptake of small liposomes (mean diameter≈70nm) from the injection site (s.c. injection) is typically completed within 12 hours [29]. After lymphatic uptake there is only one opportunity for liposomes to travel through the LNs before entering the blood stream. Lymph nodes internalize approximately 1-2% of the liposome dose within that one pass through, and more than 50% of that uptake occurs within the first 4 hours post injection [29]. The association of liposomes with LN cells 4 hours post injection was considerably higher than that 24 hours post injection ($p<0.05$), with the exception of GM1-lip uptake by DCs at 4 and 24 hours. This suggests that mouse LN cells internalize liposomes within a relatively short amount of time, which is in agreement with the timeline discussed above. The low fluorescence of LN DCs and macrophages after 24 hours could be a result of the degradation of the fluorescent lipid marker used in all studies. OregonGreen488-DHPE, composed of the OregonGreen488 fluorophore conjugated to the phospholipid DHPE, is an analog of fluorescein that overcomes some of the limitations fluorescein possesses. Unlike fluorescein, OregonGreen488 is pH-insensitive at physiological pH, with a pKa of 4.7. However, internalized liposomes would ultimately expose the fluorophore to a $pH<5$ in the late

stages of the endocytic pathway [55], which would result in a reduced fluorescence. Furthermore, the highest cell fluorescence values exhibited after 4-hours uptake is associated with the lowest fluorescence values at the 24-hour time point, suggesting high initial uptake of liposomes results in reduced readable fluorescence after 24 hours.

Interestingly, GM1-lip exhibited similar uptake by LN DCs 4 and 24 hours post injection. Macrophage uptake of GM1-lip had been significantly reduced from 4 to 24 hours, however, uptake of GM1-lip was still significantly higher than PI-lip. These results imply GM1-lip persist in the lymphatic system and/or lymph nodes for longer periods of time, compared to PI-lip. We have shown in this report, and many others have published that liposomes containing GM1 are superior to liposomes containing PI at reducing macrophage uptake [21, 24, 33, 34, 43]. Therefore, it is probable that GM1-lip had improved ability to evade extensive cell uptake initially and be retained in the LN. This most likely allowed for GM1-lip to be internalized, albeit at a lower capacity, over an extended period of time.

The pH-sensitive liposome, composed of PE:CHEMS is a valuable delivery system that has been studied extensively in our lab for cytosolic delivery [40, 56, 57]. In combination with the hemolysin from *Lm*, LLO, this lipid formulation is especially useful as a vaccine carrier to stimulate cell-mediated immunity due to cytosolic delivery of antigen [39]. To determine if PE:CHEMS compositions could be applied along with the preferential DC uptake exhibited by PI-liposomes, we evaluated the uptake of ePE:CHEMS:PI liposomes in macrophages and dendritic cells. Because CHEMS is negatively charged at neutral pH, PE:CHEMS liposomes bear a high negative surface

charge and were highly internalized by both BMDCs and BMMs. The presence of PI in ePE:CHEMS liposomes did not show an advantage in promoting uptake by DCs. Remarkably, ePE:CHEMS liposomes containing GM1 (10mol%) retained relatively reduced uptake by macrophages compared to PE:CHEMS liposomes. However, GM1 at this mol% likely abrogated the pH-sensitivity. Partial pH-sensitivity can be retained with low amounts (<5%) of GM1 lipid in a pH-sensitive lipid formulation, although this low mol% of GM1 is not sufficient to noticeably improve the circulation times of liposomes [23, 58]. Thus, using PE:CHEMS composition was determined to not be suitable in our approach to promote liposome uptake specifically by DCs.

CONCLUSION

We have investigated the macrophage and DC uptake of two glycolipid-containing liposome formulations. *In vitro* studies determined DOPC:Chol liposomes containing 10mol% PI glycolipid displayed distinct uptake behavior by DCs, which was not seen in macrophages. The presence of 10mol% PI was suitable for promoting liposome uptake by DCs whilst having no effect on macrophage uptake. This characteristic of 10mol% PI-liposomes was not observed when 10mol% GM1 glycolipid was used in the liposome formulation, or when PEG-liposomes were compared. *In vivo* studies determined that the enhanced uptake of PI-liposomes was not DC-specific. PI-liposomes and GM1-liposomes represent two alternative approaches to enhancing DC uptake. In the context of vaccine design, higher initial uptake of PI-liposomes by LN macrophages and DCs would increase initial antigen uptake by APCs, which may

prompt immune responses. Contrastingly, the lower uptake of GM1-liposomes would expose lesser amounts of antigen to cells initially, but this exposure would continue over an extended period of time. Based on *in vivo* results, both PI-lip and GM1-lip formulations may be beneficial as vaccine carriers for enhancing antigen delivery to DCs.

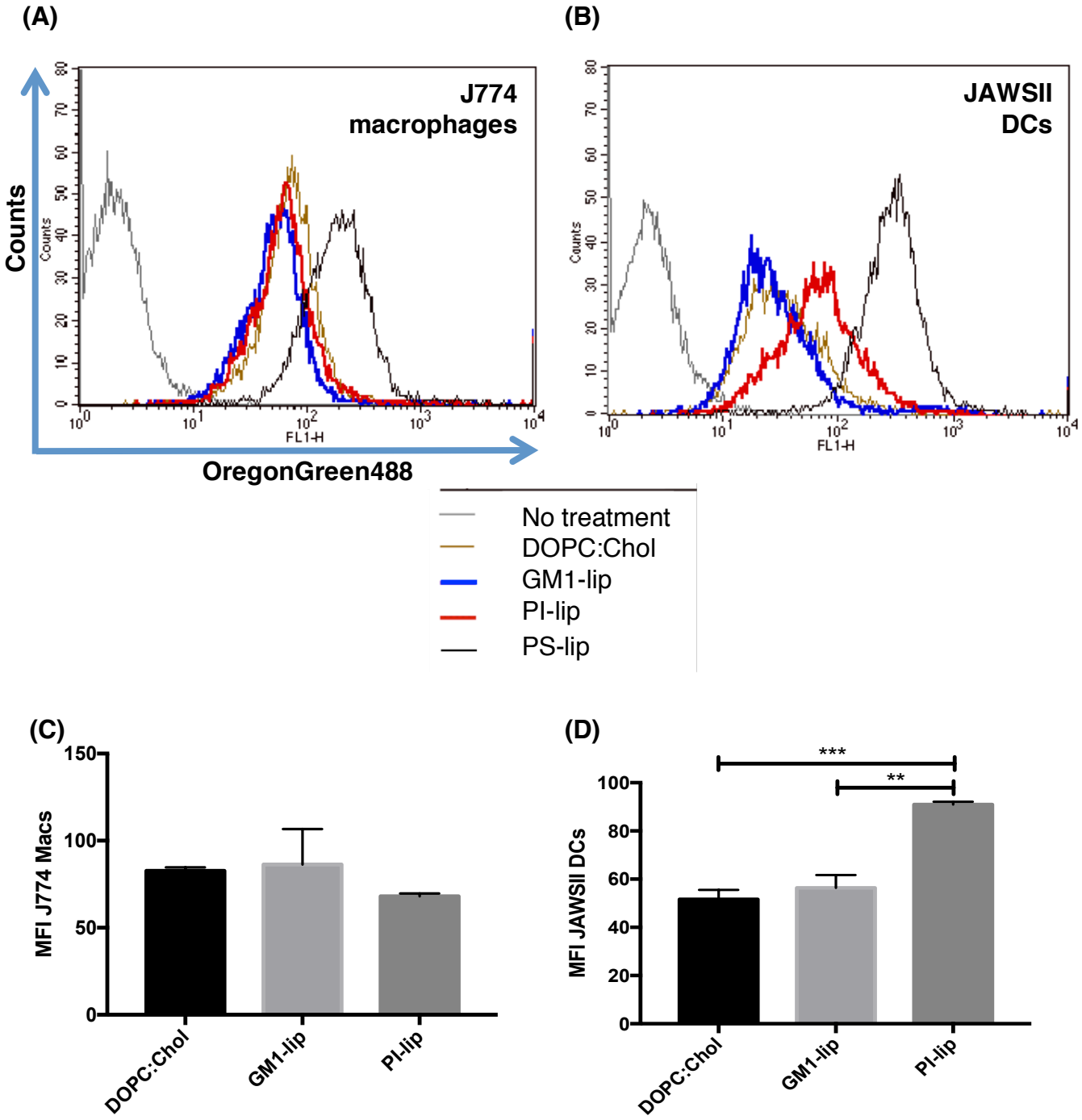


Figure 2.1 JAWSII dendritic cells exhibit preferential uptake of PI-liposomes
Liposome uptake by J774 macrophages and JAWSII DCs was assessed using flow cytometry. Cells were pulsed with 100µM OregonGreen488 fluorescently labeled liposomes for 1 hour at 37°C. Upper panel shows representative histograms of indicated groups in (A) J774 macrophages and (B) JAWSII DCs. Lower panel shows mean fluorescence intensities (MFI) of (C) J774 macrophages and (D) JAWSII DCs with indicated liposome treatment. Data shown represent mean ± SEM, n=3 (**p<0.01, ***p<0.001, analyzed using one-way ANOVA and Tukey's post-hoc test).

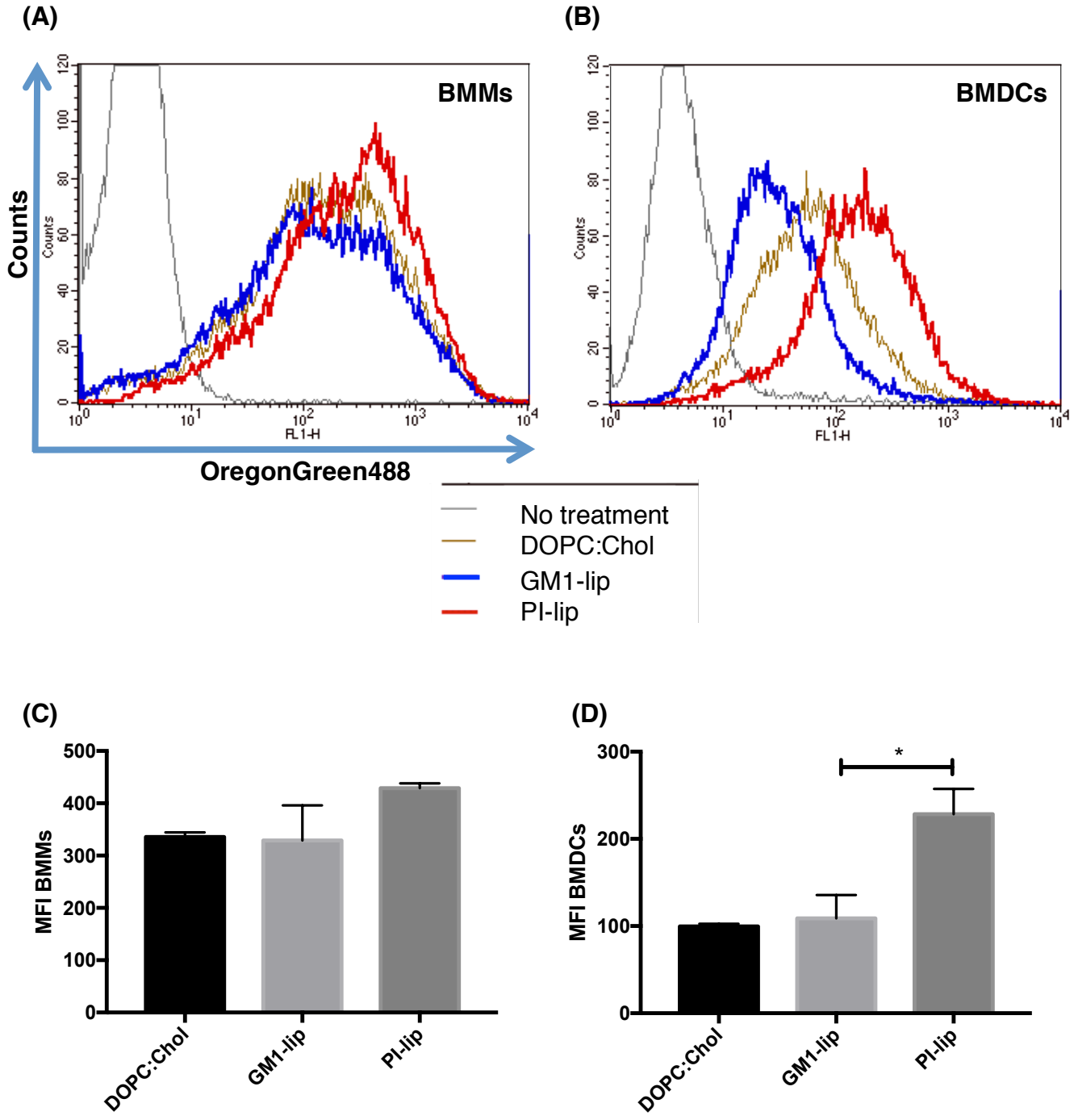


Figure 2.2 Uptake trends in primary bone marrow-derived cells resemble those presented in cell lines

Liposome uptake by BMMs and BMDCs was assessed using flow cytometry. Cells were pulsed with 100µM OregonGreen488 fluorescently labeled liposomes for 1 hour at 37°C. Upper panel shows representative histograms of indicated groups in BMMs (A) and BMDCs (B). Lower panel shows mean fluorescence intensities (MFI) of BMMs (C) and BMDCs (D) with indicated liposome treatment. Data shown represent mean ± SEM, n=2 (*$p<0.05$, analyzed using one-way ANOVA and Tukey's post-hoc test).

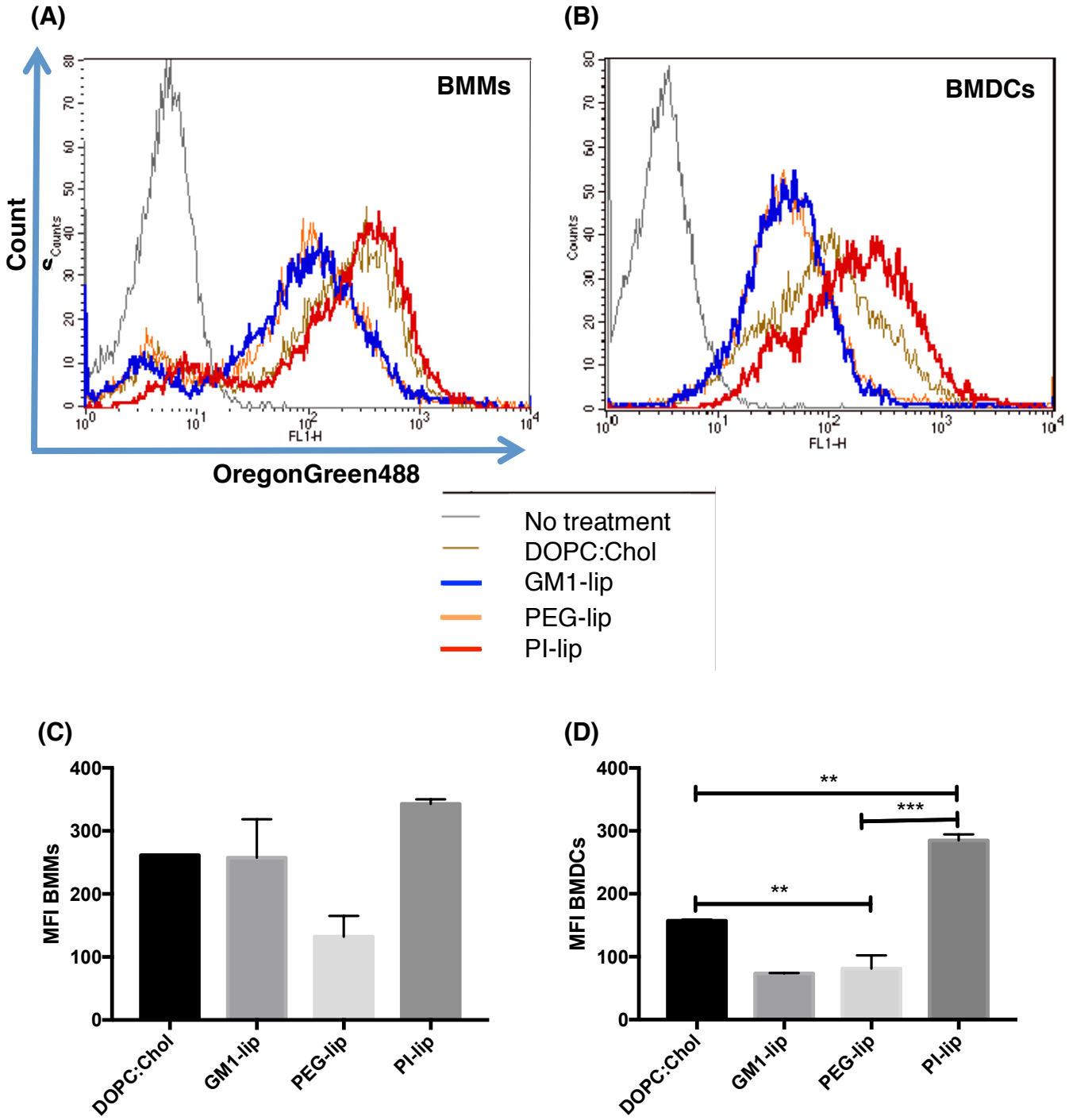


Figure 2.3 Uptake of PEG-lip is comparable to GM1-lip, in BMMs and BMDCs
Liposome uptake by BMMs and BMDCs was assessed using flow cytometry. Cells were pulsed with 100µM OregonGreen488 fluorescently labeled liposomes for 1 hour at 37°C. Upper panel shows representative histograms of indicated groups in BMMs (A) and BMDCs (B). Lower panel shows mean fluorescence intensities (MFI) of BMMs (C) and BMDCs (D) with indicated liposome treatment. Data shown represent mean ± SEM, n=2 (**p<0.01, ***p<0.001, analyzed using one-way ANOVA and Tukey's post-hoc test).

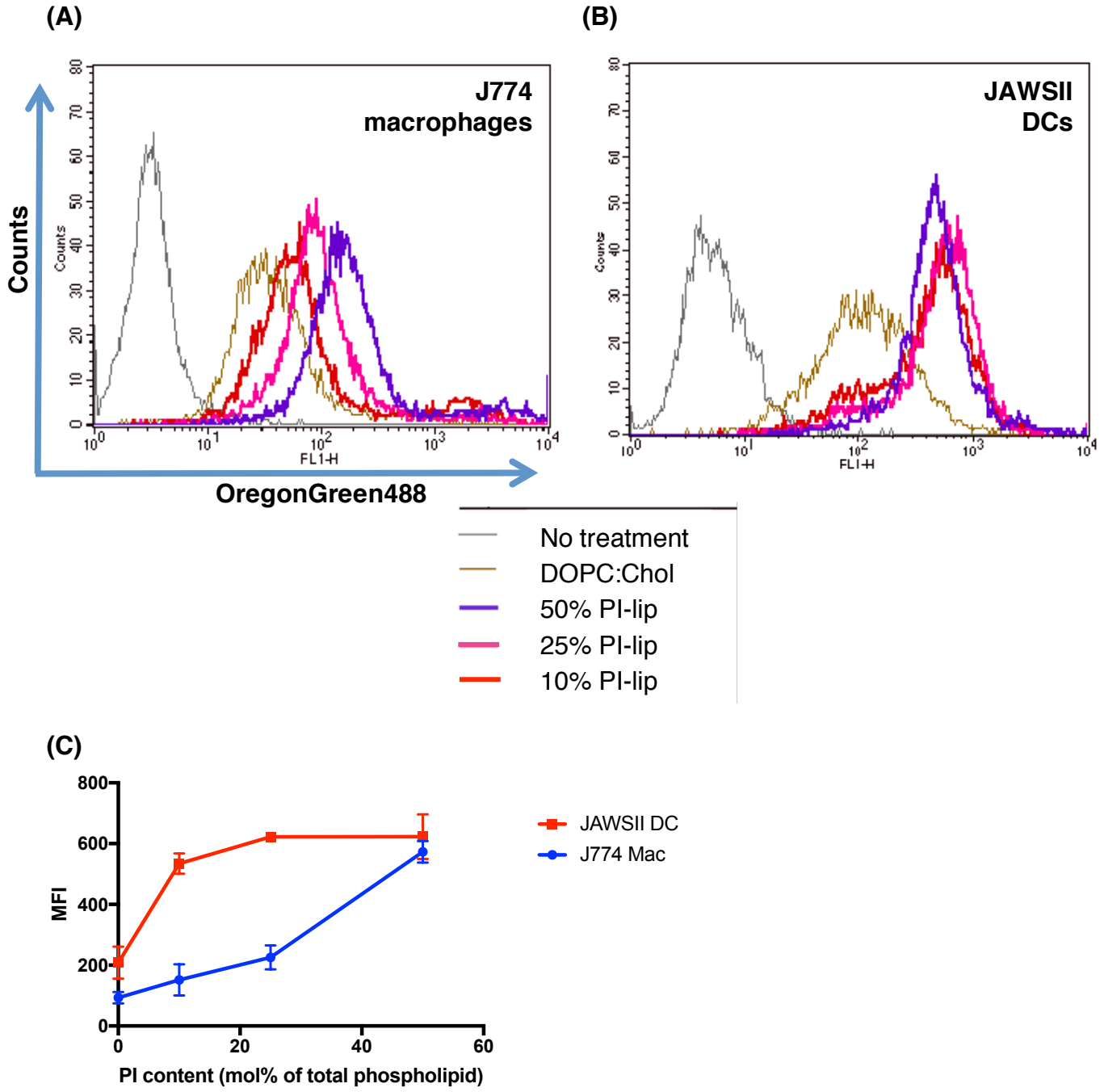


Figure 2.4 Effect of PI density on the liposome uptake by macrophages and DCs
Liposome uptake by J774 macrophages and JAWSII DCs was assessed using flow cytometry. Cells were pulsed with 100µM OregonGreen488 fluorescently labeled liposomes for 1 hour at 37°C. Upper panel shows representative histograms of indicated groups in (A) J774 macrophages and (B) JAWSII DCs. (C) Mean fluorescence intensities (MFI) of corresponding histograms plotted against increasing PI mol% present in the liposome formulation. Data shown represent mean of two independent experiments ± SEM

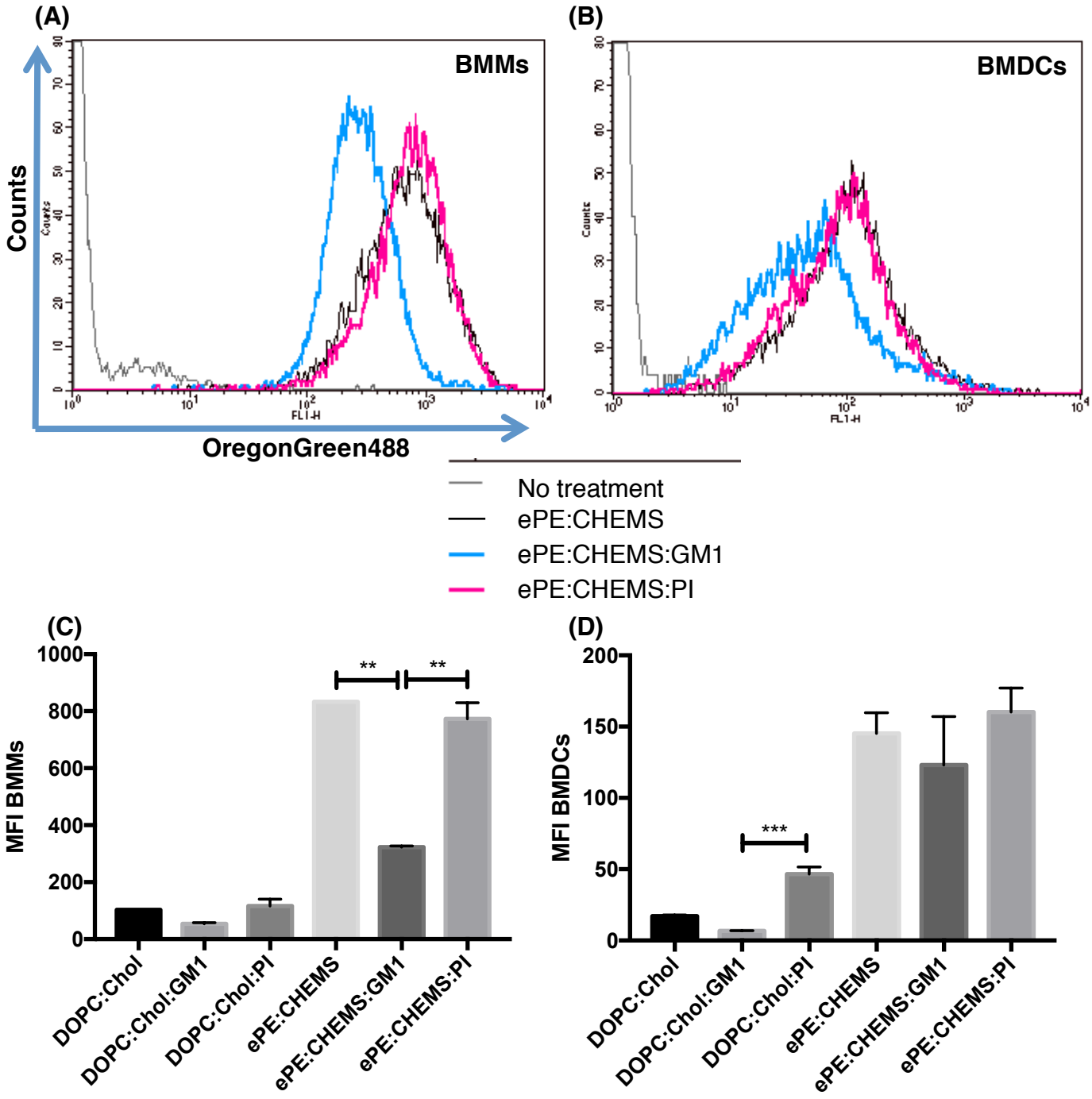


Figure 2.5 ePE:CHEMS liposome compositions dissolve liposome uptake trends
Liposome uptake by BMMs and BMDCs was assessed using flow cytometry. Cells were pulsed with 100µM OregonGreen488 fluorescently labeled liposomes for 1 hour at 37°C. Upper panel shows representative histograms of indicated groups in BMMs (A) and BMDCs (B). Lower panel shows mean fluorescence intensities (MFI) of BMMs (C) and BMDCs (D) with indicated liposome treatment. Data shown represent mean ± SEM, n=3 (**p<0.01, ***p<0.001, analyzed using one-way ANOVA and Tukey's post-hoc test).

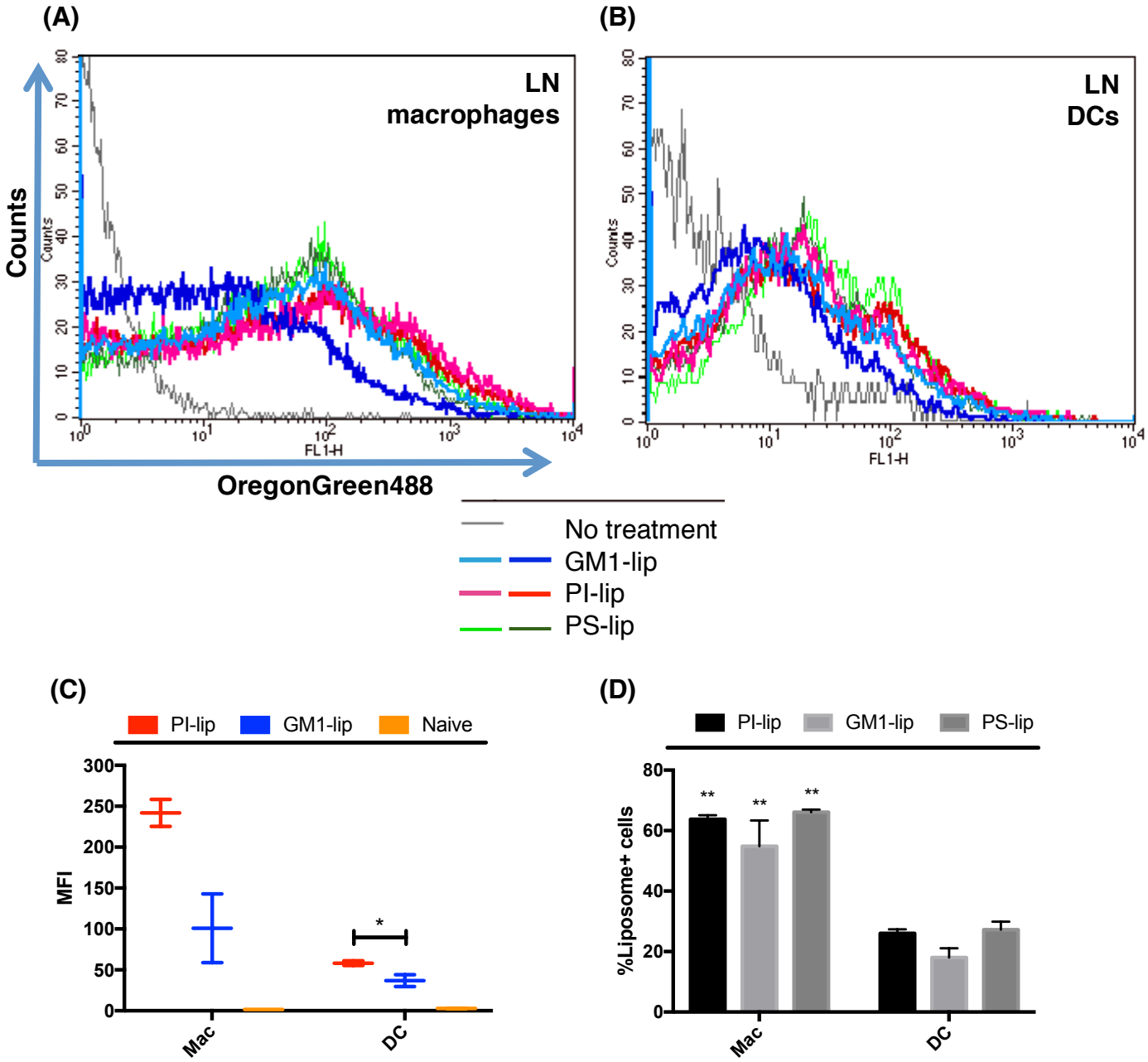


Figure 2.6 PI-lip promote uptake by macrophages and DCs present in the LN
C57BL/6 mice (n=2 per formulation) were subcutaneously injected with 250nmoles of the indicated OregonGreen488 fluorescently labeled liposomes in the hind flank near the tail base. After 4 hours, inguinal LN cells were stained with fluorescent anti-CD11b and anti-CD11c and analyzed by flow cytometry. Upper panel shows histograms of indicated groups in (A) $CD11b^{+}CD11c^{-}$ LN macrophages and (B) $CD11b^{+}CD11c^{+}$ LN DCs. (C) Corresponding mean fluorescence intensities (MFI) in cell types. *p<0.05, analyzed using one-way ANOVA and Holm-SIdak's post-hoc test (D) Percentage of indicated cells positive for liposomes **p<0.01, analyzed using two-way ANOVA with Bonferroni's post-hoc test. Data shown represent mean ± SEM, n=2

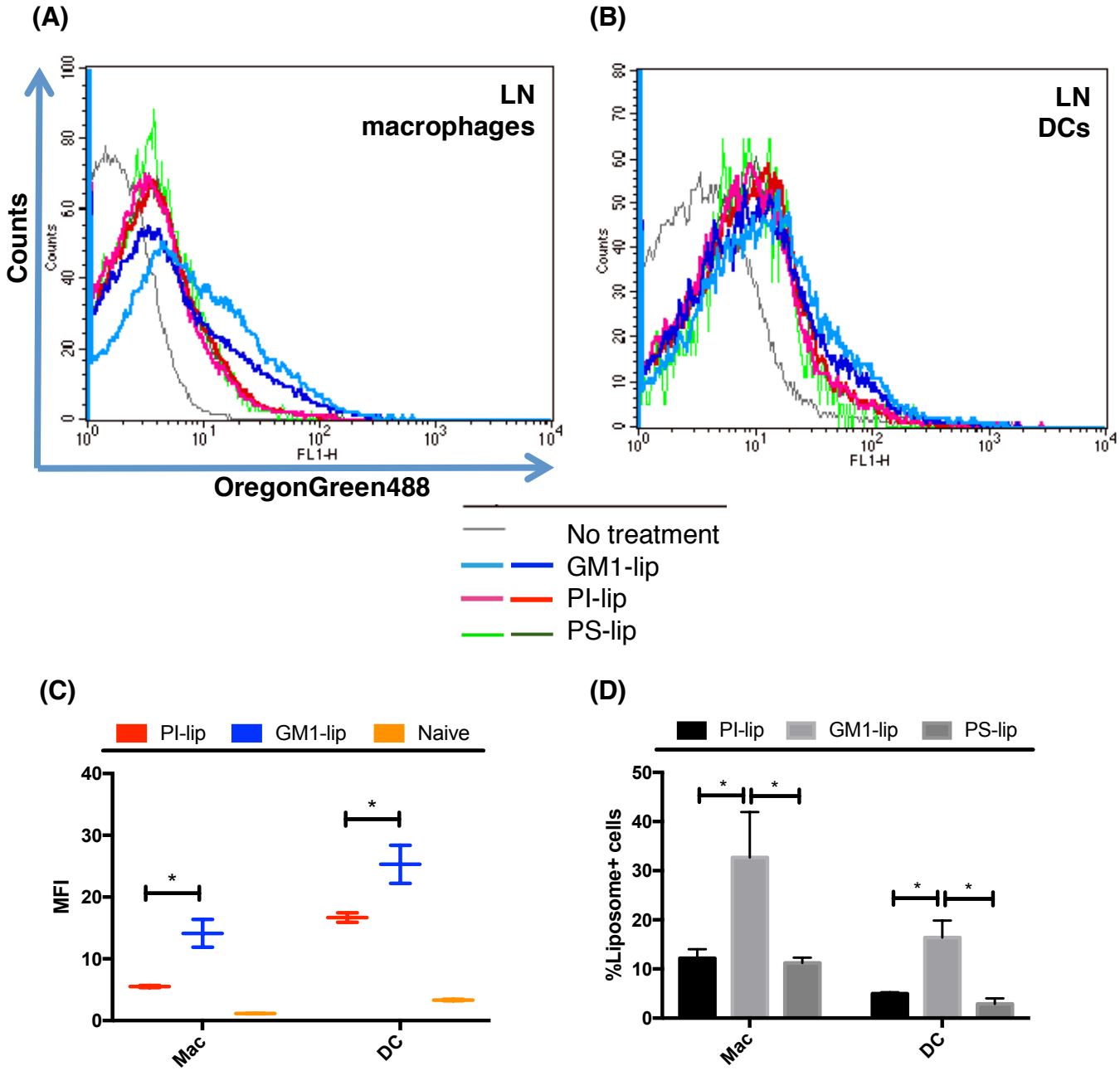
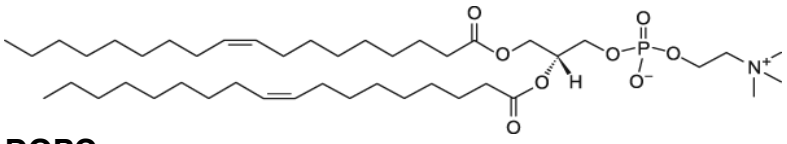


Figure 2.7 GM1-lip has prolonged uptake by cells in the LN

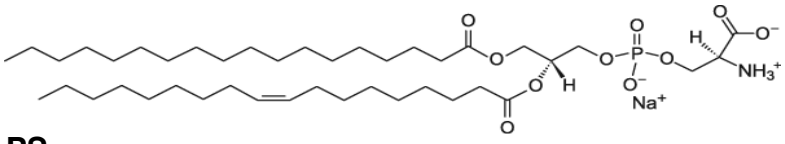
C57BL/6 mice (n=2 per formulation) were subcutaneously injected with 250nmoles of the indicated OregonGreen488 fluorescently labeled liposomes in the hind flank near the tail base. Twenty-four hours post injection, inguinal LN cells were stained with fluorescent anti-CD11b and anti-CD11c and analyzed by flow cytometry. Upper panel shows histograms of indicated groups in (A) $CD11b^{+}CD11c^{-}$ LN macrophages and (B) $CD11b^{+}CD11c^{+}$ LN DCs. (C) Corresponding mean fluorescence intensities (MFI) in cell types. *$p<0.05$, analyzed using one-way ANOVA and Holm-SIdak's post-hoc test (D) Percentage of indicated cells positive for liposomes *$p<0.05$, analyzed using two-way ANOVA with Bonferroni's post-hoc test. Data shown represent mean ± SEM, n=2

Liposome composition	Mole ratio	Size* (nm)	Poly dispersity index (PDI)	Zeta potential (mV)
DOPC:Chol	2:1	111.1	0.190	-4.98 ± -.361
DOPC:Chol:PI (10mol% PI-lip)	9:5:1	105.9	0.104	-9.62 ± -.074
DOPC:Chol:PI (25mol% PI-lip)	7.5:5:2.5	112.4	0.022	-14.1 ± -1.09
DOPC:Chol:PI (50mol% PI-lip)	1:1:1	116.7	0.014	-22.6 ± -1.74
DOPC:Chol:GM1 (10mol% GM1-lip)	9:5:1	108.6	0.090	-7.93 ± -.621
DOPC:Chol:PS (10mol% PS-lip)	9:5:1	104.9	0.099	-12.3 ± -0.946
DOPC:Chol:PS (50mol% PS-lip)	1:1:1	109.5	0.107	-26.9 ± -2.07
DOPC:Chol:PEG-PE (PEG-lip)	9:5:1	120.1	0.046	-1.43 ± -0.110

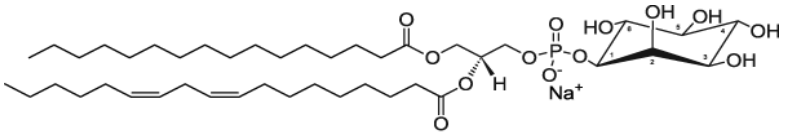
Table 2.1 Composition, size, PDI and zeta potential of liposomes
Representative particle size, poly dispersity index (PDI) and zeta potential data of liposome formulations are shown. Size (diameter; nm) was determined by dynamic light scattering and zeta potential was determined by electrophoresis and laser Doppler velocimetry. PDI is a dimensionless measure of the width of particle size distribution.
*Size listed is Z-average particle size



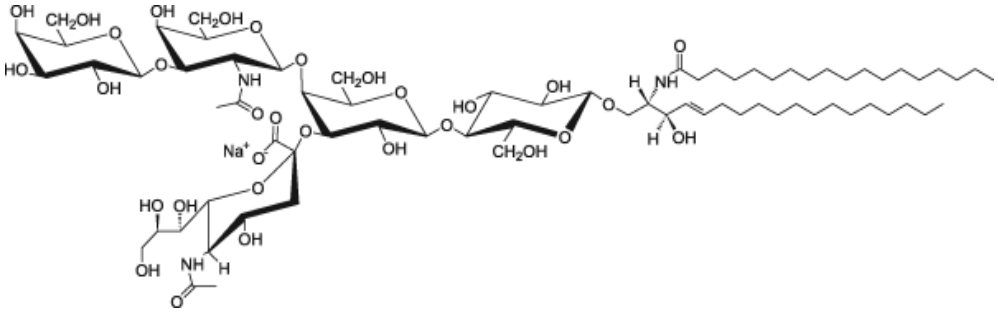
DOPC



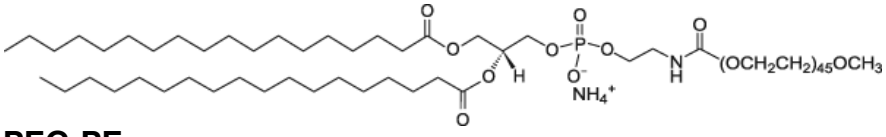
PS



PI



GM1



PEG-PE

Figure 2.8 Lipid structures
Structures of the predominant species in lipid product

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CHAPTER 3

Evaluation of DOPC Liposomes Containing Glycolipid as Vaccine Carriers to Stimulate Cell-Mediated Immunity

SUMMARY

In Chapter 2, two glycolipid-containing liposome formulations were examined as potential vaccine carriers to enhance antigen uptake by dendritic cells (DCs). DCs are prime targets for stimulating immune responses from protein vaccine preparations because of their unique abilities to stimulate T cells and coordinate innate and adaptive immune responses. Liposomes containing phosphatidylinositol (PI) or monosialotetrahexosylganglioside (GM1) glycolipid encapsulating the model protein antigen ovalbumin (OVA) and the hemolysin listeriolysin-O (LLO) were examined for their potential to generate antigen-specific cell-mediated immune responses in additional to humoral, antibody responses. Protein subunit vaccines in general have failed to generate cell-mediated immunity, especially the activation of $CD8^+$ T cells. Therefore, we focused most of our efforts on monitoring the generation of $CD8^+$ T cells after immunization with the liposome formulations. Herein we have described glycolipid-containing dioleoylphosphatidylcoline (DOPC) liposomes encapsulating OVA and LLO generated antigen-specific $CD8^+$ T cells after immunization. This is likely a result of increased DC uptake of liposomes, especially for PI containing liposomes, as well as

cytosolic delivery of antigen with the aid of LLO. Liposomes composed of DOPC and PI (DOPC:PI liposomes) produced higher mean titers of antigen-specific serum IgG1 and IgG2a antibodies compared to DOPC liposomes containing GM1 (DOPC:GM1 liposomes). However, despite distinctly different uptake by lymph node macrophages and DCs, all antigen-specific cell-mediated immune responses monitored were similar between the two glycolipid liposome formulations.

INTRODUCTION

The immune system is categorized into two main responses, innate immunity and adaptive immunity. The innate immune system is used to distinguish self from non-self. Cells of this immune system recognize unique non-self motifs termed pathogen-associated molecular patterns (PAMPs) by various pattern-recognition receptors (PRRs) on the outside and inside of the cell [1]. Innate immune responses are essential for early inhibition of infection and viral/bacterial proliferation. The innate immune system functions to produce molecules that stimulate the immune system (e.g., chemokines, cytokines and interferons (IFNs)) and activate leukocytes (e.g., macrophages) in response to infection [2]. Based on the innate immune system's determination of non-self, the adaptive immune response develops antigen-specific immune responses [3]. The adaptive immune system is further divided into two arms termed humoral immunity and cell-mediated immunity. Humoral immunity is defined by the production of antibodies by B lymphocyte cells, which detect pathogens in blood or fluids. Cell-mediated immunity refers to the activation of $CD4^+$ T helper (Th) cells and $CD8^+$ T

lymphocyte cells, also known as cytotoxic T lymphocytes (CTLs). The adaptive immune response is also responsible for the generation of antigen-specific memory B and T cells. Memory cells are crucial for recognizing re-exposures to a pathogen, facilitating quicker host immune responses to infection [4].

Live attenuated vaccines are extremely effective at preventing infection because they elicit strong cellular and humoral immune responses. These strong responses mimicked the protective immunity a person would develop in response to a natural infection [5]. However, due to safety concerns of live vaccinations, current vaccine development has shifted from using live attenuated pathogens to protein subunit vaccines. Protein subunit vaccines rely on protein antigens, which inherently have low immunogenicity, to generate protective immunity. It is not a surprise then that most of these protein subunit vaccines have failed to generate sufficient protective immune responses [6, 7]. Specifically, cell-mediated immune responses have been lacking in protein subunit vaccines. Cellular immune responses are primarily directed at intracellular pathogens, which persist inside host cells protected from antibody recognition and opsonization. Many of the most needed vaccines against HIV, cancer, hepatitis C and tuberculosis will require cell-mediated responses to clear the infection or disease [7-11]. Therefore the development of enhanced cellular responses to protein subunit vaccine preparations is an important topic of research.

A proposed way to stimulate enhanced cell-mediated responses is through directing of antigen to the lymph nodes. Lymph nodes (LN) are rich in macrophages and dendritic cell (DCs), as well B and T cells [12, 13]. The high density of DCs in close

proximity to T cells is why LN targeting is an attractive option for stimulating cell-mediated immune responses from protein subunit vaccinations. Access to the lymph nodes is most importantly dependent on size [14]. Nanoparticle vaccine formulations must be small (<0.2 μm) in order to drain into the lymphatic capillaries and travel to the lymph nodes [15]. Small liposomes (mean diameter <0.1 μm) can gain access to lymph nodes quicker, but they also display higher blood concentrations suggesting they pass through the lymphatic system with low uptake by DCs in the lymph nodes [14]. Larger nanoparticle formulations may still gain access to the lymph nodes if internalized by DCs at the injection site and subsequently migrate to the lymph nodes [14, 15]. Most commonly, subcutaneous (s.c.) injection of nanoparticle formulations, including liposomes, is used to facilitate delivery to the lymph nodes. The uptake study performed with these liposome formulations in Chapter 2 of this thesis, is the only known study to characterize the uptake behavior of PI- or GM1-containing liposomes after s.c. administration. The use of these liposome formulations has previously been limited to small molecule delivery to tumor after intravenous injection.

From a vaccination standpoint, DCs represent a prime target for stimulating robust cellular immune responses towards delivered antigen(s). DCs are unique in their ability to “cross-present” antigens to T cells and induce T cell activation *in vivo* [16-18]. Cross-presentation allows exogenously delivered antigen to be loaded on major histocompatibility complex (MHC) class I molecules resulting in presentation to $CD8^+$ T cells, which are key mediators of cell-mediated immune responses [19, 20]. DCs, unlike macrophages, are able to prime naïve CTLs and helper $CD4^+$ T cells using cross-

presented antigens [16, 17, 21]. Activation of naïve $CD4^+$ and $CD8^+$ T cells, leads to T cell proliferation and their transition into effector cells [22, 23]. Two studies carried out with DC-deficient mice, have shown DCs are a critical component for antigen cross-priming to $CD8^+$ T cells as well as protection against the intracellular pathogen *Listeria monocytogenes (Lm)* [16, 24]. One additional study with mice outfitted with mutant DCs unable to cross-present antigen observed that mice were severely compromised in their ability to generate antigen-specific $CD8^+$ T cells after infection with *Lm* and concluded that DC cross-priming plays an essential role in T cell responses against *Lm* [25]. Targeting material(s) to DCs for uptake can be additionally beneficial since dendritic cells that take up antigen or particulate antigen at injection sites can also migrate to lymph nodes in order to stimulate T cell responses [26]. This provides material (or nanoparticles encapsulating material) access to lymph nodes where effector T and B cells reside. DC's characteristic shape also enables them to interact with multiple T cells simultaneously [27]. *In vitro* it has been estimated that one mature DC can activate 100-3,000 T cells [28]. In conclusion, increasing antigen delivery to DCs is one approach to stimulating more robust immune responses, especially T cell mediated responses.

$CD8^+$ T cells are activated by antigen-presenting cells (APCs), such as macrophages and DCs, displaying antigen in the context of MHC class I antigen presentation [27]. In order to present antigen on MHC I molecules, antigen needs to be delivered to the cytosol of cells. To gain access to the cytosol is not an easy feat for exogenous antigen. Even formulating antigen into liposomes will still result in endocytosis of liposomes and ultimately lead to destruction of delivered components in

the lysosome [29]. The hemolysin listeriolysin-O (LLO) *from Listeria monocyotgenes (Lm)* has been used in conjunction with liposomes co-encapsulating antigen to promote cytosolic delivery of liposomal antigen. LLO is maximally active at pH~5.5, conditions present in the endocytic pathway [30]. Active LLO forms pores in the endosomal membrane, thus facilitating the delivery of liposomal antigen to the cytosol of cells. However, for effective cytosolic delivery of antigen via LLO, pH-sensitive liposomes are generally utilized [29]. pH-sensitive liposomes contain an amphipathic lipid, typically phosphatidylethanolamine (PE), which is unable to form stable bilayers at physiological pH, and a second stabilizing amphipathic lipid such as cholesteryl hemisuccinate (CHEMS) that conditionally stabilizers the bilayer dependent on pH. At physiological pH, PE:CHEMS liposomes form stable lipid bilayers. However, at acidic conditions present in the endocytic pathway CHEMS loses its negative charge and its therefore unable to stabilize the PE lipid bilayer [31]. Thereafter, pH-sensitive liposomes lose their membrane integrity, which allows LLO access to the endosomal compartment.

We had previously examined whether pH-sensitive liposomes could be utilized along with the glycolipid-liposome formulations, however the PE:CHEMS compositions high negative surface charge resulted in enhanced cell uptake by both macrophages and DCs. Liposomes composed of dioleoylphosphatidylcoline (DOPC) with no cholesterol (Chol) content to stabilize the membrane are known to lose membrane integrity more quickly than their cholesterol-containing counterpart [32, 33]; thus, we hypothesized that once internalized and introduced to the acidic endocytic pathway, DOPC liposomes would breakdown [34]. Thereafter, LLO would be released into the

endosome, as is the case with pH-sensitive liposomes, and antigen could be delivered to the cytosol.

$CD4^+$ T cells are another component of cell-mediated immunity. These cells are activated by APCs that present antigen via MHC class II molecules. MHC class II molecules are present in all APCs, including B cells, macrophages and DCs [27]. $CD4^+$ cells are known as helper T cells due to their roles in promoting immune responses from all cells in the immune system. APCs, B cells and $CD8^+$ T cells all receive direction from $CD4^+$ T cells. $CD4^+$ T cells are subdivided into T helper 1 (Th1) and T helper 2 (Th2) cells that either enhance or inhibit cell-mediated immunity, respectively [35]. Th1 cells promote cell mediated-immunity by secretion of cytokines such as IL-12, TNF-α/β and IFN-γ. These cytokines enhance the activation of CTLs and macrophages, and promote the differentiation of B cells [35, 36]. B cells that are activated by Th1-type cytokines secrete IgG2a, an opsonizing antibody associated with viral clearance. Skewing $CD4^+$ T cells to differentiate into Th2 cells results in IL-4, IL-5, and IL-6 production and the proliferation of B cells that secrete IgG1, a neutralizing antibody [35, 37, 38]. A Th2 type response promotes humoral immunity [36].

MATERIALS AND METHODS

Listeriolysin-O (LLO) expression and preparation

The *hly* gene encoding for LLO was inserted into the bacterial expression plasmid pET29b with a polyhistidine tag. Recombinant LLO was purified from *E. coli* as described by Mandal et al. [39], with the following exceptions. Protein expression was

induced with 1mM isopropyl β-D-thiogalactopyranoside and grown fro 4-6 hours. After which the cell pellet was collected and resuspended in wash buffer (50mM sodium phosphate, 300mM sodium chloride, 20mM imidazole, pH 8) containing 2mM PMSF and 1mM 2-mercaptoethanol and then lysed using a French press. Lysate supernatant was incubated with Ni-NTA agarose (Qiagen, Valencia, CA) for 2 hours and the Ni-NTA extensively washed with wash buffer. Polyhistidine-tagged LLO was eluted in wash buffer containing 400mM imidazole, then extensively dialyzed against HEPES-buffered saline containing EDTA (HBSE) (10mM HEPES, 140mM NaCl, 1mM EDTA, pH 8.4) at 4°C. Protein purity was assessed by SDS-PAGE and activity by hemolysis assay, as previously described [39]. Aliquots of LLO were stored at -80°C.

Liposome preparation

All lipids were obtained through Avanti Polar Lipids (Alabaster, Alabama). Liposomes were prepared at a 9:1 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Δ9-cis) (DOPC)) and one of the following three lipids: L-α-phosphatidylinositol (Soy) (PI), monosialotetrahexosylganglioside (ovine brain) (GM1), or L-α-phosphatidylserine (porcine brain) (PS). This resulted in DOPC liposomes containing 10mol% of PI, GM1 or PS. L-α-phosphatidylethanolamine (egg, chicken) (ePE) and cholesteryl hemisuccinate (CHEMS) (Sigma-Aldrich) were prepared at a 2:1 molar ratio. Lipid mixtures were dried completely using a Buchi Rotavapor R-200 rotary evaporator to create a lipid film. Lipid films were rehydrated with isotonic (290 ± 10 mmol/kg osmolality) HBSE, pH 8.4 containing soluble ovalbumin (OVA) (Grade VI,

Sigma-Aldrich) at 25 mg/ml (*in vivo* studies) or 30 mg/ml (*in vitro* studies) and LLO at 250 µg/ml (*in vivo* studies) or 150 µg/ml (*in vitro* studies). Hydrated lipid films were vortexed, subjected to 5 freeze/thaw cycles and sonicated 8x1min cycles using a water bath sonicator. Unencapsulated protein was removed by size exclusion chromatography using a 1x25cm Sepharose CL-4B column (GE Healthcare). The size of the liposomes and zeta potential was determined using a Malvern ZS90 ZEN 3600 zetasizer. Phospholipid content of liposomes was quantified using Bartlett's phosphate assay [40]. Determined phospholipid concentrations signified the concentration of liposomes and were used in the doing calculations in the antigen presentation assay. GM1 does not have a phosphate group and thus the phospholipid assay concentrations determined do not include GM1 content of the liposomes; dosing calculations were adjusted to account for this. Quantification of encapsulated protein was determined using SDS-PAGE. Proteins were resolved on a 4-20% Tris-glycine gel (Invitrogen) with 1X electrode buffer (250mM Trizma, 2M glycine) containing 0.1% SDS. Gels were stained with Krypton protein stain (Pierce) and protein band intensities were measured on a Typhoon 9200 (Molecular Dynamics). Protein content was quantified using ImageQuant (GE Healthcare). Prepared liposome samples were stored at 4°C and used within 10 days of preparation

Cell culture

All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. All cells were maintained in in a humidified incubator at

37°C and 5% CO_2. B3Z cells, an OVA SIINFEKL peptide-specific $CD8^+$ T-cell hybridoma (CD8 OVA, H-2Kb-restricted), were maintained in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2mM L-glutamine, 1mM sodium pyruvate, 100µg/mL streptomycin, 100U/mL penicillin, 50µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 25mM HEPES.

Bone marrow was harvested from femurs and tibia of female C57BL/6 mice and differentiated into bone marrow-derived macrophages (BMMs) and bone marrow-derived dendritic cells (BMDCs). BMM media (DMEM supplemented with 20% HI-FBS, 30% L-929 cell conditioned media containing macrophage colony stimulating factor (M-CSF), 2mM glutamine, 100µg/mL streptomycin, 100U/mL penicillin and 55µM 2-mercaptoethanol) was used to maintain the BMM cultures. BMMs were derived as described previously by Stier et al [41]. In short, precursor cells were cultured for 6 days in the presence of M-CSF, a component of the L-cell media supplement. Cells were replenished with new cell media on day three of culture. BMMs were then harvested on day six of culture and stored in liquid nitrogen until needed. BMDC media (RPMI-1640 supplemented with 10% HI-FBS, 100 µg/mL streptomycin, 100U/mL penicillin, and 50µM 2-mercaptoethanol) was prepared and murine GM-CSF was added fresh each time to the BMDC media at 20ng/ml immediately before use. Cells were cultured in the presence of GM-CSF for 6 days, exchanging the cell media every 2 days, after which loosely adherent and non-adherent cells were harvested and stored in liquid nitrogen until needed.

In vitro antigen presentation

In vitro antigen presentation was performed as described previously in Andrews *et al* [42]. $2x10^5$ cells/well were added to 96-well plates in serum-containing DMEM the night prior. BMM and BMDCs were washed with serum-free DMEM and incubated with liposome samples diluted in serum-free DMEM for 2 hours. Cells were dosed based on liposomal OVA content, with OVA concentration measuring at 30 μg/ml per well. Between all cell washings, the plates were centrifuged at 1500 rpm for 5 minutes to reduce cell loss due to washing steps. Cells were washed in serum-containing DMEM and incubated for 3 hours. Before fixing, the cells were washed twice in serum-free media. Cells were fixed by adding 1% paraformaldehyde in PBS for 15 minutes on ice. The 1% paraformaldehyde solution was prepared by dissolving 96% paraformaldehyde in warm 1X PBS by drop wise addition of 0.1M NaOH. The solution was pHed to 7 prior to fixing cells. Paraformaldehyde was quenched by the addition of excess 0.2 M lysine in DMEM for 20 minutes at RT. The cells were washed in serum-containing DMEM twice and in B3Z media once after quenching. $2x10^5$ cells/well of B3Z cells in B3Z media were added and incubated for 15 hours. Cells were washed with RT 1X PBS, and the β-galactosidase substrate (0.15mM chlorophenol red-β-Dgalactopyranoside (CPRG) (Calbiochem, Gibbstown, NJ), 100μM 2-mercaptoethanol, 9mM MgCl2, 0.125% NP40 (Calbiochem, Gibbstown, NJ) in 1X PBS) was added to measure production of chlorophenol red by β-galactosidase due to BMM/BMDC antigen presentation of SIINFEKL to B3Z cells. The CPRG substrate was incubated with cells for 9 hours, after which the absorbance of chlorophenol red was measured at 595 nm on a Synergy plate

reader (BioTek, Winooski, VT). BMMs and BMDCs treated with the positive control, OVA-CD8 peptide SIINFEKL, consistently had similar chlorophenol absorbance values.

Immunization protocol

C57BL/6 (7-8 weeks old; Jackson Laboratories, Bar Harbor, ME) were used in this study and handled according the University of Michigan Institutional Animal Care guidelines. C57BL/6 mice possess the H-2K^b MHC haplotype. Mice were injected subcutaneously on each side of the hind flank near the tail base with liposomes encapsulating a total of 10μg OVA (1.16-2.89μg LLO) or soluble OVA (10μg) in 100μL volume. Naïve mice were injected with the same volume of HEPES buffered saline (HBS), pH 8.4. Day 0 mice were injected with prime immunization and on Day 10 injected with boost immunization. A blood draw was performed before boost injections on Day 10 to analyze IgG titers post-prime only. Day 21 mice were euthanized and a final blood collection was performed via cardiac puncture.

CD8$^+$ T cell tetramer staining

Seven days after Day 0 and Day 10 immunizations, blood was collected from the cheeks of mice where the retro-orbital and superficial temporal vein meet as described in Golde *et al*. [43]. K_2EDTA coated tubes (BD Biosciences, Franklin, NJ) were used for the blood collection. Blood samples were pipetted gently to evenly distribute the cells then red blood cells were lysed twice using ACK buffer (Life Technologies). Cells were centrifuged at 1500g for 5 min at 4°C between red blood cell lysis steps. Cells were

washed with 1X phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and centrifuged again. The cell pellet was resuspended with CD16/32 Fc block (eBioscience) and incubated for 10 minutes at room temperature (RT) before flow cytometry cell staining. A small volume of each sample was removed to establish negative and single-color controls. Cells were incubated with T-select $H2K^b$ – OVA Tetramer-SIINFEKL-PE (MBL, Japan) for 30 minutes at RT then stained with anti-CD8 only (prime immunization samples) or additionally with anti-CD44, and anti-CD62L (boost immunization samples) for 20 minutes on ice. Cells were washed two times with 1X PBS/1%BSA and additionally stained with DAPI to distinguish live and dead cells. Cell fluorescence was analyzed by flow cytometry and the data was analyzed in FlowJo.

Interferon (IFN)-γ ELISPOT

The Immunology Core at the University of Michigan Cancer Center prepared the enzyme-linked immunospot (ELISPOT) plates used in this assay. ELISPOT plates were coated and blocked using a standard ELISPOT protocol. Briefly, plates were coated with anti-mouse IFN-γ capture antibody (5 µg/mL) overnight at 4°C followed by washing and blocking with serum-containing media. Prepared ELISPOT plates were obtained from the Immunology Core on Day 21 of the immunization study. The same day, splenocytes from immunized mice were isolated and added to the plates at two densities ($5x10^5$ and $2.5x10^5$ cells/well) and stimulated for 18 hours with either cell media only (in duplicate), 2.5 µg/mL $OVA_{257-264}$ peptide (SIINFEKL) (in triplicate) or 1.25 µg/mL concanavalin A (in duplicate). Plates were washed two times with PBS and then transferred back to the

Immunology Core for completion of the assay and imaging of the plates. In brief, the plates were then washed two more times with 1X PBS containing Tween 20 (PBS-Tween20) then incubated with biotinylated anti-mouse IFN-γ detection antibody at RT. Avidin-HRP was added to the wells at RT followed by the addition of the enzyme substrate solution (3-amino-9-ethylcarbazole (AEC)). Color development was stopped by washing with distilled water. The plates were dried completely at RT after which the number of spot-forming units (SFU) in each well was determined using a computerized CTL ImmunoSpot Image Analyzer.

Anti-OVA IgG1 and IgG2a ELISA

Blood samples were collected on Day 10 and Day 21 in microtainer tubes with serum separator (BD Biosciences, Franklin, NJ). Sera were collected after centrifuging the blood samples at 10000xg for 5 minutes. Sera were used immediately or stored at -80°C until needed. Maxisorp NUNC plates were coated with 10 µg/mL OVA in 0.1M sodium phosphate, pH 9.0 buffer overnight at 4°C. Plates were washed 6x with PBST (1x phosphate buffered saline, 0.05% Tween) before adding 1X PBS/1%BSA blocking buffer. Plates were blocked for a minimum of 2 hours at RT or overnight at 4°C. The plates were washed 6x with PBST and incubated with serial dilutions of sera (in duplicate) in 1X PBS/1% BSA/0.05% Tween 20 dilution buffer overnight at 4°C. Anti-OVA isotype-specific secondary antibodies (goat anti-mouse IgG2a-biotin conjugated or goat anti-mouse IgG1-biotin conjugated) (Southern Biotech, Birmingham, AL) were detected with Avidin-HRP (eBioscience, San Diego, CA). Plates were developed with

TMB substrate (KPL, Gaithersburg, MD) and color development was stopped with the addition of 2N sulfuric acid. Absorbance was read at 450nm on a Synergy plate reader (BioTek, Winooski, VT) and the data were fit to a 4-parameter curve on the Gen5 data analysis software (Biotek, Winooski, VT). Titer was defined as the reciprocal of the highest dilution where absorbance read above the mean absorbance + 3 standard deviations of the negative control sera (naïve mice sera).

RESULTS

DOPC:PI and DOPC:GM1 liposomes encapsulating OVA and LLO can deliver OVA to the cytosol of BMMs and BMDCs

Liposomes composed of DOPC and glycolipid were formulated with LLO and the model antigen ovalbumin (OVA) and evaluated for their ability to deliver OVA to the cytosol of cells. For LLO to be active, the liposomes must break down sufficiently enough for LLO to be released into the endosome. Thereafter, LLO facilitates the formation of pores in the endosomal membrane, allowing for cytosolic delivery of OVA. Although these DOPC-glycolipid liposomes are not pH-triggered to deform, as some pH-sensitive liposome formulations are, these liposomes are, apparently, still able to release LLO into the endosome for action. Antigen presentation is monitored by the substrate conversion to chlorophenol red, which is mediated by B3Z T cells activated via MHC Class I antigen presentation of the OVA CD8 peptide, SIINFEKL (Figure 3.1). This assay is specific for MHC Class I antigen presentation, however, it does not discern between antigen delivered via LLO to the cytosol from cross-presented exogenous

antigen on MHC I molecules. The glycolipid incorporated in the liposome formulation did not affect the cytosolic delivery of OVA, as both DOPC:PI and DOPC:GM1 liposomes resulted in similar antigen presentation by bone marrow-derived macrophages (BMMs) and bone marrow-derived dendritic cells (BMDCs).

Glycolipid liposome formulations generate OVA-specific $CD8^+$ T cells and $CD44^+/CD62L^+$ memory $CD8^+$ T cells in immunized mice

C57BL/6 mice were immunized via subcutaneous injection on Day 0 and Day 10 with 10 μg OVA in soluble or liposomal formulations (Figure 3.2). Table 3.1 displays physical characteristics of the liposome vaccine formulations. An MHC tetramer assay was used to detect and quantify antigen-specific $CD8^+$ T cells generated as a result of immunizations. MHC-I-SIINFEKL tetramers bind to $CD8^+$ T cells displaying the specific matching T cell receptor for SIINFEKL. Cells collected from the blood of immunized mice were stained with fluorescent MHC-I-SIINKEFL tetramers and fluorescent cell markers (i.e. anti-CD8, anti-CD44, anti-CD62L) then analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMCs) isolated from the blood of immunized mice were monitored for OVA-specific $CD8^+$ T cells on Day 7 (7 days post-prime) and Day 17 (7 days post-boost), in addition to OVA-specific $CD44^+/CD62L^+$ memory $CD8^+$ T cells on Day 17 (Figure 3.3A and 3.3B).

Seven days post prime immunization, the mean percentage of OVA-specific $CD8^+$ T cells was 0.49% ± 0.03% for DOPC:PI liposomes and 0.29% ± 0.02% for DOPC:GM1 liposomes. Both DOPC:PI and DOPC:GM1 liposome formulations

generated higher percentages of $CD8^+$ T cells compared to soluble OVA (0.145% ± 0.01%) ($p<0.01$ and $p<0.05$, respectively). Comparing glycolipid liposome formulations, DOPC:PI liposomes produced a statically significant higher frequency of antigen specific $CD8^+$ T cells compared to DOPC:GM1 liposomes ($p<0.01$) (Figure 3.3A). In addition to our glycolipid liposome formulations, we also immunized mice with pH-sensitive ePE:CHEMS liposomes (n=4 mice), a liposome formulation well studied for cytosolic antigen delivery. ePE:CHEMS liposomes generated 1.00% ± 0.25% of OVA-specific $CD8^+$ T cells (data not shown).

Boost immunizations with soluble OVA did not alter the percentage of antigen-specific $CD8^+$ T cells (prime: 0.145% ± 0.01%; boost: 0.165% ± 0.01%). However, boost immunizations with liposome formulations did improve antigen-specific $CD8^+$ T cell frequency. $CD8^+$ T cell frequencies after boost were 2.22% ± 0.93% for mice immunized DOPC:GM1 formulations and 3.96% ± 1.05% for DOPC:PI formulations (Figure 3.3A). There is an obvious trend of increasing mean% of $CD8^+$ T cells in the order of soluble OVA<DOPC:GM1<DOPC:PI. However, the reported $CD8^+$ T cell frequencies from soluble OVA, DOPC:PI or DOPC:GM1 immunized mice were not considered to be statistically different from one another (ANOVA $p=0.09$), which is due to the inter-subject variance of mice immunized with the liposome formulations. Mice immunized with ePE:CHEMS liposomes also exhibited variable T cell frequency with an observed mean percentage of 4.57% ± 1.29% (data not shown). Remarkably, immunization with DOPC:PI and DOPC:GM1 liposome formulations generate a similar percentage of OVA-specific $CD8^+$ T cells when compared to the ePE:CHEMS liposome

formulation, the latter being specifically designed for cytosolic delivery of encapsulated material for presentation to $CD8^+$ T cells. Overall the data indicate DOPC:PI and DOPC:GM1 liposomes can improve antigen-specific $CD8^+$ T cell frequency in mice, indicating both liposome formulations promote adaptive, cell-mediated immune responses.

On Day 17, antigen-specific $CD8^+$ T cells obtained from the blood of mice were additionally probed for $CD44^+$ and $CD62L^+$, indicators of memory cells developed from the expanded $CD8^+$ T cell population. Immunization with PI- or GM1- containing liposome formulations generated memory T cells in equal quantities (Figure 3.3B). Encapsulating OVA inside DOPC:PI or DOPC:GM1 liposomes co-encapsulating LLO, stimulated significant enhancements in memory T cell generation compared to immunization with only soluble OVA antigen ($p<0.05$). Immunizations with soluble OVA developed some antigen-specific memory T cells, although, this amount was not considered to be statistically different compared to mice that received no OVA antigen (naïve).

Immunization with DOPC:PI or DOPC:GM1 liposomes produce similar numbers of OVA-specific IFN-γ secreting cells

An ELISPOT assay was performed to quantify the antigen-specific IFN-γ producing cells. Monitoring the number of cells secreting IFN-γ in response to CD8 peptide stimulation gives an indication of the $CD8^+$ T cell response generated by immunization. Cells were plated in triplicate, at two cell densities, to assess spot

formation after OVA-CD8 peptide (SIINFEKL) stimulation. The data shown in Figure 3.4 are the results of the higher cell density wells, although the results at the lower cell density were in agreement. The average number of spots formed per $1x10^6$ cells was 143.5 ± 26.86 and 138.5 ± 26.99 for mice immunized with DOPC:PI liposomes and DOPC:GM1 liposomes, respectively. ePE:CHEMS liposomes produced similar numbers of IFN-γ producing cells with mean spots formed equaling 145.2 ± 27.87 (data not shown). Mice immunized with soluble OVA produced spots (SFU≤5) at the same level as background (naïve mice). These results confirm the expansion of antigen-specific $CD8^+$ T cells as a result of liposomal immunizations we reported using MHC I tetramer staining and additionally indicate that the T cells generated produce IFN-γ in response to OVA peptide re-stimulation to aid the cellular immune response.

DOPC:PI liposomes improve Anti-OVA IgG1 and IgG2a titers

Antibody titers are an important measure of humoral protection provided by immunization(s). Moreover, the antibody subclasses produced can indicate Th immune responses, where IgG1 is associated with a Th2 response and IgG2a associated with a Th1 response. Sera collected on Day 10 and Day 21 from immunized mice were analyzed for IgG1 and IgG2a by ELISA. After prime immunizations only, no significant differences between the liposome formulations existed, but overall, immunizations induced primarily IgG1 with lower IgG2a titers. The respective ratios of anti-OVA IgG1/IgG2a was 8.9 and 13.8 for DOPC:PI and DOPC:GM1 liposomes (data not shown). After boost immunization, antibody titers indicate mice immunized with

DOPC:PI liposomes evoke a stronger antibody response in comparison to DOPC:GM1 liposomes, with higher mean titers of anti-OVA IgG1 and IgG2a antibodies ($p<0.01$) (Figure 3.5). We corroborated previous data from our lab indicating ePE:CHEMS liposomes induce high IgG1 titers with low IgG2a titers; in this study we determined the IgG1/IgG2a ratio after ePE:CHEMS boost immunization was 10 (data not shown). Both glycolipid liposome formulations improved that IgG1/IgG2a ratio to 3.3 indicating these formulations can promote Th1 immunity. While immunization with ePE:CHEMS liposomes produced similar antigen-specific serum IgG1 titers as the glycolipid liposome formulations ($p>0.05$), the serum IgG2a titers were lower than those produced from immunizations with PI-lip ($p<0.0001$) and GM1-lip ($p<0.05$). Immunization with soluble OVA produced no detectable titers after prime or boost immunizations at the lowest tested dilutions.

DISSCUSION

Stimulating cell-mediated immunity via protein subunit vaccine formulations is challenging due to the low immunogenicity of soluble protein antigens. Encapsulating soluble protein antigen in liposomes can protect the antigen from degradation and also deliver antigen to cells of the immune system, specifically APCs. We sought to compare glycolipid-containing liposomes for their ability to stimulate immune responses with focus on the cell-mediate immune response. Liposomes composed of DOPC phospholipid and either PI or GM1 glycolipid encapsulating OVA and LLO were found to (1) deliver OVA to the cytosol of macrophages and DCs for MHC class I antigen

presentation to T cells (2) generate antigen-specific $CD8^+$ T cells and memory T cells *in vivo* (3) stimulate B cell production of anti-OVA IgG1 and IgG2a antibodies. The glycolipid formulations generated similar immune responses for all those that were monitored, with the exception that liposomes containing PI glycolipid produced the highest titers of IgG1 and IgG2a. We also show that immunization with soluble OVA alone does not stimulate $CD8^+$ T cell responses, in alignment with general conclusion that exogenous soluble protein alone is not able to enter the cytosol of cells, or be cross-presented by DCs *in vivo* [19, 44].

The studies herein establish DOPC:PI and DOPC:GM1 formulations as viable vaccine delivery formulations to promote antigen-specific immune responses. Our results indicate DOPC:PI and DOPC:GM1 liposomes are capable of stimulating cell-mediated immune responses in addition to antibody responses after vaccination. Furthermore, antibody responses generated after vaccination with DOPC:PI or DOPC:GM1 liposome formulations included IgG2a antibodies, which indicates Th1 type immunity that further promote cell-mediated responses. In comparison to ePE:CHEMS immunized mice, which are known to produce primarily high anti-OVA IgG1 titers (dominant Th2 immunity), DOPC:PI and DOPC:GM1 immunized mice had significantly increased IgG2a antibodies, further demonstrating that the glycolipid-containing liposome vaccine formulations examined promote Th1 immunity.

$CD8^+$ T cell tetramer staining and ELISPOT monitoring of IFN-γ producing cells show DOPC:PI and DOPC:GM1 liposomes generate similar numbers of antigen-specific $CD8^+$ T cells after prime-boost vaccination. Following boost injections, the T cell MHC

tetramer staining was more variable between subjects, which prevented statistically significant conclusions from being drawn. Tetramer staining post-boost immunization with DOPC:GM1 liposomes indicated two high responders and 3 low responders, with minimal variability within the two groups. However, the underlying reasoning as to why DOPC:GM1 immunizations produced high and low responses could not be related to the formulation of the liposomes or the administration of the immunizations, and therefore is presently unclear. It is worthwhile to note that liposomes containing GM1 have constantly produced more variable results *in vivo* and *in vitro*. ELISPOT analysis of IFN-γ producing cells was additionally performed as a separate measure of T cell activation. *In vivo,* $CD8^+$ T cells will secrete IFN-γ, a cytokine that promotes Th1 immunity and cell-mediated responses. The ELISPOT results align with $CD8^+$ T cell tetramer staining, indicating DOPC:PI and DOPC:GM1 formulations equally generate antigen-specific $CD8^+$ T cells.

PE:CHEMS liposomes encapsulating OVA and LLO have been previously shown to generate antigen-specific $CD8^+$ CTL responses [29, 39]. We report that pH sensitive, ePE:CHEMS liposomes show no advantage compared to DOPC:PI and DOPC:GM1 liposomes in stimulating $CD8^+$ T cell responses. This could be a result of DOPC:PI and DOPC:GM1 having increased antigen delivery to DCs, similar capability of intracellular delivery of antigen, or a combination of both characteristics both of which are further discussed below.

pH-sensitive PE:CHEMS liposomes containing LLO are remarkably more efficient at delivering macromolecules into the cytosol of cells compared to non-pH-sensitive

formulations [29, 45]. Therefore the ability of DOPC:PI and DOPC:GM1 liposomes to produce equal amounts of $CD8^+$ T cells as ePE:CHEMS liposomes is an interesting observation. Liposomes composed of purely ePC or DOPC lipids exist in a fluid membrane state and have been reported to leak liposomal contents within short periods of time in buffer or plasma [32, 33, 46]. While the addition of glycolipid to the DOPC liposome composition stabilized the liposome formulation at physiological pH [32, 47], these PC liposomes would lose membrane integrity in the acidic endocytic pathway [34]. Similarly, bilayer destabilization due to acidifying pH is how ePE:CHEMS liposomes release their encapsulated contents [31]. Liposomes composed of DOPC:PI or DOPC:GM1 have not previously been evaluated for their cytosolic delivery of antigen. However we have performed *in vitro* antigen presentation experiments that indicated DOPC:PI and DOPC:GM1 liposomes encapsulating LLO and OVA result in OVA presentation to $CD8^+$ T cells, while DOPC:Chol:PI and DOPC:Chol:GM1 containing LLO and OVA liposomes do not (data not shown). Previous studies that have reported PE:CHEMS enhances cytosolic delivery compared to non-pH-sensitive liposomes made this observation when comparing to DOPC:CHEMS non-pH-sensitive liposomes [29, 34]. In the DOPC:CHEMS liposome formulation, CHEMS still acts as a bilayer stabilizer especially at the high % of liposome composition used. Based on these studies and the *in vitro* antigen presentation studies mentioned, it is reasonable to assume that the addition of a stabilizer such as cholesterol or CHEMS impairs the ability of DOPC liposomes to deliver antigen into the cytosol. The addition of glycolipid to stabilize DOPC liposomes used here may have had a slight negative effect on ability for cytosolic

delivery, however the effect would presumably be less drastic than cholesterol or CHEMS addition to DOPC liposomes because glycolipid was present at 10mol% phospholipid (or 6.6% total lipid), whereas cholesterol or CHEMS typically comprises 30% or more of total lipid content. Therefore the DOPC:PI and DOPC:GM1 liposomes used in this report may facilitate lipid membrane disruption in the acidifying endosome, similar to pH-sensitive ePE:CHEMS formulation.

The hemolysin LLO component of these liposomal vaccine formulations may have also played a role in the immune responses monitored in this immunization study. The usage of LLO in liposome formulations was primarily to induce cytosolic delivery of antigen, however, LLO has also been realized to have adjuvant-like effects. The inherent ability of LLO to disrupt endosomal membrane bilayers is cause for alarm in infected cells and this causes a multitude of cellular responses after exposure to LLO [48, 49]. Cell exposure to LLO-expressing bacteria or exogenously added LLO, results in numerous cell-signaling events including those that trigger T cell and DC death [50, 51], production of pro-inflammatory cytokines IL-1 and IFN-γ by DCs and macrophages [52-54], and phosphorylation of MAP kinases in HeLa cells, which plays roles in cytokine responses and cell proliferation [49, 55]. LLO has been shown to have an adjuvant effect, stimulating pro-inflammatory cytokine production from APCs and resulting in decreased tumor volume, when either fused to or co-injected with HPV-16 E7 protein [56]. Therefore it is probable we also observed an adjuvant effect due to the LLO protein component in the liposomal vaccine formulations examined.

We have previously described that DOPC liposomes containing PI and GM1 are internalized by DCs *in vivo*, with initially enhanced DC uptake using PI lipid, and lower yet extended uptake of liposomes bearing GM1 lipid. Although we have not directly compared the *in vivo* DC uptake of these glycolipid formulations with ePE:CHEMS liposomes, the latter show predominantly high uptake by macrophages with lesser ability to enhance DC uptake *in vitro* (Chapter 2, Figure 2.5). Therefore, increased antigen delivery to DCs using glycolipid-containing liposome formulations may have stimulated enhanced $CD8^+$ T cell responses based on DCs' unique abilities to cross present antigen and activate naïve T cells. Interestingly, Collins *et al*. has described a non-pH-sensitive liposome composed of DOPC and dioleoylphosphatidylserine (DOPS), DOPS being another anionic lipid, which generated MHC Class I restricted T cell responses to OVA delivery *in vivo* [57]. In addition, these authors did not see this response *in vitro* when using macrophages. The MHC Class I OVA response observed was inferred to be a result of DC cross-presentation *in vivo*. These results additionally demonstrate how it may be possible for DOPC:PI and DOPC:GM1 liposomes to generate antigen-specific $CD8^+$ T cells as a result of internalization by DCs.

In relation to our *in vivo* uptake study previously discussed in Chapter 2, it appears that the immunological outcomes measured here are not drastically affected by the differences in glycolipid-liposome uptake we observed by lymph node cells. The initially enhanced macrophage and DC uptake we saw of PI- liposomes nor the more extended macrophage and DC uptake observed of GM1-liposomes resulted in significantly enhanced antigen-specific $CD8^+$ T cells generated from prime-boost

vaccinations. The only advantage of immunization with DOPC:PI liposomes was the increased anti-OVA IgG1 and IgG2a antibody titers.

CONCLUSION

We clearly show that soluble OVA co-encapsulated with LLO inside DOPC:PI or DOPC:GM1 liposomes are capable of producing $CD8^+$ T cells, a measure of cell-mediated immunity. DOPC:PI and DOPC:GM1 liposomes generated similar $CD8^+$ T cell frequencies and T cell functionality. DOPC:PI and DOPC:GM1 liposome formulations stimulate B cell production of IgG2a, a sign of Th1 immunity induced by vaccination. In addition, the glycolipid vaccine formulations did not negatively effect the production of anti-OVA IgG1. These glycolipid vaccine carriers show promise even when compared to ePE:CHEMS liposomes, a liposome formulation designed to deliver antigen to the cytosol for increased $CD8^+$ T cell responses.

Liposome composition	Mole ratio	Size (nm)	PDI	Zeta potential (mV)
ePE:CHEMS	2:1	164.7	0.235	-21.1 ± -1.614
DOPC:PI	9:1	162.8	0.281	-10.4 ± -.8001
DOPC:GM1	9:1	170.8	0.296	-10.5 ± -.8084

Table 3.1 Summary of representative size, PDI and zeta potential of liposomes
The particle size, poly dispersity index (PDI) and zeta potential of liposomes were determined after sonication. Size (diameter) was determined by dynamic light scattering and zeta potential was determined by electrophoresis and laser Doppler velocimetry. PDI is a dimensionless measure of the width of particle size distribution.

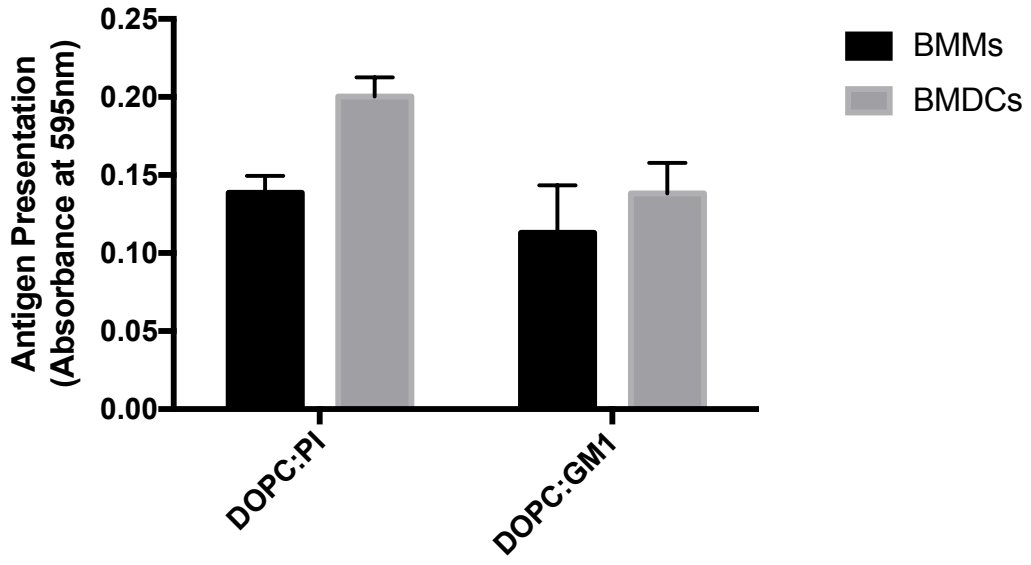

Figure 3.1 DOPC:PI and DOPC:GM1 liposomes encapsulating OVA and LLO can deliver antigen to the cytosol of BMMs and BMDCs

BMMs and BMDCs were plated in equal numbers one day prior to the addition of liposomes. DOPC:PI or DOPC:GM1 liposomes encapsulating OVA and LLO were administed to cells while matching the OVA dose at 6 μg/well. Cells were incubated with liposomes for 2 hours. After which, liposomes were washed off and cells were further incubated for 3 hours and the fixed. B3Z cells, a T cell hybridoma recognizing MHC I-SIINFEKL complexes in the context of $H2k^b$, were added to fixed cells for 15 hours. The antigen presentation of BMMs and BMDCs to B3Z T cells was monitored by the conversion of CPRG substrate to chlorophenol red in the activated B3Z cells and measured by absorbance at 595nm. Data shown represent mean ± SEM, n=3. Two-way ANOVA detected no significant differences in antigen presentation ($p=0.06$).

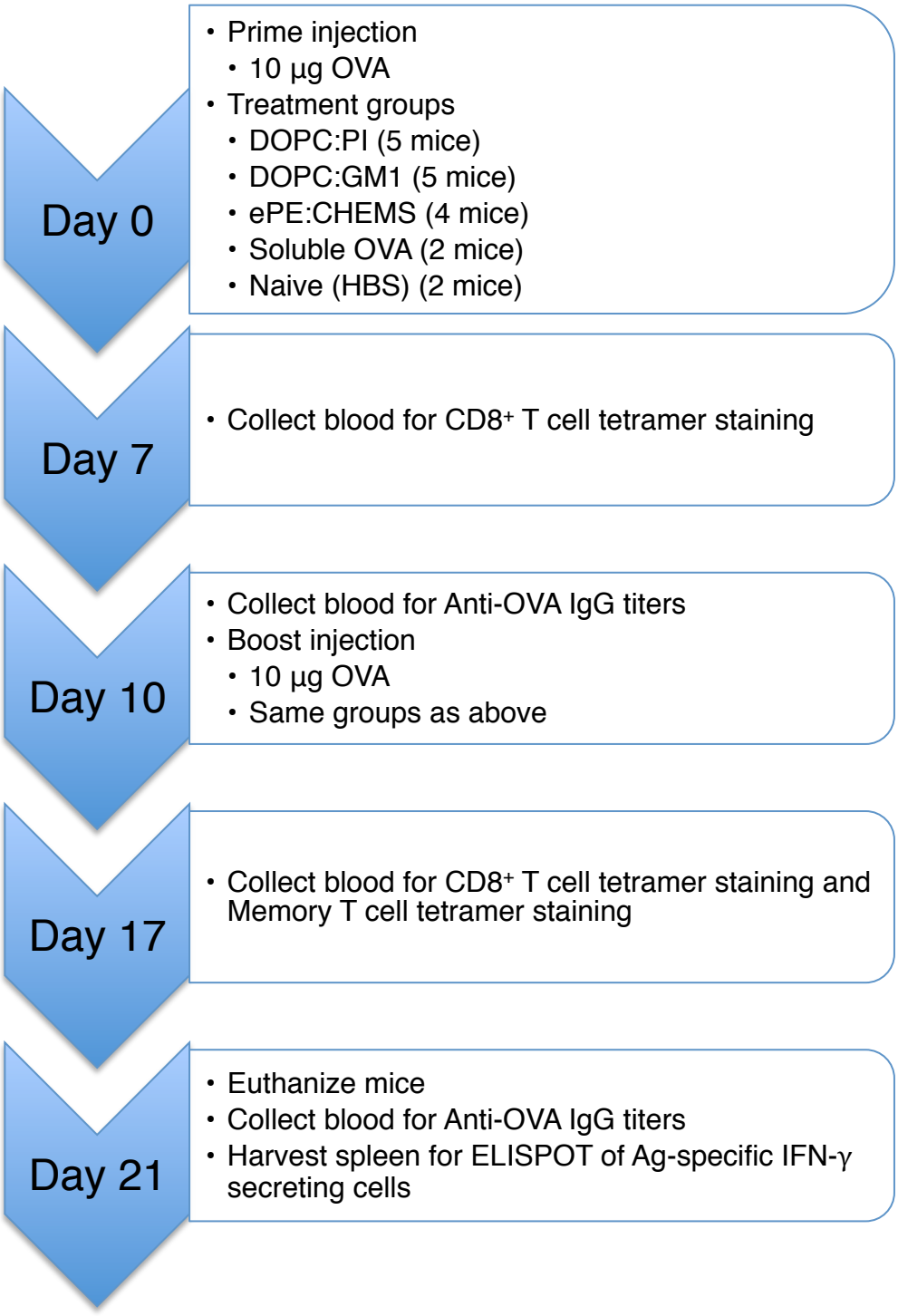

Figure 3.2 Flowchart for immunization study

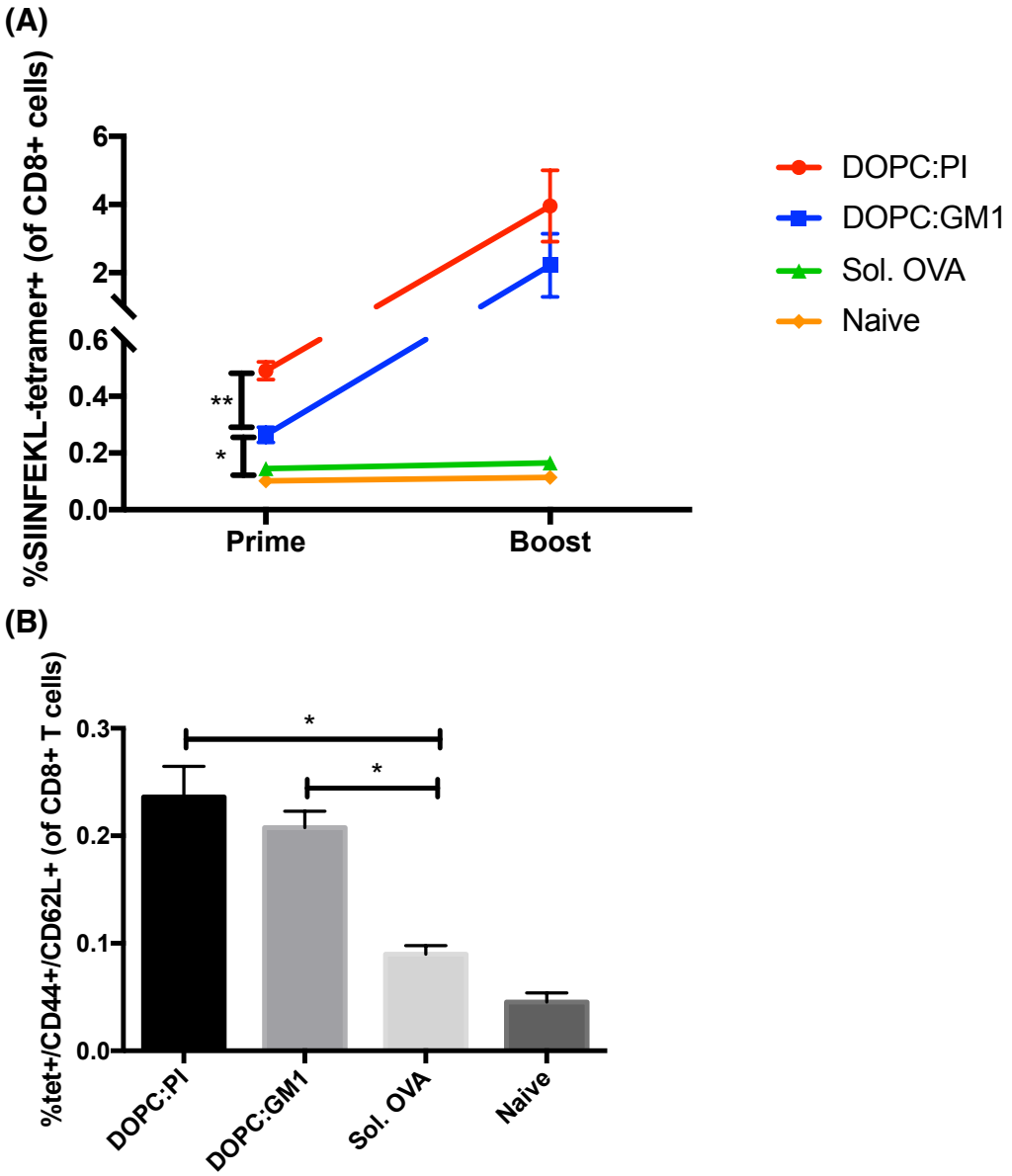

Figure 3.3 Glycolipid liposome formulations generate OVA-specific CD8$^+$ T cells and CD44$^+$/CD62L$^+$ memory CD8$^+$ T cells in immunized mice

Female C57BL/6 mice were injected subcutaneously with 10µg OVA in liposome or soluble formulations on Days 0 and 10. LLO in the liposome formulations ranged from 1.16-2.35µg (prime) and 1.89-2.89µg (boost). The percentage of antigen-specific CD8$^+$ T cells was determined by flow cytometry analysis seven days post injections. PBMCs in the blood were evaluated for OVA-specific CD8$^+$ T cells by staining with fluorescent SIINFEKL-MHC tetramers. (A) Percentage of OVA-specific CD8$^+$ T cells after prime and boost immunizations. Data shown represent mean ± SEM, n=2-5 mice/group (B) Percentage of OVA-specific CD8$^+$ memory T cells after boost immunization. Data shown represent mean ± SEM, n=2-5 mice/group (*p<0.05, **p<0.01 analyzed by one-way ANOVA and Tukey's post-hoc test).

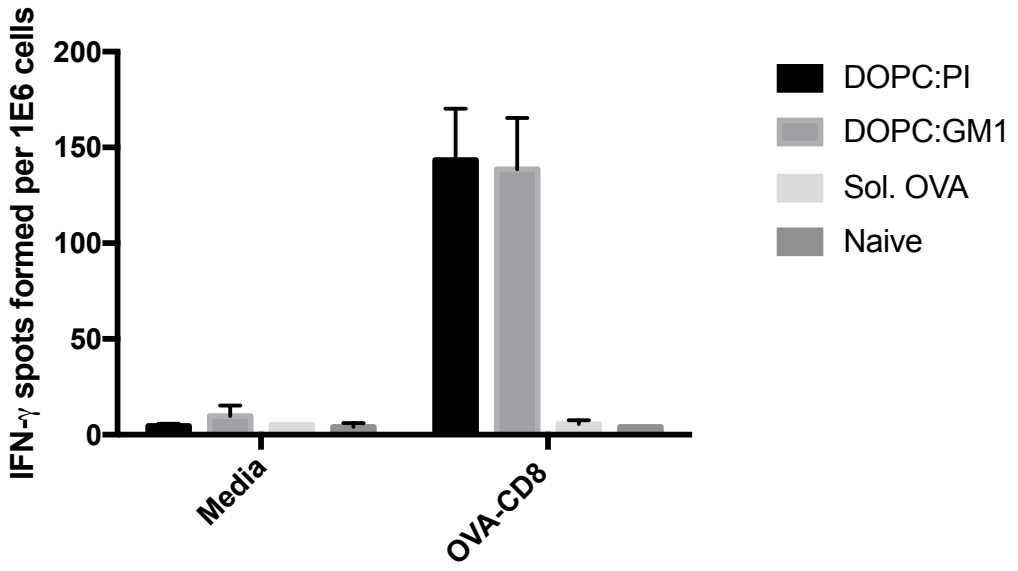

Figure 3.4 Immunization with DOPC:PI or DOPC:GM1 liposomes produce similar numbers of OVA-specific IFN-γ secreting cells

Female C57BL/6 mice were injected subcutaneously with 10μg OVA in liposome or soluble formulations on Days 0 and 10. LLO in the liposome formulations ranged from 1.16-2.35μg (prime) and 1.89-2.89μg (boost). Day 21, mice were sacrificed and splenocytes analyzed for OVA-specific IFN-γ producing cells via ELISPOT. Splenocytes were stimulated with 2.5 μg/ml SIINFEKL (OVA-CD8 peptide) or cell media only for 18 hours. As a positive control, cells were also incubated with Con A, a non-specific stimulant, for 18 hours. There was no statistical difference between the numbers of spots formed when cells were stimulated with ConA. Data shown represent the mean number of IFN-γ spot forming units (SFU) ± SEM, n=2-5 mice/group. Analyzed by two-way ANOVA and Tukey's post-hoc test.

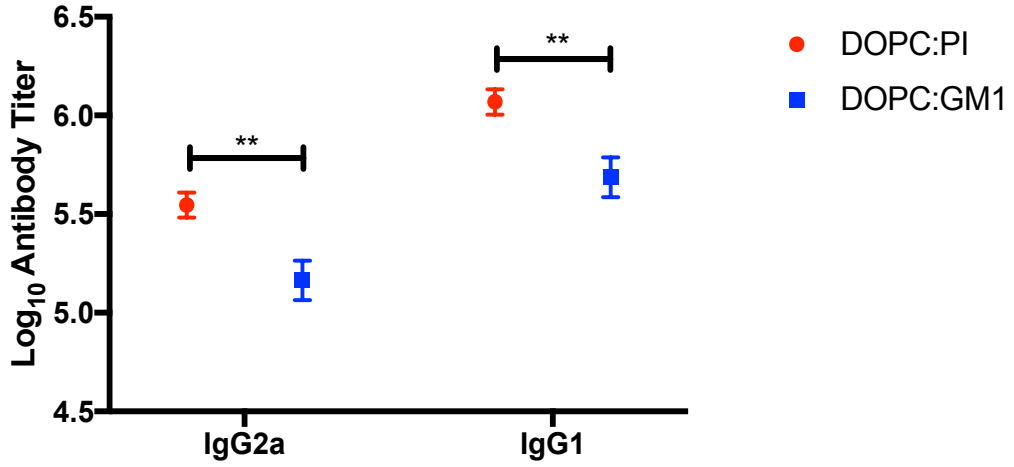

Figure 3.5 DOPC:PI liposomes improve Anti-OVA IgG1 and IgG2a titers
Female C57BL/6 mice were injected subcutaneously with 10µg OVA in liposome or soluble formulations on Days 0 and 10. LLO in the liposome formulations ranged from 1.16-2.35µg (prime) and 1.89-2.89µg (boost). Mice were sacrificed on Day 21 to analyze anti-OVA IgG titers. Sera collected from the blood of immunized mice were analyzed by ELISA for OVA-specific IgG1 and IgG2a antibodies. Mice immunized with soluble OVA did not read above background at the lowest dilution tested and the data are not displayed. Data shown represent the log transformation of antibody titers ± SEM, n=5 mice/group. (**$p<0.01$ analyzed by one-way ANOVA and Tukey's post-hoc test).

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CHAPTER 4

Significance and Future Directions

SIGNIFICANCE

This thesis presents the first known study to investigate the uptake of liposomes containing either phosphatidylinositol (PI) or monosialotetrahexyosylganglioside (GM1) glycolipid by dendritic cells, and further compare this uptake to that by macrophages. The two liposome formulations considered in this thesis have previously been realized to reduce liposome uptake by macrophages in the mononuclear phagocyte system (MPS) after intravenous (i.v.) injection of the liposome formulation [1, 2]. The only recognized purpose of these liposome formulations was delivery of small molecules to peripheral tissues (i.e. tumor) after i.v. injection [3, 4]. The uptake of these liposome formulations after subcutaneous administration had not been previously examined. This thesis also serves as the first known body of work that describes PI- or GM1-containing long-circulating liposomes as vaccine delivery vehicles, specifically after subcutaneous administration.

Macrophage clearance is generally accepted as the main route of liposomal clearance from the body. Even long-circulating liposomes, such as PI-liposomes GM1-liposomes and PEG-coated liposomes will eventually be cleared by macrophages in mice [5-7]. This research was stimulated by the notion that the decreased initial

macrophage uptake described for PI or GM1-containing liposome formulations may facilitate increased interactions with and uptake of these liposomes by another major phagocytic cell type, dendritic cells (DCs). The use of a DC-targeting ligand or DC-specific antibody conjugated to the liposomal membrane is typically required to augment liposomal uptake by DCs. However, we have shown that incorporating PI glycolipid in the liposome composition can promote liposomal uptake by DCs. We also have demonstrated that liposomes containing 10mol% PI have enhanced uptake by DCs in comparison to liposomes containing 10mol% GM1, while macrophage uptake of these two liposome formulations were similar. These are important observations, which show that complex targeting approaches may not be necessary when seeking improved DC uptake of a lipid nanoparticle formulation. With trends in vaccine research and development delving into more complex approaches in an effort to stimulate greater immune responses, this research suggests that complexity may not always be warranted. It may be possible to stimulate necessary immune responses just by directing nanoparticles to lymph nodes, areas rich in immune cells.

Previous uses of glycolipid- containing long-circulating liposome formulations focused on small molecule drug delivery to peripheral tissues after intravenous administration [4, 8]. Intravenous injections are not an ideal route of administration for vaccinations, therefore subcutaneous injection was the route of administration chosen in *in vivo* studies. The liposome formulations of primary interest in this thesis had not previously been administered subcutaneously and thus, the results presented are the first to describe their uptake behaviors after subcutaneous administration. We show that

subcutaneous administration of liposomes could result in uptake by draining lymph node (LN) cells, which provides the generation of significant $CD8^+$ T cell responses. These PI- or GM1- glycolipid liposome formulations were initially of interest as vaccine carriers because of their ability to deter initial macrophage uptake and potentially increase liposomal antigen delivery to DCs. The targeting of DCs may promote enhanced immune responses from the delivered protein antigen [9-12]. We predicted that the higher DC uptake observed with PI-containing liposomes would augment cell-mediated immune responses when this liposome formulation was utilized as a vaccine carrier. However, it appears that this enhanced DC uptake had little effect on the cell-mediated immune responses generated. Still, both PI-containing liposomes and GM1-containing liposomes were considered effective vaccine carriers for stimulating cell-mediated immune responses from the protein antigen. This is true even when the glycolipid liposome formulations were compared to ePE:CHEMS liposomes, a liposome formulation designed to deliver encapsulated material to the cytosol for increased $CD8^+$ T cell responses.

We have also demonstrated that glycolipid-containing liposomes formulated without cholesterol and encapsulating the hemolysin, listeriolysin-O (LLO), can be used to deliver the protein antigen ovalbumin (OVA) into the cytosol of macrophage and dendritic cells. Liposomes are most typically formulated with cholesterol in order to stabilize the lipid membrane. In some cases, however, stabilizing the liposome may thwart liposomal delivery of encapsulated material to cells. The studies presented in this

thesis exemplify the idea that simple manipulations of liposome compositions can produce significant effects on liposome uptake and liposomal delivery.

FUTURE DIRECTIONS

The glycolipid liposome formulations presented have shown promise as vaccine delivery systems that generate cell-mediated immune responses from protein antigen delivery. Further development of these liposome formulations as vaccine carriers should include the use of an adjuvant for enhanced cell-mediated immune responses [13]. The molecular adjuvant consisting of CpG oligodeoxyribonucleotides (ODNs) contains unmethylated CpG sequences that mimic bacterial DNA. These sequences are recognized by TLR9, a pattern-recognition receptor (PRR) located in endosomes [14]. The location of TLR9 is fitting for liposomal delivery of encapsulated cargo into the endosome. CpG-ODNs have been characterized as an adjuvant in multicomponent liposomal formulations previously and were shown to induce robust Th1 immunity (promoted by $CD4^+$ Th1 T cells) and $CD8^+$ cell-mediated immune responses [15]. Alternatively, another experimental adjuvant, poly(I:C), a synthetic double stand RNA resembling viral material, could be used to skew Th1 responses. Poly(I:C) is recognized by multiple PRRs, including TLR3 in the endosome, and RIG-I and MDA-5 in the cytosol of cells. Recognition of poly(I:C) in the endosome or cytosol results in the production of type I interferons promoting cellular immunity [16, 17]. The multiple receptors of poly I:C make it an attractive option for addition to a multicomponent vaccine delivery system. TLR3 recognition in the endosome during liposome deformation, as well as RIG-I and

MDA-5 recognition in the cytosol after LLO-mediated cytosolic delivery may provide an enhanced or possibly synergistic adjuvant effect.

In addition, future immunization studies with glycolipid containing liposomes should investigate immune responses from $CD4^+$ T cells. The immunization studies presented focused heavily on the induction of $CD8^+$ T cell responses. However, $CD4^+$ T cells play an important role in directing and promoting cell-mediated immunity. The ELISPOT assay and $CD4^+$ tetramer staining could be implemented after immunizations to assess $CD4^+$ T cell responses. Moreover, IL-12 and IL-5 ELISA experiments would give indications of the cytokine environment developed from vaccination. The presence of cytokines such as IL-12 and IFN-γ are critical for maintaining cellular immune responses, while IL-5 indicates Th2 immune responses (promoted by $CD4^+$ Th2 cells) that stimulates humoral immunity [18].

The analysis of these glycolipid-containing liposomes as vaccines carriers may also benefit from more functional assays to describe the effectiveness of vaccination. The ELISPOT assay can be used as a functional assay, however, there are a few drawbacks. For example, ELISPOT assays to quantify $CD8^+$ IFN-γ producing cells may provide misleading results since certain non-cytotoxic cells can secrete IFN-γ whereas $CD8^+$ cytotoxic T lymphocytes (CTLs) with proven lytic activity do not always secrete IFN-γ. Moreover, there is a poor correlation between the IFN-γ ELISPOT assay and clinically relevant immune responses in humans [19]. An *in vivo* CTL assay would be additionally beneficial for analyzing the quality of the CTL response.

Further investigations may also assess the role of the Class B scavenger

receptors, CD36 and SR-BI in the recognition and uptake of PI-liposomes and GM1-liposomes. CD36 and SR-BI have been previously described in the uptake of anionic liposomes containing PS or PI lipids [20, 21]. The interaction(s) of these receptors with GM1 lipid or GM1-liposomes to our knowledge has not been described. Uptake experiments similar to those described in Chapter 2, with PI- or GM1- fluorescent liposome formulations, could be performed after pre-incubation of macrophage and dendritic cell types with anti-SR-BI or anti-CD36. Importantly, these studies should be performed in macrophage and DC types from different sources (e.g. cell lines, primary cells, *ex vivo* naïve cells) as expression of the scavenger receptors may differ based on the cell source/type. The results from these studies would indicate if CD36 and/or SR-BI scavenger receptors are involved in the recognition of PI or GM1 lipids and the cell-mediated endocytosis of liposomes. Since class B scavenger receptors can influence cell signaling pathways and additionally are involved in innate immunity that leads to pro-inflammatory responses [22-24], the results of such cell pathways after liposome treatment/uptake could also be analyzed to describe the impact of PI-liposome and GM1-liposome uptake on cell function and signaling.

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APPENDIX A

Competition Uptake Studies with Glycolipid-Containing Liposome Formulations

SUMMARY

The glycolipid liposome formulations previously studied were analyzed for their uptake after cells had been pre-treated to non-fluorescent (competing) liposomes. These studies were performed to distinguish which liposome formulations may compete for binding and uptake sites on cells. Liposomes containing 10mol% phosphatidylserine (PS) (PS-liposomes; PS-lip) negatively affected subsequent uptake of liposomes containing 10mol% phosphatidylinositol (PI) (PI-liposomes; PI-lip) in both JAWSII dendritic cells and J774 macrophages, but had no effect of the uptake of liposomes containing 10mol% monosialotetrahexosylganglioside (GM1) (GM1-liposomes; GM1-lip). A separate observation was pre-treatment of cells with PI-lip negatively affected subsequent uptake of PI-lip in JAWSII dendritic cells (DCs), but this was not the case in J774 macrophage cells. PI-lip uptake was not affected by pre-treatment of cells with GM1-lip. Furthermore, GM1-lip uptake was unaffected by pre-treatment with GM1-lip or PI-lip. These results indicate that PS-lip and PI-lip compete for binding sites in JAWSII DCs and J774 macrophage cells. PI-lip and GM1-lip appear to have different recognition sites on JAWSII DCs and J774 macrophages.

INTRODUCTION

Endocytosis is a means of cell uptake that takes the forms of phagocytosis, receptor-mediated endocytosis and pinocytosis. Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), are proficient in endocytosis. Two primary functions of APCs are to endocytose material and then present it to T cells as part of the immune response or tolerance. Macrophages are particularly adept at internalizing many forms of materials including soluble proteins, whole cells, and liposomes [1, 2]. DCs are less active than macrophages in endocytosis and are more tailored for the antigen presentation role. However, DCs are considered to be highly active in pinocytosis [3, 4]. The ability of DCs to capture material is also dependent on the cells state of maturity. Immature dendritic cells (iDCs) are highly efficient at endocytosis while mature DCs (mDCs) down regulate this activity [5, 6].

The primary uptake mechanism of liposomes is endocytosis [7]. More specifically, the liposomes in the presented studies are presumed to undergo receptor-mediated endocytosis [7, 8]. The uptake of PI-liposomes, GM1-liposomes and PS-liposomes are dramatically different *in vitro* and in different cell types, which would presumably be a result of receptor-mediated uptake by recognition of specific lipid head groups, rather than a non-specific method of uptake [8].

As previously discussed, PI-liposome show distinct uptake behavior in macrophages and DCs. Where the presence of only 10mol% PI lipid in a liposome composition can promote liposomal uptake by JAWSII DCs, J774 macrophages required 50mol% PI to reach comparably high uptake levels of PI-liposomes. The

increased recognition and uptake of PI-liposomes by JAWSII DCs was hypothesized to be a result of some cell-surface receptor or other binding site that recognized PI lipid. The Class B scavenger receptors SR-BI and CD36 have been indicated in the uptake of anionic lipids such as PI and PS [9, 10], however there is no dedicated PI, PS, or GM1 receptor that has been described to date.

MATERIALS AND METHODS

Liposome preparation

Cholesterol (Chol) was obtained from Calbiochem (La Jolla, California). All non-fluorescent lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama). Oregon Green® 488 1,2-Dihexadecanoyl-*sn*-Glycero-3 Phosphoethanolamine (Oregon Green 488-DHPE) was obtained through Life Technologies (Grand Island, New York). Liposomes were prepared at a 2:1 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Δ9-cis) (DOPC) to Chol. In uptake experiments, Oregon Green 488-DHPE lipid was incorporated at 1mol% of the total phospholipid. L-α-phosphatidylinositol (Soy) (PI), monosialotetrahexosylganglioside (ovine brain) (GM1), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE) or L-α-phosphatidylserine (porcine brain) (PS) were incorporated at indicated mol% of the total phospholipid. Lipid mixtures were dried into a lipid film using a Buchi Rotavapor R-200 rotary evaporator. Lipids were dried completely then rehydrated with isotonic (290 ± 10 mmol/kg osmolality) HEPES-buffered saline containing EDTA (HBSE) (10mM HEPES, 140mM NaCl, 1mM EDTA, pH 7.4). Hydrated

lipid films were vortexed, subjected to 5 freeze/thaw cycles and extruded using double-stacked polycarbonate 100 nm diameter filters (GE). The size of the liposomes and zeta potential was determined using a Malvern ZS90 ZEN 3600 zetasizer. Phospholipid content of liposomes was quantified using Bartlett's phosphate assay [11]. Determined phospholipid concentrations signified the concentration of liposomes and were used in the dosing calculations. GM1 does not have a phosphate group and thus the phospholipid assay concentrations determined do not include GM1 content of the liposomes; dosing calculations were adjusted to account for this. Prepared liposome samples were stored at 4°C and used within 10 days of preparation

Cell culture

All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. All cells were maintained in in a humidified incubator at 37°C and 5% CO_2. J774A.1 mouse macrophages (ATCC TIB-67) and JAWSII mouse dendritic cells (ATCC CRL-11904) were purchased from ATCC (Manassas, Virginia). J774 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS (HI-FBS) and 100µg/mL streptomycin, 100U/mL penicillin. JAWSII cells were maintained in alpha minimum essential medium with ribonucleosides and deoxyribonucleosides supplemented with 20% HI-FBS, 100 µg/mL streptomycin, 100U/mL penicillin, 2mM L-glutamine and 5ng/ml murine GM-CSF (PeproTech, Inc. Rocky Hill, New Jersey).

In vitro cell uptake

Equal counts of cells suspended in serum-free DMEM were aliquoted into Eppendorf tubes. Increasing concentrations of the non-fluorescent, competing liposomes (100μM, 250μM, 500μM, or 1mM [phospholipid]) were incubated with cell samples for 30 minutes at 4°C, unless otherwise noted. After treatment, any non-cell bound liposomes were removed by centrifugation and three subsequent cell washes in serum-free DMEM. Next, OregonGreen488 fluorescently labeled liposomes were added (100μM final [phospholipid]) to cell samples suspended in serum-free DMEM for 1 hour at 37°C. After treatment any non-cell bound liposomes were removed by centrifugation and three subsequent cell washes in phosphate buffered saline (PBS) pH 7.4. Cell fluorescence was obtained using a BD FACSCalibur flow cytometer (San Jose, CA). Cell Quest software was used to plot fluorescence values and obtain the mean fluorescence intensity (MFI) of samples.

RESULTS AND DISCUSSION

To further characterize the uptake of the glycolipid-containing liposome formulations a series of competition experiments were performed. Cells were pre-treated with a liposome formulation (competitor) for a short amount of time before being exposed to secondary fluorescently labeled liposome formulation whose uptake was of interest. During pre-treatment, in the case that liposomes bind to a cell-surface site or receptor, those cell-bound liposomes would then be endocytosed by the cell. This would leave less of these binding sites or receptors on the cell surface, which would affect the

uptake of subsequent liposomes added to the cell sample if the two liposome formulations examined were to share these sites for uptake (i.e., competition). We also studied the impact of pre-treatment concentrations on subsequent cell liposomal uptake of fluorescent liposomes. The highest competition ratio studied was 10:1; with 10 times more non-fluorescent liposomes pre-incubated with cells than subsequent fluorescent liposome concentration incubated with the cells.

We first investigated the possibility of PS-lip and PI-lip being recognized by the same cell-surface receptor or cell-surface component. Cell samples were pre-treated with non-fluorescent PS-liposomes before being washed and treated with fluorescent PI-liposomes. In the presence of increasing concentrations of PS-liposomes, PI-liposomes showed reduced uptake by JAWSII DCs and J774 macrophage cells (Figure A.1A). This suggests that PS-lip was binding some cell-surface site(s) on macrophages and DCs, which also facilitates uptake of PI-lip. When the same competition experiment was performed with non-fluorescent PS-lip and fluorescent GM1-lip there were no obvious negative effects on GM1-uptake. This was observed in both J774 macrophages and JAWSII DCs. Previously, even at 100μM PS-lip treatment concentration, JAWSII DCs and J774 macrophages exhibited similarly high uptake of PS-lip; therefore the increased concentrations used in this competition experiment (500μM or 1mM) most likely resulted in the internalization of a large amount of binding sites and cell membrane due to PS-lip uptake. The observation that PS-lip pre-treatment of cells did not affect the uptake of GM1-lip in the same cells suggests these liposome formulations do not compete for cell-surface binding sites (Figure A.1B).

In a separate set of similar competition experiments, we identified that GM1-lip and PI-lip did not negatively affect the uptake of one another mediated by either JAWSII DCs or J774 macrophage cells (Figure A.2A and A.2B). The uptake of interest was unaffected even when a high, 1mM concentration, of competing liposome was pre-incubated with cells. This result indicates that PI-lip and GM1-lip bind different cell-surface binding sites for uptake or are recognized and internalized by different cell-surface receptors.

In continuing with the competition studies, PI-liposomes were observed to compete with each other for uptake by JAWSII DCs but not by J774 macrophage cells (Figure A.3A). The same result was observed whether pre-treatment was carried out at 4°C (permits liposome binding only) or at 37°C (permits liposome internalization) (37°C pre-treatment condition data not shown). The decreased DC uptake of fluorescent PI-liposomes of interest could to be a result of previously described high DC uptake of PI-liposomes. The increased binding of PI-lip during pre-treatment likely diminished the binding sites available on DCs for following PI-liposomes. This is presumably not a problem for PI-lip uptake by J774 cells. However, this cannot be explained by low uptake of PI-lip during pre-treatment, because while PI-lip does enhance DC uptake of liposomes, the overall uptake of PI-lip is similar between macrophages and DCs due to macrophages' enhanced capacity for uptake. The concept of quicker receptor 'recycling' to the surface of macrophages for enhanced fluorescent PI-lip uptake is also not a viable explanation since pre-treatment was performed at 4°C, a temperature at which only liposome binding occurs. An increased amount of binding sites for PI-lip on

macrophages may explain this result observed in J774 macrophages. This explanation seems to directly conflict previous *in vitro* uptake results, which indicate PI-lip do not promote liposomal uptake by macrophages. However, it is possible that macrophages possess more binding sites, albeit with less specificity for PI-lip.

GM1-liposomes do not compete with each other for uptake in either cell type (Figure A.3B). This result was also observed when pre-treatment was carried out at 4°C or 37°C (37°C pre-treatment condition data not shown). It is interesting that GM1-liposome pre-treatment presented no observable effect on uptake of liposomes in any of the competition experiments performed (Figure A.2A and A.3B). This may be a result of the inherent long-circulating property of GM1-lip in both DC and macrophages cell types. GM1-containing liposomes exhibit consistent, low uptake over a large dose range *in vivo* [12]. The increasing concentrations of GM1-lip introduced in these studies may have had little change in the amount of liposomes that were internalized by cells, therefore presenting no challenge to the secondary liposome formulation's uptake.

CONCLUSION

JAWSII DCs and J774 macrophages appear to have different sites of recognition for PI-liposomes and GM1-liposomes. The uptake of the glycolipid formulations was not affected by competition with one another. PI-lip does compete with PS-lip for uptake in JAWSII DCs and J774 macrophages cells. PS-lip uptake may diminish binding sites on cells that are necessary for PI-lip uptake. Interestingly, GM1-lip uptake was unaffected by pre-treatment of cells with PS-lip, PI-lip or GM1-lip, even at the highest competition

ratios examined. Generally low GM1-lip uptake by cells may allow GM1-lip uptake to be unaltered even if there are less binding sites present on cells following liposome pre-treatment. Since PI-liposomes seemingly compete with each other for uptake by JAWSII DCs but not in J774 macrophage cells, there appears to be a more specific recognition and uptake mechanism for PI-lip on DCs.

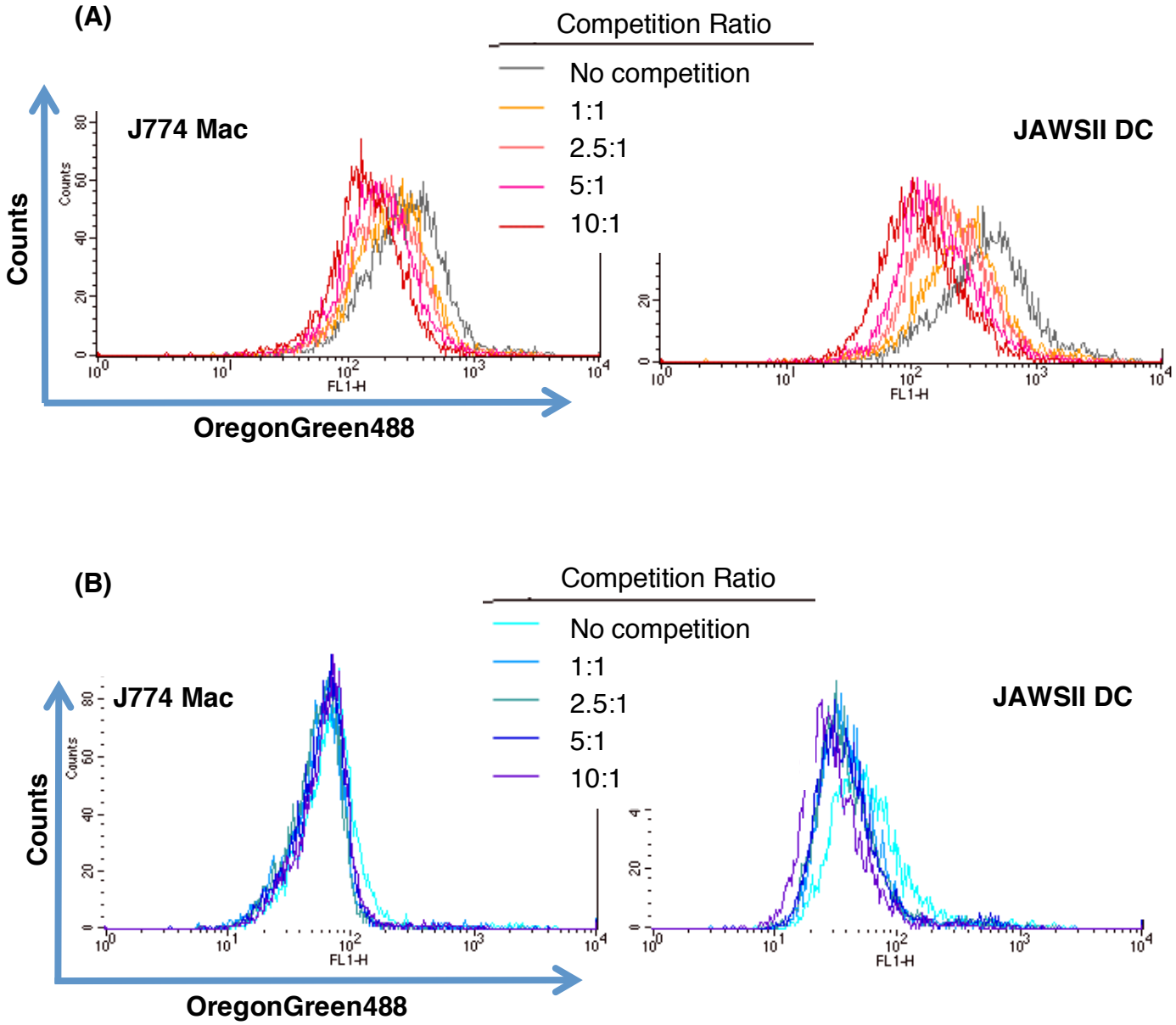

Figure A.1 PI-lip compete with PS-lip for uptake by macrophages and DCs
Cells were pre-treated for 30 min at 4°C with the non-fluorescent competing liposome formulation (PS-liposomes) at increasing concentrations from 100 μM (1:1 competition) to 1 mM (10:1 competition). Cells were washed to remove non-associated PS-liposomes. Then fluorescently labeled liposomes of interest were incubated with cells for 1 hour at 37°C. The liposome uptake of interest was monitored by OregonGreen488-DHPE fluorescence of cell samples. The x-axis represents fluorescence of cells (A) Competition of PS-liposomes with (A) PI-liposomes or (B) GM1-liposomes in J774 macrophages and JAWSII DCs.

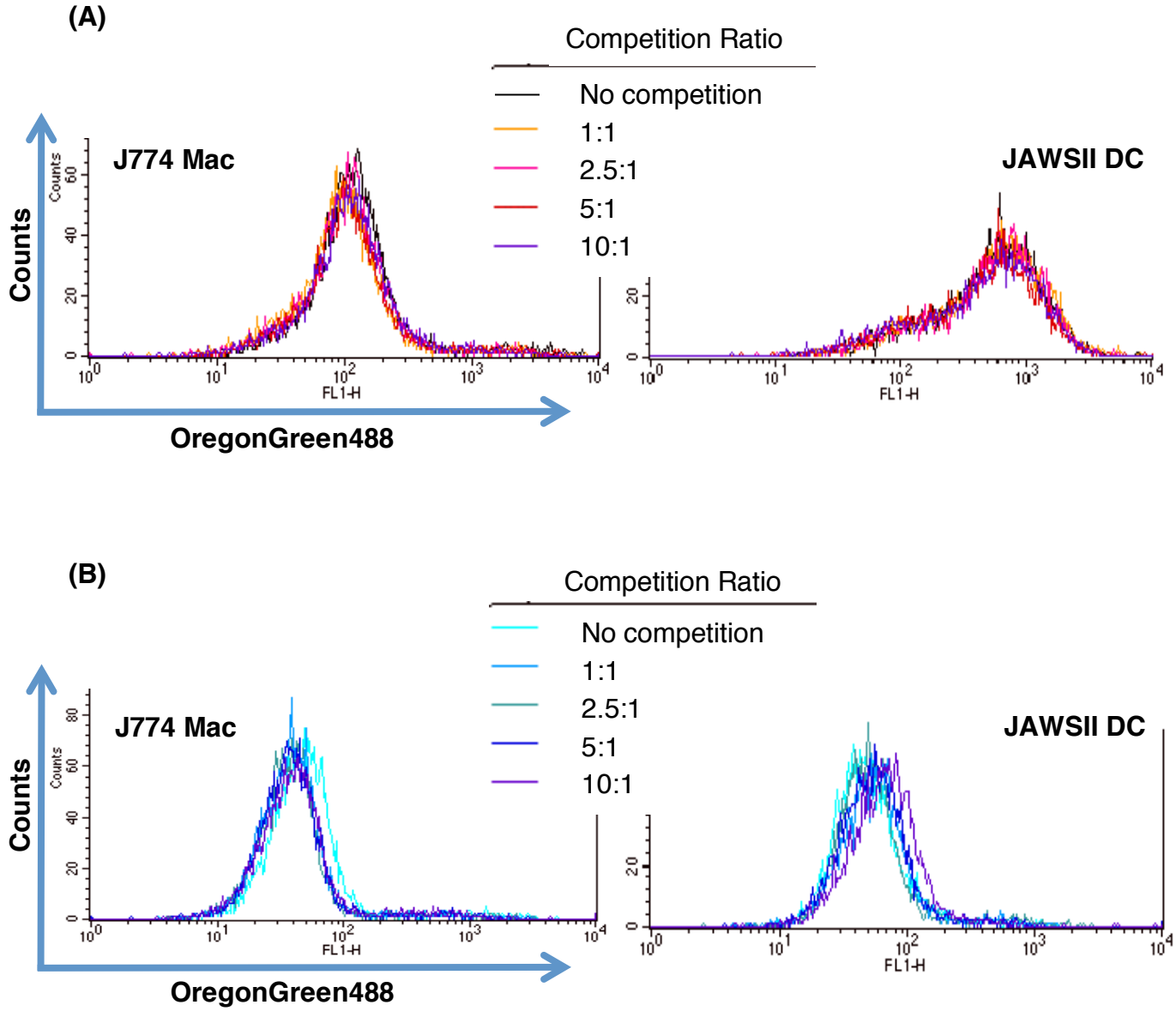

Figure A.2 PI-lip and GM1-lip do not compete for binding sites on macrophages and DCs

Cells were pre-treated for 30 min at 4°C with the non-fluorescent competing liposome formulation at increasing concentrations from 100 µM (1:1 competition) to 1 mM (10:1 competition). Cells were washed to remove non-associated liposomes. Then fluorescently labeled liposomes of interest were incubated with cells for 1 hour at 37°C. The liposome uptake of interest was monitored by OregonGreen488-DHPE fluorescence of cell samples. The x-axis represents fluorescence of cells (A) GM1-lip (pre-treatment) and fluorescent PI-lip uptake (B) PI-lip (pre-treatment) and fluorescent GM1-lip uptake in J774 macrophages and JAWSII DCs.

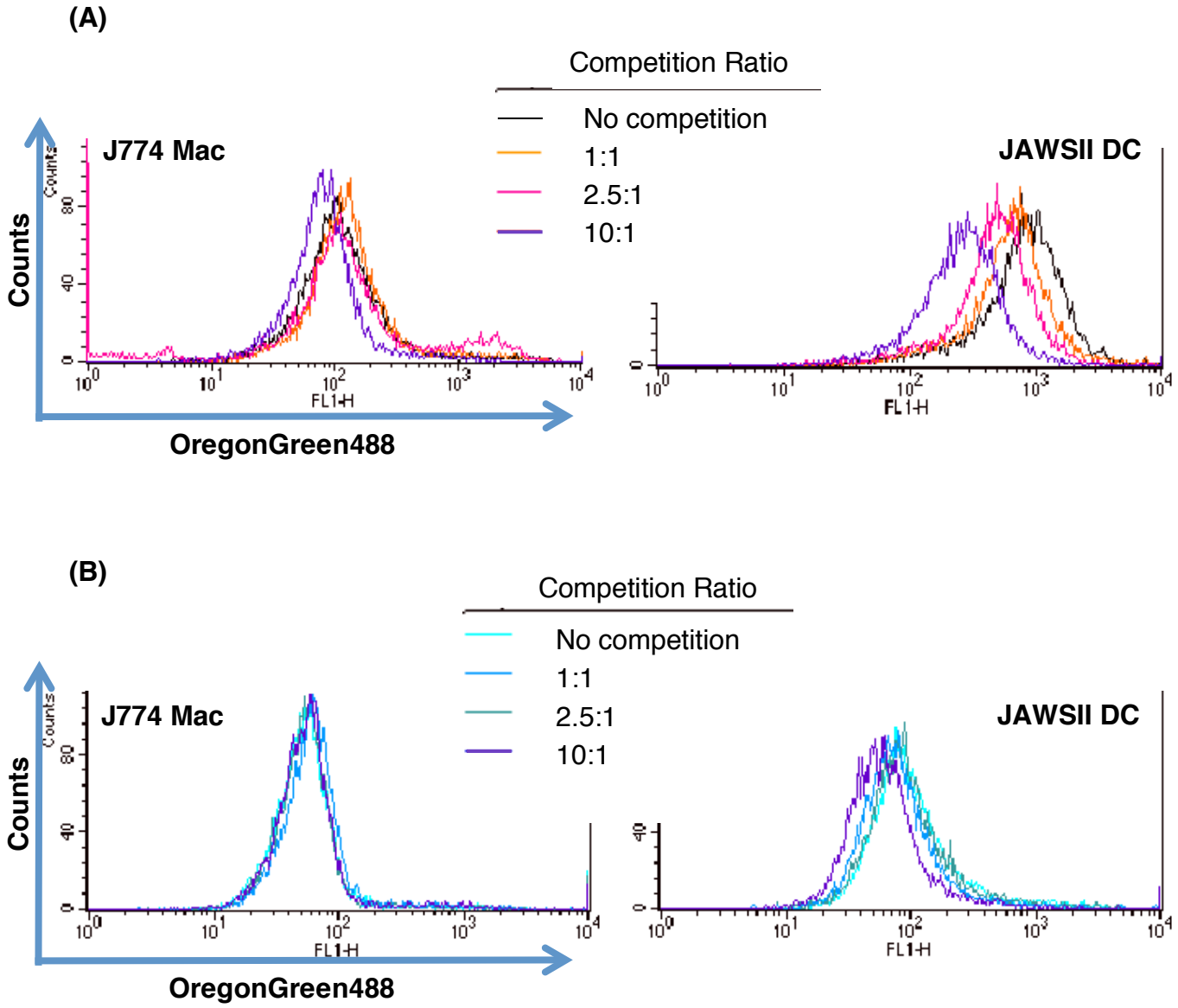

Figure A.3 Pre-treatment of JAWSII DCs with PI-lip inhibits subsequent uptake of PI-lip

Cells were pre-treated for 30 min at 4°C with the non-fluorescent competing liposome formulation at increasing concentrations from 100 μM (1:1 competition) to 1 mM (10:1 competition). Cells were washed to remove non-associated liposomes. Then fluorescently labeled liposomes of interest were incubated with cells for 1 hour at 37°C. The liposome uptake of interest was monitored by OregonGreen488-DHPE fluorescence of cell samples. The x-axis represents fluorescence of cells (A) PI-lip (pre-treatment) and fluorescent PI-lip uptake (B) GM1-lip (pre-treatment) and fluorescent GM1-lip uptake in J774 macrophages and JAWSII DCs. Pre-treatment was also performed at 37°C to permit liposome internalization and the results were in agreement with those shown.

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APPENDIX B

Characteristics of Glycolipid-Containing Liposomes

SUMMARY

The glycolipid-containing liposomes presented in this thesis are easily prepared and have reproducible liposome characteristics. These are two important factors that potentiate the further development of these liposome formulations as vaccine delivery carriers. Liposomes were sized down by either sonication or extrusion methods, both of which produced liposomes of a suitable size (<200nm) for subcutaneous injection and local lymph node targeting of liposomes. Furthermore, liposome preparations had poly dispersity index (PDI) values in the ideal range (<0.3) for nanoparticle formulations. PDI is a measurement of the width of size distribution, which indicates the heterogeneity of nanoparticle formulations. In order to make accurate calculations of encapsulated material and the amount of liposomes needed to provide a therapeutic effect, PDI must be low and reproducible from batch-to-batch. Independent preparations of liposomes consistently had similar zeta potential values, indicating glycolipid composition was comparable. Liposomes had low encapsulation efficiency of ovalbumin (OVA), with typically 2-3% encapsulated. Methods could be improved upon to (1) decrease liposome loss during extrusions, and (2) increase encapsulation of OVA in liposomes. Incorporation of phosphatidylinositol (PI) glycolipid in DOPC liposomes has been shown

in this thesis to promote dendritic cell uptake of liposomes and produce $CD8^+$ T cell-mediated immune responses, indicating them as beneficial vaccine delivery systems. Further benefit of using PI lipid is it's plentiful sourcing from plants (soy), as opposed to monosialotetrahexosylganglioside (GM1) glycolipid sourcing from brain tissues. Nevertheless, either PI- or GM1-containing liposomes are simple to prepare and possess reproducible liposome characteristics, which is necessary for cost-effective vaccine development.

INTRODUCTION

Size and surface charge are important physicochemical factors that can be easily and quickly measured to predict liposome uptake behaviors. Dynamic light scattering (DLS) determines size of liposomes and provides a measure of size range, known as the poly dispersity index (PDI). The size of nanoparticles is one of the most important factors for liposome delivery to peripheral tissues and uptake by certain cells [1-5]. The charge surrounding a particle, known as zeta potential, is another parameter than can drastically affect liposome uptake as well as stability. Anionic particles have zeta potentials below -10mV, while particles with zeta potentials in the range of -10mV to +10mV are considered approximately neutral [6]. Negatively charged lipids, such as phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidyglycerol (PG) added to a liposome formulation can increase stability of liposomes in solution [7-9], however these lipids' negative charge also enhances liposome uptake by cells [10-12]. Sterically-stabilized or long-circulating liposomes utilize phosphatidylinositol (PI),

monosialotetrahexosylganglioside (GM1), or poly(ethylene) glycol (PEG)-conjugated lipids to increase stability of liposomes *in vitro* and *in vivo* [13-16]. Although these lipids are also negatively charged, their bulky hydrophilic head groups contribute to stability and deters rapid uptake of these liposomes by macrophages [2, 11, 17].

Other important characteristics of liposomes include phosphate concentration, indicating lipid recovery from preparation, and the concentration of encapsulated materials in liposomes. These factors are of critical importance in development of nanoparticles for vaccine delivery, as extensive loss of liposome and/or antigen(s) would likely increase costs of production.

MATERIALS AND METHODS

Liposome preparation

Cholesterol (Chol) was obtained from Calbiochem (La Jolla, California). All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama). Liposomes were prepared at a 2:1 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Δ9-cis) (DOPC) to Chol. L-α-phosphatidylinositol (Soy) (PI) and monosialotetrahexosylganglioside (ovine brain) (GM1) were incorporated at 10 mol% of the total phospholipid. pH-sensitive liposomes contained L-α-phosphatidylethanolamine (egg, chicken) (ePE) and cholesteryl hemisuccinate (CHEMS) (Sigma-Aldrich) at a 2:1 molar ratio. Lipid mixtures were dried into a lipid film using a Buchi Rotavapor R-200 rotary evaporator. Lipids were dried completely then rehydrated with isotonic (290 ± 10 mmol/kg osmolality) HEPES-buffered saline containing EDTA (HBSE) (10mM HEPES, 140mM NaCl, 1mM EDTA, pH

7.4). Alternatively, liposomes to be used for immunization studies had lipid films rehydrated with isotonic (290 ± 10 mmol/kg osmolality) HBSE, pH 8.4 containing soluble ovalbumin (OVA) (Grade VI, Sigma-Aldrich) at 25 mg/ml and LLO at 250 µg/ml. Hydrated lipid films were vortexed and subjected to 5 freeze/thaw cycles. Liposomes prepared by extrusion (empty liposomes) were extruded 4 times using double-stacked polycarbonate 100 nm diameter filters (GE). Liposomes prepared by sonication (liposomes containing OVA/LLO) were subject to 8x1min cycles of sonication using a water bath sonicator. Size exclusion chromatography using a 1x25cm Sepharose CL-4B column (GE Healthcare) was used to remove unencapsulated protein in liposome formulations prepared with protein in the lipid film hydration solution. Prepared liposome samples were stored under argon gas at 4°C. Liposomes were used within 10 days of preparation.

Liposome characterization

The size of the liposomes and zeta potential was determined using a Malvern ZS90 ZEN 3600 zetasizer. Liposomes were diluted in HBSE buffer, pH 7.4 for the sizing and zeta potential measurements. Phospholipid content of liposomes was quantified using Bartlett's phosphate assay [18]. Absorbance was read on a Molecular Dynamics Emax plate reader at 805 nm. Phospholipid concentrations of liposome samples were determined based on a standard curve of known phosphate concentration(s) generated for each assay. Determined phospholipid concentrations signified the concentration of liposomes, which were used in the dosing calculations. GM1 does not have a phosphate

group and thus the phospholipid assay concentrations determined do not include GM1 content of the liposomes; dosing calculations were adjusted to account for this. Quantification of encapsulated protein was determined using SDS-PAGE. Proteins were resolved on a 4-20% Tris-glycine gel (Invitrogen) with 1X electrode buffer (250mM Trizma, 2M glycine) containing 0.1% SDS. Gels were stained with Krypton protein stain (Pierce) and protein band intensities were measured on a Typhoon 9200 (Molecular Dynamics). Protein content was quantified using ImageQuant (GE Healthcare) based on known protein content run on the same gel. Measured total encapsulated protein amount was divided by total protein amount added to liposomes in the hydration solution, x100 to obtain encapsulation efficiency (%).

Lipid calculations

The surface area of a sphere, $4\pi r^2$ was used to measure the outer liposomal surface area. The outer surface area of the liposome was divided by the relative surface area (nm^2) of lipid head group(s) for the indicated lipid composition at specified mole ratios, providing the total number of lipids estimated to be in the outer leaflet of the lipid bilayer. The head group surface areas of lipids used are as follows: DOPC: 0.62 nm^2 [19]; PI: 0.7 nm^2; GM1: 1.0 nm^2 [20-22]. The PI lipid head group surface area was estimated based on the surface area of phosphatidylglycerol (PG) head group=0.66 nm^2 [19].

RESULTS AND DISCUSSION

The liposome formulations outlined in Table B.2 are the main liposome formulations studied *in vitro* and *in vivo* in this thesis. The average diameter(s) of liposomes prepared by extrusion through 100nm polycarbonate filters was ~120nm with less than 10% deviation in diameter between differing formulations and between individual preparations of identical liposome formulations. The heterogeneity of the sample, indicated by the poly dispersity index (PDI) was more variable, but remained lower than 0.3, which is ideal for nanoparticle formulations. PDI values can range from 0 to 1, where values less to 0.05 are rarely seen other than with highly monodisperse samples, and a value above 0.7 indicates a highly broad size distribution. Preparation of liposomes by sonication is preferred when encapsulating listeriolysin-O (LLO). Previous work with LLO-containing liposomes has proven extrusion of these liposome formulations to be problematic (unpublished data). In these experiments we used bath sonication, as to avoid a probe-type sonicator that may shed material into the sample during sonication. Alike extrusion, sonication of liposomes produces liposomes of a smaller diameter. However, sonication of liposome preparations is more time-consuming, does not size the liposomes used in the studies below ~150nm diameter and results in a higher PDI value (Table B.2). Nevertheless, the liposomes formulations prepared by sonication were similar in size and PDI, and these values were reproducible in batch-to-batch liposome preparations. Although these liposomes are larger than those prepared by extrusion, their size (<200nm) is sufficient to drain into the lymphatic system after subcutaneous injection [1, 23].

The measured zeta potential of PI- and GM1- liposomes was typically between -9mV and -11mV. This suggests these glycolipid-containing liposomes are only very slightly anionic, as a zeta potential between -10mV and +10mV is approximately neutral [6]. PI-liposomes had slightly lower zeta potential which is likely a result of the charge shielding effect previously described of PI- and GM1-containing liposomes [14]. The bulky carbohydrate groups of these glycolipids in the liposome formulation have been theorized to shield the negative charge present in the glycolipid head groups. From the zeta potential measurements in Table B.2, it appears the bulkier GM1 lipid head group may better shield the negative charge located in its head group, resulting in a less negative zeta potential compared to PI-containing liposomes. The ability of GM1 to better shield negative charge would also explain why GM1-liposomes are more capable of deterring rapid macrophage uptake *in vitro* and *in vivo* compared to PI-liposomes.

The phospholipid concentration determined by Bartlett's assay was another reproducible feature of these liposome formulations (Table B.3). This assay specifically measures the concentration of phosphate (in phospholipids). Lipid mixtures contained 10µmol of total lipid (DOPC, PI, GM1) hydrated in 500µL HBSE buffer, resulting in a 20mM phosphate concentration before extrusion. The exception to this is GM1, which does not contain a phosphate group. Since GM1 is not reactive in the phosphate assay and therefore is not monitored, the phosphate concentration of GM1-containing liposomes is lower than liposome formulations such as DOPC:Chol or DOPC:Chol:PI, which contain only phospholipids (Table B.3). Liposomes that contain only DOPC:Chol have 10µmol DOPC added, whereas the glycolipid-containing liposomes

(DOPC:Chol:PI or DOPC:Chol:GM1) contain 9µmol DOPC and 1µmol of either PI or GM1. This 1µmol glycolipid content of the total 10µmol lipid content is why glycolipid-containing liposomes are labeled as 10mol% PI-liposomes or 10mol% GM1-liposomes. The 10mol% GM1 in the liposomes that is not monitored in the phosphate assay is confirmed in the liposome formulation in zeta potential measurements, which show a more negative surface charge compared to the neutral DOPC:Chol liposomes and a similar zeta potential to 10mol% PI-containing liposomes (Table B.2). After extrusion, liposomes are much less concentrated than 20mM indicating some liposomes are lost in the extrusion process. The addition of glycolipid to DOPC:Chol liposome formulation does not negatively impact the liposome recovery from extrusion. Liposomes that were formulated encapsulating material (e.g. LLO and OVA) were passed through a Sepharose CL-4B column to remove unencapsulated protein, which largely affects phosphate concentration. Liposome [phosphate] concentration is substantially diluted in the column flow-through buffer. Concentrated liposome samples collected from the column had a typical final phosphate concentration ranging from 2 nmol/µl to 5 nmol/µl (mM). The volume of concentrated samples was typically ~1-2ml. More dilute liposome samples could also be collected from the column (<2nmol/µl; ~1ml).

The outer surface appearance (i.e. outer lipid membrane leaflet components) of the liposomes is important for cell recognition. As established in Chapter 2 and in Appendix A, the presence of different lipids in the liposome formulation can have drastic effects on liposome uptake by cells. Table B.4 summarizes the appearance of the outer liposome membrane leaflet in terms of total lipid number and total glycolipid number.

The outer surface area of liposomes prepared by extrusion is approximately 4.522×10^{-14} nm^2 for the 120nm diameter liposome. Liposomes of 120nm diameter and composed of DOPC, Chol, and PI had approximately 72,006 lipids in the outer membrane leaflet, of which 7,200 are PI glycolipid. There are a slightly smaller number of lipids, 68,723, when the larger GM1 head group is incorporated in the DOPC, Chol bilayer of liposomes. Thus, GM1-containing liposomes contain approximately 6,872 glycolipids in the outer membrane leaflet, a less than 5% difference in number of glycolipids compared to PI-containing liposomes. Liposomes that were prepared by sonication and used in the immunization studies presented in Chapter 3 are slightly larger in area with an outer surface area of 1.075×10^{-13} nm^2 (based on avg. diameter of 185nm). In these larger liposomes, the total number of lipids in the outer liposome membrane leaflet in the DOPC:PI liposome is 169,826 and in DOPC:GM1 liposomes, 163,373. The total number of glycolipids would be 16,982 in DOPC:PI liposomes and 16,337 in DOPC:GM1 liposomes, more than twice that estimated to be in the outer membrane leaflet of 120nm diameter liposomes. The difference in glycolipid number becomes smaller as size of liposomes increases, where in 185nm diameter liposomes the difference is less than 4%.

Both PI and GM1 lipids extend perpendicularly from the lipid bilayer into the aqueous space [24, 25]. The hydration of these large hydrophilic lipid head groups results in a large bulky surface area. For PI-containing liposomes this can result in defects in the packing of PC lipids in the liposome bilayer, resulting in increased water penetration in the liposome [25]. A less tightly packed lipid bilayer when PI is

incorporated would suggest there are less lipids in a PI-containing liposome of the same size as a liposome containing only DOPC, for example [25]. Alternatively, GM1 in the lipid membrane can increase the packing of different lipids resulting in less aqueous leakage from liposomes [26-28]. These differences in bilayer packing are not only a result of the glycolipid head group, but also of the lipid hydrocarbon chain length and saturation, which are summarized in Table B.1. PI from soy has a mixed unsaturated (16:0) and saturated (18:2) fatty acid composition that could contribute to less tightly packed lipid bilayers. The carbon double bonds are found in the middle of the hydrocarbon chain, which form kinks in this carbon tail region, resulting in less van der Waals interactions with other lipid's hydrocarbon tails in the bilayer. GM1 consists of 18:0 and 18:1 fatty acid components, and the one carbon double bond is located near the lipid head group, allowing for its longer hydrocarbon tails to have increased van der Waals interactions with other lipids in the bilayer. The increased chain length and van der Waals interactions with GM1 can form more stable and rigid bilayers.

GM1 has been found asymmetrically distributed in lipid bilayers because of its large hydrated head group [29]. Although, in that study, liposomes had an average of only 20nm diameter, the small size of which likely affected the ability of bulky GM1 head groups (1.2nm length [24]) to distribute in the inner membrane leaflet. Nonetheless, steric hindrance and high hydration caused by the GM1 head group may still play a role in the distribution of GM1 in the liposome membrane. So while the mol% of glycolipids was matched in GM1- or PI-liposome formulations studied here, there may be slightly

more GM1 glycolipid located on the outer membrane leaflet, increasing the predicted glycolipid value given in Table B.4.

As these liposome formulations investigated in this thesis have the potential to be used as vaccine carriers, it was imperative to determine how much antigen and other components are encapsulated in liposome preparations. A drawback to using liposomes in any delivery context is that they have typically low encapsulation of materials. Lipid films were hydrated with 25mg/ml OVA but prepared liposome formulations contained approximately 0.2mg/ml OVA (Table B.5). The determined encapsulation efficiency was ~3% for OVA. The protein LLO was encapsulated at a much higher efficiency than OVA, with typically ≥50% of total LLO added encapsulated. The lack of encapsulation of OVA may be remedied in a few ways. More OVA may become encapsulated if lipid films are simply hydrated with less volume, thereby allowing more of the added volume to be encapsulated in liposomes. Another approach to increase OVA concentration inside the liposomes may be to increase the freeze/thaw cycles during liposome preparation [30]. Alternatively, the reverse-phase evaporation method for preparing liposomes may increase encapsulation efficiency of hydrophilic molecules [31]. Changing lipid features such as hydrocarbon length and degree of saturation can also affect encapsulation of material in liposomes [32]; however, changing lipid content is also likely to have effects on stability, uptake of liposomes, as well as the liposomal delivery of material inside cells. The glycolipid incorporated in the liposome composition did not significantly affect ratios of OVA to LLO encapsulated in the liposomes; there was typically ~3-5X more μg OVA than μg LLO encapsulated in either glycolipid-containing liposome formulation.

This ratio was additionally similar to the OVA:LLO ratio when pH-sensitive ePE:CHEMS liposome composition was compared.

The bulky carbohydrate head groups and negative charge of PI- and GM1-glycolipid-containg liposomes are speculated to contribute to decreased fusion and aggregation of liposomes in solution [17, 33-35]. DLS measurements support this, showing one single narrow peak indicative of one vesicle size population with a narrow size range (Figure B.1) Furthermore, uptake studies as described in Chapter 2 generated similar results when liposomes were prepared up to 10 days prior. So while the impact of storage on liposomes was not directly monitored, reproducible results could be obtained with liposome up to 10 days post-preparation, suggesting liposomes are stable for a short time as prepared. Liposome aggregates or liposomes that had fused would likely have different uptake characteristics [14, 36]. However, assays to monitor aggregation as well as leakage of glycolipid-containing liposomes over time would add significant value in the characterization of these liposomes delivery vehicles.

CONCLUSION

The characteristics of these glycolipid-containing liposome formulations presented are highly reproducible batch-to-batch. They are similar in terms of size, zeta potential, phosphate concentration and liposomal surface glycolipid content. Liposome preparation by sonication methods could be improved upon to provide higher encapsulation of OVA. Overall, glycolipid-containing liposomes are of easy preparation

with reproducible characteristics, adding further value to their future potential as liposomal vaccine carriers.

Lipid name	Acronym	Lipid source	Purity	Carbon chain length:double bonds; fatty acid name(s)[u]	Molecular Weight (g/mol)	Charge at pH 7
1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Δ9-cis)	DOPC	Synthetic*	>99%	18:1; oleic acid	786.113	Neutral
L-α-phosphatidylethanolanime	ePE	Chicken, egg	>99%	18:1; oleic acid 18:0; stearic acid	748.608	Neutral
L-α-phosphatidylinositol	PI	Soy	>99%	18:2; linoleic acid 16:0; palmitic acid	866.647	Negative
L-α-phosphatidylserine	PS	Porcine, brain	>99%	18:1; oleic acid 18:0; stearic acid	824.966	Negative
Monosialotetrahexosyl-ganglioside	GM1	Ovine, brain	>99%	18:0; stearic acid 18:1; sphingosine	1568.805	Negative
1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]	PEG-PE; PEG-DSPE	Synthetic*	>99%	18:0; stearic acid	2805.497	Negative[37]

Table B.1 Lipid characteristics

* Synthetic phospholipids are prepared from glycerophosphocholine (GPC) derived from plant or animal sources. The typical plant source of GPC is soybean lecithin.

◆ Predominant fatty acid species in lipid product

Information provided by Avanti Polar Lipids, lipid manufacturer, expect where noted

Liposome composition	Size* (nm) (SD)	Poly dispersity index (PDI)(SD)	Zeta potential (mV) (SD)
Preparation by extrusion			
DOPC:Chol:PI (10mol% PI-lip)	115.54 (9.87)	0.086 (0.079)	-11.29 (0.87)
DOPC:Chol:GM1 (10mol% GM1-lip)	118.32 (10.53)	0.078 (0.044)	-8.29 (0.64)
DOPC:Chol	123.06 (8.38)	0.119 (0.058)	-5.56 (0.49)
Preparation by sonication			
DOPC:PI	196.83 (27.75)	0.275 (0.025)	-10.24 (0.79)
DOPC:GM1	186.74 (25.19)	0.280 (0.064)	-9.20 (0.68)
ePE:CHEMS	173.73 (6.36)	0.214 (0.028)	-23.94 (1.94)

Table B.2 Liposome size, poly dispersity index and zeta potential
The particle size and poly dispersity index (PDI) of liposomes were determined after liposome preparation by extrusion or sonication. Size (diameter; nm) was determined by dynamic light scattering at 22°C. PDI is a dimensionless measure of the width of particle size distribution. Zeta potential measured in HBSE, pH 7.4 at 22°C Zeta potential was determined by electrophoresis and laser Doppler velocimetry. All measurements were performed by a Malvern Zetasizer ZS90. Data shown is representative of 8 separate preparations of liposomes by extrusion, or 3 separate preparations of liposomes by sonication.
SD: standard deviation
*Size listed is Z-average particle size

Liposome composition	Phosphate concentration (mM) (SD)
DOPC:Chol:PI (10mol% PI-lip)	13.02 (3.36)
DOPC:Chol:GM1 (10mol% GM1-lip)	11.03 (2.19)
DOPC:Chol	14.51 (2.34)

Table B.3 Phosphate content of liposome preparations
Phosphate content of liposomes was quantified using Bartlett's phosphate assay. Absorbance was read at 805nm. Data shown is representative of 10 separate preparations of liposomes by extrusion. SD: standard deviation

Liposome composition	Mole ratio	# of outer surface lipids	Outer surface area (nm^2) of liposome	# of outer surface glycolipid
Preparation by extrusion				
DOPC:Chol:PI (10mol% PI-lip)	9:5:1	72,006	$4.522x10^{-14}$	7,200
DOPC:Chol:GM1 (10mol% GM1-lip)	9:5:1	68,723	$4.522x10^{-14}$	6,872
DOPC:Chol	2:1	72,935	$4.522x10^{-14}$	NA
Preparation by sonication				
DOPC:PI	9:1	169,826	$1.075x10^{-13}$	16,982
DOPC:GM1	9:1	163,373	$1.075x10^{-13}$	16,337
ePE:CHEMS	2:1	173,387	$1.075x10^{-13}$	NA

Table B.4 Features of liposomal surface
Lipid head group areas are as follows: DOPC: 0.62 nm^2; PI: 0.7 nm^2: GM1: 1.0 nm^2. The total number of outer surface lipids and total number of outer surface glycolipid were calculated based on a relative lipid head group size for the indicated lipid composition at specified mole ratios. Liposomes prepared by extrusion had an average 120nm diameter. Liposomes prepared by extrusion had an average 185nm diameter.
NA: not applicable

Liposome composition	Liposomal OVA concentration (µg/µl) (SD)	Liposomal LLO concentration (µg/µl) (SD)	OVA:LLO ratio
DOPC:PI	0.162 (0.059)	0.041 (0.010)	4.0
DOPC:GM1	0.228 (0.059)	0.046 (0.001)	5.0
ePE:CHEMS	0.202 (0.063)	0.035 (0.004)	5.8

Table B.5 OVA and LLO encapsulation in liposomes
Liposome preparations used for immunizations were analyzed for their liposomal OVA and LLO content. The concentration of OVA and LLO shown is the mean and standard deviation (SD) for two separate liposomes preparations. OVA:LLO ratios were calculated based on total µg of OVA and LLO determined in liposome samples. The ratio shown is an averaged ratio from 6 liposome samples in 2 independent liposomes preparations.

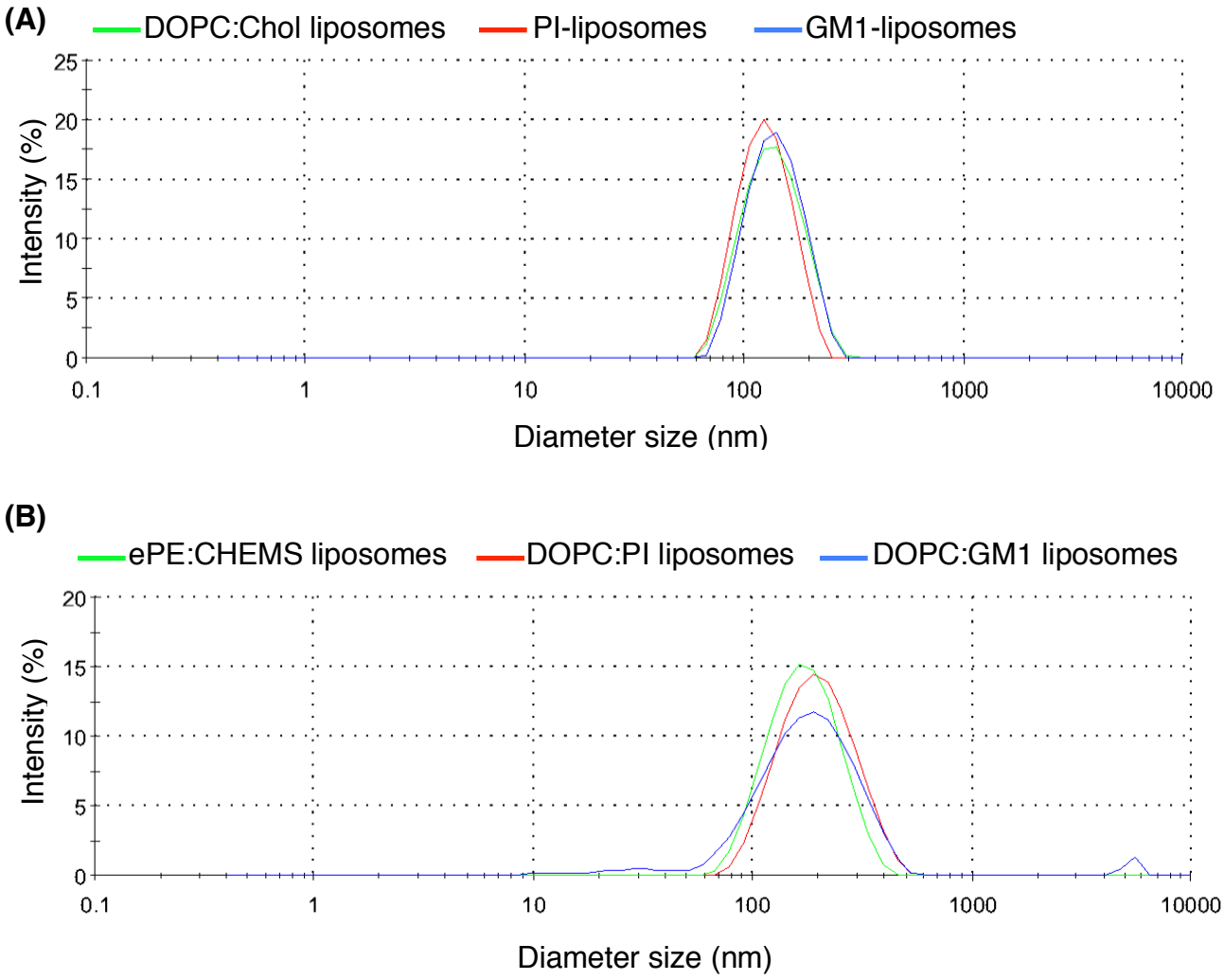

Figure B.1 Representative DLS size measurement plots
Representative dynamic light scattering (DLS) size measurement plots from liposomes prepared by extrusion through double-stacked 100nm polycarbonate filters (A) or sonication 8x1min cycles (B).

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