CHARACTERIZATION OF FLAGELLIN-FUNCTIONALIZED LIPOSOMES AS A VACCINE CARRIER AND ADJUVANT

by

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A Dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmaceutical Sciences)
in the University of Michigan
2015

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For my parents
Funso & Ronke Adeniyi

for bearing the brunt of my random ill-humor at your many innocuous combinations of "how", "when", and "your research"-it wasn't always bad

ACKNOWLEDGEMENTS

I will like to express my gratitude to my advisor and dissertation chair, Dr Kyung-Dall Lee. It was Dr Lee who first knew of my interest in the doctoral program as a P1. I'm thankful he managed not to laugh in derision at that interest, considering I was at his office to argue for two points on a true false question on a pharmaceutics exam. I'm grateful for his skillful direction in my research, for his patience and foresight when nothing seemed to work, and of course, providing a conducive intellectual space to train as a young scientist. I thank Dr Duxin Sun, Dr Mary O'Riordan, and Dr James Moon for serving on my dissertation committee. Their commitment, feedback and critical reviews were instrumental in how I contemplated questions and difficulties, which allowed me to take a more comprehensive approach to vaccine delivery and its applications.

I will like to thank past Lee lab members for their important contributions to my scientific experience. They include: Dr. Chester Provoda, for being a trusty guru available to offer assistance ranging from scientific editing and regular technical discussions to troubleshooting equipment failures; Emily Rabinsky, whose kind words, impeccable memory, and experience with surface-modified liposomes I could always count on even across the campus; Zach Walls for my early introduction to molecular biology; Chasity Andrews for the foundation she laid that was valuable in setting up immunologic assays-even years later; and Nahyung Kim for her support on the DNA vaccines project.

I thank other Lee lab members for the pleasure of working with them, Juhee Lee, Kefeng Sun, Allison Matyas, Laura Radecki and others. I would also like to thank Dr Yasuhiro Tsume for his generous help with animal studies and useful discussions. This paragraph would be incomplete without mentioning the numerous lunchtime shenanigans

of some of those listed above, Chester Provoda, Hiro Tsume, Kefeng Sun, and others that provided necessary distractions that fostered the collegial ease that made the fourth floor tick.

I will like to extend my thanks to other people in the college, who one way or another supported my work and progress. I'm grateful for Luckasz Ochyl's kind assistance and invaluable expertise in the *in vivo* studies and tetramer staining. I thank Dr. Cherie Dotson, for her unstinting support that started even before I began the doctoral program. I thank her for her versatility to be a lot of things from a champion, to mentor or even just a listening ear. I extend special thanks to Gail Beninghoff, and Patrina Hardy for making life simpler many times. I acknowledge the funding support for my work, which was provided through the following: Rackham Merit Fellowship, the American Foundation for Pharmaceutical Education, the Pharmacological and Biorelated Chemistry Training grant and the Upjohn Fellowship at the College of Pharmacy.

I'm grateful for the camaraderie of many people who I've shared this journey with over the years; Yea Min Huh, Ronak Shah, Byumseok Koh, Yehua Xie, Hanna Song, Maya Lipert, Karthik Pisuparti and many others. I thank Brittany Bailey for being there for the laughs and the many a-wining, a-bouqueting, and venting sessions. We tried, and I'd say we achieved some measure of success at staying sane.

Finally, I will like to pay tribute to my family and loved ones who kept me grounded in the "center of the universe" even when I was far out in Ann Arbor, and those who looked forward to dressing to the hilt when this day arrived. I may have sabotaged your attempts to attend any ceremonies to mark this occasion, which would have been a joy for you, but I

hope this dissertation is a suitable installment towards your faith and enduring support of the seemingly unending years in Ann Arbor.

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
BMM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CFA	Complete freunds adjuvant
CHEMS	Cholesteryl hemisuccinate
CPRG	Chlorophenol red-β-D galactopyranoside
CTL	Cytotoxic T lymphocyte
DAMPs	Danger-associated molecular patterns
DNA	Deoxyribonuecelic acid
DPX	p-xylene-bis-pryidinium bromide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
H2A	Histone H2A
HBG	HEPES-buffered glucose
HBS	HEPES-buffered saline
Hi-FBS	Heat-inactivated fetal bovine serum
HPTS	8-hyrdoxypyrene-1, 3, 3-trisulfonic acid
IL	Interleukin
LDH	Lactate dehydrogenase
LLO	Listeriolysin O
LLOLPDII	Anionic LLO liposome-polycation-DNA
LPDII	Anionic liposome-polycation-DNA

LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Ni-lipid	1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)
Ni-NTA	Nickel-chelated nitriloactetic acid
NLR	NOD-like receptor
NLRC4	NOD-like receptor –containing a caspase activating and recruitment domain
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBA	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05% Tween
pDNA	Plasmid DNA
PE	Phosphatidylethanolamine
PN	Protamine
PRRs	Pathogen-associated molecular pattern-recognition receptor
ROS	Reactive oxygen species
T3SS	Type 3-secretion system
T4SS	Type 4-secretion system
Th	T helper cell
TLR	Toll-like receptor
YFP	Yellow fluorescent protein

ABSTRACT

Since the recognition that the adjuvant capacity of flagellin is better harnessed when both flagellin and the antigen are delivered to the same cell, there has been a need to exploit flagellin in ways that fulfill this constraint. We propose a liposomal delivery system functionalized with *Salmonella typhimurium* flagellin (fliC) as a way to meet this need. Our goal is to characterize the fliC-functionalized liposome as a vaccine adjuvant and evaluate its ability to target cells expressing Toll Like receptor 5 to enhance the vaccine potential of a liposome-encapsulated antigen. Proinflammatory cytokine secretion and preferential cell association were evaluated in murine alveolar macrophage cell line and bone marrow-derived macrophages *in vitro*. Caspase-1 activation and IL-1β secretion were used to determine inflammasome activation in studies employing LLO to gain cytosolic access. After a prime-boost immunization regimen, humoral and $CD8^+$ T cell adjuvant effect of functionalized liposomes *in vivo* were determined by quantifying antigen-specific IgG1 and IgG2c and tetramer staining of antigen-specific $CD8^+$ T cells. We report that fliC-functionalized liposomes are able to elicit the proinflammatory cytokine, IL-6, with comparable efficacy to soluble protein in a TLR5-mediated manner from an alveolar macrophage cell line but not from bone marrow-derived macrophages. FliC-functionalized liposomes also demonstrate the capacity to preferentially associate with flagellin-responsive cells, enhance MHC class I –restricted peptide presentation *in vitro,* and elicit IgG1 and $CD8^+$ T cell response specific to liposome-encapsulated antigen. Using LLO-encapsulating flagellin-bearing liposomes, we demonstrate that fliC delivery to the cytosol enhances inflammasome activation and fliC-functionalized LLO liposomes are able to stimulate antigen-specific IgG1 in immunized mice. The physicochemical stability of the

flagellin-functionalized liposome and the immune profile it elicits recommend fliC-functionalized liposomes as feasible for vaccine carrier and adjuvant function.

CHAPTER 1 Vaccine delivery to engage the innate and adaptive immune systems

INTRODUCTION

The goal of vaccination

We are generally able to survive a host of pathogenic invasions because of the natural defenses put up by our immune system. However, there are times when the invading organism is able to evade these defenses, and this is consequently manifested as a disease process; vaccines insure the host against such an evasion should one ever be exposed to the corresponding pathogen. The global eradication of small pox is a result of a successful global-scale vaccination and diseases such as, diphtheria, tetanus, yellow fever, pertusis, *Hemophilus influenza* type b, measles, mumps, rubella, and typhoid- have been largely controlled by vaccinations (1). The impact of vaccines on the global population has been so substantial that with the exception of the production of safe drinking water, no other intervention has had such an impact on reduction of mortality and population growth (1).

Vaccination has been a cornerstone of disease prevention for years; while traditional vaccines have been associated with prevention of infectious diseases, with increasing morbidity and mortality from various diseases, the search for new vaccines has not been limited to those for use in combating infectious disease. The range of vaccine for preventable or treatable diseases has widened to include non-communicable diseases such as nicotine addiction, obesity, hypertension and cancers-with the hypothesis that active

vaccination against molecules implicated in the disease process will offer a chance to treat such disease (2).

Despite the advances made in vaccine development, there are still diseases such as HIV and malaria, for which no vaccines exist; there are also existing vaccines that are suboptimal in their defense against their corresponding diseases. Several factors are responsible for these inadequacies in vaccine development, one of which is the lack of immunogenic potency of some vaccines (3). While each vaccine type has unique challenges associated, considering that the main goal of vaccination is to generate an immune response potent enough to create lasting immunological memory (3), a failure in this endeavor represents a major setback for any vaccine. In most natural infections, a repertoire of antigens from a pathogen stimulates a variety of interactions with the host's immune system to create such a response. Thus, the ultimate aim for many vaccine research studies is to replicate the antigen exposure that occurs during natural infection without inducing the disease process seen in a natural infection.

IMMUNE RESPONSE TO ANTIGENS

The innate immunity presents the first line of defense that is activated within minutes of recognizing an invading pathogen (4) The mechanisms of innate defenses are mediated by chemical, physical and cellular processes for example stomach acidity, secretion of mucus on epithelial cells to prevent pathogen adherence, and activation of macrophages respectively (4). Upon infection by a pathogen, The innate immune system is alerted through the recognition of molecular motifs called pathogen- associated molecular patterns (PAMPs) by the pathogen recognition receptors (PRRs) expressed by the cells of the innate immune system. PAMPs such as bacterial lipopolysaccharide, lipoproteins and

mannose-containing molecules found in viruses and bacteria etc. are expressed constitutively by invading pathogens and not found in eukaryotes; the PRRs such as the scavenger receptors, mannose-binding lectin and the Toll-like receptors (TLRs) have evolved to enhance recognition of foreign bodies by the immune system (5, 6).

When effector cells, such as macrophages residing in submucosal tissue layers and circulating neutrophils recognize a pathogen, they phagocytose the pathogen and secrete chemokines and cytokines, such as interleukins 1, 6 and 12, and a local inflammatory response is induced to recruit other effectors to augment the actions of the activated macrophages. Because the innate defenses are dependent on recognizing predetermined receptors, this defense mechanism is characterized by a rapid response but also by lack of specificity and inability to generate immunological memory (3). However, the production of cytokines and other effector molecules during the innate immune response, along with the antigen presenting function of macrophages and dendritic cells, induce and segue into the second line of defense, provided by the adaptive immune response.

Unlike the PRRs that mediate recognition in the innate immune response, recognition in the adaptive immune response is through antibodies and T cell receptors. The adaptive immune response is generated and directed against the specific offending pathogen and usually results in protective immunity against re-infection with that same pathogen; this is mediated by T and B lymphocytes providing cell-mediated and humoral responses respectively (4). During the innate immune response, PRRs on macrophages and dendritic cells (DCs) recognize PAMPs and the antigens are displayed in association with the major histocompatibility complex (MHC) class I or MHC class II molecules. Since they possess the ability to migrate to a lymphoid organ, the APCs present this antigen to

stimulate the amplification, maturation and differentiation of naive lymphocytes (7). As pathogens can vary from intracellular bacteria, parasites and viruses to extracellular parasitic worms, bacteria and fungi, the cellular site of an infection leads to varying effector mechanisms of the adaptive immune response to remove the pathogen (4, 7).

Processing of extracellular and intracellular pathogens and their antigens

Extracellular and intravesicular pathogens are inaccessible to the proteasome complex in the cytosol, and are degraded within the acidified endolysosomal pathway, where the peptides of the antigen are loaded on to MHC class II molecules and displayed on the cell surface. As $CD4^+$ T cells have a high affinity for MHC class II molecules, these cells recognize the peptides in the context of the MHC molecules (8). The T helper 1 and 2 (Th1 and Th2) functional subsets of $CD4^+$ T cells produce cytokines to determine the outcome of the activation and are essential to induce high affinity antibodies and immunological memory (9). The Th17 and Tregs are relatively newly identified as subsets of $CD4^+$ T cells that that help regulate protection against certain classes of pathogens (10). Factors that influence the differentiation to this subset of cells vary, but the cytokines that dominate at the time of the initial T cell priming has been shown to be the important determinant. Whereas the presence of the pro-inflammatory IL 12 dictates a Th1 differentiation and the presence of the anti-inflammatory IL 4 predisposes differentiation towards a Th2 response (5, 6). Th1 cytokines support activation of macrophages (BMM) and generation of T cells, while the Th2 cytokines support activation of B cells, production of antibodies and eradication of extracellular pathogens (11). In summary, activation of $CD4^+$ T cells leads to: (1) activation of other effector cells to eradicate intravesicular pathogens and (2) activation of B lymphocytes to produce antibodies against extracellular pathogens.

On the other hand, antigens from intracellular pathogens in the cytosol are degraded by the proteasome, translocated into the endoplasmic reticulum, where they are bound to MHC class I molecules and shuttled through the Golgi apparatus and ultimately presented on the cell surface. $CD8^+$ T cells, have a high affinity for peptides presented in the context of the class I molecules, hence they become activated leading to the death of the cell presenting the peptide MHC class I complex on its surface (4). The cytokine milieu enabled by $CD4^+$ T helper cells is important in priming and the generation of $CD8^+$ T cells and the protective antibody response.

HOW VACCINES WORK, TRENDS IN VACCINE DESIGN

Vaccines serve to mimic a pathogenic invasion without actually inducing the disease state; thus the goal is to create immunological memory that can act as surveillance against future exposure to pathogens. Vaccine design has taken multiple approaches to generate appropriate immune responses to achieve this goal. Approximately 75% of vaccines licensed for use against human viral diseases are live- attenuated vaccines, which include vaccines against measles, mumps, rubella, and zoster (12). These vaccines are developed by adapting the virus to replicate in its non- natural host and as a result, emerge weakened in virulence (12). These vaccines most closely mimic natural infections, and thus have several advantages including (1) the ability to replicate in the host (2) frequent ability to target antigen presenting cells, (3) containing all the antigens of the pathogen in the native form and as such are able to elicit strong innate immune responses (4) when replicating intracellularly, they are also able to elicit cytotoxic $CD8^+$ T cell responses, and eventually produce a large pool of memory B and $CD8^+$ T cells (12).

With these advantages come disadvantages that include a possible reversion to a pathogenic form and a limitation on potential recipients, for example, immunocompromised patients (4). The approach using vaccination with an inactivated whole organism or a protein subunit vaccine bypasses this disadvantage in that these vaccines cannot replicate in the host and thus cannot revert to a more virulent form. However, this safety assurance comes at a cost to desired immunogenicity, with many requiring the addition of an adjuvant to bolster the immune response and the need for booster doses to maintain immunity against the pathogen (4, 12), a major drawback to providing vaccination to people without regular access to healthcare.

The need for enhanced activation of immune responses and cytotoxic T lymphocyte response

It is now accepted, that for successful vaccines against diseases such as HIV, malaria and tuberculosis, robust T cell responses of sufficient magnitude and quality will need to be generated (13-15) Studies have shown that macaques challenged with simian immunodeficiency virus were partially protected by vaccines that stimulate $CD8^+$ T cell immunity (15-17). Additionally, and one of the salient lessons from the failure of several HIV vaccines in clinical trials is the need, not only for neutralizing antibodies against HIV, but also HIV- specific $CD8^+$ T cells response to generate persisting T cell responses and suppress the virus (15). Vaccines that stimulate a T cell response can ensure a more rapid, targeted immune response to eliminate viruses in the early stages of an infection. Other studies have shown a variety of infections for which $CD8^+$ T cell responses are essential, such as in other retroviruses (18).

Naturally, what constitutes a desired immune response profile necessary for the control of a pathogen will vary from pathogen to pathogen; however, most licensed

vaccines have relied on antibody titers as a strong immune correlate of protection (11, 19). It is clearer now, however, that a humoral response alone is not sufficient as an immunological outcome for certain diseases. Whatever the case, the option to a generate cell-mediated immune response to any pathogen for which it is required is of importance and getting access to the cytosolic pathway, MHC class I, which favors the activation of T cells is of priority in vaccine design for such diseases. Th1 cells secret IFNγ to promote activation of $CD8^+$ T cells.

VACCINE ADJUVANTS AND IMMUNOPOTENTIATORS

The notion of improving vaccine response by the addition of certain compounds has been demonstrated by the addition of aluminum compounds to vaccines for about 70 years (20) and is present in vaccines against Hepatitis A, diphtheria and tetanus and more. Oil-in-water emulsions, AS03 and MF59 are licensed for use in Europe, and AS04, a combination adjuvant of monophosphoryl lipid A (a TLR ligand) adsorbed onto alum, was recently licensed as a vaccine adjuvant in the United States (20). Materials under study for their adjuvant activity vary widely in properties and they range from small molecules, emulsions, mineral salts to bacterial compounds (21) A reflection of the classical definition of the adjuvant as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone" (22).

Logically, studies have shown that when antigens are co-administered with vaccine adjuvants, the resulting immune response is more potent. While adjuvants have been shown to improve immune response against co-administered antigens, broaden protection or potentially reduce the dose of antigen needed, or providing cross protection against antigens related to the administered antigen (23), the mechanisms by which they do are

not well elucidated. For example, mechanisms suggested for the adjuvant behavior of alum includes, forming a depot of antigen at inoculation site allowing gradual introduction of adsorbed antigen, presentation of adsorbed antigens in a more immunogenic multivalent form and induction of a local inflammatory environment that leads to recruitment of lymphocytes (21, 24). The primary mode of action of an adjuvant can be used to classify it in to two broad categories. Immunostimulatory adjuvants have direct immunostimulatory effects on antigen presenting cells (APCs) and particulate adjuvants, such as emulsions, liposomes and virus-like particles, are those that function as delivery systems to enhance the uptake of antigens (22).

Toll and NOD-like agonists as vaccine adjuvants

It was observed that many successful vaccines seem to exhibit intrinsic adjuvant activity from motifs that are known to stimulate the toll like receptors and other pathways of the innate immune system (20), for example, the presence of lipopolysaccharide, a TLR 4 ligand in the typhoid vaccine, the presence of single stranded RNA which is now known to stimulate TLRs 7 and 8 in the inactivated polio vaccine (25). TLR agonists have been identified and studied as vaccine adjuvants even though they have been present in vaccine formulations albeit unwittingly. This presents evidence that the search for a better adaptive cell-mediated response does not entirely discount the role of the innate immune system to reach this end.

Toll-like agonists

Intracellular pathogens, for example viruses, need the cooperative interaction between the innate and the adaptive response to be eradicated. Toll like receptors, a main component of the innate immune system, and TLR recognition of PAMPs has been shown to

lead to signaling pathways that result in induction of inflammatory cytokines, trigger dendritic cell maturation that leads to increased antigen capacity to help direct adaptive immune response to antigens (8). The TLR9 agonist, CpG has been tested to enhance response in chronic viral infections and cancer (22). The TLRs are the best characterized class of PRRs in humans and appear to specialize in the recognition of bacterial (TLRs 1, 2, 4, 5) and viral (TLRs 3, 7, 8, 9) (26) PAMPs. Toll-like receptors (TLRs) are widely expressed by various immune cells including dendritic cells are among the main cells using this class of PRRs as a means for recognizing pathogens which may carry multiple TLR ligands (26). *In vitro* studies have shown that TLR agonists are able to directly activate the dendritic cells to stimulate T cell activation and differentiation (21, 26, 27) suggesting that the quality of the immune response induced against a particular pathogen may depend on engaging the specific PRRs expressed by cells at the infection site (23). There are studies exploring the potential of a variety of TLR agonists alone or in combination as adjuvants (Table 1.1) in hopes of finding strategies to enhance the immune response.

Nod-like agonists

The Nod-like receptors (NLRs) are comprised of 22 human genes and more in mouse. Analysis of the NLR family reveals 3 subfamilies, the NODs, NLRPs and the IPAF families, which share similarities in domain structure (28). NLRP3 is the best characterized of the NLRs and it responds to range of compounds and particulates that have been implicated in enhancing immune response such as silica, chitosan, ATP and uric acid crystals(29). Because of the diversity and lack of common patterns of apparent agonists, NLRs are generally considered to perform cytosolic surveillance for danger-associated molecular patterns (DAMPs), such as reactive oxygen species (ROS), or PAMPs secondary

to the different stimuli (28-30). Muramyl dipeptide (MDP) of bacterial cell wall peptidoglycan is recognized by NOD2 and was found to be responsible for the ability of Complete Freund's Adjuvant (CFA) to mount an optimal humoral and cellular response. MDP has been suggested to play a critical role in priming $CD4^+$ T cell cells toward specific Th profiles and several derivatives have been synthesized and researched to enhance its adjuvant effect while managing its pyrogenicity (29). It is thought that NLR-mediated peptidoglycan synergizes with TLRs to elicit optimal adaptive immune response (31), prompting studies of combination of NLR and TLR agonists as a way to modulate Th1 or Th2 activity. However, there has been evidence to show that such combinations could potentially annul the effect of the component agonist sand or lead to anergy (32).

Flagellin as both TLR and NLR agonist

Flagellin is the main structural unit of the flagellum, the motility organelle in many bacteria such as *Pseudomonas, Serratia, Proteus, Escherichia* and others (33). Analysis of Gram- positive and -negative bacteria reveal It is a highly conserved at the amino and carboxy termini while the interspacing region, known as the hypervariable region exhibits substantial variability and length (34, 35). The 55 KDa protein has four major domains: (1) D0, which comprises approximately 50 residues each of the N and C termini involved in the NLR signaling (2), D1, contains highly conserved regions involved in signaling recognized by the TLR receptor, (3) D2 and (4) D3 the latter two which span the hypervariable region to which antibodies are directed (33, 35, 36) (Figure 1.1).

Flagellin signals via TLR5, which is expressed on dendritic cells, endothelial cells, epithelial cells, and monocytes (37) (Table 1.2). The signaling cascade upon flagellin and TLR5 ligation is well studied. Ligation mobilizes nuclear factor NF-κB and stimulating

production of proinflammatory cytokines, and maturation and migration of dendritic cells to secondary lymphoid sites (5, 37). Like TLR 2, 4, 5, 7, 8 and 9, TLR5 signals through the IL-1R associated kinase (IRAK) signaling pathway governed by the adapter protein MyD88 to activate various transcription factors (23, 34). Signaling through this adapter protein leads to the activation of type 1 interferons (α and β) required for control of viral infections and production pro-inflammatory cytokines such as tumor necrosis factor α (23). Intracellular flagellin, as in an infection with a flagellated bacterium, signals via IPAF, also called Nod-Like Receptor C4 (NLRC4), and Naip5 (38), making flagellin both a TLR and an NLR agonist. Flagellin exhibits a couple of advantages that makes it attractive for use in vaccines such as, its effectiveness at low doses, lack of IgE production and flexibility with which it can be manipulated- in terms of fusion proteins- without any loss of signaling via TLR5 (39-42).

USE OF NANOPARTICLES IN VACCINE DELIVERY

Particulate delivery systems typically are of similar dimensions as the pathogen and can be used in combination with immunostimulatory adjuvants to affect the magnitude of the response of direct a certain immune profile (22). Due to their ability to be targeted to immune cells of interests or loaded with immunostimulatory agents, nanoparticles have been of interest in vaccine delivery (43). Inorganic nanoparticles such as gold, silicate of calcium phosphate nanoparticles are being examined though they are non-degradable and stay in the tissues for extended periods of time (43, 44). Polymeric nanoparticles ranging from naturally derived polymers such as hyaluronic acid to polyesters such as poly (lactic-co-glycolic acid) have been also been exploited in vaccine delivery to various degrees (22, 43, 45). As biologically inspired systems, biomolecular materials such as virus-like

particles, micelles and liposomes offer great advantages to vaccine delivery, they can be used to deliver encapsulated cargo, and their surface can be manipulated to display different moieties, which offers an advantage in targeting (43) Clinically viral particles have been used to deliver vaccines, with the vaccine against the human papillomavirus (HPV) being a recent one (46). This licensed vaccine contains the major capsid protein of HPV and an alum salt adjuvant. It has been determined to reduce the infection of HPV by 90% (44). This demonstrates the potential and attractiveness of bimolecular nanoparticles as vaccine carriers. Liposomes also present a benefit in delivery and there are a number of licensed liposome-based drugs on the market. Because of their relative immunogenicity, liposomes are considered the most useful for vaccine delivery (44). As spherical vesicles with a phospholipid bilayer, they are able to encapsulate either antigen or adjuvant and can be rendered immunogenic by functionalizing the surface for presentation to antigen presenting cells (APCs) (43, 44).

Antigen presenting cells as a vaccine delivery targets

Dendritic cells (DCs) and macrophages are antigen-presenting cells that play a major role in orchestrating responses of the innate and adaptive immune systems. Their important role in instructing the immune system makes them strategic targets of vaccine delivery. Antigens have been targeted towards DC surface receptors *in vivo* after *ex vivo* loading (48) and protein antigens have been used to target the DEC-205, a dendritic cells receptor to enhance MHC class I antigen presentation (49).

Surface modification of liposomes to enhance delivery to antigen presenting cells

Studies of antigen attachment to liposomal vaccines show that physical association in some form to the liposome is required and surface conjugated antigen generally elicits greater antibody responses than encapsulated antigens, although, CTL responses are the same (50). In using liposomes are delivery vehicles; various parameters have to be considered. Parameters such as vesicle size, lamellarity, surface charge, lipid composition, fusogenicity, membrane fluidity and antigen attachment are important to the design of liposomes as vaccine carriers (50). For the purpose of targeting, antigen attachment on the surface of the liposome is of particular importance and the antigen has to be physically or chemically associated with the liposome. These attachment strategies include covalent conjugation to the lipid surface via a lipid moiety, non-covalent surface attachment using biotin, NTA-Ni (II)-hexahistidine or exploiting antibody–epitope interactions and surface adsorption.

Covalent conjugation is predominantly done via disulfide, amide or thioester bonds (50, 51) and usually involves multiple steps with buffers of varying pH that increases the potential of damaging protein activity and immunogenicity (52). Surface adsorption as a mode of protein attachment has not been comprehensively studied (50). The use of non-covalent attachment such as that of NTA-mediated protein association can be a good investigative tool. It only requires a simple modification of the protein- adding histidine groups -and it is mild enough to not affect the activity of the protein (52). This strategy exploits the relatively high affinity of histidine residues and Ni^{2+}, which can be utilized in liposomes containing lipids with a Ni-chelating head group to immobilize hexahistidine-tagged proteins (53-55). Understandably, the strength of the interaction between the protein and the chelated nickel on the liposome surface is of importance to its ability to

make the desired interaction. While some studies have raised the possibility that this interaction may not remain in the serum-containing environment *in vivo* (52), several other studies have demonstrated its usefulness *in vivo* (55, 56). Indeed, the desorption kinetics of the histidine-tagged protein from chelator lipids have been explored experimentally and shown to be governed by the valency of protein surface binding, where polyvalently bound proteins have a significantly longer desorption time than monovalently bound proteins. This valency can be regulated by incubation times of the his-tagged protein with the lipid platform (57). This effect of the incubation times can easily be taken into account in preparing surface modified liposomes using this non-covalent strategy.

CONCLUSION

The delivery of antigens and adjuvant on a liposomal delivery system requires various considerations. Notably, the behavior of the protein and the various interactions that will lead to desired response and also the potential of the liposome to serve as a suitable platform for attachment and delivery vehicle. We explore functionalization of liposomes with an adjuvant while considering the immunological relevance of the functionalization for both liposomes and the adjuvant.

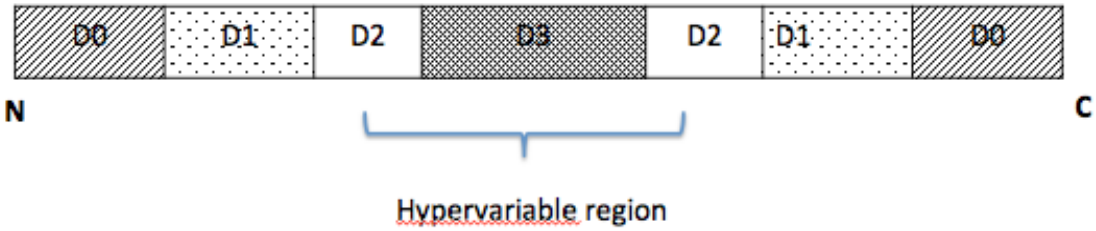

Figure 1.1 Flagellin domains and associated activity

D0 and D1 domains are conserved regions (D0 (IPAF) recognition site, D1: TLR5 recognition site) D2 and D3: Hypervariable region, recognized by antibodies

Name	Components	Receptor/pathway	Disease target
Alum*	Aluminum salts (aluminum hydroxide, aluminum phosphate)	NLRP3 uric acid, DNA	Diphtheria, tetanus, pneumococcus, HAV, HBV, anthrax, tick-borne encephalitis, MenC, MenB, HPV
MF59*, AS03*, AF03, SE	Oil-in-water emulsion squalene oil plus surfactants	MyD88, ASC ATP	Seasonal and pandemic influenza
Virosomes*	Liposomes plus influenza HA	Unknown	HAV
AS04*	Alum, MPL	TLR4	HBV, HPV
RC-529*	Alum, TLR4 agonist	TLR4	HBV
Imiquimod	Small molecule Imidazoqinoline	TLR7	Cancer
CpG	Synthetic DNA alone or formulated with Alum	TLR9	HBV, malaria, influenza, anthrax, cancer
Poly ICLC	Synthetic double strand RNA	TLR3, MDA5	Cancer, HIV
Flagellin	Linked to HA	TLR5	Influenza
AS01	Liposomes, MPL, QS21	TLR4	Malaria
AS02	Oil-in-water emulsions, MPL, QS21	TLR4	Malaria, TB, cancer
AS15	Liposomes, MPL, CpG, QS21	TLR4 and TLR9	Cancer
Iscomatrix	Saponins, cholesterol	Unknown	HCV, influenza, HPV, cancer
IC31	DNA, peptides	TLR9 agonist	TB
CAF01	Trehalose-dibehenate, cationic liposomes	C-type lectins Mincle and MCL	TB
GLA-SE	Oil-in-water emulsion, synthetic MPL	TLR4	Influenza
Montanide (ISA51, ISA720) IFA	Water-in-oil emulsion mineral oil, surfactants	Unknown	Malaria, HIV, cancer, influenza
CT, LT, LTK63	Bacterial toxins	GM1	Influenza (intranasal), ETEC (patch), cholera (oral)

ETEC, enterotoxigenic *Escherichia coli*; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; MenB, Meningococcal B, MenC, Meningococcal C; Mincle, macrophage inducible Ca2+-dependent (C-type) lectin; TB, tuberculosis. (* adjuvants in licensed vaccines) (29)

Table 1.1: Clinically tested human vaccine adjuvants

Several studies have explored the use of TLR agonists and adjuvants; some are currently being tested for human vaccines

	Cell type	Species	TLR5 mRNA	TLR5 protein	Respond to flagellin
Monocytes	Peripheral Monocytes	Human	Yes	Yes	Yes
	U1, U38 promonocytic cell lines	Human			Yes
	THP1 promonocytic cell line	Human			Yes
	Alveolar macrophages murine	Murine	Yes		
	Peritoneal macrophages	Murine	Yes/No		
	J774.1 macrophage cell line	Murine	Yes		
	HeNC2 macrophage cell line	Murine	Yes		
	GG2EE macrophage cell line	Murine			Yes
	10ScNCr/23 macrophage line	Murine			Yes
	RAW264.7 macrophage line	Murine	No		Yes*
Dendritic cells	Langerhans cells	Human			Yes
	Primary CD4+ blood DC	Human			Yes
	Monocyte-derived DC	Human	Yes		Yes
	Peripheral plasmatoid DC	Human	No		
	Splenic DC	Murine	Yes		No
	Bone marrow derived DC	Murine	Yes		Yes
	D2SC/1 splenic DC line	Murine	Yes		Yes
	FSDC fetal skin DC cell line	Murine	Yes		

Table 1.2: Expression of TLR5 and responsiveness to flagellin in monocytes and dendritic cells

TLR5 shows variable expression across cell types and cell lines, adapted from (34)

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CHAPTER 2 Characterization of flagellin-functionalized liposomes as a vaccine carrier and adjuvant

SUMMARY

Since the recognition that the adjuvant capacity of flagellin is better harnessed when both flagellin and the antigen is delivered to the same cell, there has been a need to exploit flagellin in ways that fulfill this constraint. We propose a liposomal delivery system functionalized with *Salmonella typhimurium* flagellin (fliC) as a way to meet this need to engage both the innate and adaptive immune response. Our goal is to characterize flic-functionalized liposomes as a vaccine adjuvant and evaluate their ability to simultaneously target cells expressing TLR5 to ultimately enhance vaccine potential of a liposome-encapsulated antigen. We report that fliC-functionalized liposomes are able to elicit the proinflammatory cytokine IL-6 with comparable efficacy to soluble antigen in an alveolar macrophage cell line (MH-S), and the ability of the cells to respond to the liposomes is mediated by TLR5. The functionalized liposomes exhibit preferential cell-association with MH-S cells. *In vivo*, the functionalized liposomes are able to enhance antigen-specific $CD8^+$T cell response and antigen-specific IgG1 response.

INTRODUCTION

Many successful vaccines seem to exhibit intrinsic adjuvant activity from motifs that are known to stimulate the Toll-like receptors and other pathways of the innate immune system (1). These motifs include lipopolysaccharide, a TLR4 ligand used in the typhoid vaccine, and the TLR7 and TLR8 ligand, single-stranded RNA, in the inactivated polio

vaccine (2). TLR agonists have been identified and studied as vaccine adjuvants even though they have been present in vaccine formulations, albeit inadvertently. Toll-like receptors play an important role in the assessment of pathogen at the cell surface and act as mediators between the innate and adaptive immune system by (a) stimulating signaling pathways that result in the induction of inflammatory cytokines, and (b) triggering dendritic cell maturation to direct adaptive immune responses to antigens(3). *In vitro* studies have shown that TLR agonists are able to directly activate dendritic cells to stimulate T cell activation and differentiation (4-6), suggesting that the quality of the immune response induced against a particular pathogen may depend on engaging the specific pattern recognition receptors (PRRs) expressed by cells at the infection site (5).

As the only TLR known to recognize a protein ligand, TLR5 is of interest in this study for the ease with which its ligand may be manipulated for the study of vaccine adjuvants. TLR5 is expressed on dendritic cells, epithelial cells, and monocytes (7). Its ligand flagellin, a 55 KDa protein, is known to prime the innate immune system in response to flagellated bacteria. As one of the best-characterized PRRs, the signaling cascade generated downstream of a TLR5/ligand interaction has been identified as an activation that mobilizes NFκB and stimulates proinflammatory cytokine production (8, 9). Studies have shown that the C- and N-terminal domains of flagellin are required for interaction with TLR5 (10, 11). Signaling through this adapter protein leads to the activation of type 1 interferons (α and β) required to control viral infections and production of proinflammatory cytokines such as tumor necrosis factor α (5) Other than directed DC activation, the adjuvant activity of flagellin is thought to be due to a number of other processes such as generalized recruitment of T and B lymphocytes to secondary lymphoid

sites, direct activation of T lymphocytes, and the induction of proinflammatory cytokines and chemokines (12). The flagellin from *Salmonella typhimurium* has been widely used in studies of flagellin's adjuvant activity since flagellin from *Salmonella* strains is generally one of the most potent inducers (13). When flagellin-expressing cells were co-administered with a plasmid that encodes for ovalbumin (OVA), it led to an enhanced humoral and cell-mediated antigen-specific response after two DNA boosts (9). A main limitation to the co-administration approach is that co-administration does not guarantee that the same cells that take up the plasmid are primed. Although some studies of flagellin co-administered with antigen report enhanced responses, studies utilizing a fusion protein of an antigen and flagellin in comparison to the former approach suggest a greater benefit. Additional studies show that priming the same cells that receive the antigen may actually be necessary to stimulate any response (12, 14).

Other studies of flagellin as an adjuvant in the context of flagellin-antigen fusion proteins have demonstrated $CD8^+$ T cell and $CD4^+$ T cell adjuvant effects (15-17). The usefulness of flagellin as an adjuvant for specific antigens can be restricted by limitations arising from their fusion proteins, the context in which flagellin has mainly been recognized for enhanced adjuvant properties. While antigen-flagellin fusion proteins, particularly antigens inserted in the hypervariable region, have demonstrated adjuvant ability, there are limitations specific to the antigen used, such as difficulty of producing the chimeric protein or inability to generate antibodies recognizing the native antigen (12). In spite of flagellin's general flexibility in creating antigen–fusion proteins, this presents a quintessential drug delivery challenge to harness the adjuvant capacity of flagellin in such cases.

The liposomal approach offers the benefit of being a vehicle to deliver and protect its cargo from protease degradation (18). As a delivery platform, liposomes have been used in vaccine delivery systems due to their general lack of immunogenicity, ability to encapsulate antigens to enhance cell uptake, and capacity to be functionalized on the surface (19). A study demonstrated that liposomes engrafted with flagellin-related peptides (two peptides from the conserved N terminal regions of *Salmonella typhimurium* flagellin) enhanced liposomal binding to dendritic cells and further induced their maturation. Vaccination of mice with the engrafted liposomes containing ovalbumin (OVA) yielded an OVA-specific T cell response and an increased number of $CD8^+$ T cells producing IFNγ (20) without contribution from the C motif. Liposomes engrafted with peptides from the C motif exhibited little binding to TLR5 (20). This study made a number of important observations, but did not expressly include portions of flagellin that may function independently of TLR5. The evidence that points to a TLR5 independent enhancement of antigen-specific $CD8^+$ T cell response, underscores the role of TLR5 independent processes in the adjuvant capacity of flagellin.

Considering that preexisting immunity to flagellin does not appear to result in immune suppression upon subsequent immunizations (21-23), it is worth examining flagellin as a full protein in the context of a liposomal delivery system to evaluate the adjuvant capacity of the full protein in both TLR5-dependent and independent pathways. In view of the established role of flagellin as an adjuvant for both innate and adaptive immunity and the need for simultaneous delivery to APCs, we sought to evaluate the role of a liposomal carrier functionalized with the full flagellin protein as both an innate and adaptive immunopotentiator. In the current study, the effect of the flagellin on properties

of the liposomes, its ability to target the antigen presenting cells, and the resulting immune responses are examined. We hypothesize that this functionalization serves multiple purposes. Firstly, expression of the ligand on the surface of the delivery vehicle allows for interaction of flagellin peptides with TLR5, an interaction found to be necessary for the desired proinflammatory immune response (10, 13). Secondly, to perhaps preferentially target TLR5-expressing cells while enhancing the delivery of flagellin to the cytosol of cells primed with an antigen to achieve enhanced adaptive immunity. Finally, to immunupotientiate a liposome-encapsulated antigen delivered to APCs in the style of flagellin fusion proteins.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

BL21 (DE3) RIPL were used as host strains for expression of the recombinant proteins fliC–his and YFP-his, both with a C terminal hexahistidine tag. The *E. coli* strains carrying pET28a plasmids with FliC used in these studies was a gift from Dr. Russell Vance (University of California, Berkeley, CA) and the construct for the YFP citrine variant was a gift from Dr. Joel Swanson (University of Michigan, Ann Arbor, MI). The adenine nucleotide was inserted into the sequence via quik-change site-directed mutagenesis to orient the hexahistidine tag with the reading frame. The cells were transformed with DNA of fliC-his in pET28a and YFP-his in pET29b. Cells were grown in 37 ° C in TB broth. Protein expression was induced with 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 30 ° C and grown for another 6 hours. Cells were centrifuged at 4000xg for 30 min at 4 ° C. Cell pellets were store at -80 ° C till use. The pellets are suspended in wash buffer (50mM

sodium phosphate dibasic, 300mM sodium chloride, and 20mM imidazole) containing 1mg/ml lysozyme and 1mM phenylmethylsulfonyl fluoride (PMSF) incubated for 30 minutes on ice.

Purification of fliC: After centrifugation at 20000xg for 30 minutes, the pellet was homogenized in 1M urea and 1% triton twice, followed by two washes in buffer containing 1mM PMSF. The wash steps were performed between centrifugation steps at 20000xg at 4 ° C for 30 minutes. The pellet was homogenized in 6M guanidine hydrochloride containing 1mM PMSF and centrifuged at 100000 x g at 4 ° C for 1 hour. The supernatant was applied to Ni-NTA agarose resin (Qiagen, MD) and incubated for 2 hours at 4 ° C. Bound his-tagged protein was eluted from a Ni-resin column against 400mM imidazole and dialyzed in 1x PBS pH 6.4 for 16 -20 hours.

Purification of Yellow Fluorescent protein: YFP-his pellet in 1mg/ml lysozyme was lysed using a Digital Sonifier Cell disruptor (Emerson, Danbury, CT) eight times at 50% amplitude with a 30 second interval of sonication and 30 second incubation on ice. The lysate was centrifuged at 12000 x g for 1 hour at 4 ° C and the supernatant incubated with the Ni-NTA agarose resin (Qiagen, MD) for 2 hours on a shaker at 4 ° C. Bound his-tagged protein was eluted from a Ni-resin column against 400mM imidazole and dialyzed in 1x PBS, pH 8.4, for 16-20 hours.. Protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein purity was evaluated using SDS gel electrophoresis and visualized using Krypton fluorescent stain (Thermo Scientific) per the manufacturer's instructions and a Typhoon 9200 imager (GE Healthcare).

Removal and quantification of endotoxin

The resulting protein was purified of endotoxin using a polymyxin B column (Pierce, Rockford IL) and further clarified of DNA and viral particles using an acrodisc Mustang Q filter (Pall, Ann Arbor MI). Endotoxin was quantified using the Limulus Amebocyte Assay (Associates of Cape Cod, MA). The endotoxin content was determined to be < 0.05EU/μg protein.

Preparation of liposomes and protein-coated liposomes

The lipids 18:1 (Δ9-Cis) PE (DOPE) 1,2-dioleoyl-*sn*glycero-3 phosphoethanolamine, cholesteryl hemisuccinate (CHEMS), 18:1 DGS-NTA(Ni) 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)(Ni-NTA) were mixed at a 2:1:0.02 ratio and dried down on a rotary evaporator and afterwards stored under vacuum for 12-16 hours. The dried lipid films were suspended in HEPES-buffered saline (HBS) (10mM HEPES, 140mM NaCl, pH 8.4) for subsequent protein coating. For liposomes encapsulating OVA, OVA was encapsulated at a concentration of 20mg/ml. Fluorescent liposomes for cell uptake were made using 18:1 (Δ9-Cis) PC (DOPC) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, cholesterol, Ni-NTA lipid and 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine (Oregon Green® 488 DHPE) at 2:1:0.02:0.01 ratios. All aforementioned lipids were acquired from Avanti Lipids, Alabaster, AL except for Oregon Green DHPE from Life technologies, Gaithersburg, MD. The resulting lipid films where re-suspended in HBS, passed through 4 freeze/thaw cycles, and sonified in a bath sonicator 4-5 times in 1-minute cycles. To coat with protein, the resulting liposomes were incubated with protein at a 20:1 Ni lipid to protein molar ratio for 1-2 hours at 4 ° C (with the exception of studies varying the Ni-NTA to fliC molar ratios, the Ni-NTA: fliC used in other

studies was 20:1). The free protein was purified from the liposomes via size exclusion chromatography on a CL4B (GE Healthcare) column and the eluted liposomes were evaluated by SDS gel electrophoresis to confirm the presence of protein and quantify the amount of protein on the liposomes. Liposome diameter was determined using dynamic light scattering on a Zetasizer instrument (Malvern, Westborough, MA). Phospholipid content of liposomes was quantified using Bartlett's method of phosphate quantification (24).

Trypsinization of functionalized liposomes

Functionalized liposomes that were subsequently stripped of the protein coating were prepared by applying the liposomes to trypsin TPCK immobilized on agarose beads (Thermo Sci.) and incubated at 37° C for 8 hours according to manufacturer's instructions.

Membrane leakage and pH sensitivity assay

PE: CHEMS: Ni lipid films were formed as previously described and rehydrated in 8-hydroxypyrene-1, 3,6-trisulfonic acid (HPTS) and p-xylene-bis-pyridinium bromide (DPX) (35mM HPTS, 50mM DPX, 20mM HEPES, 0.5M NaOH, 290mOsm/kg). After four freeze-thaw cycles, the rehydrated lipid film was sonicated in a bath sonifier four times in 1-minute pulse and rest cycles. The unencapsulated HPTS/DPX were separated from encapsulated HPTS/DPX on a Sephadex G-50 (GE health care) gel filtration column and phospholipid content quantified before protein functionalization at different Ni-NTA to protein ratios. Unencapsulated HPTS fluorescence associated with 10nmols of lipid in HBS pH 8.4 was determined using kinetic measurements every 10 minutes on a Synergy plate reader (BioTek, Winooski, VT) using the filters passing light at the excitation and emission

wavelengths of 400(30) nm and 508(20nm), respectively. Triton X-100 was added at a final concentration of 2% to lyse the liposomes and determine total fluorescence.

To evaluate pH sensitivity, the liposomes were incubated in pH 5.5 (MES buffer) and HBS, pH 7.4, and the fluorescence of encapsulated HPTS was determined as previously described in the membrane leakage assay.

Cell lines and Tissue culture

Tissue culture media was purchased from Invitrogen (Carlsbad, CA) and all cells were maintained in a humidified incubator at 37° C and 5% CO_2, unless otherwise stated. MH-S cell line (ATCC, Manassas, VA) was maintained in ATCC modified RPMI 1640 (with 2mM L-glutamine, 25mM L-glucose, 1mM sodium pyruvate and 10mM HEPES) (Life technologies CA), supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100µg/ml streptomycin, 100u/ml penicillin, and a final concentration of 50mM β-mercaptoethanol.

Generation of bone marrow-derived macrophages

Bone marrow was harvested from femur and tibia of 7-8 week old C57BL/6 mice as described in Stier *et al.* (25). The bone marrow cells were differentiated into bone marrow-derived macrophages (BMM) in DMEM containing 20% heat-inactivated fetal bovine serum (Hi-FBS), 30% L-929 cell conditioned media containing macrophage stimulating factor, 100µg/ml streptomycin, 100u/ml penicillin, and 55µM β-mercaptoethanol. The cells were replenished with new medium on day 3, harvested on day 6 and stored in liquid nitrogen until use. Upon thawing, cells were maintained in DMEM (Invitrogen, CA), supplemented with 10% Hi-FBS, 100µg/ml streptomycin, 100u/ml penicillin and 2mM L-glutamine.

In vitro cytokine secretion analysis by ELISA

2x 10^5 MH-S cells and BMM from C57BL/6 mice were plated in 96-well plates in respective media 16-20 hours before liposome treatment. The cells were incubated with liposomes for 3 hours, the treatment discarded, and the cells incubated in their respective media for 21 h. Cell supernatants were collected and analyzed via ELISA for IL-6 (eBioscience, San Diego, CA) secretion according to the manufacturer's instructions.

Cell association and uptake study by flow cytometry

1x10^6 cells/ml of staining buffer (1xPBS with 10% Hi-FBS) were treated with fluorescent liposomes and dosed according to flagellin concentration. YFP coated and non-coated fluorescent PC: CHOL: Ni liposomes were matched by intensity and used as controls. Cells were treated with liposomes at either 4 ° C or 37 ° C for 1 hour. Control cells were treated with either 1000ng/ml anti-mTLR5 (Invivogen, CA) or 1000ng/ml rat IgG2a for isotype control (Invivogen, CA) at 37 ° C for 1 hour before treatment with liposomes. Cells were fixed with 2% paraformaldehyde, washed in ice-cold staining buffer, and centrifuged at 500xg at 4 ° C. The wash step was repeated twice before cell pellets were suspended in staining buffer for flow cytometry analysis on FACSCALIBUR (BD biosciences, San Jose, CA).

In vitro antigen presentation

In vitro antigen presentation was performed as previously described in (26). Briefly, 2 x 10^5 cells/well were plated in 96-well plates overnight before the day of the assay, cells were washed in serum-free media and treated with liposomes in serial dilutions with the highest concentration at 200μM phosphate in serum-free media for 2 hours. BMM were washed and incubated in complete DMEM for 3 hours. The cells were fixed in 1%

paraformaldehyde for 15min at 4° C. The paraformaldehyde solution was prepared in warm 1X PBS and dissolved by adding drops of 0.1M NaOH; the pH of the solution was adjusted to 7, and the solution filtered through a 0.45µm filter. The paraformaldehyde was quenched by 0.2M lysine in DMEM for 20 minutes at RT. The cells were washed in DMEM after quenching, and 2 x 10^5 cells/well of B3Z cells in B3Z media (RPMI 1640 supplemented with 2mM L-glutamine, 25mM L-glucose, 1mM sodium pyruvate and 25mM HEPES) were added per well and incubated for 15 hours at 37° C and 5% CO_2. The plates were centrifuged at 1500rpm for 5 minutes, media was carefully removed and the cells washed in RT 1X PBS and centrifuged again. The PBS supernatant was removed carefully. CPRG substrate (0.15 mmol/L chlorophenol red-β-D-galactopyranoside (Calbiochem), 9 mmol/L $MgCl_2$, and 0.125% NP40, and 100 mmol/L β-ME in PBS) was added to measure production of β-galactosidase by B3Z cells in response to BMM presentation of SIINFEKL in H-2K^b. The plates were incubated for 4 hours at 37°C and absorbance values were measured at 595nm using a spectrophotometer (BioTek, Winooski VT).

Mice and Immunization protocol

C57BL/6 mice, 8-10 weeks old (Jackson labs, Bar Harbor, ME) were used in this study and were handled according the University of Michigan Institutional Animal Care guidelines. Mice were immunized subcutaneously at the base of the tail with OVA-encapsulated liposomes (OVA) and fliC -functionalized OVA ((OVA)-fliC) containing 10µg OVA and 7-8µg fliC, on day 0 and day 10. Naïve mice were immunized with same volume of HEPES buffered saline pH 8.4 (HBS). Mice were euthanized on day 21 and blood was collected via cardiac puncture.

$CD8^+$ T cell tetramer staining

Seven days after immunization, blood was collected via the superficial temporal vein of immunized mice. The blood was collected in dipotassium EDTA coated microtainer tubes (BD, biosciences, Franklin, NJ). After blood collection, the cells were resuspended by gentle pipetting. Red blood cells were lysed with ACK lysis buffer (Life Technologies) twice. The cells were centrifuged at 1500 x g for 5 minutes at 4° C between each lysis. The cells were washed in FACS buffer (Ix PBS containing 1% BSA) and centrifuged at 1500 x g for 5 minutes at 4° C. The resulting pellet was stained for flow cytometry after CD16/32 Fc block (eBioscience, San Diego CA) and incubated for 10 minutes at room temperature (RT). A small volume of each sample was collected for FACS negative and single controls. Cells were incubated with T-select H2Kb – OVA-tetramer SIINFEKL PE (MBL, Japan) for 30 minutes and stained with anti-CD8, anti-CD44, and anti-CD62L. The incubation was followed by DAPI staining to discriminate live from dead cells. Cell fluorescence was evaluated via flow cytometry.

Measurement of Antibody titers

Blood was harvested from euthanized mice via cardiac puncture in microvette 500 centrifuge tubes (Sarstedt, Germany). The sera were isolated by centrifugation at 10000 x g for 5 minutes and analysed by ELISA or stored at -80° C until use. To determine serum antibody titers, briefly, Maxisorp Nunc immunoplates (eBioscience, San Diego, CA) were coated with 10µg/ml OVA (Sigma-Aldrich, St Louis, MO) in 0.1M sodium phosphate pH 9.0, coating buffer overnight (12-16 hrs). The plates were washed in PBST (1x Phosphate Buffered Saline, 0.05% Tween) and then blocked overnight at 4° C with PBST containing 1% BSA (blocking buffer). The plates were washed and incubated with serial dilutions of

sera in blocking buffer overnight at 4° C. OVA-specific biotinylated goat anti-mouse IgG1 and goat anti-mouse IgG2c (Southern Biotech, Birmingham, AL) were detected with Avidin-Horseradish Peroxidase (eBioscience, San Diego, CA) and finally TMB substrate (KPL Inc., Gaithersburg, MD). Plates were washed 5 times in PBST between each step. Conversion of substrate to colorimetric product was stopped by 2N sulfuric acid and absorbance at 450nm was determined. Data were fit to a 4-parameter curve on the Gen5 data analysis software, (Biotek, Winooski, VT) to determine titer, defined as the dilution factor that yields an absorbance of 0.5. The lowest dilution used was reported for samples below the limit of detection.

RESULTS

Liposomes functionalized with flagellin retain membrane integrity and maintain pH-sensitivity

In spite of the versatility of liposomes in drug delivery, it is essential to ensure that the surface functionalization does not alter its ability to serve as a delivery vehicle. To evaluate the effect of flagellin functionalization on the physicochemical properties of the nickel-containing liposomes, we evaluated the membrane integrity and the pH-sensitivity of these liposomes after functionalization. Liposomes were encapsulated with HPTS, a highly water-soluble membrane-impermeant pH indicator, and its cationic collisional quencher, DPX, and the membrane integrity was monitored by the change in HPTS fluorescence over time. As the content of the liposome leaks, DPX-mediated quenching of HPTS fluorescence is reduced due to DPX dilution into the surrounding buffer. The liposomes were functionalized at different Ni-NTA lipid: fliC molar ratios. The fluorescence

measurements from 10nM of liposomes show that the lower the Ni-NTA:fliC functionalization ratio, the higher the fluorescence observed in the liposomes, indicating a higher fraction of these liposomes leaked their content in comparison to liposomes coated at higher Ni-NTA:fliC ratios. However, the results are noteworthy for the stable fluorescence over time in the different functionalization ratios tested (Fig. 2.1A), which suggests that the functionalization process does not adversely affect the membrane integrity.

To evaluate the effect of functionalization of liposomes on the pH-sensitivity, uncoated liposomes and liposomes functionalized at a various Ni-NTA:fliC molar ratios were compared. All tested liposome samples contained the HPTS/DPX fluorescent dye, and fluorescence in pH 5.5 buffer mimicking endosomal pH conditions was compared to buffer at physiologic pH 7.4. Both coated and uncoated liposomes released little HPTS (<10%) in pH 7.4 buffer, however, rapid HPTS release occurred within ten minutes of adding both the uncoated and coated liposomes to pH 5.5 buffer (Fig. 2.1B). Addition of liposomes to pH 4.5 buffer yielded >90% release of both functionalized and uncoated liposomes. While functionalization of the liposomes may reduce the pH sensitivity in comparison to uncoated liposomes, liposomes remain structurally stable and remain responsive to low pH upon functionalization with fliC.

MH-S cells respond to flagellin

The stimulation of TLR5 by flagellin initiates a signaling cascade that leads to NFκ B activation and subsequent secretion of proinflammatory cytokines such as IL-6 and TNFα. The variability of expression of TLR5 on different cell-types is documented in the literature (27, 28). Alveolar macrophages have been identified as a macrophage type expressing

TLR5 based on proinflammatory cytokine responsiveness (29, 30). We therefore sought to evaluate the alveolar macrophages cell line, MH-S, as a possible cell type to evaluate the effects of flagellin on macrophages using the secretion of IL-6 as a marker of flagellin response (Fig. 2.2B). To confirm that the proinflammatory IL-6 secretion observed was due to a protein-specific signal, and not endotoxin content of the protein, we compared recombinant flic (Fig. 2.2A) to equimolar concentrations of recombinant YFP, which was purified from *E. Coli.* and purified of endotoxin as was fliC. To ensure the cytokine secretion capability of the cells tested, equimolar concentrations of LPS were tested in comparison. Both BMM and MH-S are able to secrete IL-6 after exposure to 18nM LPS. In contrast, neither cell type was able to exhibit IL-6 secretion in response to 18nM soluble YFP, confirming that both flagellin and YFP do not contain detectable endotoxin, the response to which could have been mitigated by TLR4. MH-S cells secreted significant amounts of IL-6 in response to flagellin while unresponsive to YFP treatment. In our studies, BMM were unresponsive to flagellin in terms of IL-6 secretion, in agreement with previous studies that suggest that BMM do not express TLR5. Taken together, these results confirm the flagellin-specific response of the alveolar macrophage cell-line and therefore its utility in evaluating the flagellin response in macrophages.

Functionalized liposomes stimulate proinflammatory cytokine response in a protein-specific manner

Given the response of MH-S cells to flagellin, we evaluated the effect of the flagellin-functionalized liposomes on MH-S cells. Functionalized liposomes exhibited a similar response to soluble flagellin over the 10μg/ml-10ng/ml range (Fig. 2.2C). To ascertain that the IL-6 response was due to the attached protein, we evaluated the response after

digestion with trypsin immobilized on agarose beads. SDS-PAGE staining revealed a reduction of at least 82% in fliC content of functionalized liposomes after trypsin treatment (data not shown). The cytokine production as a response to functionalized liposomes was significantly diminished after cells were treated with functionalized liposomes that had been treated with trypsin (Fig. 2.3A). The result is consistent with the diminished response seen when MH-S cells are incubated with trypsin-digested soluble recombinant flagellin, confirming that the response to the liposomes is due to the attached protein on the surface of the liposomes. Response to YFP was unchanged regardless of trypsin digestion, an indication that the flagellin is responding as a result of a specific interaction on MH-S cells.

Proinflammatory response to functionalized liposomes is mediated by TLR5

To evaluate the specificity of functionalized liposome response, we needed to determine if the IL-6 response seen in MH-S cells could be attributed to the flagellin interaction with cell-surface TLR-5. We treated MH-S and BMM with mouse TLR5 neutralizing antibody and assayed IL-6 cytokine secretion in response to the liposomes. There was an anti-TLR5 concentration-dependent reduction in IL-6 response to flagellin in MH-S cells (Fig. 2.3B), but not in BMM (data not shown). Treatment of anti-mTLR5 inhibited cells with equimolar doses of soluble YFP or YFP-functionalized liposomes did not lead to changes in the minimal IL-6 response secreted, indicating that response or lack of response to YFP-his is not TLR5 mediated.

FliC density on liposomes can be regulated by varying the Ni-lipid content to fliC ratio

To evaluate the possibility that the protein density on the liposomes had an effect on the proinflammatory response from alveolar macrophages, we tested 1μg/ml of liposomes

functionalized at varying Ni-NTA lipid to fliC ratios, ranging from 20:1 to 100:1. After functionalization, the number of molecules per liposome was determined. The number of liposomes in solution was calculated as

$N_{lipo} = ((M_{lipid} \times N_A)/N_{tot} \times 1000))$

where $N_{tot} = 17.69 \times [(d/2)^2 + ((d/2)-h^2)]$ in which d = diameter of the liposomes in nm and h= thickness of the bilayer, estimated to be about 5nm. M_{lipid} is molar concentration of lipid, N_A is Avogadro's number, and N_{tot} is number of lipid molecules in unilamellar liposome (31). FliC coating density decreased with decreasing Ni-NTA lipid to fliC ratio: for liposomes in the 140nm range, 200 fliC molecules per liposome in the 20:1 ratio to about 20 fliC molecules per liposome in the 100:1 functionalization ratio (Fig. 2.2D). This underscores the range of achievable coating densities while keeping the Ni-NTA lipid concentration constant. The proinflammatory response was comparable in the 20-75:1 ratio, an indication that available TLR5 receptors may be saturated with fliC at this dose. However, at the same dose, the response elicited by the 100:1 functionalization ratio was significantly reduced when compared to the 20:1 ratio. The non-functionalized control groups for each treatment group did not elicit any measurable response from the MH-S cells (data not shown), an indication that the corresponding increase or decrease in the lipid amount presented to the cells was not responsible for the response observed. Our data showed that at various incubation ratios, the density of flagellin molecules/liposome could be achieved using a single Ni lipid concentration. This is important for keeping the Ni lipid content low and still achieve variable coating density since the biological effect of Ni-NTA presence is not well studied, hence a desire for inclusion of a minimal amount in vaccine formulations (32). The results demonstrate that the functionalization process can

be used to control the protein density on the liposomes and could serve as a method of fine-tuning the innate immune response.

Non-specific flagellin functionalization of liposomes yield proinflammatory response

To test the effect of the specific his-tagged interaction with liposomes containing the Ni-chelated phospholipid head group, liposomes lacking in the Ni-NTA phospholipid were compared to NI-containing liposomes. At a 20:1 (Ni-NTA) to flagellin molar ratio, the protein is attached to the liposomes with similar coating efficiency. Liposomes without phospholipid to mediate a specific protein interaction are able to elicit IL-6 response of similar magnitude as liposomes bearing flagellin via the specific Ni-6-his interaction over a dose range of 1μg/ml-1ng/ml. While the proinflammatory response is similar to that seen in liposomes functionalized via the specific interaction, the possibility that dissociated protein from the liposome is causing the interaction, as would be in cells tested with soluble flagellin, is not tested. Given the poorer membrane integrity of these liposomes over time (data not shown) these liposomes were not used in further studies.

Functionalized OVA liposomes preferentially associated with flagellin-responsive cells

To evaluate the ability of the liposomal-anchored flagellin to preferentially target its receptor, we tested the association of liposomes containing phospholipids with fluorescently labeled head group to MH-S and BMM. Given the inability of BMM to produce IL-6 in a TLR-5-mediated fashion, the cells were treated as a control. To eliminate the risk of non-specific interaction mediated by negatively charged CHEMS in both functionalized and uncoated liposomes, we used the neutral DOPC:cholesterol-based liposomes to eliminate indiscriminate binding and discern actual differences in specific interactions

occurring with the cells. The fluorescent liposomes exhibit fluorescence quenching when coated with proteins. As such, doses of the control liposomes were determined by matching the fluorescence intensity of the flagellin-functionalized liposomes with uncoated and YFP-coated liposomes of corresponding intensity. Uncoated liposomes were used as fluorescent intensity-matched control and YFP coated liposomes were also intensity matched as a protein-coated control. BMM and MH-S cells were treated with 1μg/ml of flagellin functionalized liposomes dosed according to flagellin. MH-S cells demonstrated increased cell association with fliC-functionalized liposomes in contrast with the BMM that do not exhibit any fliC-influenced cell-association (Fig. 2.4). To ensure that the cell-specific association is mediated by binding to TLR5, we attempted to test the effect of anti-TLR5 treatment on the ability of liposomes to associate with the cells. This, however, showed no effect. The commercially acquired anti mouse TLR5 is described as a TLR5-neutralizing antibody and most commercially available antibodies are raised against the cytoplasmic intracellular domains of TLR5, and of no use in binding the TLR5 receptor.

Flagellin functionalized liposomes activate OVA-specific $CD8^+$ T cells

We sought to evaluate the implication of our observations on the adaptive immune response *in vivo*. Mice were immunized subcutaneously ten days apart according to a prime/boost regimen with liposomal OVA (OVA) and fliC-functionalized OVA liposome ((OVA)-fliC) at 10ug OVA. OVA-specific $CD8^+$ T cell response was analyzed by direct tetramer staining of OVA-specific $CD8^+$ T cells in peripheral blood mononuclear cells 7 days after immunization to determine the strength of $CD8^+$ T cell response. Mice immunized with functionalized liposomes exhibited a moderate but significant increase in the frequency of circulating OVA-specific $CD8^+$T cells (Fig. 2.6A). This effect was discovered to

be protein-specific as this enhancement holds true when compared to mice immunized with YFP-functionalized liposomes (data not shown).

FliC-functionalized liposomes enhance OVA-specific Th2-mediated antibody isotype, IgG1 production

Several studies have demonstrated that soluble flagellin overwhelmingly induces Th2 type responses. To evaluate the adjuvant capabilities of the flagellin-functionalized liposomes as a delivery system, we tested whether mice immunized with functionalized OVA liposomes could develop an OVA-specific antibody response. Eleven days after the booster immunization, sera from mice immunized with fliC-functionalized liposomes exhibited significantly enhanced OVA-specific titers of IgG1 (Figure 2.6B). In contrast, the immunized mice generated low levels of IgG2c anti–OVA titers, and the addition of fliC to OVA liposomes did not enhance the low response observed (data not shown).

Functionalized liposomes enhance MHC I specific antigen presentation *in vitro*

To understand the $CD8^+$ T cell enhancement mediated by flagellin, we wanted to see if the response could be seen *in vitro* and explained by antigen presentation. To this effect, we cultured BMM with OVA-containing pH-sensitive liposomes with and without functionalization with fliC. We determined whether the treated macrophages expressed the SIINFEKL-MHC-complex recognizable by B3Z cells, a lacZ-inducible $CD8^+$ T cell hybridoma cell line specific for $OVA_{257-264}$ (SIINFEKL) presented on the murine H-$2K^b$ MHC class I molecule. We found that (OVA)-fliC liposome-treated cells show enhanced conversion of the β-galactosidase substrate, CPRG, to chlorophenol red as a surrogate for B3Z β-galactosidase production. The measured absorbance of chlorophenol red at 595nm was

significantly enhanced ($p<0.005$) in functionalized liposomes in comparison to liposomal OVA (Fig. 2.7), though the amounts of OVA were comparable. This ability of fliC-functionalized liposomes to present SIINFEKL-MHC complex to B3Z cells is independent of known cytosolic pathways because flic is unable to activate caspase-1 and IL-1β independently of any cytosolic delivery mediator (data not shown). However, the functionalized liposomes enhance MHC class I –specific antigen presentation. In addition, considering BMM presumably lacks TLR5, these data reaffirm reports that neither TLR5 nor IPAF is required for flagellin-mediated $CD8^+$ T cell effect against an accompanying antigen.

DISCUSSION

In comparison to coadministration of antigen and flagellin, colocalized delivery of flagellin and antigen has been observed to offer superior adjuvant properties, meanwhile some fusions have been observed to either be intractable to purification or the protective antibodies generated towards them do not recognize the native antigens(12). Having previously explored the various functional capacities of liposomes, including delivery of adjuvants (26), we studied liposomes as a platform to enable the simultaneous delivery of flagellin using ovalbumin as an antigen in a multifunctional delivery system. We monitored the ability of fliC-functionalized liposomes to retain their carrier ability and the ability of fliC attached to the liposomes to mediate innate and adaptive immune response *in vitro* and *in vivo*. We observed that fliC-functionalized liposomes (a) retain the chemical and physical characteristics of the liposomes in terms of pH sensitivity and membrane integrity, respectively, (b) are able to induce fliC-mediated proinflammatory cytokine response in a

TLR5-specific fashion, (c) are able to preferentially associate with fliC-responsive cells, (d) and are able to enhance OVA-specific $CD8^+$ T cells and mediated OVA-specific IgG1 production.

Several studies suggest that both humoral and $CD8^+$T cell adjuvant properties of flagellin are independent of TLR5 (33, 34). We anchored the full soluble protein on the liposomes to harness both the TLR5 dependent- and independent-potential of flagellin on a multifunctional delivery platform. While the concept of multifunctional liposomes is not new, surface modification of liposomes introduces the risk of compromising the bilayer integrity and other properties of the liposomes. We had previously studied the effect of different methods of protein coating on the performance of liposomes including using the affinity his-tag of YFP-his and a Ni-chelating lipid in the liposomal membrane (35). These studies revealed that coating the liposome via this non-covalent method did not destabilize the membrane and did not alter the serum stability of the liposomes. In the case of pH-sensitive liposomes, the protein coating did not significantly weaken the pH-sensitivity. In the present study, fliC-functionalized liposomes were found to retain the membrane integrity and pH sensitivity of the uncoated liposomes revealing that fliC-functionalized liposomes are reliable carriers of encapsulated cargo.

The induction of chemokines and cytokines is a key step in the adjuvant effect of flagellin and this induction is mediated by TLR5. TLR5-mediated signaling induces activation of NF-κB, which regulates the expression of proinflammatory cytokines such as IL-6, however the priming of other TLRs can result in this proinflammatory cytokine secretion as well. It has been proposed that the presence of endotoxin in flagellin preparations across studies may be responsible for the disparities in the flagellin effect on

murine dendritic cells and its precursors reported in the literature (12). As a recombinant protein purified form *E. coli*, we eliminated the potential of endotoxin or nucleic acid priming of other TLRs to isolate the effect of flic in functionalized liposomes. We observed that the murine alveolar–derived macrophage cell line secrets proinflammatory IL-6 to fliC and fliC-functionalized liposomes in a protein- and TLR5-specific fashion, in contrast to BMM that serve as a control. This observation demonstrated the comparable ability of functionalized liposomes to fliC to induce the innate immune response over a wide dose range, showing that the attachment of liposome to the C terminal hexahistidine fliC had no effect on its interaction with or recognition by TLR5. This flexibility has been noted in many studies of immunopotentiating flagellin-antigen fusion proteins that have been created as N or C terminal fusions or by insertion into the hypervariable region of flagellin (12).

Targeting to enhance drug delivery to a select group of cells, either cancer cells or antigen presenting cells, has long been one of the main aims of modifying liposomes at the surface(19). For example, the HER2–target liposomes are in clinical trials to deliver doxorubicin to HER-2 positive cells after seeing increase in HER-2 positive cell binding and internalization of the anti-Her2 liposomes (36). Relatedly, we observed that fliC-functionalized liposomes showed a significant preferential association to the fliC-responsive MH-S cells and not in fliC-unresponsive BMM, further supporting the hypothesis that BMM do not express TLR5. We did not confirm that TLR5 mediated the interactions of the fliC-functionalized liposomes, which we would have been able to determine if the increased cell association could be reversed by addition of free anti-TLR5, but most commercially available anti-TLR5 antibodies are raised against cytoplasmic domains of the receptor. However, this interaction was specific to fliC in comparison to interactions with

liposomes coated with YFP under similar functionalization conditions, as was seen in proinflammatory cytokine secretion.

Previous reports have associated the $CD8^+$ T cell adjuvant ability of flagellin with the ability of fusion proteins to prime APCs with both the adjuvant and the antigen. For example, Cuadros *et al.* showed that flagellin-EGFP fusion protein developed EGFP-specific T cell responses in BALB/c mice immunized with the fusion protein or adenovirus-EGFP, but not EGFP alone (16). Another study showed that recombinant flagellin (fljB, the phase variant to fliC in *Salmonella typhimurium*) and OVA fusion was superior to the same doses of fljB administered with OVA in terms of antigen-specific antibody response and protective CD8 T^+ cell response (14). Our observations of fliC-mediated enhanced antigen-specific $CD8^+$ T cell response is consistent with our hypothesis that the fliC-functionalized liposomes can exploit the adjuvant ability of flagellin in the context of antigen co-localized delivery. Given the cell association data, a possible explanation is that this is mediated by increased internalization through TLR5 *in vivo,* where the functionalized liposomes selectively target TLR5-expressing APCs, which in turn selectively present OVA to $CD8^+$ T cells. Our findings *in vitro* show that this explanation is deficient. Indeed, when fliC-functionalized liposomes encapsulating OVA are exposed to BMM, liposomes are able to enhance MHC class I-specific antigen presentation. We propose this ability is independent of TLR5-mediated enhanced antigen delivery because these macrophages are presumed to lack TLR5, seen as both inabilities to elicit proinflammatory cytokine to fliC and to engender preferential association to functionalized liposomes. The recognition that the theory of TLR5-mediated enhanced internalization is inadequate has also been raised in the literature. There have been reports of liposome-mediated cross presentation (37),

however, in comparison to OVA liposomes, we were able to determine that the enhanced $CD8^+$ T cell presentation is mediated by fliC. Bates *et al.* found that flagellin fusion proteins promoted antigen-specific $CD8^+$ T cell response independently of TLR5 and its signaling adaptor protein MyD88. In their studies, flagellin OVA fusion proteins were able to promote OVA-specific $CD8^+$ T cell response in $TLR5^{-/-}$ and $MyD88^{-/-}$ mice. In addition, *in vitro,* both WT and $TLR5^{-/-}$ APCs were able to elicit significant OVA-specific $CD8^+$T cell response that was significantly diminished by the proteasome inhibitor, lactacystin, but not the lysosomal acidification inhibitor choloroquine, suggesting the $CD8^+$ T cell response involves proteasome-mediated antigen processing (33). Endogenous antigens are subject to proteasome processing and the derived peptides are routed to the classical MHC class I pathway, where then will end up on MHC class I to be recognized by $CD8^+$ T cells on the cell membrane. Considering the MHC class I-specific OVA peptide, SIINFEKL, was presented, our theory was that by virtue of the fliC being anchored on the liposome, if OVA were delivered in the cytosol, so would fliC, therefore we used markers of fliC in the cytosol, IL-1β and active caspase-1, as surrogates for cytosolic delivery of OVA. From our observations, the $CD8^+$ T cell response is also independent of the flic-functionalized liposomes' ability to enhance cytosolic delivery, since functionalized liposomes are unable to elicit Il-1β secretion in BMM, even after priming by LPS, and also unable to activate caspase -1(data not shown). Outside the classical MHC class I and MHC class II antigen processing pathway, cross presentation has been recognized as a process that enables antigens derived from extracellular sources, such as liposomal antigens, (38) to be presented to $CD8^+$ T lymphocytes via MHC class I (39-41). While the mechanisms of cross presentation have not yet been fully defined, proposed models of cross presentation include, simply, a cytosolic

pathway where exogenous antigens gain access to the cytosol and are sensitive to proteasome inhibition or a vacuolar pathway where antigen processing onto MHC class I occurs in the endocytic compartment and sensitive to inhibition of lysosomal proteolysis (41). The source of MHC class I molecules in these models remains elusive (39, 41, 42). The finding by Bates *et al*., which eliminates the likelihood of an endosomal model of cross presentation, does not account for the presence of flagellin fusion proteins in the cytosol, which would precede proteasome degradation. We did not probe the mechanism with which the fliC-functionalized liposomes enhance $CD8^+$ T cell response, but the lack of cytosolic fliC markers and published evidence of fliC-enhanced proteasome-mediated processing may be a reflection of the classic conundrum of the mechanism of cross-presentation. Additionally, TLR agonists have been reported to induce cross presentation in dendritic cells. While flagellin was not reported to be one (43) it is worth noting that it was studied by coadministering the OVA and flagellin (44). Nevertheless, our results demonstrating that functionalized liposomes are able to enhance $CD8^+$ T cell response aligns with reports of flagellin co-delivered with antigen as fusion proteins enhancing CD8 T^+ cell response.

Furthermore, the benefits realized from the codelivery of antigen and adjuvant has not only been realized in the form of $CD8^+$ T cell responses. Fischer *et al.* reported this phenomenon in the context of other adjuvants and antigen combination in a nickel-containing nanolipoprotein delivery platform. In their study, they found that incorporating MPLA and the his-tagged recombinant influenza hemagluttinin 5 (H5) and CpG with his-tagged LcrV of *Yersinia pestis* on Ni-chelating nanodiscs enhanced IgG production *in vivo* relative to coadministered formulations and non-adjuvanted nanodiscs (45). We observed

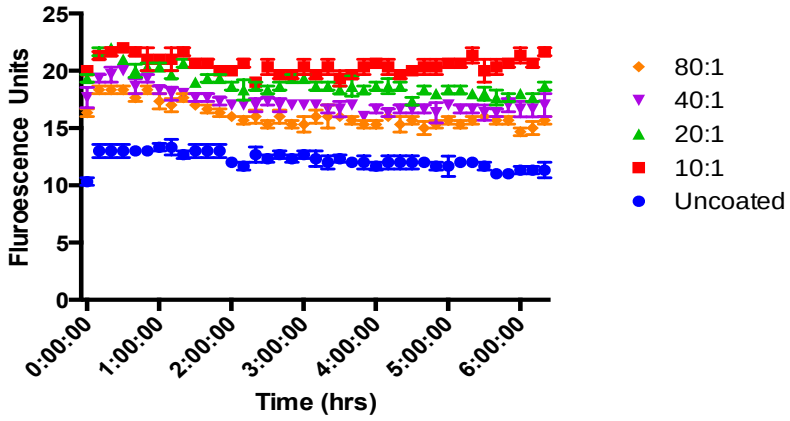
that fliC-functionalized liposomes demonstrated a high titer of OVA-specific IgG1 and little IgG2c production. This profile is consistent with reports of soluble fliC inducing a predominantly Th2 antibody phenotype and Th2-type $CD4^+$ T cell cytokine response (16, 46, 47). Operating under the presumption that functionalized liposomes have no access to the cytosol *in vivo*, and, as such, do not engage NLRC4, our data lends credence to the observation that NLRC4 is dispensable in flagellin-mediated humoral immunity as NLRC4-deficient mice elicit comparable antibody titers as wild type mice (48).

The desire for antigen and adjuvant delivery to the same APC for enhanced immune response against the antigen presents a vaccine delivery challenge. The *in vitro* and *in vivo* data presented in this report demonstrate that fliC-functionalized liposomes present a delivery option to meet this need, particularly in the context of protein antigens that can be encapsulated in liposomes.

CONCLUSION

We have evaluated the characteristics of fliC-functionalized liposomes as a carrier and an adjuvant. Taken together, our results show that fliC-functionalized liposomes can be used successfully in the delivery of both antigen and adjuvant and can be used to deliver antigens where the adjuvant activity of flagellin is desired to enhance antigen-specific $CD8^+$ T cell response and IgG1-response. Given the discoveries of TLR5 and NLRC4-responsive sites on flagellin, and their role or lack thereof in the adjuvant activity of flagellin, it was important to study flagellin as an intact protein. This study is significant as the first study to evaluate the usefulness of fliC as a full protein on a liposomal delivery vehicle for the delivery of antigen and adjuvant to APCs.

(A)



(B)

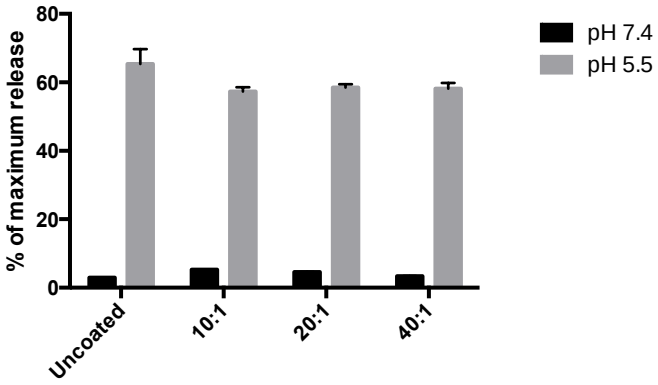


Figure 2.1: Liposomes functionalized with fliC retain membrane integrity and pH-sensitivity

Liposomes functionalized with flagellin retain membrane integrity over time and retain similar pH sensitivity as uncoated liposomes: 10nM of uncoated and functionalized PE: CHEMS: Ni liposomes encapsulating HPTS/DPX were tested for HPTX/DPX leakage. Membrane integrity was assessed by increased HTPS fluorescence due to the dilution of the collisional quencher DPX upon leakage. (A) Liposomes functionalized at various NTA (Ni):fliC molar ratios were tested and compared to uncoated liposomes and HBS buffer, pH 8.4. Fluorescence measurements were taken for one second every ten minutes over 6 hours. (B) Functionalization does not alter pH-sensitivity of PE: CHEMS: Ni liposomes. Fluorescence associated with liposomes in buffer with pH 7.4 or pH 5.5 was measured. The measurements were normalized to the maximal fluorescence measurement taken from liposomes lysed in 2% Triton–X 100. Data displayed as mean ± SEM.

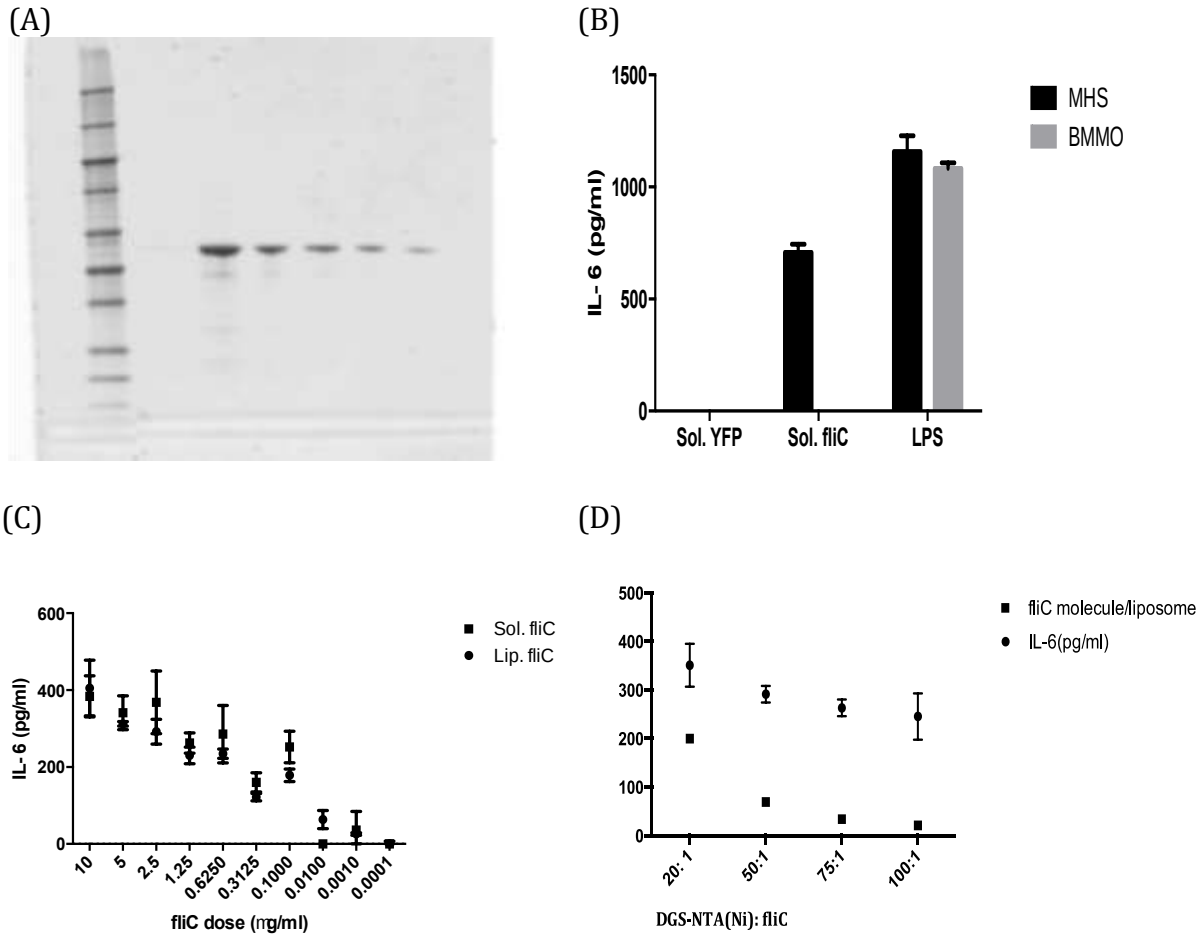
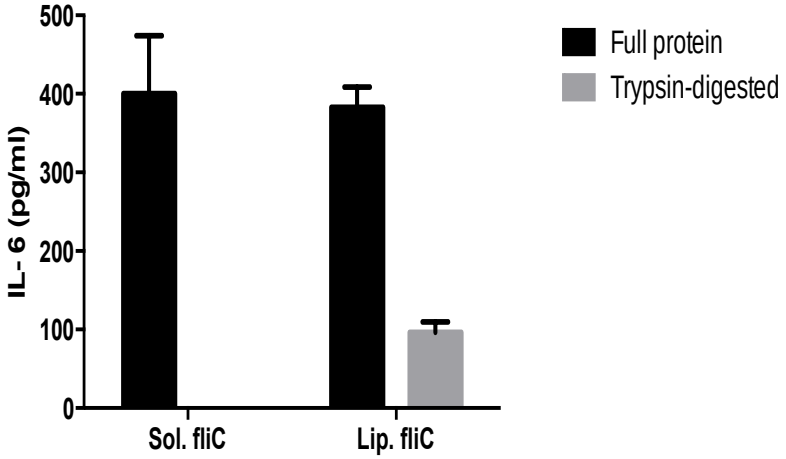


Figure 2.2: Alveolar macrophage cell line responds to fliC

(A) Various dilutions of purified recombinant flagellin visualized by Krypton. (B) MH-S cells and bone marrow-derived macrophages were incubated with 18nM soluble recombinant flagellin and soluble YFP for 3 hours. Cells were also pulsed with equimolar amount of LPS as a positive control. The treatment was discarded and the cells were incubated in complete medium for 21 hours. (C) Liposomal fliC elicited comparable IL-6 to soluble fliC over a wide concentration range in MH-S cells. (D) Number of fliC molecules per liposome as a function of Ni-NTA:flagellin ratio. The coating density of liposomes and the associated proinflammatory response to functionalized liposomes-Liposomes were functionalized in the indicated Ni lipid:fliC ratio. MH-S cells are treated with 1 μg/ml of functionalized liposomes for 3-4 hours at 37° C. After discarding the treatment, the cell supernatant was then collected after 20-21 hours and assayed for IL-6. Cell supernatants were assayed for IL-6 by ELISA. Data represents average of triplicates ± SEM.

(A)



(B)

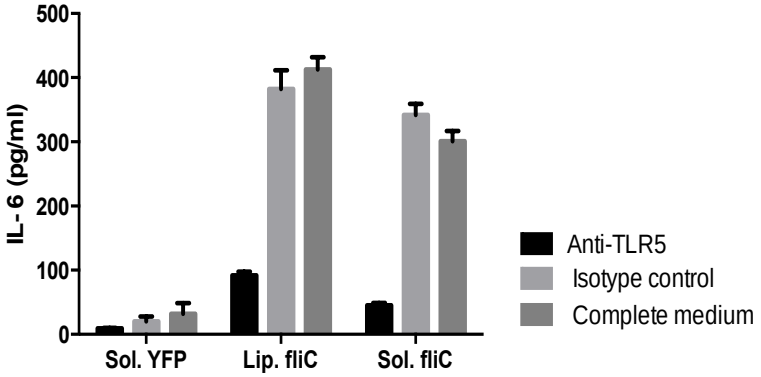


Figure 2.3: Trypsin and anti TLR5 treatment significantly diminish IL-6 secretion

Trypsin and anti-TLR5 treatment significantly diminished pro-inflammatory response from fliC-functionalized liposomes**.** (A) FliC-liposomes that were stripped of the protein coating were prepared by applying the liposomes to trypsin TPCK immobilized on agarose beads and incubated at 37 ° C for 6 hours. Cell culture supernatants were analyzed for IL 6 secretion using ELISA. (B) Anti-TLR5 antibody significantly reduces IL-6 secretion to fliC-functionalized liposomes in MH-S cells. MH-S cells were treated with complete medium, 1 µg/ml anti-mTLR5, or 1µg/ml isotype control for 1 hour at 37° C. Cells were then incubated with 1µg/ml soluble fliC, functionalized fliC (Lip. fliC), and equimolar amount of soluble YFP for 3 hours. The treatment was discarded and cells were further incubated in complete medium for 21 hours. Data represents average of triplicates ± SEM.

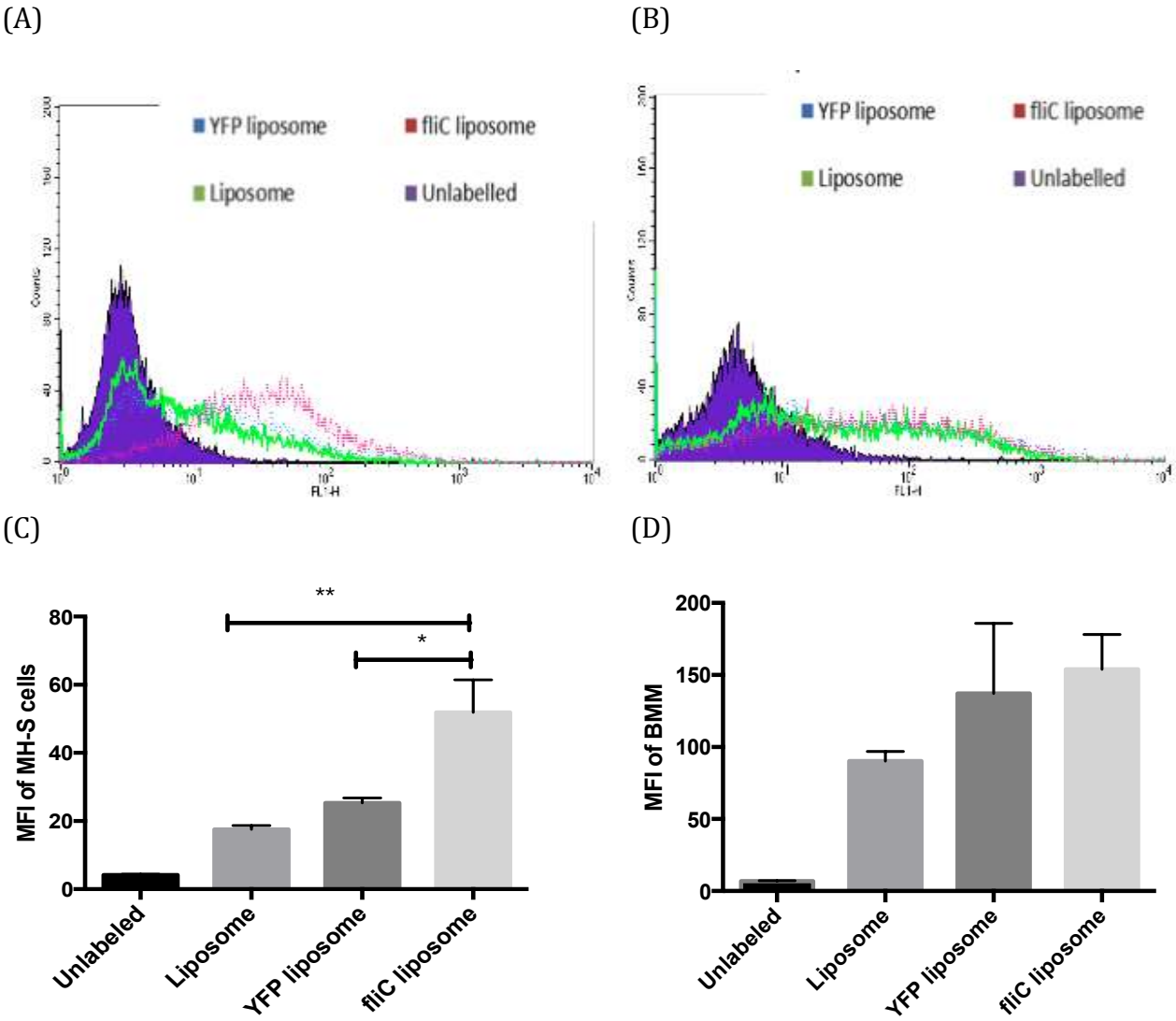


Figure 2.4: FliC liposomes preferentially associate with flagellin responsive cells

FliC-liposomes preferentially associate with cells responsive to flagellin. Cell-specific association was assessed by flow cytometry after treatment of cells with fluorescent Oregon-Green 488 labeled DOPC:CHOL liposomes and labeled liposomes functionalized with fliC and YFP (fliC liposomes and YFP liposome respectively). Control treatment with uncoated and YFP liposome matched the fluorescent intensity of the 1µg/ml fliC liposome treatment. The cells were incubated with the liposomes at 4° C for I hour. The cells were washed and the degree of cell-associated liposomes was determined using flow cytometry. Upper panel shows overlaid histograms from (A) MH-S cells and (B) BMM. The lower panel shows mean fluorescence intensity (MFI) levels from (C) MH-S staining and (D) BMM staining with designated liposomes. Data represents mean ± SEM from an experiment representative of at least two independent experiments. (* $p<0.05$, ** $p<0.01$ analyzed by one-way analysis of variance)

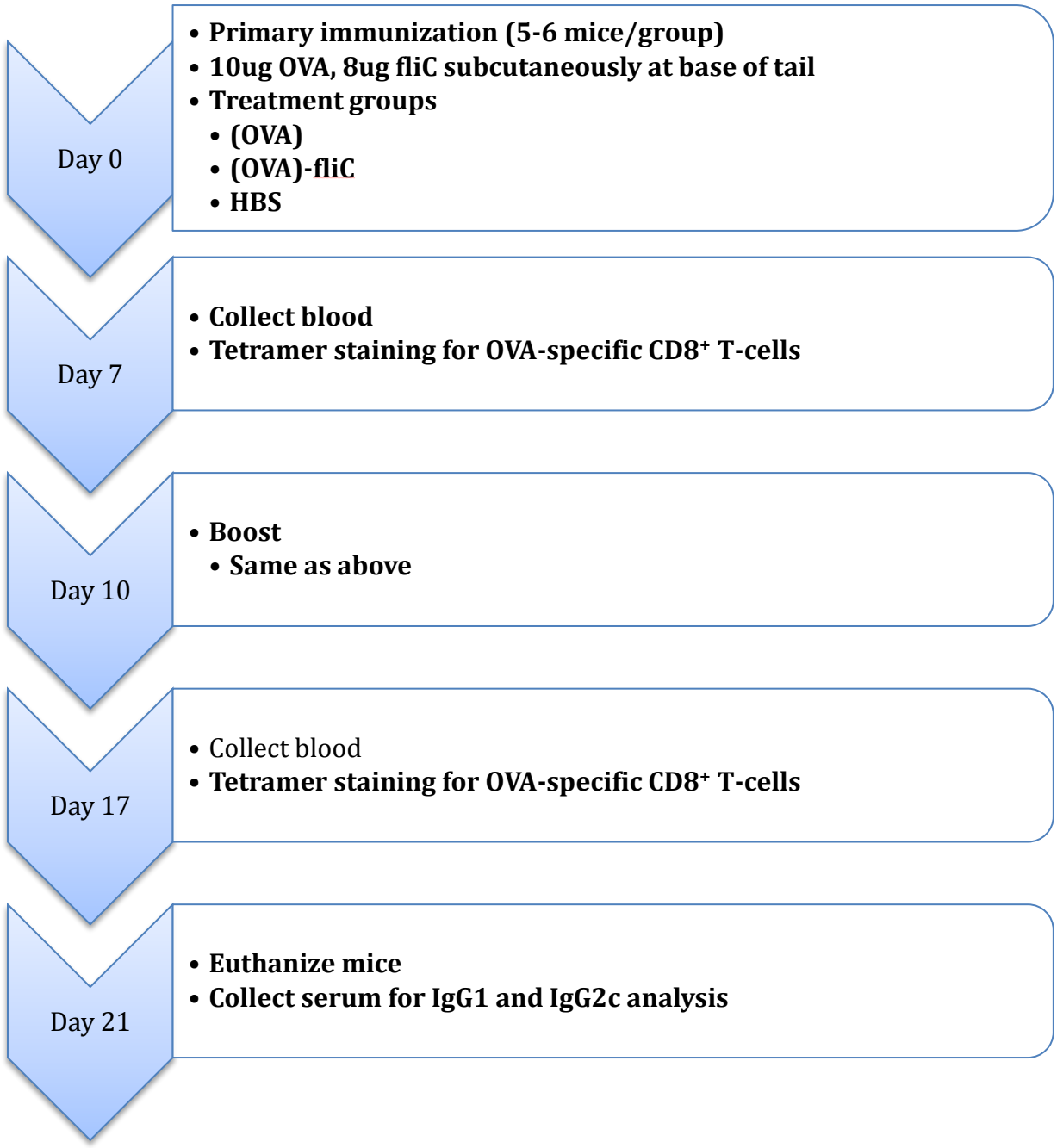


Figure 2:5 Flowchart for immunization protocol

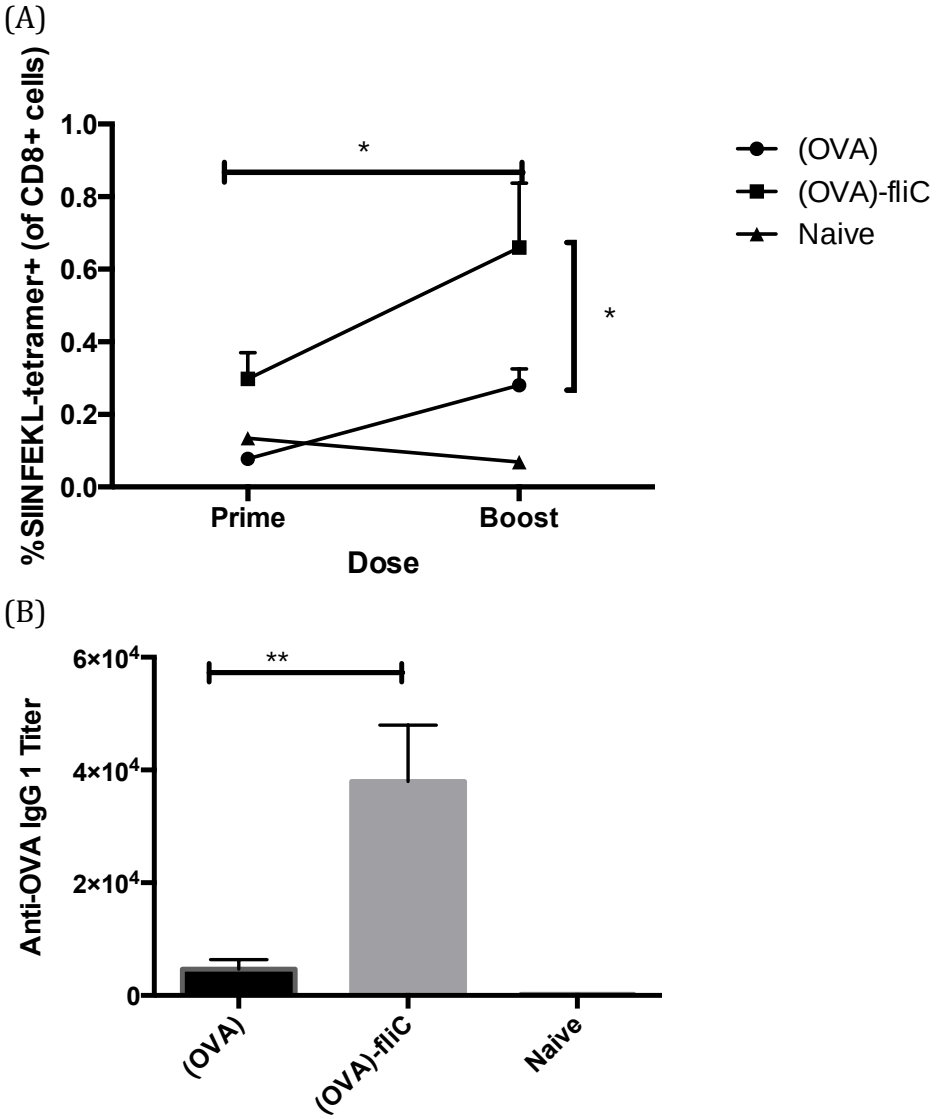


Figure 2:6: Flagellin functionalized liposomes enhance frequency of OVA-specific CD8⁺ T cells

Flagellin functionalized liposomes enhance the frequency of OVA-specific CD8⁺ T cells**.** C57BL/6 mice were immunized s.c. with liposomal OVA alone and (OVA)-fliC-functionalized liposomal OVA. Dose was normalized to 10μg OVA and 5-7μg fliC. Nine days later, the animals were boosted with the same formulation. On Day 16, frequencies of OVA-specific CD8⁺T cells were evaluated by MHC-1 tetramer staining via flow cytometry. (A) Average percentage of OVA-specific CD8⁺T cells are shown as mean ± SEM, n=4 (*$p<0.05$, two-way analysis of variance). (B) Flagellin functionalized liposomes encapsulating OVA induce IgG1 antibody production. Sera from immunized mice were assayed for anti-OVA IgG1 and IgG2c (not shown) 21 days after immunization. Data represents mean ± SEM from one experiment representative of two independent experiments. (**$p<0.005$, one-way analysis of variance, Tukey's multiple comparisons test)

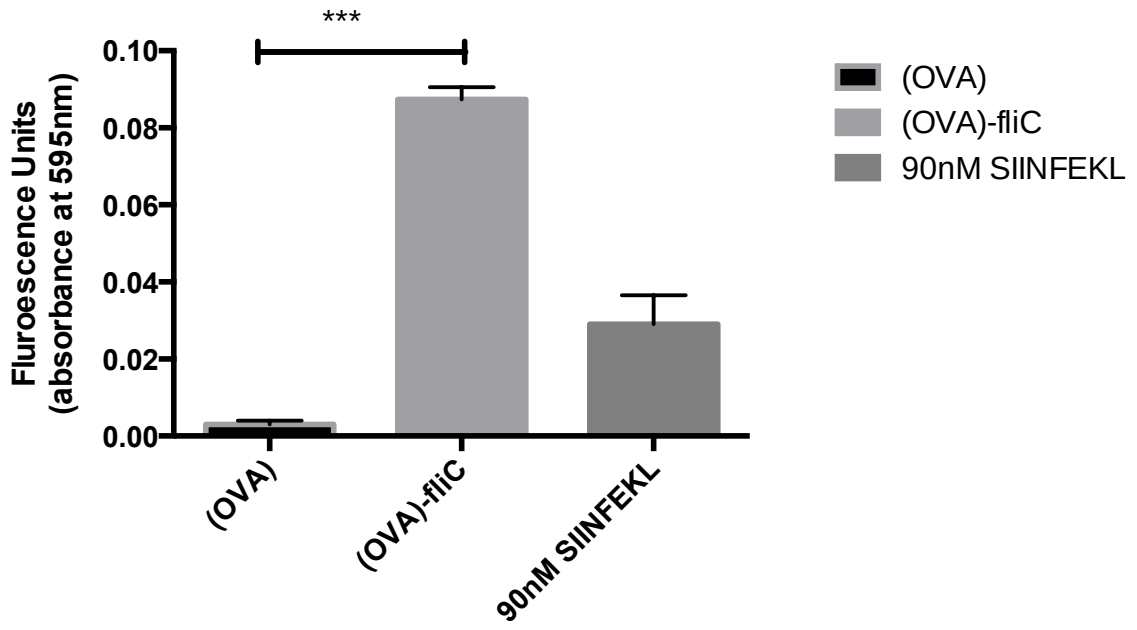


Figure 2.7: FliC-functionalized liposomes enhance MHC class I-restricted peptide presentation

FliC-functionalized liposomes are able to deliver OVA to cytosol of BMM for MHC Class I–specific antigen presentation**.** BMM were pulsed with 200nM liposomes with (13µg/ml OVA) liposomal OVA (OVA), and 11 µg/ml OVA fliC-functionalized liposomal OVA (OVA-fliC) for 2 hours. 90nM SIINFEKL was used as positive control. Cells were washed and further incubated for 3 hours and fixed. The fixed cells were incubated with B3Z cells for 15 hours. Presentation of SIINFEKL –MHC complex to B3Z cells was monitored by the conversion of CPRG substrate to chlorophenol red in primed B3Z cells, and absorbance at 595nm was measured. Data represents mean ± SEM from one experiment representative of two independent experiments. (* $p<0.05$ analyzed by one-way analysis of variance, Tukey's multiple comparisons test).

Liposomes	Diameter (nm) (PDI)
Unfunctionalized (DOPE: CHEMS: DGS NTA (Ni)	182.6 (0.225)
FliC-functionalized (DOPE: CHEMS: DGS NTA (Ni)-fliC)	191.3 (0.173)
Trypsinized flic-functionalized liposomes	177.1 (0.167)

Table 2.1: Summary of representative liposomes size

Summary of representative liposome size after functionalization and subsequent trypsinization of attached fliC. Hydrodynamic diameter was measured by dynamic light scattering and the polydispersity index (PDI), measure of width of particle size distribution, was calculated by square of standard deviation/mean diameter.

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CHAPTER 3 Evaluation of the TLR5-independent contribution to fliC-functionalized liposomes response *in vitro* and *in vivo*

SUMMARY:

The evidence that points to a TLR5-independent enhancement of antigen-specific humoral and $CD8^+$ T cell response underscores the role of other signaling pathways in the adjuvant capacity of flagellin. Flagellin elicits response through another pathway in the cytosol via NLRC4. Using liposomes encapsulating the pore-forming protein, LLO, we propose to gain access to the cytosol to deliver surface-attached fliC to NLRC4. Our goal is to evaluate the contribution of the cytosolic recognition of fliC to its adjuvant capacity, and simultaneously introduce the model antigen ovalbumin to the cytosol to enhance its access to the MHC class I pathway and augment cell-mediated response. We report that LLO liposomes deliver flagellin to the cytosol to activate the inflammasome, seen as an enhanced activation of capase-1 and IL-1β secretion in LPS-primed BMM. *In vivo*, the functionalized liposomes are able to enhance OVA-specific IgG1 response of LLO liposomes, however, and this enhancement is not seen in circulating $CD8^+$ T cells.

INTRODUCTION

Apart from TLRs that sense conserved molecular patterns from a wide variety of pathogens, another class of pathogen recognition receptors is NOD-like receptors (NLRs). NLRs sense stimuli of microbial origins and endogenous markers of cellular damage in the cytosol, acting as a second line of defense in the cytosol for pathogens that evade the extracellular surveillance PRRs (1, 2). The inflammasome is formed in response to breaches of the cytosol by several pathogenic molecules and serves as a platform for pro-caspase-1 cleavage into active caspase-1, which in turn induces maturation of IL-1β and IL-18 from their pro forms (2, 3). Each inflammasome also contains either a caspase activation and recruitment domain (CARD) or a pyrin domain (PYD) that mediates the signaling event (4); hence the classic inflammasome contains an NLR, an PYCARD or ASC adaptor molecule and procaspase-1. The NLR involved in the inflammasome varies depending on the pathogen involved and non-NLR inflammasomes containing proteins such as AIM2 have also been described (3, 5). Caspase-1 activation through a variety of inflammasomes such as NLRP3, NLRC4 andAIM2 has been shown to induce pyroptosis, a form of cell death.

NLRP3 is a widely studied inflammasome that has been implicated in the adjuvant activity of alum. Until recently, alum was the only adjuvant licensed for use in human vaccines. It was determined that alum induced IL-1β and activated caspase-1 through the NLRP3 inflammasome, as LPS-primed BMM from NLRP3 $^{-/-}$ mice were unable to secret IL-1β or activate caspase-1 (6). This study however reported that NLRP3 was dispensable for adjuvant activity *in vivo*. In another example, Li et al. reported that antigen-specific antigen production in vaccine containing alum was significantly diminished in NLRP3 deficient mice and suggested targeting NLRP3 activation as a way to enhance adjuvant efficacy (7).

While these differences may be attributed to differing routes of immunization in the selected studies, the contradictory findings highlights the elusive understanding of the role the inflammasome plays in mediating adjuvant activity, in this case, of alum.

Intracellular flagellin, as would be present in an infection with a flagellated bacterium, signals via IPAF, also called Nod-Like Receptor C4 (NLRC4) (8-10)The NLRC4 inflammasome is activated by gram-negative bacteria, such as *Salmonella typhimurium*, *Legionella pneumophila* and *Pseudomonas aeruginosa,* with functional type III (T3SS) and type IV secretion systems (T4SS) (8, 9). While cytosolic flagellin was the first identified NLRC4 ligand, there is some evidence that some non-flagellated bacteria also induce the NLRC4 inflammasome (11). Flagellin within the cytosol of macrophages triggers a cascade of events that helps to mount a response against an invading flagellated pathogen. Chief mediators of the immune response subsequent to cytosolic invasion include, the proinflammatory cytokines, IL-1β and IL-18. Their activity is controlled by expression and secretion. The first as a result of priming by a TLR, resulting in the NF-κB-mediated secretion of the precursor forms, proIL-1β and proIL-18. The maturation and secretion is mediated by inflammasomes.

Some flagellin-mediated adjuvant effects have been reported to be independent of the conserved regions of flagellin, known to be essential for TLR5 binding. However, the contribution of signaling outside this pathway to the adjuvant effect of flagellin is not commonly examined (9). Given our observation of the immune stimulating effect of fliC-functionalized liposomes, in the current study, we examine the TLR5 independent contribution of the flagellin presented in context of liposomes using the pore-forming protein listeriolysin O (LLO). The LLO-containing liposomes have been shown to enhance

cytotoxic T lymphocyte activity when used to deliver immunostimulatory CpG oligonucleotides by virtue of LLO's ability to provide access to the cytosol (12). With flagellin fusion proteins, the adjuvant activity of which is a motivation for this investigation, fusion proteins also can't access the cytosolic pathway, whereas the LLO liposomes acts as a truly multifunctional vehicle allowing s for compartmentalized delivery to gain access to the cytosol. We hypothesized that using LLO-containing liposomes with flagellin anchored to the liposome surface serves a dual purpose. First, the presence of LLO mediating cytosolic delivery allows for delivery of antigen to the cytosol and MHC-class I pathway to enhance cell-mediated immune response. Secondly, in delivering flagellin to the liposomes, we would be able to observe the effect of functionalized liposomes in the context of the non-TLR5 flagellin recognition pathway and the effect of activating the NLRC4 inflammasome on the antigen-specific immune profiles observed.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

BL21 (DE3) RIPL were used as host strains for expression of the recombinant LLO protein. The construct for the recombinant LLO with an N-terminal His tag and AcTEV affinity tag (His-TEV-LLO) was a gift form Dr Jiayan Liu (University of Michigan, Ann Arbor, MI). His-TEV-LLO were expressed and purified as described by Mandal *et al.* with some modifications (13)Cells were grown in 37 ° C in TB broth. Protein expression was induced with 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 30 ° C and grown for another 6 hours. Cells were centrifuged at 4000xg for 30 minutes at 4 ° C. Cell pellets were stored at -80 till use. The pellets were suspended in wash buffer (50mM sodium phosphate

dibasic, 300mM sodium chloride, and 20mM imidazole) containing 1mg/ml lysozyme and 1mM phenylmethylsulfonyl fluoride (PMSF) incubated for 30 minutes on ice.

His-TEV-LLO pellet in 1mg/ml lysozyme was lysed using a Digital Sonifier cell disruptor (Emerson, Danbury, CT) four times at 50% amplitude with a 30 second interval of sonication and 30 second incubation on ice. The lysate was centrifuged at 12000 x g for 1 hour at 4 ° C and the supernatant incubated with the Ni-NTA agarose resin (Qiagen, MD) for 2 hours on a shaker at 4 ° C. Bound his-tagged protein was eluted from a Ni-resin column against 400mM imidazole and dialyzed in 1x PBS, pH 8.4 for 16 -20 hours. To remove the hexahistidine tag from the purified protein, his-TEV-LLO was added to ProTEV protease (Promega, Madison, WI) according to the digestion protocol given in the manufacturer's instructions and incubated at room temperature overnight (12-16 hours). The eluate from the incubation of a Ni-Resin and digestion mixture incubated at 4 ° C for 2 hours was collected as the purified LLO. Protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL) according to manufacturer's instructions. Protein purity was evaluated using SDS gel electrophoresis and visualized using Krypton fluorescent stain (Thermo Scientific) per the manufacturer's instructions and with a Typhoon 9200 imager (GE Healthcare)

Hemolysis assay of recombinant LLO

LLO activity was determined by its ability to lyse sheep red blood cells (Lampire Biologicals, Pipersville, PA). Serial dilutions of LLO were made in MBSE (10mM MES pH 5.5, 140mM NaCl and 1mM EDTA pH 5.5) containing 1mg/ml BSA. The RBCs were washed in 1X PBS, then diluted in MBSE and plated at 2 x 10^7 cells/100µl/well in a 96-well plate, DTT was added to the cell solution at a concentration of 2mM before plating the cells. The

LLO dilutions were added to the RBCs and the plate was sealed and incubated for 15 minutes at 37° C while rotating. Cell debris was pelleted by centrifuging the plate at 1400 x g for 15 minutes at 4° C. 150µl of the supernatant were transferred in to a clean 96-well plate and the absorbance at 450nm was measured. Values were compared to the absorbance of the positive control LLO –his standard. LLO was heat-inactivated by incubating at 70° C for 10 minutes.

Preparation of liposomes and protein-coated liposomes

The lipids 18:1 (Δ9-Cis) PE (DOPE)1,2-dioleoyl-*sn*glycero-3 phosphoethanolamine, cholesteryl hemisuccinate (CHEMS), and 18:1 DGS-NTA(Ni) 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)(Ni-NTA) were mixed at a 2:1:0.02 ratio and dried down on a rotary evaporator and afterwards stored under vacuum for 12-16 hours. The dried lipid films were suspended in HEPES-buffered saline (HBS) (10mM HEPES, 140mM NaCl, pH 8.4) for subsequent protein coating.. The resulting lipid films were re-suspended in HBS, passed through 4 freeze/thaw cycles sonified in a bath sonicator 4-5 times in 1-minute cycles. For liposomes encapsulating a protein, the lipid films were suspended in a solution of LLO or fliC-his at a 0.3 µg/ml and 20mg/ml OVA protein concentration. All aforementioned lipids were acquired from Avanti Lipids, Alabaster, AL. To coat with protein, the resulting liposomes were incubated with protein at a 20:1 Ni lipid to protein molar ratio for 1-2 hours at 4 ° C. The free protein was purified from the liposome via size exclusion chromatography on a CL4B column (GE Healthcare) and the eluted liposomes were evaluated by SDS gel electrophoresis to confirm and the presence of protein and quantify the amount of protein on the liposomes. Liposome diameter was determined using dynamic light scattering on a Zetasizer instrument

(Malvern, Westborough, MA). Phospholipid content of liposomes was quantified using Bartlett's method of phosphate quantification.

Cell lines and Tissue culture

Tissue culture media was purchased from Invitrogen (Carlsbad, CA) and all cells were maintained in a humidified incubator at 37° C and 5% CO_2, unless otherwise stated.

Generation of Bone marrow-derived macrophages

Bone marrow was harvested from femur and tibia of 7-8 week old C57BL/6 mice as described in Stier *et al.* (14). The bone marrow cells were differentiated into bone marrow-derived macrophages (BMM) in DMEM containing 20% heat-inactivated fetal bovine serum (Hi-FBS), 30% L-929 cell conditioned media containing macrophage stimulating factor, 100µg/ml streptomycin, 100u/ml penicillin, and 55µM β-mercaptoethanol. The cells were replenished with new medium on day 3, harvested on day 6 and stored in liquid nitrogen until use. Upon thawing, cells were maintained in DMEM (Invitrogen, CA), supplemented with 10% Hi-FBS, 100µg/ml streptomycin, 100u/ml penicillin and 2mM L-glutamine

In vitro antigen presentation

In vitro antigen presentation was performed as previously described in Andrews *et al.* (12) with a few modifications. Briefly, 2 x 10^5 cells/well were plated in 96-well plates overnight before the day of the assay, cells were washed in serum-free media and treated with liposomes in serial dilutions with the highest concentration at 200µM phosphate in serum-free media for 2 hours. BMM were washed and incubated in complete DMEM for 3 hours. The cells were fixed in 1% paraformaldehyde for 15 minutes at 4° C. The paraformaldehyde solution was prepared in warm 1X PBS and dissolved by adding drops

of 0.1M NaOH; the pH of the solution was adjusted to 7, and the solution filtered through a 0.45μm filter. The paraformaldehyde was quenched by 0.2M lysine in DMEM for 20 minutes at RT. The cells were washed in DMEM after quenching, and 2 x 10^5 cells/well of B3Z cells in B3Z media (RPMI 1640 supplemented with 2mM L-glutamine, 25mM L-glucose, 1mM sodium pyruvate and 25mM HEPES) were added per well and incubated for 15 hours at 37° C and 5% CO_2. The plates were centrifuged at 1500rpm for 5 minutes, media was carefully removed and the cells washed in RT 1X PBS and centrifuged again. The PBS supernatant was removed carefully. CPRG substrate [0.15 mmol/L chlorophenol red-β-D-galactopyranoside (Calbiochem), 9 mmol/L $MgCl_2$, 0.125% NP40, and 100 mmol/L β-ME in PBS] was added to measure production of β-galactosidase by B3Z cells in response to BMM presentation of SIINFEKL in H-2K^b. The plates were incubated for 4 hours at 37°C and absorbance values were measured at 595 nm using a spectrophotometer (BioTek, Winooski VT).

***In vitro* cytokine secretion analysis by ELISA**

2x 10^5 BMM were played 12-16 hours in BMM media (DMEM supplemented with 30% L-cell conditioned supernatant, 20% Hi-FBS, 2mM L-Glutamine, 0.055mM β-Me, and 100ug/ml streptomycin and 100u/ml penicillin), 16-20 hours before liposome treatment. The cells were incubated with liposomes for 3-4 hours, the treatment removed and the cells incubated in their respective media for 21 hours. Cell supernatants were collected and analyzed via ELISA for Il-1β (eBioscience) secretion according to the manufacturer's instructions.

Lactate Dehydrogenase assay

After a 4-hour treatment of cells, the cell supernatant was applied to a 96-well plate and tested for LDH according to manufacturer's instructions (Pierce, Rockford, IL). Briefly, the absorbance of at 490nm was measured and the % of maximum LDH released was calculated thus:

((Experimental release - spontaneous release)/ (Maximum release - spontaneous release))

where maximum release is the absorbance obtained from lysis of the macrophage the Triton X-100-based lysis buffer, and spontaneous release is the absorbance obtained from LDH released to the cytosol of untreated macrophages to account for serum effect.

Measuring activation of Caspase-1

Activation of inflammasome via activation of caspase-1 was measured as described by Jakobs *et al.* (15). Briefly, 2x 10^5 BMM from C57BL/6 mice were plated in 12-well plates in their respective media for 12-16 hours the day before treatment. In BMMs primed with LPS before treatment, the cells were incubated in 200ng/ml LPS for 4 hours before treatment. The cells are treated with various groups in corresponding 500µl serum-free media. for 6 hours. In the case of LPS positive control, the cells were treated with 200ng/ml LPS for 4 hours followed by an addition of 5mM ATP for 2 hours. After 6 hours, cell supernatant was collected. The supernatant was clarified of cell debris by centrifugation at 1000 X g for 5 minutes. Protein was precipitated from the cell supernatant using methanol chloroform extraction. Briefly, 500µl methanol was added to the collected supernatant, followed by 125µl chloroform, the mixture was vortexed vigorously, and centrifuged for 5 minutes at 7500xg. The solvent layer was discarded and the protein layer was resuspended in 500µl methanol and vortexed vigorously. The protein precipitate was centrifuged at

7500xg for 5 minutes. The methanol was discarded and the resulting pellet was dried for 10 minutes at 55° C. The pellet was resuspended in 40µl 1x SDS sample buffer). Equal amounts of the extracted protein was run on SDS-PAGE on 10% BIS-TRIS gel (Life Technologies) in 1x MES buffer at 200V for 1 hour. The protein was transferred on the PVDF membrane (BioRad, Hercules, CA) at 200mA for 1 hour. The membrane was blocked for 90 minutes in 3% casein at room temperature, and incubated in anti-caspase-1 p20 (Adipogen, San Diego, CA) in 1% casein for 12-16 hours at 4° C. The membrane was incubated in anti-mouse HRP for 2 hours at RT. The membrane was washed 4 times in 1X PBS with 0.05% Tween between incubation steps. The membrane was incubated with ECL substrate (Pierce, Rockford, IL) for 5 minutes and chemifluorescence was detected on the Typhoon 9200 imager (GE Healthcare) using the 457 nm excitation laser and 520nm BP 40 emission filters.

Immunization protocol

C57BL/6 mice, 8-10 weeks old (Jackson labs, Bar Harbor, ME) were used in this study and were handled according the University of Michigan Institutional Animal Care guidelines. Animals were immunized subcutaneously at the base of the tail with OVA-encapsulated liposomes (OVA), OVA LLO encapsulating liposomes (OVA; LLO), and fliC-functionalized liposomes (OVA LLO), containing 10µg OVA and 8µg fliC, on day 0 and day 10. Naïve mice were immunized with the same volume of HEPES buffered saline pH 8.4 (HBS). Mice were euthanized on Day 21 and blood was collected via cardiac puncture.

$CD8^+$ T cell tetramer staining

Seven days after immunization, blood was collected via the superficial temporal vein of immunized mice. The blood was collected in dipotassium EDTA coated microtainer tubes (BD, Biosciences, Franklin, NJ). After blood collection, the cells were resuspended by gentle pipetting. Red blood cells were lysed with ACK lysis buffer (Life Technologies) twice. The cells were centrifuged at 1500 x g for 5 minutes at 4° C between each lysis. The cells were washed in FACS buffer (Ix PBS containing 1% BSA) and centrifuged at 1500 x g for 5 minutes at 4° C. The resulting pellet was stained for flow cytometry after CD16/32 Fc block (eBioscience, San Diego CA) and incubated for 10 minutes at RT. A small volume of each sample was collected for FACS negative and single controls. Cells were incubated with T-select H2Kb – OVA-tetramer SIINFEKL PE (MBL, Japan) for 30 minutes and stained with anti-CD8, anti-CD44, and anti-CD62L. The incubation was followed by DAPI staining to discriminate live from dead cells. Cell fluorescence was evaluated via flow cytometry.

Measurement of antibody titers

Blood was harvested from euthanized mice via cardiac puncture in microvette 500 centrifuge tubes (Sarstedt, Germany). The sera were isolated by centrifugation at 10000 x g for 5 minutes and analyzed by ELISA or stored at -80° C until use. To determine serum antibody titers, briefly, Maxisorp Nunc immunoplates (eBioscience, San Diego, CA) were coated with 10µg/ml OVA (Sigma-Aldrich, St Louis, MO) in 0.1M sodium phosphate pH 9.0, coating buffer overnight (12-16 hours). The plates were washed in PBST (1x phosphate buffered saline, 0.05% Tween) and then blocked overnight at 4° C with PBST containing 1% BSA (blocking buffer). The plates were washed and incubated with serial dilutions of sera in blocking buffer overnight at 4 ° C. OVA-specific biotinylated goat anti-mouse IgG1

and goat anti-mouse IgG2c (Southern Biotech, Birmingham, AL) were detected with Avidin-Horseradish Peroxidase (eBioscience, San Diego, CA) and finally TMB substrate (KPL Inc., Gaithersburg, MD). Plates were washed 5 times in PBST between each step. Conversion of substrate to colorimetric product was stopped by 2N sulfuric acid and absorbance at 450nm was determined. Data were fit to a 4-parameter curve on the Gen5 data analysis software, (Biotek, Winooski, VT) to determine titer, defined as the dilution factor that yields an absorbance of 0.5. The lowest dilution used was reported for samples below the limit of detection.

RESULTS

Flagellin functionalized LLO liposomes enhances caspase-1 activation and secretion of IL-1β

We examined the role of the cytosolic flagellin-signaling pathway through NLRC4 in its adjuvant properties in the context of the functionalized liposomes. It is well documented in the literature that flagellin triggers activation of the NLRC4 inflammasome in the cytosol. We wanted to evaluate the ability of functionalized liposomes to activate the inflammasome when flagellin is introduced into the cytosol with the aid of the pore-forming listeriolysin O encapsulated in the liposomes (referred to as LLO liposomes). We have previously confirmed the ability of LLO liposomes to mediate access to the cytosol to deliver liposomal cargo. Flagellin functionalized LLO liposomes and heat-inactivated LLO liposomes where compared to reveal LLO-mediated delivery of fliC into the cytosol. Liposomal LLO activated the inflammasome and functionalizing LLO liposomes with flagellin enhanced inflammasome activation seen as the presence of a subunit of activated caspase-1, p20 (Figure 3.1A). Separate treatment with soluble LLO and soluble fliC and the

combined treatment with soluble fliC and soluble LLO display the same enhancement pattern (data not shown) that is absent in soluble fliC treatment alone. When BMM without LPS priming before exposure to different treatments are activated, the activation pattern seen remains the same (Figure 3.1B), an indication that TLR priming is inconsequential to the process of caspase-1 activation. The enhancement in the p20 subunit, while slight, can be observed visually, however we went further to examine IL-1β secretion.

The fliC-mediated enhancement of caspase-1 activation translates disproportionately into IL-1β secretion from LPS treated BMM exposed to functionalized liposomes (Figure 3.2A), where we demonstrate that access to the cytosol is mediated by LLO, which induces slight but significant IL-1β by itself, but the secretion of IL-1β is significantly enhanced by the presence of fliC in the cytosol. The magnitude of caspase-1 p20 in the LPS+ATP treated cells is similar to fliC-functionalized LLO liposomes, while the secreted IL-1β in both treatment groups are not wide apart with LPS-ATP secreting > 22 times more IL-1β than the functionalized LLO liposomes. The disproportionate translation of caspase-1 activation to IL-1β secretion may not be of consequence as different inflammasomes are involved in LPS and ATP activation, but this can perhaps be attributed to varying efficiencies of different inflammasomes in the processing of IL-1β. Together, these results demonstrate the ability of fliC-functionalized liposomes to activate the NLRC4 inflammasome when given access to the cytosol. In the absence of LPS priming, the BMM were not able to secret IL-1β, a demonstration of the principle of the two-signal process necessary for IL-1β secretion (Figure 3.2B).

To ensure surface-attached fliC remained active in the cytosol, we compared it to liposomes encapsulating LLO and fliC. We found that fliC-functionalized on the surface of

liposomes is able to activate the inflammasome as determined by similar caspase-1 activation and IL-1β secretion as the LLO and fliC encapsulated liposomes. The alveolar macrophage cell line, MH-S, did not activate caspase-1 or secret IL-1β even in response to positive controls, LPS and ATP (data not shown). Considering that it is known that activation of the inflammasome is independent of TLR5, this comparison is evaluated in BMM, which presumably lacks TLR5. Hence, this does not indicate that post-TLR5 interaction does not alter the activity of fliC in the cytosol, but it does show that surface-functionalized fliC remains active after passing through the endosome before it gains access to the cytosol.

Encapsulation of LLO in liposomes protects macrophages from LLO-induced cell death

Since the activation of inflammasomes is known to lead to pyroptosis, a form of cell death dependent on caspase-1, we measured the release of lactase dehydrogenase (LDH) as a marker of pyroptosis after the treatment of cells for 4 hours. We observed that soluble fliC did not mediate significant LDH release, while soluble LLO by itself, and in combination with soluble fliC, elicited high amounts of LDH from BMM irrespective of 4-hour LPS priming. LDH elicited from liposomal formulation of these proteins was significantly diminished. Liposomes prepared for immunization with encapsulated OVA showed a similar pattern (Fig. 3.3B).

Functionalized LLO liposomes promote MHC class I-restricted presentation of OVA-specific peptides by Bone Marrow macrophages

In our previous studies using LLO to mediate cytosolic delivery, we showed the ability of pH-sensitive LLO liposomes to mediate delivery of antigens into the cytosol to

elicit an immune response via MHC class I presentation. We wanted to explore the effect that the presence of flagellin in the cytosol would have on MHC class I presentation of antigen encapsulated in fliC-functionalized LLO liposomes. To this effect, we cultured BMM with OVA-containing pH-sensitive LLO liposomes with and without functionalization, and OVA-containing liposomes functionalized with fliC. We determined whether the treated macrophages expressed the SIINFEKL-MHC-complex recognizable by cells B3Z, a lacZ-inducible $CD8^+$ T cell hybridoma cell line specific for $OVA_{257-264}$ (SIINFEKL) presented on the murine H-$2K^b$ MHC class I molecule. As expected, we found that OVA LLO liposome-treated cells showed enhanced conversion of the β-galactosidase substrate, CPRG, to chlorophenol red as a surrogate for B3Z β-galactosidase production. The measured absorbance of chlorophenol red at 595nm was normalized to OVA concentration in respective treatment groups. However, cells exposed to the fliC-functionalized counterpart did not enhance β-galactosidase production from B3Z cells, though the liposomes exhibited similar β-galactosidase induction and had equivalent OVA to LLO ratios encapsulated (Fig. 3.4). We did not observe any additional fliC effect in presenting MHC class I–restricted peptides over a range of doses and did not determine the contribution of fliC to this response.

Functionalized liposomes do not enhance OVA-specific $CD8^+$ T cell response in the presence of LLO

We have previously demonstrated the efficacy of LLO liposomes to promote a Th1-type response and enhance $CD8^+$ T cell lytic activity. It is accepted that the circulating $CD8^+$ T cells may not necessarily reflect functional cellular immune response as we have shown in the past. Given the accessibility to the MHC class I pathway, the expectation that OVA

LLO liposomes would enhance the frequency of SIINFEKL tetramer positive $CD8^+$ T cells is reasonable. However this expectation was not realized in the results of the tetramer staining 7 days after the booster immunization, as it appears that activation of the inflammasome correlates with a reduced frequency of OVA-specific $CD8^+$ T cells (Fig. 3.5A). One different variable in the LLO liposomes used in this study than our previous studies is the presence of the NTA (Ni) lipid component used for the non-covalent surface attachment to the his-tagged fliC. Evidently, this also does not correlate with the results seen in MHC class I SIINFEKL presentation data that shows significantly better MHC class I presentation in OVA LLO liposomes and functionalized OVA LLO liposomes than liposomal OVA.

Addition of LLO to OVA-containing functionalized liposomes enhances the fliC-mediated IgG1 production

Several studies have demonstrated that soluble flagellin overwhelmingly induces Th2 type responses. To evaluate the adjuvant capabilities of the flagellin functionalized liposomes as a delivery system, we tested whether mice immunized with functionalized OVA liposomes could develop an OVA-specific antibody response. Eleven days after the booster immunization, sera from mice immunized with fliC-functionalized liposomes, with and without LLO, exhibited significantly enhanced OVA-specific titers of IgG1 (Fig. 3.5B). While the addition of LLO and, by extension, activation of the inflammasome, significantly enhanced the IgG1 response in fliC-functionalized LLO liposome-immunized mice, this response was independent of LLO. In contrast, the immunized mice generated low IgG2c anti–OVA titers, and the addition of fliC to LLO liposomes did not enhance the low response observed (data not shown). These results are consistent with the studies that show fliC-mediated humoral responses are independent of IPAF. Our data demonstrate that fliC-

functionalized liposomes exhibit similar humoral characteristics as flagellin antigen fusion in the literature.

DISCUSSION

The goal of this study was to investigate the effect of fliC-functionalized liposomes on the immune response against encapsulated antigen, ovalbumin, in the context of fliC delivered in to the cytosol. We proposed that by using pH-sensitive LLO liposomes, LLO-mediated cytosolic delivery of ovalbumin and identified adjuvant fliC would engage the fliC cytosolic signaling pathway and augment the $CD8^+$ T cell response engendered by the increased access of OVA to the MHC class I antigen presentation pathway. Although flagellin has been shown to display a predominantly Th2- biased humoral response (16-18), we also proposed that the humoral response to enhancing the cytosolic delivery of OVA would be skewed towards a Th1 type. We determined that fliC-functionalized LLO liposomes enhanced caspase-1 activation in BMM and secretion of IL-1β from LPS-primed BMM. Furthermore, fliC-functionalized liposomes encapsulating OVA and LLO enhanced the OVA-specific IgG1 response but did not have an augmenting effect on the frequency of circulating antigen-specific $CD8^+$ T cells.

The pH-sensitive liposomes used in this study were instrumental in the delivery of encapsulated cargo. CHEMS (cholesteryl hemisucccinate), a component of the liposomes, was used to engender pH-sensitivity to the liposomes. It serves to stabilize PE (phosphoethanoloamine) in a bilayer state and it is negatively charged at neutral pH. In the acidifying environment of the endosome, CHEMS is protonated at pH < 6.0, which destabilizes the liposomes by promoting the formation of the hexagonal phase (19). We have previously used LLO-containing liposomes as a strategy to enhance $CD8^+$ T cell response against encapsulated antigen and even of more relevance to this study, to deliver protein antigen and, adjuvant to the same APC (12, 13). The rationale behind this approach

is that liposomes would be taken up via endocytosis, and, as the endosome acidifies, the pH-sensitive liposomes lose their membrane integrity and expose LLO to allow it breach the endosome to deliver its contents to the cytosol. In this study, the presence of the endosomal content in the cytosol serves a dual purpose: first to introduce the cargo, ovalbumin, to the classical MHC-class 1 antigen presentation pathway, and secondly to allow for the fliC to be accessible in the cytosol to activate the inflammasome.

As the mediator of cytosolic access, we observed that LLO liposomes are able to activate caspase-1. Presumably, LLO itself mediates inflammasome activation. *Listeria monocytogenes*, from which LLO is derived, has been implicated in the activation of multiple inflammasomes, NLRP3, AIM2 and NLRC4 (20-22). Furthermore, fliC-functionalized LLO liposomes slightly enhanced caspase-1 activation mediated by LLO. Although we did not ascertain the NLRC4 contribution to this enhancement, flagellin is widely regarded an inducer of the NLRC4 inflammasome (10, 23, 24). Likewise, LPS-primed BMM produced modest and significant IL-1β in response to LLO liposomes when compared to empty liposomes. The magnitude of this response was significantly enhanced in the presence of fliC. And this observation occurred over a dose range of LLO liposomes (not shown). We propose that the active caspase-1 and IL-1β enhancements are indications of activating the NLRC4 inflammasome.

Caspase-1 activation through inflammasomes has been shown to lead to pyroptosis, indicated by membrane pore formation and rupture that leads to loss of intracellular materials and proteins (25, 26), such as LDH (27), which was used as a marker for cell-death. We observed varying levels of LLO-mediated toxicity regardless of LPS-priming, This toxicity was not enhanced by the presence of fliC on the liposomes even at the dose at

which fliC enhances IL-1β. We saw that encapsulating LLO in liposomes minimized LLO-mediated cell death when compared to soluble protein, an observation that aligns with past observations (28). LLO is has been involved in both caspase-1 and caspase-3 dependent cell death (29, 30), however, we determined this secretion is driven by pyroptosis because caspsase-3 mediated cell-death is apoptotic, a process which is not considered inflammatory or lytic (26, 31), hence would not expect to see LDH form other sources of LLO-mediated toxicity (Table 1). Overall, we observed that by measuring markers of inflammasome activation, active caspase-1 and IL-1β secretion, LLO liposomes activate the inflammasomes, and the functionalization with fliC, enhances the activation of inflammasomes. Incidentally, our observation of soluble LLO-mediated cell death is in contrast with the reports of Molofsky et al. who reported that soluble LLO induced similar levels of LDH as soluble flagellin from different sources (32). The differences in dose, they used 10 times lower LLO concentration, and exposure time, 2 versus 4 hours in our study, may be responsible for the differing observation. As mentioned above, we did not observe the fliC- enhanced cell death from the liposomal formulation of this protein either.

In addition, we observed that IL-1β secretion was not proportional to cell death. Both LLO liposomes and functionalized LLO liposomes elicit similar levels of LDH, yet significantly different levels of IL-1β. This observation supports the hypothesis that the cell death that mediates the clearance of invading pathogens is independent of IL-1β secretion (4, 33).

In step with this fliC-mediated enhancement we observed, *in vivo*, that fliC functionalization of (OVA;LLO) led to high titers of anti-OVA IgG1 in immunized mice, an indication that fliC mediates antibody response in the presence of inflammasome activation

and that inflammasome activation, and this may be beneficial to the humoral adjuvant activity of fliC. Mice immunized with (OVA;LLO) produced significantly higher IgG1 than (OVA), a confirmation of previously observed data by Andrews *et al*. Production of IgG2c, while slight, was enhanced in (OVA;LLO) as compared with (OVA) but was not enhanced by functionalization. With regard to frequency of circulating $CD8^+$ T cells, mice immunized with (OVA;LLO)-fliC had similar circulating $CD8^+$ T lymphocytes as (OVA;LLO) and (OVA). Without the LLO and apparent activation of the inflammasome, we had observed that fliC enhanced the circulating $CD8^+$ T cell frequency, leading us to consider that activation of the inflammasomes was responsible for the lack of response seen in mice immunized with (OVA;LLO)-fliC. Furthermore, the lack of LLO response in mice immunized with (OVA;LLO) was unexpected and is in conflict with previously published reports from our group (12, 13).

We expected enhanced $CD8^+$ T cell response in these mice because we proposed that the LLO liposome would deliver OVA into the cytosol, enhance OVA's access to the MHC class I pathway, and improve $CD8^+$ T cell response. To ensure that the LLO remained functional and able to mediate delivery into the cytosol, we evaluated the ability of BMM treated with the LLO liposomes to present antigen to T cells in the context of the MHC class I peptide complex *in vitro*. The treated BMM were able present class-I restricted OVA peptide to T cells in an LLO-mediated fashion. We have identified differences between the liposomal vehicle and recombinant LLO from the differences between previous studies versus the current study. In contrast to previous studies, the liposome formulation contained the Ni-chelating lipid and the LLO did not contain the hexahistidine tag. While we observed that the LLOhis liposomes presented antigens better *in vitro* in comparison to

the same dose of LLO, the LLO liposomes were still able to significantly mediate delivery of OVA into the cell (data not shown). The concentration of encapsulated LLO used in the current study was increased to account for this observation (300μg/ml versus 200μg/ml in previous studies). The other difference was in the method of determining $CD8^+$ T cell response, which had been determined in previous studies via functional assays measuring cytokine secretion and antigen-specific lysis. However, we have previously employed tetramer staining as we did in the current study to examine antigen-specific $CD8^+$ T cell response to a clinically relevant antigen, Influenza nucleoprotein (NP). In this case, LLOhis liposomes mediated NP-specific $CD8^+$ T cell response in comparison to liposomal NP alone. To further complicate the comparisons, a study using NP-specific cytotoxic T cell lysis and *ex vivo* IFNγ secretion to monitor the $CD8^+$ T cell response did not observe an LLO effect (34).

The presence of Ni-chelating lipid in the liposomes presents a prime variable in this study. Indeed, recent studies have implicated Ni^{2+} in the NLPR3 inflammasome pathway and even TLR4 activation (35-37). Interestingly, the effect has been seen as activating NLRP3 inflammasomes. Multi-walled carbon nanotubes were reported to elicit increasing IL-1β from primary alveolar macrophages (primed with LPS) with increasing Ni^{2+} contamination. The ability of the nanotubes to elicit this response is attenuated by caspase-1 inhibition and cathepsin B inhibition. This study proposed that Ni^{2+} is able to mediate this activation by contributing to the rupture of the phagolysosome to release the lysosomal protease, which has been proposed as a ligand for NLRP3 inflammasome in the cytosol (35). Similarly, in another recent study, the authors found that PMA-primed THP1 cells and LPS-primed human PBMC, murine BMM and BMDC produced increasing IL-1β to increasing

Ni^{2+} concentrations in a capase-1, NLRP3 and ASC-dependent fashion but independently of the phagolysosomal pathway (36).

Cathepsin B, a ligand for the NLRP3 inflammasome, was reported to be the medium through which particulates such as aluminum salts, silica and asbestos activate the NLRP3 inflammasome (38). These particulates are thought to induce lysosomal rupture, which exposes cathepsin B to the cytosol. Being an endosomal pore-forming protein, this implies LLO has a potential to mediate a similar effect. Indeed, the purpose of LLO is to mediate the release of endosomal contents, which include cathepsin B into the cytosol. In fact, this has been reported to be the case, where human PBMCs infected with *Listeria monocytogenes* displayed an LLO-dependent release of cathepsin B, which led to IL-1β secretion (20). More relevantly, this study also noticed purified LLO-mediated IL-1β release was independent of cathepsin B (20). Some of the studies showing independence of cathepsin B, particularly when using cathepsin B-deficient mice, have been able to allow other researchers to infer that the cathepsin B inhibitors used in studies may have off-target effects on cathepsin B, hence the main ligand resulting from the particulate-mediated inflammasome activation may be unidentified (38). While we do not observe inflammasome activation in non-LLO nickel containing liposomes, we cannot discount the role that Ni^{2+} may play in the presence of other activators of the inflammasome. The biological fate and effect of Ni^{2+} in a formulation such as the one used in this study has not been characterized in detail. It was assumed that the observations from our previous studies that used non-nickel containing LLOhis liposomes would apply to this study, which used nickel-containing LLO liposomes. However, there is no experimental basis for this assumption; a detailed comparison particularly in the context of inflammasome activation

is needed to check this notion. We observe activation of the inflammasome in the presence of LLO and this was enhanced by the presence of fliC in the cytosol, the mechanism notwithstanding.

The foregoing reflects the intricate nature of the mechanisms and contributors in the activation of an inflammasome; it has however not proffered any rationale for the discordance in data we observed *in vitro* and *in vivo.* Although we observed inflammasome activation and enhanced presentation of MHC class I-restricted antigen *in vitro*, this enhancement did not translate to enhanced $CD8^+$ T cells in immunized mice, which prompts a consideration of the role of the inflammasome, perhaps NLRP3 and NLRC4 in (OVA;LLO)-fliC and (OVA;LLO) *in vivo.* Many pathogens have developed strategies to avoid the effects of activating the inflammasome to promote their survival and virulence. These evasion strategies include inhibiting caspase-1 activity or activation, inhibiting IL-1β and preventing inflammasome recognition of the virulence factor (5). Based on this, activation of the inflammasomes is thought to be required for protective immunity against a number of pathogens, including *Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and Influenza A (39).

With regard to NLRC4 inflammasomes, a notable study by Sauer *et al.* has indicated that *Listeria monocytogenes* engineered to activate NLRC4 inflammasomes decreased the induction of antigen-specific T-cells (40). In their study, *L. monocytogenes* was engineered to ectopically secrete *L. pneumophila* flagellin restricted to expression in cytosolic bacteria. These engineered bacteria activated the inflammasome, and the intracellular growth was severely attenuated in BMM *in vitro* and *in vivo* where the engineered bacteria produced fewer colony-forming units in the spleen and liver of immunized mice; this attenuation is

rescued in NLRC4$^{-/-}$/Naip5$^{-/-}$ mice. While activation of the inflammasome controlled the infection, they further observed that mice immunized at both low and high doses of the engineered bacteria showed decreased protection to subsequent *L. monocytogenes* challenge in terms of bacterial burden in both spleen and liver, when compared to mice immunized with an unengineered strain. They also reported that mice immunized with the engineered strain induced defective CD8^{+} T cell response as determined by *ex vivo* stimulation of splenocytes with peptides and tetramer staining. Both the defective protective response and defective T cell development were reversed in caspase-1 $^{-/-}$ mice, correlating the response with inflammasome activation (40). Observations of the Sauer *et al.* study supported the hypothesis that intracellular pathogens evade activating the inflammasome to promote their virulence and reported the role the inflammasome plays in infection control *in vitro* and *in vivo.* However, in alignment with our observation that mice immunized with inflammasome-activating formulations exhibit no significant antigen-specific CD8^{+}T cells, they also demonstrated that inflammasome activation was deleterious to generating protective immunity.

Irrespective of fliC functionalization, in our study comparing both (OVA;LLO) and (OVA;LLO)-fliC, of the triad of parameters we evaluated (IL-1β secretion, caspase-1 activation and pyroptosis) the inefficient pyroptosis (and ranging from < 20% after 4 hours of incubation versus < 10% in formulations used in cytokine studies) was instigating. Our observation may have been mediated by exposure times. In the presence of active caspase-1, which is understood to be required for cytokine secretion in NLRC4 inflammasomes and presumably led to IL-1β secretion, we did not observe efficient cell-death. In the NLRC4 inflammasome, pyroptosis can occur independently of caspase-1 processing as Broz *et al.*

determined that pyroptosis can be triggered by catalytically active but unprocessed caspase-1 (33). Pyroptosis functions to eliminate the replicating intracellular pathogens, making them susceptible to phagocytosis and killing by a secondary phagocyte, notably neutrophils (4, 26).

We propose that *in vitro*, antigen-presenting BMM not primed with LPS – as was in the antigen presentation assay- would not produce IL-1β since there is no pro-form of IL-1β. BMMs do not express TLR5, hence the need to prime with LPS to generate pro-IL-1β in our cytokine secretion studies. However, the caspase-1 activation is independent of TLR priming and unaffected by the lack of TLR5 in BMM, so in the presence of LLO, caspase-1 is activated. According to our observations, there is caspase-1 activation; potentially inefficient pyroptosis at four hours and the cells are not subject to any effect of IL-1β. Because these BMM are able to present class I –restricted antigens to T cells in this scenario, this leads us to propose caspase- 1 activation may not be of significance in the antigen presentation observed *in vitro* and the BMM presents the cytosolic content as it would, while the lack of IL-1β in this environment cues us to its potentially important role in the lack of response seen *in vivo*. The role of IL-1β and IL-18 in innate and adaptive immunity have been relatively well studied. IL-1β induces the expression of many genes, including IL-6 and TNFα, and plays a vital role in the induction of Th17 in humans and the antigen-driven expansion and differentiation of CD4 T cells (39). Depending on the cytokine milieu, IL-18 in combination with IL-12 or IL-2 can induce differentiation of either Th1 or Th2 cell types (39, 41). When introducing the formulation *in vivo*, where presumably the (OVA;LLO)-fliC encounter TLR5-expressing APCs, ligation of fliC and TLR5 leads to the pro-form of IL-1β present for cleavage by caspase-1 upon inflammasome

activation. Since pyroptosis mediated by this formulation appears diminished, we propose that, in the absence of pyroptosis, IL-1β could dictate the inflammatory milieu that governs the course of cell-mediated immunity. (OVA; LLO) *in vivo* would not interact with TLR5 to produce the precursor to IL-1β and our observation is not accommodated in our proposed model. Considering we are unsure what inflammasome is being activated in this formulation, there are perhaps other unknown elements driving the immune profile observed but this remains unanswered. Studying the effect of inflammasome activation results in an intricate web of determinants that is not easily deconstructed especially in the face of activating multiple inflammasomes.

CONCLUSION

Our results have demonstrated that in the presence of LLO and, by extension, activation of the inflammasome, fliC-functionalized liposomes enhance IgG1 production. The inflammasome-activating formulations minimized pyroptosis, enhanced MHC-class I-restricted antigen presentation *in vitro*, and did not engender the $CD8^+$ T cell response in immunized mice. Understanding the role of pyroptosis *in vivo* may be significant in understanding how delivery of fliC in the cytosol affects its $CD8^+$ mediated immunity, particularly in the context of a delivery vehicle that minimizes pyroptosis.

(A) (B)

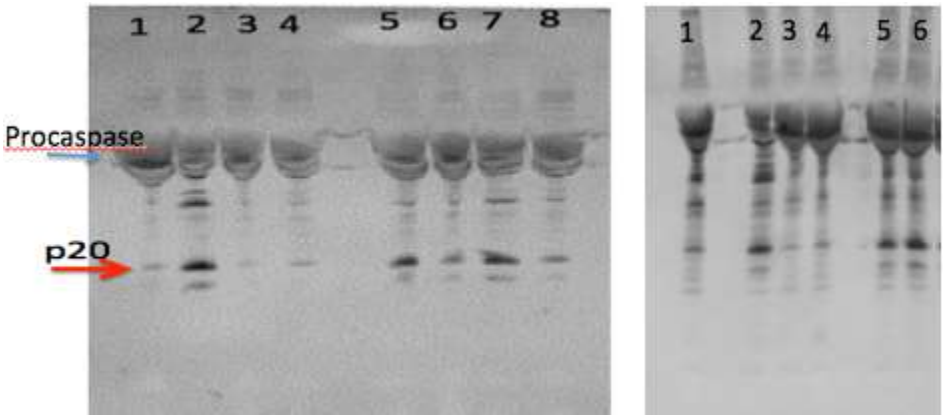
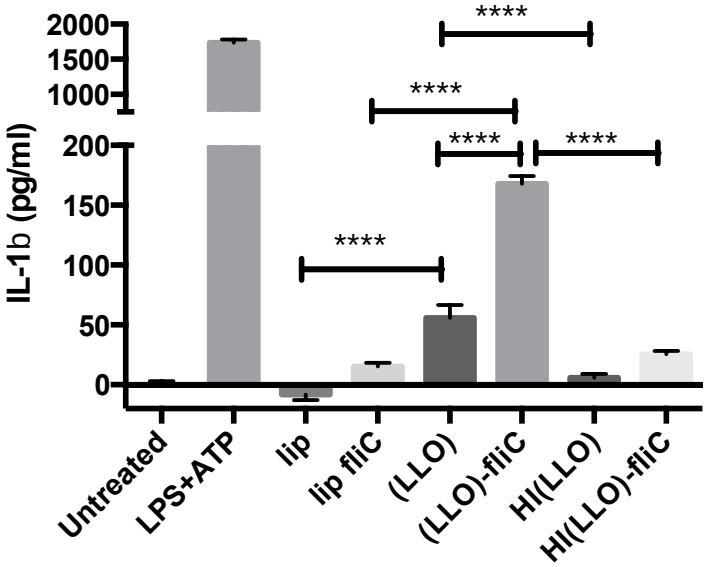


Figure 3.1: Flagellin enhances LLO-mediated activation of caspase-1

Flagellin-enhances LLO-mediated activation of caspase-1.- BMM were stimulated according to LLO and fliC concentration (10μg/ml), and caspase-1 extracted from cell-free supernatants was immunoblotted on the following lanes, **lane 1**: untreated cells; **lane 2**, 200ng/ml LPS for 4 hours, followed by a 2 hour 5mM ATP incubation; **lane 3**, blank liposomes; **lane 4**, fliC-functionalized liposomes; **lane 5**, LLO liposomes; **lane 6**, heat-inactivated LLO liposomes; **lane 7**, fliC-functionalized LLO liposomes; and **lane 8**, functionalized heat-inactivated LLO liposomes. LLO-inactivated liposomes were made by incubating LLO liposomes at 70 ° C for 10 minutes. Encapsulated content of the liposomes is in parentheses, and protein attached after formation of liposomes is represented by hyphens. (B) BMM not primed with LPS. **Lane 1**: untreated cells; **lane 2**, LPS+ATP; **lane 3**, blank liposomes; **lane 4**, fliC-functionalized liposomes; **lane 5**, LLO liposomes; and **lane 6**, fliC-functionalized LLO liposomes.

(A)



(B)

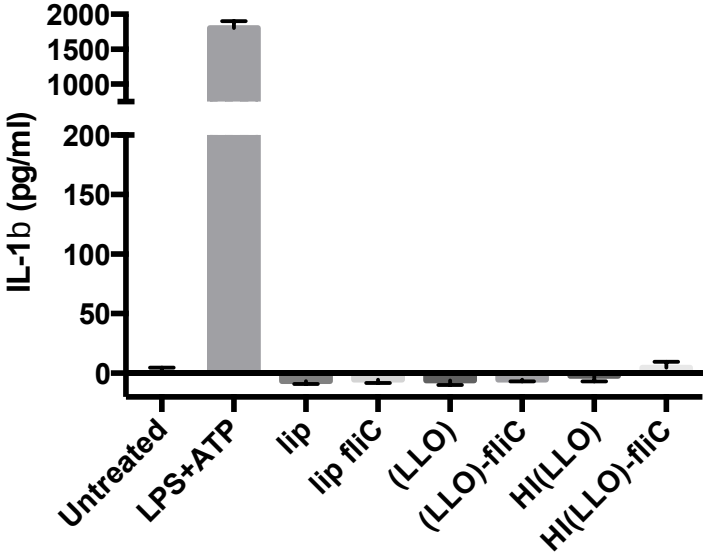
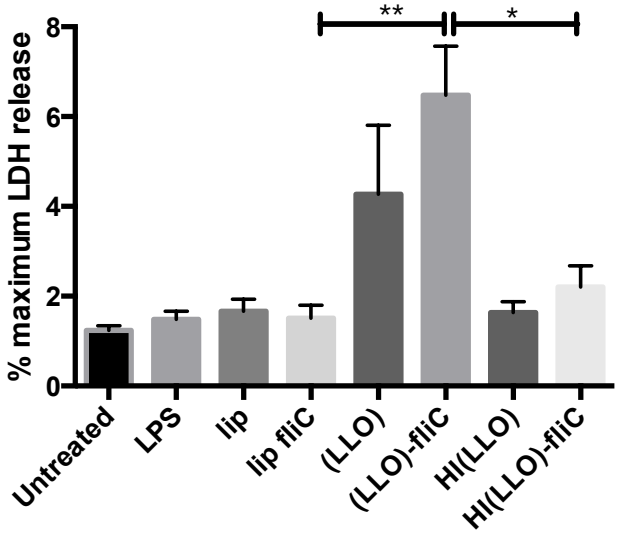


Figure 3.2: FliC enhances LLO-mediated secretion of IL-1β

Flagellin-enhances LLO-mediated secretion of IL-1β. A) Bone-marrow derived macrophages were treated with 200ng/ml LPS for 4 hours and the primed cells were exposed to 5mM ATP, 10μg/ml LLO, and 8ug/ml fliC for 4 hours. B) IL-1β is mediated by LPS priming. BMM were not primed with LPS before the same treatment was applied. The treatment was discarded, cells were pulsed in complete media, and the cell supernatants were assayed for IL-1β via ELISA after 20 hours. Data shows mean of triplicates ± SEM and analyzed by one-way analysis of variance (*$p<0.05$).

(A)



(B)

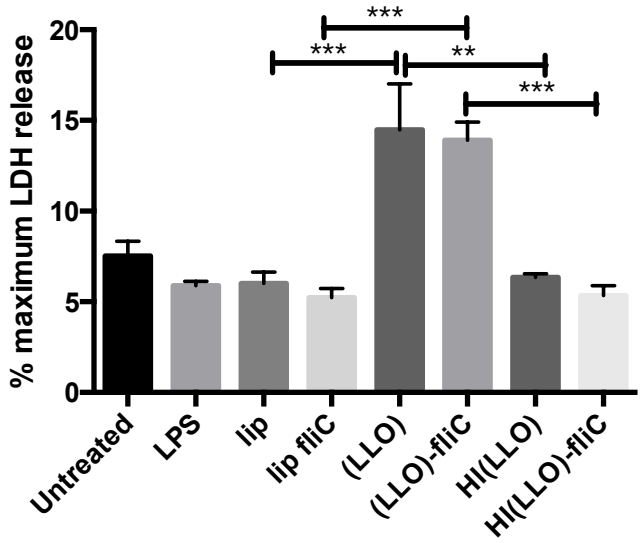


Figure 3.3: FliC does not enhance LLO-mediated toxicity regardless of LPS priming

FliC does not enhance LLO-mediated cell death regardless of LPS-priming. (A) Bone-marrow derived macrophages were treated with 200ng/ml LPS for 4 hours and the primed cells were exposed to 10µg/ml LLO and 8ug/ml fliC for 4 hours. (B) BMM were not primed with LPS before the same treatment was applied. The cell supernatants were assayed for LDH. Data shows mean of triplicates ± SEM and analyzed by one-way analysis of variance (*$p<0.05$).

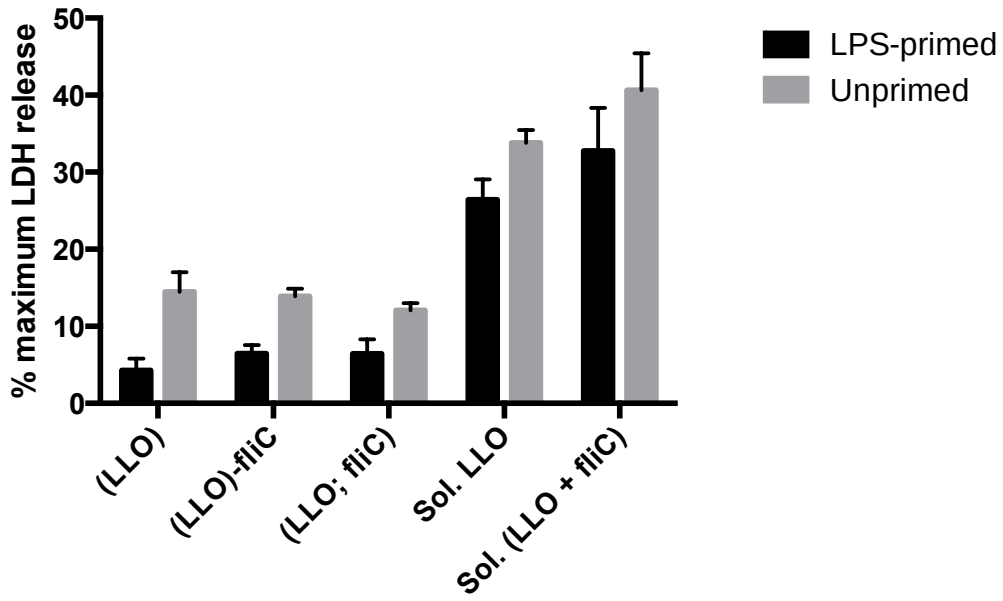


Figure 3.4: Encapsulation in liposomes minimizes toxicity of soluble LLO

Encapsulation in liposomes minimizes toxicity of soluble LLO: Bone-marrow derived macrophages were treated with 200ng/ml LPS for 4 hours and the primed cells were exposed to formulations with 10µg/ml LLO and 8µg/ml fliC either in soluble form or in liposomes for 4 hours. The cell supernatants were assayed for LDH.

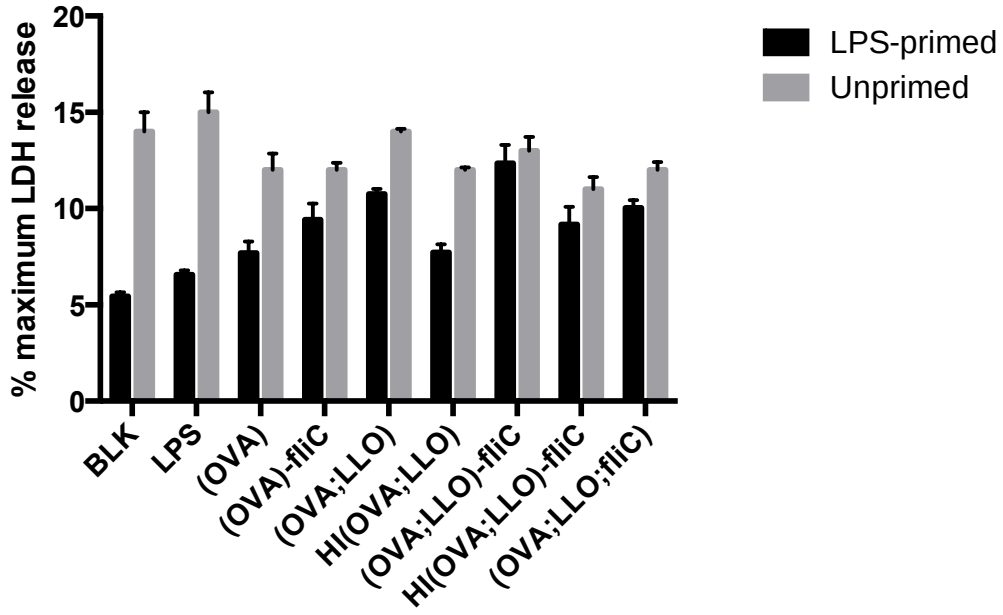


Figure 3.5: In the presence of OVA, LLO-mediated toxicity is not apparent

In the presence of OVA, LLO-mediated toxicity is not apparent. Bone-marrow derived macrophages were treated with 200ng/ml LPS for 4 hours and the primed cells were exposed to formulations with 10µg/ml LLO and 8µg/ml fliC for 4 hours. The cell supernatants were assayed for LDH. Data shows mean of triplicates ± SEM

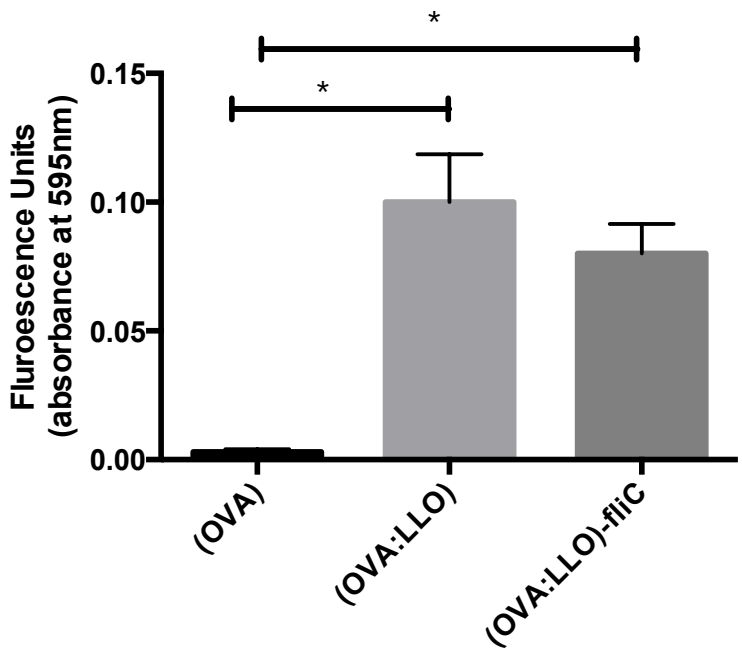


Figure 3.6: Functionalization with fliC does not inhibit LLO-mediated cytosolic OVA delivery *in vitro*

BMM were pulsed with liposomal OVA (OVA), OVA-LLO liposomes (OVA; LLO), fliC-functionalized liposomal OVA, and fliC-functionalized LLO OVA liposomes [(OVA; LLO)-fliC] for 2 hours. Cells were washed and further incubated for 3 hours and fixed. The fixed cells are incubated with B3Z cells for 15 hours. Presentation of SIINFEKL–MHC complex to B3Z cells was monitored by the conversion of CPRG substrate to chlorophenol red in primed B3Z cells and absorbance at 595nm. Data shows mean of triplicates ± SEM of one representative experiment of two independent experiments and analyzed by one-way analysis of variance (*$p<0.05$)

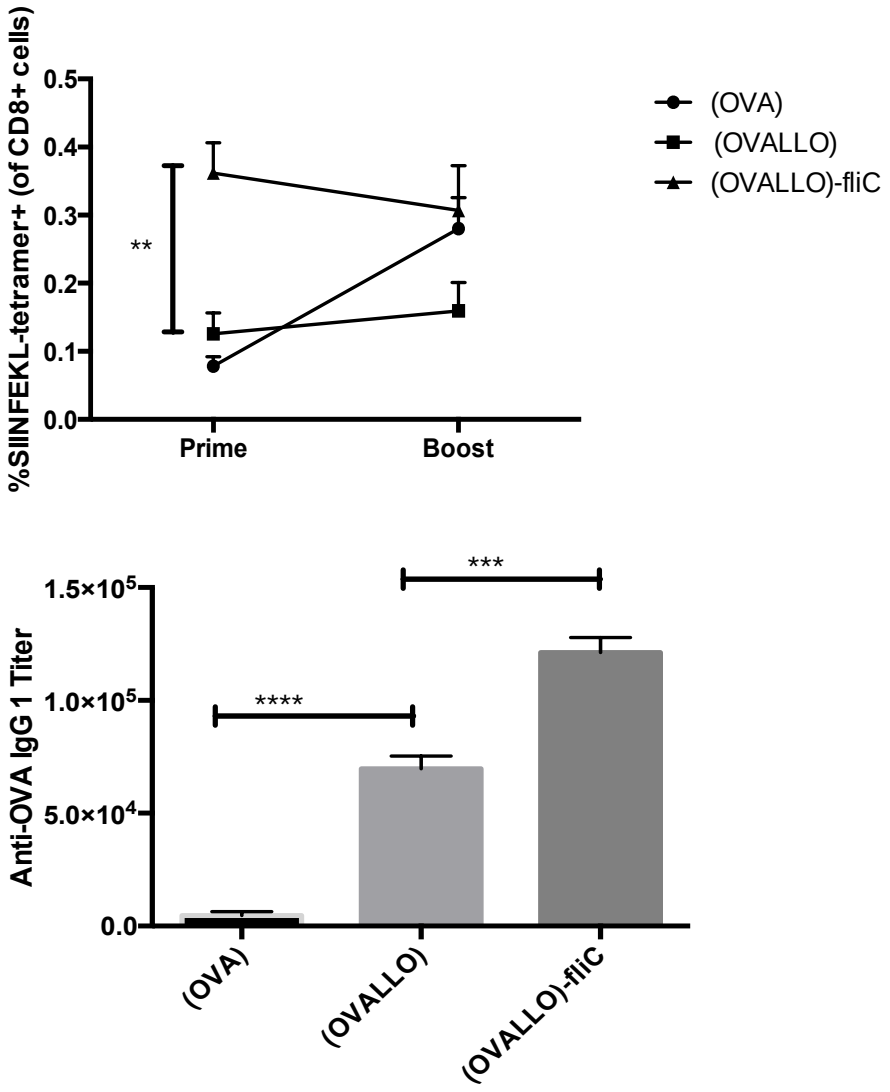


Figure 3.7: FliC-functionalized LLO liposomes enhance OVA-specific IgG1 titers

(A) C57BL/6 mice were immunized s.c. with liposomal OVA alone (OVA), fliC-functionalized liposomal OVA (OVA-fliC), OVA LLO liposomes (OVA; LLO), or fliC-functionalized OVA LLO liposomes [(OVA; LLO)-fliC]. N=4 Dose was normalized to OVA concentration at 10μg, 4-5μg LLO, and 5-7μg fliC. Nine days later, the animals were boosted with the same formulation. On day 16, frequencies of OVA-specific $CD8^+$ T cells were evaluated by MHC-1 tetramer staining via flow cytometry. Average percentage (± SEM) of OVA-specific $CD8^+$T cells from 4 mice per group is shown. (B) Flagellin functionalized liposomes encapsulating OVA induce IgG1 antibody production independently of LLO inclusion. Eleven days after the booster immunization was administered, sera were collected on day 21 and OVA-specific immunoglobulin determined by ELISA (****$p<0.0001$)

	Apoptosis	Pyroptosis
Initiating	Programmed	Programmed
Signaling pathway	Caspase-2/3/6/7/8/9	Caspase-1/11
Terminal event	Non-lytic	Lytic
Effect on tissue	Non-inflammatory	Inflammatory

Table 3.1: Comparison of apoptosis and pyroptosis

(adapted from (26))

Pyroptosis is characterized by formation of membrane pores that lead to plasma membrane lysis, releasing the cytosolic content into the extracellular space. Specific markers for this lysis are useful *in vitro*, for example, lactate dehydrogenase release is readily detected by enzyme assay. On the other hand during apoptosis, plasma membrane integrity is maintained and cellular contents are not released (31).

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CHAPTER 4 Significance and Future directions

SIGNIFICANCE

This study is the first to design and evaluate the immune profile of full flagellin, in the context of liposomes. Tactically, using the full protein affords the ability to harness properties of flagellin as both a TLR and an NLR ligand, abilities that are contingent on specific separate locations on the protein. Liposomes are particularly suited for delivery of flagellin, because their surface can be modified to display flagellin, as though it were being expressed on a pathogen, and allow flagellin interact with its receptor. In the case of fliC, not only does this interaction enhance delivery to APCs responsive to flagellin, the ligation of fliC and TLR5 initiates innate immune cascade that will produce the cytokine milieu that will govern the pattern of the adaptive immune response.

When compared to the conventional approach of using fusion proteins to target the antigen and the adjuvant to the same APC, the functionalized liposomes eliminate the limitations of a. inability to create stable fusion protein and b. altered immunogenicity of the antigen such that generated antibodies lack recognition for native antigens, which make them a suitable system to deliver virtually any antigen that can be encapsulated in liposomes to APCs while exploiting the adjuvant ability of flagellin. The functionalization process poses an inherent risk to the capabilities of both the liposomes as a vehicle and fliC as an adjuvant, however both fliC and the liposomes retain their individual characteristics. FliC-functionalized liposomes provides the benefit of targeting the adjuvant and the antigen to the APC, as flagellin-adjuvant fusions would, seen as an enhanced Th2-biased

humoral and cell-mediated response attributable to fliC and the liposomes retain their integrity and ability to deliver encapsulated cargo.

Finally the studies demonstrated the ability of LLO to mediate the delivery of surface-attached fliC to the cytosol to engage the inflammasome and evaluate flagellin actions in when present as both a TLR5 and NLRC4 ligand. Activating the inflammasome may be beneficial for fliC-mediated humoral immunity and the studies elucidate some of the complexities of trying to deconstruct the role of the inflammasome in fliC-mediated immunity.

FUTURE DIRECTIONS

The use of flagellin-functionalized liposomes in the context of a clinically relevant antigen

The foremost finding of the study presented in chapter 2, is the ability of fliC-functionalized liposomes to activate the innate immune response, enhance MHC-class I-restricted antigen presentation, and elicit humoral response and $CD8^+$ T cell response against an encapsulated antigen. Given the range of antigens that can be encapsulated in liposomes, future studies should focus on a clinically relevant protein antigen to test the range of this delivery system. In addition to antigen-specific $CD8^+$ T cell tetramer staining, functional assays should also be performed to better characterize the nature and polarity of the immune profile.

Establishing (OVA;LLO) as an appropriate positive control and exploring potential influences of experimental differences on $CD8^+$ T cell response

In the studies described in chapter 3, we expected (OVA;LLO) to serve as a positive control for OVA delivery to the cytosol and MHC class I pathway and expected to see enhanced $CD8^+$ T cell response in comparison to (OVA). We did not observe this in two independent experiments. While the different methods of monitoring $CD8^+$ T cell response may have contributed to the difference, the inability to demonstrate an LLO-mediated $CD8^+$ T cell response in vivo discouraged any subsequent functional assays and may not be a pressing source of difference at this time. The effect of differences in the formulations of previous studies and the current study, lack of hexahistidine tag and Ni-lipid content, while they appear inconsequential, will need to be probed. Being a bioactive protein, one cannot discount the role that differences such as LLO potency can have. Hemolytic activity can

vary from one purification batch to another, hence using a measure of biological effectiveness rather than absolute quantitative measure may exclude the additional complexity of varying delivery efficiency when comparing observed results to prior observations. In Chapter 3, we observed that the Ni-containing (OVA;LLO) activated an inflammasome. Since nickel has been implicated in inflammasome activation (1, 2), and the main goal of using LLO liposomes was to engage the NLRC4 inflammasome with fliC, this line of investigation would compare the formulations with regard to the ability to activate caspase-1, and the ability to secret IL-1β from LPS-primed and unprimed BMM. Secretion of LDH should also be monitored as an indication of pyroptosis. The results of this comparison will dictate if the formulations are comparable in the context of activating cell-mediated immune response.

The use of LLO liposomes presents the potential of activating multiple inflammasomes. In this scenario, regardless of the agonist, we are able to study the effect activating inflammasomes and TLR5 has on the adaptive immune system in a crude sense. At the finest, we should be able to determine the immunological relevance of combining the agonist of specifically NLRC4 inflammasome and TLR5 to be able to exploit this to optimize vaccine response. In this vein, it may be practical to determine what inflammasome is being activated in cells treated with (OVA;LLO). To start with, the caspase-1 activation measurement should be assessed in NLRC3$^{-/-}$ and NRLC4$^{-/-}$ BMMs to determine the relative contributions of each inflammasome in (OVA;LLO) and (OVA;LLO)-fliC.

Exploring the potential role of proinflammatory cytokines

In an effort to reconcile the *in vitro* antigen presentation assay results with our *in vivo* observations, we proposed that minimal pyroptosis and the lack of IL-1β allowed

MHC-class I –restricted peptides to be presented to T cells. To test the effect of IL-1β in antigen presentation, we recommend modifying the antigen presentation assay by priming BMM with LPS for 4 hours prior to the assay. The goal is to generate a pool of the pro IL-1β form, before cells are pulsed with (OVA;LLO), (OVA;LLO)-fliC and (OVA) for 4 hours (to match the exposure time we used to evaluate LDH-secretion) cells in which caspase-1 is activated, would cleave the pro IL-1β form in to IL-1β. The BMM should then be incubated for another 3 hours without treatment in media before they are exposed to T cells for antigen presentation. Unprimed BMM as we have studied in chapter 3 can serve as controls for cells with no IL-1β present. If the MHC class I- restricted peptide presentation is diminished, then IL-1β is an important link between *in vitro* and *in vivo* response observed.

Subsequently, the importance of IL-1β *in vivo* can be evaluated using IL-1β$^{-/-}$/IL18$^{-/-}$ mice. In the inflammasome activation pathway, IL-1β and IL-18 are the main proinflammatory cytokines resulting from the proteolytic activity of processed capase-1. It is understood that these two cytokines exert pleiotropic effect that can promote Th1, Th2 or Th17 responses (3). In this test, the mice will be immunized with (OVA), (OVA;LLO), (OVA;LLO)-fliC. Both the antibody titer for IgG1 and IgG2c and the CD8^{+} T cells via tetramer staining should be evaluated. The splenocytes from immunized mice can be stimulated *ex vivo* for cytokine secretion in the presence of OVA MHC class I and class II peptides to evaluate generated immune profile. If in the absence of the influence of IL-1β and IL-18, the same immunologic profile is observed, then another factor, potentially, pyroptosis is influencing T cell response. The role of the inflammasome in the observed CD8^{+} T cell response is salient when comparing response elicited from (OVA)-fliC, to the

inflammasome-activating formulations. Alternatively, the caspase-$1^{-/-}$ mice can be immunized with these treatments to confirm the role of the inflammasome.

Exploring the potential role for pyroptosis *in vitro* and *in vivo* responses

We observed minimized toxicity in comparison to soluble protein after a 4-hour exposure time. With this observation, we were not able to ascertain the practicality of measuring at that this time, so at the very least, we can suggest that encapsulated liposomes may delay the kinetics of pyroptosis and this is likely biologically relevant hence determining the relationship between exposure time to inflammasome-activating treatment and LDH secretion is worth exploring in future studies. The range of exposure time in the literature has been cited as reason for the disparity in the observations of inflammasome activation by *L. monocytogenes in vitro* (3).

In the *in vitro* antigen presentation, we observed minimal pyroptosis from the liposomal treatment, and this did not appear to affect MHC class I presentation to T cells. Pyroptosis could be insignificant *in vivo* and the cytokine response dictates the immunologic profile. What may hold the key to this answer may be determining the underlying source of how the inflammasome-dependent cytokines are released. The exact mechanism of secretion of IL-1β and IL-18 is debated. There is the suggestion that they are released during pyroptosis. In contrast, there are reports that show that while secretion may be temporally associated with caspase-1 dependent formation, secretion does not require lysis. Mechanisms like caspase-1 independent lysosome exocytosis and microvesicle shedding have been proposed (4). There has been evidence of cross talk between caapase-1- dependent and other cell death pathways that may compensate for lack of pyroptosis *in vivo*. What signifies the need to compensate for cell death in this

scenario is not well elucidated. Nevertheless, cell death in this instance has been described as kinetically slower than pyroptosis (5). A fact that suggests that if in fact pyroptosis is "rescued" *in vivo,* the secreted IL-1β and IL-18 may still govern the immunologic profile observed.

Finally, regardless of the kinetics of cell-death, we observed that in the same time frame the differences the soluble version of the LLO and LLO fliC combination has on pyroptosis. Attempts to further study the role of pyroptosis *in vivo* may aim to compare liposomal proteins with soluble protein form, with LLO liposomes, functionalized and unfunctionalized compared to fliCOVALLO and OVALLO fusion proteins respectively. To mitigate potential problems that may occur in the process of expressing the recombinant proteins, the first strategy would be to explore the main reasons that lead to lack of protein expression or lack of activity of the required protein which could be due to toxicity of the recombinant protein to the cell or insolubility of recombinant protein resulting in aggregation in inclusion bodies in the cell. Various optimization methods such as changing growth medium, temperature and length of induction or switching from the BL21 expression strain to another expression strain. If protein is being expressed, but the activity of each component is not detectable, an alternative approach to the proposed recombinant protein will be to synthesize a fusion protein that exploits the sulfhydryl of LLO needed for pore forming activity. One such approach is to utilize the lone cysteine of LLO (C484) as reported by Saito et al (6). Hence, the final protein will consist of a disulfide-bonded heterodimer that would be cleavable in the endosome, and as such the LLO would be cleaved from the fusion to exert its pore‐forming activity, while the fliCOVA would emerge from the endosome, free of the LLO. It should be noted that flagellin ovalbumin

fusions have been expressed in studies in the literature and may not pose a challenge in purification.

Correlating the presence of protein-specific immune response to other components of the vaccine vehicle to encapsulated antigen-specific immune responses

The vaccine delivery vehicle presented in this thesis is multicomponent. This is most relevant *in vivo* when two to three proteins are associated with the liposomes at a given time. In chapter 2 immunization studies, OVA and fliC and in chapter 3, OVA, LLO and fliC are present. These components are proteins, to which protein-specific immune responses can be generated. The studies only focused on the antigen-specific immune responses to the encapsulated antigen, OVA. While studies suggest that preexisting immunity to fliC does not affect the subsequent adjuvant activity of flagellin (7), and pre-existing LLO immunity does not inhibit functionality of LLO-containing vaccine formulations (8), vaccine efficacy can be limited or enhanced by preexisting carrier immunity. Hence, it may be worthwhile to explore and evaluate the anti-fliC and anti-LLO immune responses in an endeavor to correlate the anti-carrier profiles to that observed with the immune profile against encapsulated protein, specifically in an immunization regimen that consists of a booster dose.

Exploring alternative ligand attachment methods to surface of liposomes

The concept of multifunctional liposomes in the context of surface functionalization is not new. There have been many studies of liposome–associated antigens and various modes of antigen attachment to liposomes ranging from covalent conjugation to adsorption exist. These methods are generally effective in inducing immune response while some body of evidence show an advantage for covalently attached surface proteins in generating

antibody response towards the attached protein (9). The choice of using the Ni-NTA affinity for the his-tagged flagellin in this study was to have a platform for fliC to be stably tethered to interact with its receptor not to modulate the strength of the immune response against the surface protein.

Given the potentially incriminating role of Ni in inflammasome activation and the unknown effect it may have on activating $CD8^+$ T cell response *in vivo*, it is worth pursuing how to minimize the amount of nickel-containing lipid in future formulations. In chapter 2, we observed a wide range of coating densities using a single Ni-lipid concentration. Future experiments should explore a minimum concentration that elicits innate immune response but potentially non-deleterious to cell-mediated response. Regardless, the safety profile of the nickel-containing lipid is undetermined. Lipid nanoparticles with accessible nickel have been used to deliver multiple his-tagged HIV antigens, and the toxicity of these nanoparticles on a dendritic cell line was found to be reduced in comparison to charged nanoparticles (10). Similarly, *in vitro* studies suggest that NTA-functionalization of microparticles regardless of the presence of nickel does not influence toxicity in cardiac myocytes and macrophages. However, nickel nanoparticles have been implicated in enhanced lung inflammation and toxicity in inhalation exposure with a mechanism that is proposed to correlate with its capability to induced free radical damage to plasmid DNA (11).

The relevance of such fore mentioned findings on the vaccine and clinical potential of nickel-containing liposomes is unknown and have not been duly explored. Nevertheless, there are options for liposomal surface modification that can be explored to avoid the use of Ni-lipid, particularly covalent conjugation methods. Options for covalent conjugation

include conjugation through an amide bond and a disulfide or thioether bond (9). Phospholipids with amine or carboxyl functional groups such as carboxyacyl derivative of phosphoethanolamine can be conjugated to proteins (12). Lipids for disulfide or thioether conjugation, such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate (PDP-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB-PE) respectively, can be conjugated to thio-containing proteins. A comparison of PDP and MPB conjugates suggest that the MPB conjugate retain better serum stability (13). To effect functionalization via a thioether bond, the ligand to be attached to the surface of the liposomes-which contain lipids with a sulfhydryl-reactive maleimide group conjugated to the head group- should have an affinity for the maleimide moiety on the lipid. This is done either by exploiting the sulfhydryl groups present in the ligand, which in the case of fliC are lacking, or by introducing protected, yet exposable sulfhydryl groups via alternative means using reagents such as N-Succinimidyl S-Acetylthioacetate (SATA) to react with the free amines in the protein (14, 15). Alternatively, fliC can be mutated using standard PCR methods to introduce a cysteine at the C-terminus.

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APPENDIX Histone H2A enhances the capability of LLO LPDII as a nuclear delivery vector *in vitro*

INTRODUCTION

DNA vaccines, potential and limitations

DNA delivery is usually classified as viral vector-mediated or non-viral vector-mediated. The viral-mediated methods employ genetically engineered viruses as drug delivery vectors. As in live-attenuated vaccines, viral vector-mediated delivery is efficient at delivering DNA for expression; however, this efficiency comes at the cost of safety to the recipient. As demonstrated in gene therapy clinical trials, the cost of the acute immune response that is induced, the possibility of insertional mutagenesis, and immunogenicity against the carrier (1) underscored the need for alternatives to viral vector-mediated DNA delivery. Considering that DNA vaccines are able to express antigens in the cells for immunogenic purposes, DNA vaccines are well suited for targeting the MHC class I pathway. DNA-based vaccines often consist of bacterial plasmids that contain the cDNA for an antigenic protein to be transcribed and translated after administration to the subject, just as a natural infection would mediate the transcription of its genetic material in the nucleus and translation in the cytosol.

Despite the promise of the DNA-based vaccines, there is no DNA therapeutic modality licensed for human use. Three DNA vaccines have been licensed for veterinary uses including a canine melanoma vaccine and another DNA-based gene therapy product for use in pigs (2, 3). This paucity can be attributed to a number of factors that retards the

development of DNA vaccines. The ineffective delivery of DNA vaccines and lack of a potent immune response, in terms of creating the magnitude and quality of response needed for protection, in large animals and humans are the greatest hurdles to development (3). Studies are ongoing to make improvements in other areas such as optimizing DNA expression through codon modification, chemical modification of linkages to enhance resistance to nucleases and developing new manufacturing processes to produce highly concentrated DNA (3).

Strategies to enhance DNA vaccine potential

Enhancing the delivery of DNA vaccines has taken various approaches to overcoming physiological limitations imposed on a DNA vaccine introduced to the physiological space, such as barriers of the cell membrane, the endosomal membrane and the nuclear membrane and nuclease degradation in plasma and cells (1). Approaches to enhance the immune response, have led to the use of adjuvants in vaccine design, usually in the context of protein subunit vaccines (3) To enhance the immunogenicity of DNA vaccines, plasmids encoding cytokines such as interferons, interleukins, tumor necrosis factors and colony stimulating factors have been tested (4). Some of these cytokines are able to act on lymphocytes to influence patterns of Th1 and Th2 differentiation or act on antigen-presenting cells to enhance the T cell-mediated response (4, 5).

While cytokines and chemokines used as adjuvants such as IL12, IL2, IFN γ and others possess these advantages (6) there have been suggestions that they may not be able to stimulate the immune system to the same extent or in the way, as components of an infection would (Ljunggren, 2008). Other approaches to enhance the potency of the immune response have been through immunization regimens of priming the immune

system with a DNA vaccine and boosting with the protein or DNA, sometimes requiring designing two vaccines (3). A vaccine delivery system that is sufficiently immunogenic could eliminate the need for this.

Enhancing nuclear delivery of DNA vaccines

Unlike live-attenuated vaccines and vaccine delivery using viral vectors, non-viral delivery systems are unable to utilize inherent machinery to transfer of genetic material across the cell compartments for transcription in the nucleus (7). As a consequence of the multiple processes involved for an antigen encoded by a DNA vaccine to be expressed, non-viral vectors that are lacking intrinsic properties or unable to utilize host machinery are not very efficient in delivery despite the relative gain in safety when compared to viral vectors.

Transfer of DNA into the nucleus is fundamental in achieving the goal of DNA vaccination and gene therapy; as such, there have been several approaches to overcome the barriers in put in place by the cell and essentially replicate the functionalities that enable pathogens to bypass these barriers. The ideal DNA vaccine should remain stable in the extracellular space, gain access to the cytosol, be stable in the cytosolic milieu upon exposure to a myriad of enzymes, and enter the nucleus for transcription, an expectation that is checked by the presence and selectivity of the nuclear membrane (8). This ideal scenario is sabotaged by the endocytic pathway, through which the DNA will most likely be internalized by the cells.

Thus, approaches to enhance transfection efficiency of DNA vaccines have been aimed at improving the efficiency of at least one of these processes. The methods have varied from the physical, such as the use of gene guns (9) ultrasound (10) and

electroporation (11) to the more widely studied chemical methods such as cationic lipids or polymers to condense the DNA to enhance cell entry (1). The latter approach is of direct relevance to this proposal.

Many studies have employed lipids, polymers, and peptides with the goal of neutralizing and condensing the polyanionic, large molecular weight DNA to protect the DNA from enzymatic degradation and thereby increase transfection efficiency (12). Polycationic polymers and polypeptides such as polyethylenimine, poly-L-lysine, and protamine have been shown to condense DNA to yield polyplexes, which have been reported to improve transfection efficiency to varying magnitudes (1). However, the need for large amounts of these polycations to realize better transfection efficiency usually leads to cytotoxicity, the extent of which varies from one cationic polymer to another, limiting their use (1). The cationic lipids that have been explored, while biodegradable still exhibit cytotoxicity; in addition, they tend to be unstable in physiological spaces- such as in the presence of serum, reducing their potential for use in vaccine delivery systems (13, 14). Polycationic moieties enhance DNA delivery as a result of their ability to condense DNA, which leads to a reduction in DNA size, enhanced cell uptake, and increased protection from cytosolic nucleases (15, 16).

Due to their cytotoxicity, however, there is the need to further explore the use of other proteins in the delivery of plasmid DNA for therapeutic uses and vaccine purposes. The use of protein for DNA delivery provides several advantages, such as ease of production, and homogeneity in production and purity (17). In principle, an endogenous protein that possesses the ability to sufficiently condense DNA exhibits potential in enhancing safe DNA delivery.

The potential and use of histone proteins for DNA condensation and nuclear delivery

Histones, the main protein component of chromatin, are very basic proteins that consist of the linker histone H1 and four core histones H2A, H2B, H3 and H4 (18). Histones are translated in the cytoplasm and transported into the nucleus, and are rich in arginine and lysine (19), a characteristic shared with the synthetic polycations. Not only do they condense DNA, studies have shown that histones also undergo post-translational modifications such as acetylation, methylation and phosphorylation that enable them to regulate the availability of several transcription factors (20). The attractiveness of histones as a DNA delivery method lies in their properties as the endogenous DNA condensers that also possess intrinsic nuclear localizing signals that facilitate their nuclear import. Histone-mediated DNA delivery has been studied with various core histones as well as with H1, and studies point to them being able to mediate DNA delivery by condensing the DNA and enhancing cell uptake, a process termed histonefection (21).

Enhanced transfection has been associated with each of the core histones and the linker histones (22). With some studies differing in their findings on which of the histones enhances transfection, it has been suggested that these differences could be a result of the cell lines tested or histone source (17). To date, the linker histone, H1, and the core histone, H2A, have been the most studied.

The histone linker proteins have been thought of as more mobile than the core histone proteins, even referred to as the "nomads" of the nucleus (20) and in comparison to core histones have a relatively low DNA binding affinity (23). However, they have been shown to enhance DNA delivery into a variety of cell lines with and without agents such as chloroquine (17). The linker histone H1 consists of a short N- terminal domain, a central

globular domain that is highly conserved and an arginine and lysine-rich, C- terminus (23). Studies show that the sequence of the human H1 protein that imparts its gene delivery activity resides in the C terminus as GFP fusion to C-terminus of histone H1 results in a less stable (reduced binding of histone to chromatin) protein *in vivo* than fusion to the N terminus (23) and gene fragments corresponding to the C-terminus display a higher efficiency of DNA, dsRNA and siRNA transfection into mammalian cells (24).

Studies have also shown the core histone H2A from calf thymus to mediate DNA transfer in cells and generate an antitumor response in a neuroblastoma tumor model challenge after vaccination (22, 25). A fragment of H2A consisting of the N-terminal 37 amino acids has been found to be able to condense DNA and function as a nuclear localization signal. The N-terminus of H2A contains a nearly equal number of lysines and arginines clustered in this region (19). Characterization of the nuclear localization signal of H2A confirmed the transfection abilities of the N-terminus and that of the globular domain, which although it does not contain basic amino acids, is able to mediate DNA delivery and localize DNA in the nucleus, indicating the presence of two nuclear localizing signals in the H2A core protein (19).

In spite of the delivery potential of endogenous proteins like histones, these groups of proteins are not without their specific concerns. While the Puebla study showed that fragments of H1 mediated gene delivery was associated with low cytotoxicity, there are concerns associated with the safety of histones with regard to immunogenicity. It is known that patients with systemic lupus erythematosus (SLE) develop autoantibodies against histones, particularly, H1 and H2B (26) and level of anti-histone antibodies correlates with disease activity. A subsequent study localized the prominent autoantigens on histones H1

and H2B (27) while a comparatively recent study determined H1 to be the major B cell and T cell autoantigen in SLE (28). As such it will not be considered for the proposed study.

In the studies characterizing SLE patients' autoantibodies, the autoantibodies from patients' sera where directed against calf thymus histones. There is a shortage of information on the diversity of histones between species. The calf thymus histone has been popularly used to study histone-mediated delivery but the ultra-pure forms are expensive (17). Although large quantities can be generated from cultured mammalian cells, there is a limitation to the use in studies due to lack of homogeneity resulting from varied posttranslational modifications (29). This underscores the usefulness of recombinant histones expressed in bacterial systems for histone mediated DNA delivery studies (17, 29).

The knowledge of histone immunogenicity is however, being used to harness immune protection against pathogens. For example the histones of Leishmania have been found to be highly immunogenic and there have been studies investigating histone-encoding DNA as the DNA in a DNA-based vaccine against Leishmania (30, 31). Due to the association of histone proteins with SLE and the potential implication of histone immunogenicity for repeated vaccinations, it is of interest to investigate the impact of using histone from the host species for enhancing delivery and using histone from the pathogen. In principle, if the administered histones cause histone-specific responses to the pathogenic histone, any immunogenicity induced against the histone would be against the antigen, a benefit to developing a repertoire of immune cells specific for that antigen. Considering, that histones are mainly features of eukaryotic cells (32, 33) this approach may be of potential application to eukaryotic pathogens such as the malaria-causing

plasmodium or histone from a eukaryotic pathogen or an identified bacterial histone-like protein may be used to deliver viral antigen-encoding DNA such as HIV gag.

Utilizing listeriolysin O (LLO) to enhance DNA vaccination and gene delivery

Despite the potential of DNA condensing agents, the effect of these agents and the potential for useful therapeutic applications are limited if the DNA complex is trapped in the endosome and degraded in the endocytic pathway. This necessitates the use of an endosomolytic to mediate the endosomal escape of the complex. To achieve this, studies of DNA delivery have sometimes been performed in the presence of compounds such as chloroquine (34) and ammonium chloride (35). These compounds' ability to enhance endosomal escape has been attributed to the proton sponge effect. In fact, polyethylenimine is also reported to cause a buffering of the endosome to induce its rupture via this proton sponge effect (Behr, 1997). There have also been studies exploring the mechanisms by which pathogens escape the endosomes in an attempt to exploit the functionalities for use in delivery systems. Various proteins and peptides derived from bacteria, viruses, plants, and even animals have been explored to enhance the mechanisms of pore formation and fusion for endosomal escape of antigens (36).

One of such proteins is the pore-forming listeriolysin O (LLO), a pH-dependent, sulfhydryl-activated cytolysin that mediates the escape of the gram-positive bacterium, *Listeria monocytogenes,* from the acidified endosome (37) This escape is necessary for the growth and pathogenesis of *Listeria* (38). The use of LLO has been studied *in vitro* and *in vivo* when incorporated in a liposomal delivery system and has been shown to enhance cytosolic delivery of an antigenic viral protein and plasmid DNA and it has been shown to enhance antigen-specific cytotoxic T lymphocyte responses (39, 40). Because of its ability

to mediate delivery of DNA vaccines to the cytosol and the MHC class I pathway, LLO holds potential for use in any delivery system that aims to generate cytotoxic T lymphocyte response.

Considering the fore-mentioned, the histone of study for the proposed vaccine delivery system to enhance DNA condensation and nuclear delivery, will be the human core histone H2A. With LLO being able to mediate enhanced gene expression in the context of anionic liposome -PN-DNA complex LPDII *in vitro*, and the demonstrated effect on enhancing antigen-specific cytotoxic T lymphocyte response *in vivo*. Our goal was to use H2A to enhance gene expression using the previously tested LLO LPDII for potentially better immune response. The rationale being that H2A is a better mediator of gene expression than PN for it dual NLS and DNA condensing properties. If it enhances expression in the context of LLO LPDII, it would augment cytotoxic T cell response.

MATERIALS AND METHODS

Cell line, proteins

The murine macrophage cell line P388D1 (ATCC, Manassas, VA) and cultured in RPMI-1640 supplemented with 10% Hi-FBS, 100U/ml penicillin, 100µg/ml streptomycin, and 1mM sodium pyruvate. Cells were plated 12-16 hours before transfection and grown at 37 ° C in a 5% CO_2. H2A was acquired commercially (NEB, Ipwisch, MA), protamine (PN) (Sigma Aldrich, St. Louis, MO).

GFP-Luc expression

The plasmid pNGLV3 encoding firefly luciferase and green fluorescent protein, under the control of the cytomegalovirus promoter was a gift of Dr Gary Nabel (Vaccine Research

Center, MD). The plasmid was expanded in *E. coli* and purified using the Qigaen Giga Endofree plasmid purification kit (Qiagen, Valencia, CA). Concentration of the purified plasmid was determined spectrophotometrically using absorbance of 260nm and A 260 280 nm rations > 1.8.

Preparation of LLO LPDII complexes

Liposomes were prepared using the thin lipid film hydration and freeze/thaw technique at descried in Sun *et al.* (39). Briefly, Phophatidyethanolamine (PE) (Avanti Polar lipids, Alabaster, AL) and Choleteryl hemosuccinate (CHEMS)

were mixed in a 2:1 molar ratio and the organic solvent was evaporated to a thin film on a rotary evaporator and allowed to dry under vacuum for 12-16 hours. The lipid film was rehydrated in 200μg/ml LLO in 1 ml HEPES-buffered glucose (HBG; 10mM HEPES, 280mM Glucose, pH 8.4) and vortexed. The liposomes were subjected to 4 freeze/thaw cycles in ethanol bath and sonicated 5 times for 1 minute in a bath sonicator. Unencapsulated LLO was separated from encapsulated LLO on Sepharose CL4B column (GE Healthcare) equilibrated with HBG. The phosphate concentration was measured using the method of Bartlett (42).

pDNA:cationic protein complex were prepared as described previously (43) Briefly, DNA and H2A (New England Biolabs, Ipswich, MA) or PN or PN LLO where diluted from stock with HBG in equal volumes were mixed to achieve a final DNA contribution of 150μg/ml at a 1.8 amine to phosphate charge ratio. In samples containing certain percentages of % of H2A and PN, the proteins are mixed in ratios that contributed the desired percentage to the charge.

Preformed LLO liposomes were added to the DNA/cationic polyplexes at room temperature and mixed with mild vortexing to achieve the desired final component concentrations and ratios. Heat–inactivated liposomes are obtained by heating liposomes at 70° C for 10 minutes. In samples where DNA polyplexes charge ratio was evaluated, the charge ratio was defined as the amine to phosphate ratio was

Briefly, H2A, is a 15.45 KDa protein, 1μg =64.63pmol; 1 molecule contains 12 arginine residues, and 14 lysine residues making total of 26 positively charge residues/molecule; 1 μg H2A therefore contains 26 positive residues * 0.064 nmols of positive charge. = 1.66nmol positive charge/ μg. Similarly, protamine is calculated to contain 4.2nmol/ μg and 1μg pDNA has been determined to contain 3 nmols of negative charge.

In vitro transfection

P388D1 cells were plated at a density of 1.5 x 10^5 cells/well in a 24-well plate. Cells were grown at 37 °C in a 5% CO_2 humidified atmosphere for 16 hours before treatment and were typically about 70% confluent at the time of transfection. On the day of treatment, 200ul of the (LLO)LPDII containing 2μg of DNA is added dropwise to each well and supplemented with complete media for a final media containing 5% Hi-FBS. The cells were incubated for 4 hours at 37 ° C and 5% CO_2. After 4 hours, the transfection mix is discarded from the wells and wells are replenished with complete cell media. The cells were incubated for another 48 hours. The cells were washed in 1 X PBS and the luciferase gene expression is determined using a luciferase assay system according to the manufacturer's instructions (Promega, Madison, WI). The luminescence is determined on spectrophotometer (BioTek, Winnoski, VT) and the values are normalized to total protein in each well as determined by BCA assay.

Gel retardation assay

The mixture of pDNA and cationic protein were mixed together at room temperature and incubated for 1 hour to form the polyplexes. The resulting polyplex was then applied to 1% agarose gel containing 0.1μg/ml ethidium bromide, which had been poured and left to solidify. The polyplexes were electrophoresed through the gel at 100V for 30 minutes. DNA location on gel was visualized under UV light using a UV transilluminator.

RESULTS AND DISCUSSION

H2A exhibits DNA-condensing potential when in excess

We sought to evaluate DNA condensing potential of H2A in the presence of a plasmid. We demonstrated that when DNA is in excess, H2A exhibits DNA condensing potential (Figure A.1). When DNA is completely condensed, the ethidium bromide cannot intercalate into the backbone and fluoresce under UV light; hence the disappearance of bands in the wells with increasing positive charge is due to this phenomenon. However, there is the potential that at certain higher concentrations, the protein aggregates and the retardation assay may not be indicative of the behavior mentioned. At charge neutral ratios, the status of the electrostatic complex is unclear and this may be indicative of the behavior seen.

(LLO)LPDII enhances H2A-mediated transfection

We evaluated the efficiency of H2A LLOLPDII *in vitro* in comparison to the previously studied PN- LLO-LPDII. To assess the LLO-mediated enhancement of the complexes, we used liposomes encapsulating heat-inactivated LLO to condense the pDNA

polyplexes. In the presence of LLO, transfection to the macrophage cell line increased significantly in the comparison to heat-inactivated LLO liposomes.

H2A (LLO) LPDII offered an advantage to transfection efficiency than PN (LLO)LPDII, however, we demonstrated that supplementation of protamine with percentage of H2A yielded better transfection than 100% H2A (Figure A.2).

Incremental supplementation of protamine with H2A enhances transfection efficiency

Considering H2A is demonstrated to possess; 1. nuclear localization signal and 2. DNA condensing ability, we sought to evaluate the contribution of H2A and PN to DNA complexation and gene expression. The rest of the studies employed a charge ratio of 1.8. This charge ration was determined as one where differences in H2A and PN transfection efficiency were distinctive enough (data not shown). We supplemented PN with 25 or 50% with H2A, buy using H2A to contribute 25 or 50% of the positive charge to be present in the complex. We observed that H2A consistently enhances the transfection efficiency of PN-mediated transfection. PN is not known as a nuclear localizing signal, it is known to be efficient at DNA condensation because of its high density of positive charge. However, by the addition of H2A, we indicated that H2A may have bestowed an additional property to PN to cause this enhancement. Interestingly enough, this enhancement was usually seen to be better in the combinations than 100% H2A alone, an indication that H2A characteristic was benefitting from being a co-cationic protein with PN. The interaction between H2A and protamine could be responsible for this augmentation. Indeed, protamine has a higher positive charge density than H2A 4.2nmol/μg versus 1.66nmol/μg. It is possible that in the presence of a nuclear localizing signal, the increased charge density of PN outmaneuvered the benefit of H2A as a carrier of both characteristics.

Increasing net charge of lipid complex reduces transfection efficiency

In spite of being relatively poor gene-expressing mediators, an advantage of anionic liposome-based delivery system like the LPDII is better compatibility with the physiologic environment by virtue of their anionic nature. We wanted to test the effect of increasing the net charge on transfection efficiency, bearing in mind that the transfection medium contains 5% serum. We observed that the increase from -3-4 nmoles negative charge /μg of pDNA was detrimental to transfection (Figure A.3) and was comparable to transfection efficiency observed in the HI-LLOLPDII iteration of both PN and H2A. This was an indication that while some anionic charge can be beneficial, there likely exists a range at which this benefit is applicable. Further studies may be warranted in other cell types, as they may have varying requirement for interactions and may have an implication in targeting specific cell populations.

CONCLUSION

The enhancement in transfection efficiency that H2A in (LLO) LPDII is offers over PN is apparent; however the physiological significance of this was not studied. More interestingly, PN-mediated transfection in (LLO)LPDII complexes benefited from the addition of H2A. This presumably occurs by a combination of an adding a nuclear localizing signal to a protein with higher positive charge density. Further studies should look into this relationship by studying combinations of H2A with cationic proteins of varying charge densities.

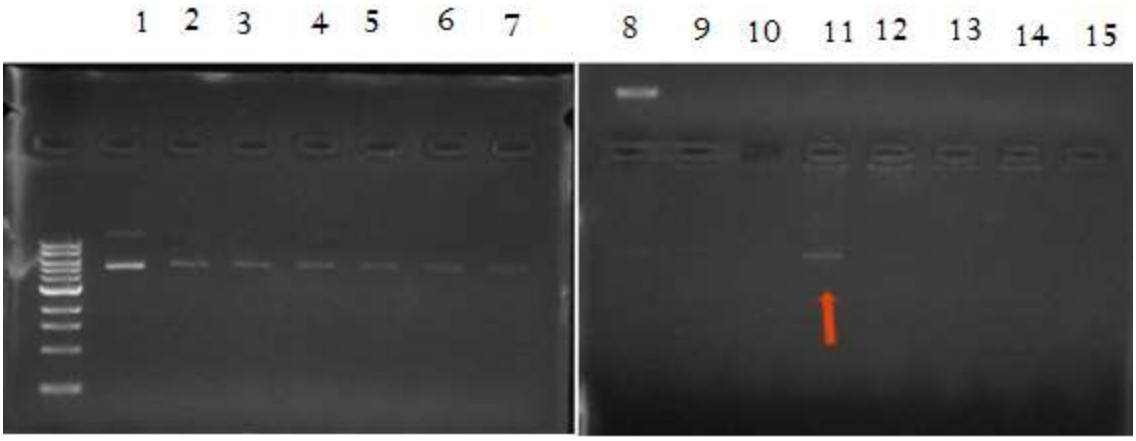

Figure A.1: Gel retardation assay of H2A condensed-pDNA

Gel retardation assay of H2A: pDNA polyplex. The samples were mixed in a w/w ratio with 1.8:1 equivalent to a charge neutral ratio, the ratio where the amines form the cationic protein at the phosphate form the pDNA are the same. There is an excess of the negative charge below charge neutral position and an excess of positive charge above the charge neutral positions. 0.25μg pDNA; **lane 1**: 0.1:1; ratio increases in an increment of 0.1 until **lane 7**: and then in increment of 0.3 till **lane 14** at 3.0 and finally **lane 15** at 3.6:1. pDNA polyplexes were run on 1% agarose containing ethidium bromide and visualized under UV light

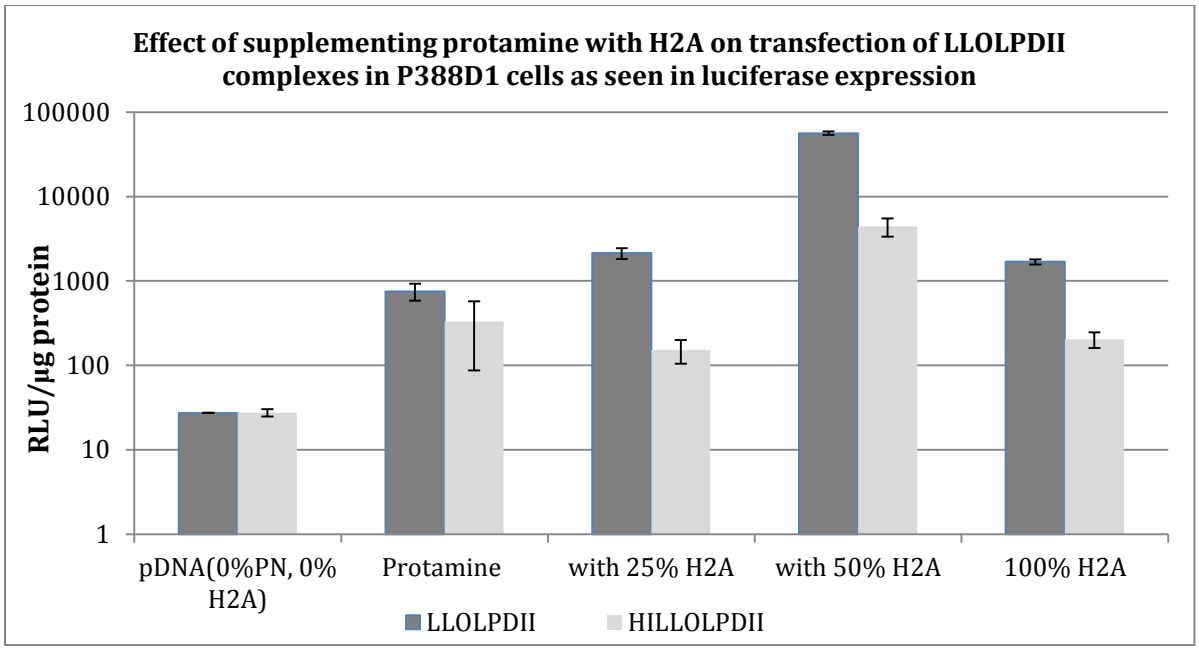

Figure A.2: Incremental supplement of PN with H2A enhances transfection efficiency

P388D1 cells were treated for four hours H2A (LLO) LPDII, PN(LLO)LPDII and PN supplemented with either 25% H2A of 50% H2A. After 4 hours, the transfection mix is dumped from the wells, and complete cell media is added to cells and incubated for another 44 hours. Luciferase expression is determined by luciferase assay system and data normalized to total protein present well.

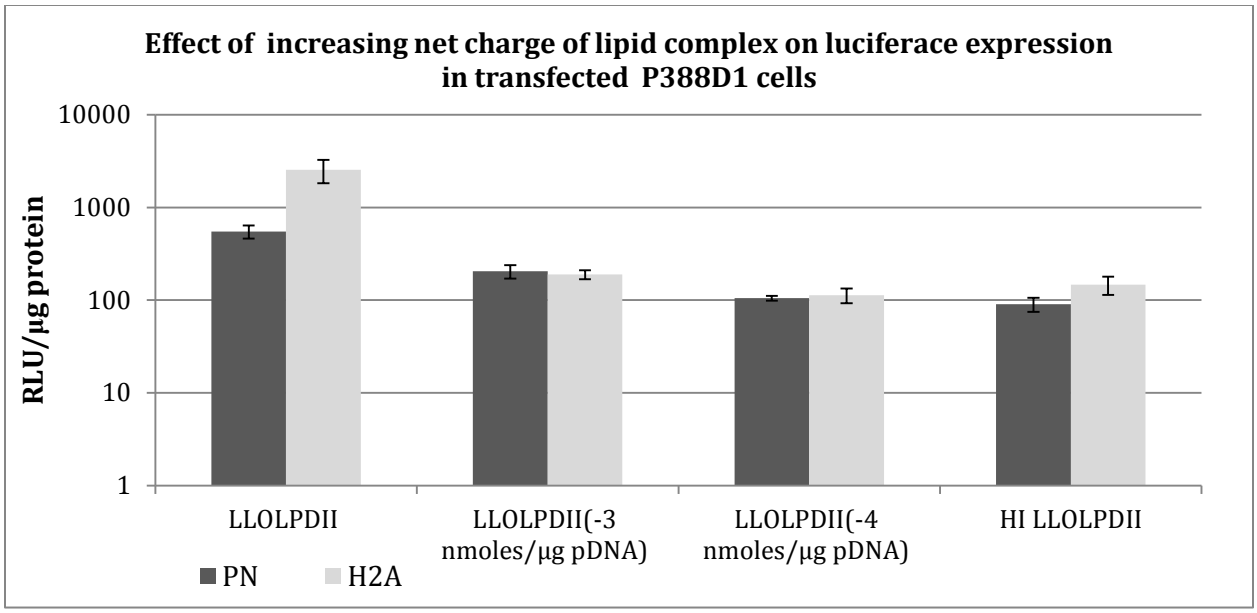

Figure A.3: Increasing net charge of lipid complex reduces transfection efficiency

P388D1 cells were treated for four hours. With the PN and H2A (LLO)LPDII. The formulations were prepared with PN or H2A with LLOLPDII with increasing concentration to have an overall theoretical charge of -3nmoles/µg pDNA or -4nmoles/ µg pDNA . The theoretical charge of the (LLO) LPDII being -2nmoles/ µg pDNA . After 4 hours, the transfection mix is dumped from the wells, and complete cell media is added to cells and incubated for another 44 hours. Luciferase expression is determined by luciferase assay system and data normalized to total protein present well.

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