Immune Modulation By Amphiphilic Oligonucleotides

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IMMUNE MODULATION BY AMPHIPHILIC OLIGONUCLEOTIDES

by

CHUNSONG YU

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2019

MAJOR: MATERIALS SCIENCE & ENGINEERING

Approved By:

Advisor

Date

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ACKNOWLEDGEMENTS

I would like to appreciate my advisor, Dr. Haipeng Liu, for his kind patience with me and considerable, continued support in my research during my entire Ph.D. training period. It gives me much honor and pleasure to learn from him and undertake research under his mentorship. Thank you for everything!

I would like to thank my committee members, Dr. Guangzhao Mao, Dr. Howard Matthew, and Dr. Kang Chen, for contributing themselves to my prospectus and dissertation. Especially, I would like to acknowledge Dr. Kang Chen and Dr. Charles Manke for their kind help in my research projects.

Myunggi An, thank you for being one of my great friends, for helping with my projects, and for sharing your ideas with me during our collaboration. The great time we spent and enjoyed together will be deeply engraved in my heart.

Dr. Meng Li, thank you for teaching me technical skills and guiding me through research projects and experimental designs. I will long remember and appreciate your help in my life as well.

Jingchao Xi, thank you for helping me with my experiments and sharing your scientific views.

I sincerely acknowledge all my friends who contribute to the fulfillment of my doctorate at Wayne State University.
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CHAPTER 1 BACKGROUND AND SCOPE OF THE DISSERTATION

1.1 Research motivation

Vaccination holds great promise in the therapeutic treatment of cancer by leveraging the host’s immune system. Significant advances in cancer immunology have led to approval of the first therapeutic cancer vaccine by FDA.\(^1\) However, the development of cancer vaccines is still infant. Maximizing therapeutic efficacy while minimizing the side effects of cancer vaccines remains challenging. One of the major goals for immunotherapy is to reverse tumor-associated immunosuppression and to restore anti-tumor immunity in patients. These challenges can be in part addressed through optimization of cancer vaccines, including formulation, rational design of antigen and vaccine adjuvants.

Oligonucleotide-based therapeutics, including CpG ODNs (oligodeoxynucleotides), siRNA, aptamer and antisense have resulted in magnificent efficacy in the treatment of cancer over the past decades. However, one of the biggest barriers in the clinical application of these functional oligonucleotide-based therapeutics is the lack of efficient delivery. For example, CpG ODNs (single-stranded synthetic ODN molecules with unmethylated deoxycytidyl-deoxyguanosine) are potent immune adjuvants and can stimulate a wide range of immune cells \textit{in vitro}. But their applications \textit{in vivo} have been compromised mostly due to the lack of efficient delivery as well as their systemic toxicity. Targeting oligonucleotide-based vaccine adjuvants to lymph nodes and limiting the systemic dissemination have been shown to greatly improve both the efficacy and safety profiles.\(^2,3\) Recently, the ‘albumin-hitchhiking’ approach demonstrated a viable and attractive delivery strategy to achieve remarkable therapeutic efficacy and reduce
systemic toxicity.\textsuperscript{2} In this approach, a lipophilic lipid tail was covalently conjugated to CpG ODN and after subcutaneous injection, amphiphilic CpG ODN-adjuvanted vaccines accumulated in draining lymph nodes (dLNs), which harbored a great number of immune cells, and induced a 30-fold increase in antigen-specific cytotoxic CD8\textsuperscript{+} T cells in mice. Meanwhile, amphiphilic ODN conjugate dramatically reduced CpG-induced toxicity, overcoming the limitations of CpG ODNs \textit{in vivo}. Here, we aim to further expand research scope of the “albumin-hitchhiking” approach in immune modulation by investigating the therapeutic efficacy of a variety of amphiphilic ODNs, and we also commit to developing novel adjuvants and drug-delivery strategies that would constitutively improve the immunogenicity of cancer vaccines and lead to tumor regression.

\textbf{1.2 Immunological background}

To understand how amphiphilic ODNs contribute to immune modulation, it is important to know how the immune system works. The immune system consists of a network of lymphoid organs and various immune cells including lymphocytes, dendritic cells, and natural killer cells. The immune system fulfills a pivotal role in defending the body against a wide variety of pathogens infections, including bacteria, virus and parasites, and in preventing the progression of cancer as well.\textsuperscript{4-6} The responses that the immune system mounts against foreign pathogens, allergies, self-damaged cells, and graft are called immune responses, which are generally classified into innate response and adaptive response.

The innate immune response, which is nonspecific immune response, detects and recognizes foreign pathogens via PAMPs (pathogen-associated molecular patterns) that are evolutionarily conserved molecular motifs expressed by a variety of microbes.\textsuperscript{7,8}
Innate immune cells, like professional antigen presenting cells (APCs) and phagocytes, detect and recognize PAMPs mainly through Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). Recognition of PAMPs leads to a cascade of signaling pathways and activates innate immune cells, triggering phagocytosis of pathogens and providing the first line of defense against various pathogens. Innate response induces rapid inflammation due to infection which gives rise to redness, swelling, heat, and pain. More importantly, innate response is crucial for the initiation of adaptive responses.

**Figure 1-1.** Schemes of how TLR ligands facilitate innate and adaptive immunity.

The adaptive immune response, on the other hand, is a specific immune response. The adaptive immune response is primarily dominated by highly specialized lymphocytes including T cells and B cells. These specialized lymphocytes are activated by APCs that have completed phagocytosis and process of pathogens or pathogen-associated antigenic molecules. Once activated, these lymphocytes proliferate and differentiate into effector cells that specifically eliminate pathogens or suppress their growth and
proliferation. Other than specificity, another unique characteristic of adaptive immune response is immunological memory which forms and develops from an original adaptive immune response to a specific pathogen and can quickly induce a more potent immune response to the same pathogen in future encounters. Although it appears that adaptive immune response is more advanced and sophisticated than innate one, innate response is generally prerequisite to the activation of adaptive response which in return can enhance innate immunity by effector molecules such as cytokines and antibodies. The role of TLR ligands in bridging innate and adaptive immunity is illustrated in Figure 1-1. TLR ligands like CpG ODNs directly activate professional APCs, creating an immune milieu that is abundant in pro-inflammatory and T helper 1 (T_{H1})-type cytokines. By enhancing the function of professional APCs, TLR ligands can facilitate the development of humoral and cellular vaccine-specific immunity.

1.3 Scope of the dissertation

![Figure 1-2. Schemes of entry of lipo ODN into the lymphatics from interstitium.](image1)
In this dissertation, we focus on expanding the scope of the “albumin-hitchhiking” approach in immune modulation and aim to improve the therapeutic efficacy of cancer vaccines. The rationale of the “albumin-hijacking” approach is shown in Figure 1-2. Briefly, small-molecule drugs or moderately sized biomolecules less than 5 nm are preferentially absorbed from the interstitial space into the blood capillaries rather than lymphatic capillaries, while increasing molecular size leads to increasing uptake via the lymphatics. We mainly studied immunological roles and physiochemical properties of a variety of amphiphilic therapeutic molecules, including lipid-conjugated ODNs and PEG (polyethylene glycol) cargos, in modulating immune activation in vitro and in vivo. We first evaluated the efficacy of three representative classes of lipid-modified CpG ODNs as vaccine adjuvants. The results showed that lipid modification on CpG ODN enhanced cellular uptake in vitro and LN accumulation in vivo. Although lipid conjugation generally compromised immunostimulatory properties of CpG ODNs in vitro, administration of lipo CpG class B and C ODNs, not lipo CpG class A ODN, with a protein antigen in mice resulted in significantly improved antigen-specific CD8+ T cells responses and antibody responses. We then evaluated the therapeutic efficacy of immunosuppressive ODN A151 and its lipid-modified form in suppressing TLR9-mediated immune responses in vitro and in vivo. The data demonstrated that lipo ODN A151 exhibited dramatically enhanced cellular uptake and increased LN accumulation compared to unmodified ODN A151. More importantly, tolerization with lipo ODN A151, not unmodified ODN A151 in mice, led to profound suppression of immune responses induced by CpG-adjuvanted vaccines. Next, we investigated the structure-based retention kinetics of lipid-based amphiphiles anchored on the plasma membranes of red blood cells (RBCs). We revealed that longer
lipid tail enabled more stable retention kinetics of amphiphiles anchored on RBCs, while shorter polyethylene glycerol (PEG) spacer was favored for markedly prolonged circulation half-life of amphiphiles inserted on RBCs in vivo. Furthermore, cationic amphiphiles covalently linked with cationic lipid tails demonstrated noticeably improved retention kinetics on RBCs in vivo compared to the anionic counterparts. Finally, we discovered a novel class of adjuvant booster that significantly enhanced the adjuvant potency of TLR7/8 agonists in vitro and in vivo. We found that lipid modification on several oligonucleotide-based TLR7 inhibitors led to remarkable upregulation of TLR7 agonist-induced NF-κB activation in the presence of serum albumin. Notably, administration of imiquimod-adjuvanted vaccine laced with the structurally-optimized adjuvant booster in mice resulted in a five-fold increase in the frequency of antigen-specific CD8+ T cells and the production of antigen-specific IgG, in comparison with the same molecular vaccine without the adjuvant booster.

In Chapter 2, we evaluated the immunostimulatory properties of amphiphilic CpG ODNs. Three representative classes of lipid-conjugated CpG ODNs were synthesized and their bioactivities as vaccine adjuvants were evaluated. We showed that lipid modification enhanced the stability of phosphodiester-backboned CpG ODN to nuclease degradation and the binding between albumin and CpG ODNs. In addition, the data revealed that lipid modification on CpG ODNs promoted cellular uptake and dramatically increased LN accumulation. Lipid-modified class A CpG (lipo CpG A) demonstrated improved stimulatory activities in murine TLR9 reporter cells and bone marrow-derived dendritic cells (BMDC) in vitro, while lipid modification on class B and C CpG ODNs decreased the level of TLR9-mediated NF-κB activation and reduced the production of
pro-inflammatory cytokines in BMDC. However, subcutaneous administration of a protein antigen ovalbumin (OVA) with lipo CpG B or CpG C, but not lipo CpG A, resulted in markedly improved OVA-specific IgG response and cytotoxic CD8$^+$ T cell response in mice compared with unmodified CpG ODN-adjuvanted vaccines. Collectively, we showed that lipid modification on three representative classes of CpG ODNs differentially modulated immune responses in vitro and in vivo. This study highlighted the significance of in vivo LN-targeting delivery of CpG ODNs in improving their efficacy as vaccine adjuvants.

In chapter 3, we investigated the therapeutic efficacy of lipid-modified immunosuppressive ODN A151 in inhibiting TLR9-mediated immune activation. Suppressive ODN A151 encoding TTAGGG motif can downregulate the production of pro-inflammatory and Th-1-biased cytokines, and therefore can markedly suppress TLR9 agonist-induced immune responses in mice. We modified ODN A151 with an albumin-binding diacyl lipid at its 5'-terminal. We found that lipid-functionalized ODN A151 showed increased cellular uptake and improved inhibitory capability of suppressing TLR9-mediated immune activation in vitro, in comparison with unmodified ODN A151. More importantly, prophylactic treatment in mice with lipo ODN A151, but not unmodified ODN A151, led to a five-fold decrease in the frequency of antigen-specific CD8$^+$ T cell and a significant reduction in the level of OVA-specific IgG in blood. Taken together, these findings suggested that targeting suppressive ODN to local LNs could be an effective approach to improving immune modulation. This approach might be utilized for treating autoimmune disorders with molecular immunomodulators.
In chapter 4, we analyzed the structure-dependent retention kinetics of lipid-based amphiphiles anchored on RBCs. We synthesized a variety of polymer amphiphiles with varying length of lipid tails and PEG spacers. Our results revealed that longer diacyl lipid chain conjugated amphiphiles exhibited more stable retention on RBCs, while shorter PEG spacers were favored for markedly prolonged circulation half-life of amphiphiles anchored on RBCs in vivo. In addition, we identified that blood flow-induced stress and serum albumin together played pivotal roles in the release of amphiphiles inserted on RBCs. Furthermore, cationic lipid modification on long PEG spacer strikingly extended the retention kinetics of amphiphiles anchored on RBCs in vivo and resulted in a 40-fold increase in circulation half-life in contrast to the anionic analogs. Our results highlighted the importance of molecular design in the dynamic stability of membrane-anchored amphiphiles.

In chapter 5, we aimed to evaluate the therapeutic efficacy of lipid-modified oligonucleotide-based TLR7 inhibitors in treating systemic lupus erythematosus. However, we discovered that several lipid-modified oligonucleotide-based TLR7 inhibitors dramatically enhanced TLR7-mediated NF-κB activation rather than suppressed it. We then revealed that it was due to the presence of albumin that lipid modification transformed oligonucleotide-based TLR7 inhibitors into TLR7 adjuvant boosters. Besides, the data showed that these adjuvant boosters were applicable to human cells and could enhance human TLR7- and TLR8-mediated NF-κB activation. More importantly, subcutaneous administration of imiquimod-adjuvanted vaccine laced with the adjuvant booster in mice resulted in a five-fold increase in the expansion of antigen-specific CD8+ T-cells and enhanced antibody response relative to the same molecular vaccine without
the adjuvant booster. These findings highlighted a novel class of adjuvant booster which could be used to dramatically improve the immunogenicity of subunit vaccines and might be broadly applicable to current vaccines.
CHAPTER 2 IMMUNOSTIMULATORY PROPERTIES OF LIPID-MODIFIED CPG ODN

2.1 Introduction

Bacterial DNA containing the unmethylated CpG dinucleotides motifs (CpG motifs) in a specific sequence can stimulate mammalian immune cells.\textsuperscript{12-14} Synthetic oligonucleotides containing CpG motifs mimic molecular signatures of bacteria and can activate the vertebrate immune cells via TLR9.\textsuperscript{12} Discovered and developed in the early 1990s, CpG ODNs have quickly drawn considerable interest due to the great promise of functioning as molecular adjuvants for vaccines. When co-administered with antigens, CpG ODNs activate professional APCs and subsequently augment antigen-specific humoral and cellular immune responses. Current clinical studies have shown that the utilization of CpG ODNs in human is safe and that CpG ODNs can improve the immune responses to co-administered antigens.\textsuperscript{10,13} CpG ODN directly stimulates B cells and dendritic cells (DCs), giving rise to the induction of both innate and adaptive immunity. TLR9 recognition is essential in this process. Once TLR9 is engaged by CpG ODNs, B cells produce IL-6, IL-12 and the CXCR3 chemokines IP-10, and secrete IgM, while DCs upregulate their expression of costimulatory molecules CD 40, CD86 and MHC molecules, and increase production of IL-1, IL-6, IL-12, IL-18, and TNF-\textalpha.\textsuperscript{15} Meanwhile, DCs differentiate to a mature stage where the antigen-presenting function is enhanced, which in turn, promotes the generation of humoral and cellular vaccine-specific immunity. Hence, CpG ODN creates an immune milieu where proinflammatory and Th1-type cytokines are enriched. Moreover, this innate immune response lays a solid foundation for antigen-specific adaptive immunity.\textsuperscript{10}
CpG ODNs trigger the secretion of T\textsubscript{H}1-type and proinflammatory cytokines and stimulate the maturation of professional APCs. These immunological effects promote the usage of CpG ODNs as potential vaccine adjuvants or “danger signal” to enhance the immunogenicity of co-administered antigens. To date, a variety of CpG ODNs have been utilized to elicit an innate immune response through the TLR9 activation pathway.\textsuperscript{10,16,17} Afterwards, innate immune responses can lead to the proliferation of cytotoxic CD8\textsuperscript{+} T cells and enhance the antigen-specific adaptive immune response against tumor antigens.\textsuperscript{10,16,17} Several preclinical studies have been carried out in mice to examine the immunogenicity of CpG-adjuvanted vaccines, and it is established that CpG ODNs are adequate to promote the humoral and cellular immune response to proteins such as ovalbumin.\textsuperscript{18,19} Several clinical studies have also exploited CpG ODNs in a combination of cancer vaccines. In melanoma studies, patients immunized with CpG ODN-adjuvanted vaccines developed >3% of circulating CD8\textsuperscript{+} T cells that were Melan-A-specific. That was an order magnitude greater than subjects treated with vaccine alone.\textsuperscript{20} In addition, in studies of Energix B, CpG ODN-adjuvanted vaccine induced a more potent and rapid antibody response. Specifically, subjects treated with CpG ODN-adjuvanted vaccine resulted in 13-fold greater primary and 45-fold greater secondary serum IgG Ab responses than those immunized with vaccine only.\textsuperscript{21,22} Collectively, CpG ODNs function as excellent adjuvants for cancer vaccines and bioagents for therapeutic treatment.

However, one of the major barriers to extensively utilize CpG ODNs as vaccine adjuvants in clinical application is lack of efficient delivery to APCs in secondary lymph organs, such as lymph nodes.\textsuperscript{2,23,24} In addition, CpG ODNs bridged by phosphodiester (PO) are vulnerable to rapid degradation by DNase. As a result, DNase-resistant CpG
ODNs synthesized by chemical modification with phosphorothioate (PS) backbone are widely studied *in vivo*.\(^{10,16,17}\) However, significant obstacles stand because of inadequate accumulation of CpG ODN in the immune system and incompetence of CpG ODN to penetrate cellular membranes. Regardless of its desirable nucleases resistance, CpG ODN cannot be readily internalized by APCs after parental immunization due to its small size, negative charge, and hydrophilic backbone. Recently, we showed that lipid conjugation at 5’ terminal of class B CpG ODN significantly promoted its cellular uptake and accumulation in APCs in LNs after subcutaneous injection.\(^2\) Lipid conjugated CpG ODN tightly bound to endogenous albumin and abundantly trafficked to draining LNs where it was physiologically filtered and accumulated in the APCs. This approach remarkably improved systemic safety as well as therapeutic efficacy of class B CpG ODN as vaccine adjuvant.\(^2\) Based on their intrinsic sequences, structural characteristics, and biological activities, CpG ODNs can be classified into three representative types.\(^{10,16}\) These classes of CpG ODNs contain a complete or partial PS backbone and a poly-G tail at either 3’ or 5’ end, or both ends, and demonstrate structure-based immunostimulatory properties.\(^{10,16}\) However, whether lipid modification can augment the biological activities of all three classes of CpG remains unclear. Here we aim to evaluate the immune stimulatory properties of all three classes of lipid-tagged CpG conjugates (lipo CpG) *in vitro* and *in vivo*, with an emphasis on their adjuvant activities when co-administered with a protein antigen. Our data confirmed that lipo CpG was able to function as a safe adjuvant and induced robust antigen-specific immunity evidenced by potent cytotoxic T cell and humoral responses. In addition, our data clearly suggested the *in vitro* immunostimulatory activities of CpG did not suffice to reproduce *in vivo* vaccine efficacy.
2.2 Materials and methods

2.2.1 Synthesis of diacyl lipid phosphoramidite

The diacyl lipid phosphoramidite was synthesized as previously described. A solution of stearoyl chloride (6.789 g, 22.41 mmol) in 1,2-dichloroethane (50 mL) was added dropwise to a solution of 1,3-diamino-2-dydroxypropane (1.0 g, 11.10 mmol) in the presence of 1,2-dichloroethane (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 h at 25 °C and then heated at 70 °C overnight. The reaction mixture was then cooled to 25 °C, filtered, and the solid was sequentially washed with CH₂Cl₂, CH₃OH, 5% NaHCO₃ and diethyl ether. The product was dried under vacuum to give the intermediate product as a white solid (yield: 90%). ¹H NMR (300 MHz, CDCl₃, ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.4-3.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3-1.2 (m, 60H), 0.9 (t, 6H). The intermediate product (5.8 g, 9.31 mmol) and N,N-Diisopropylethylamine (DIPEA, 4.2 mL, 18.62 mmol) was then suspended in anhydrous CH₂Cl₂ (100 mL). The mixture was cooled on an ice bath and 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25 °C for 1 hour, the solution was heated to 60 °C for 90 min. The solution was washed with 5% NaHCO₃ and brine, dried over Na₂SO₄ and concentrated under vacuum. The final product was isolated by precipitation from cold acetone to afford 4 g (55% yield) lipid phosphoramidite as a white solid. ¹H NMR (300 MHz, CDCl₃): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0-2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3-1.2 (m, 72H), 0.9 (t, 6H). ³¹P NMR (CDCl₃): 154 ppm.

2.2.2 Synthesis and purification of oligonucleotides
All ODN sequences were synthesized using an ABI 394 DNA synthesizer on a 1.0 micromole scale. All lipophilic phosphoramidites were conjugated as a final ‘base’ on the 5’ end of oligos. Lipophilic phosphoramidite was dissolved in dichloromethane and coupled to oligos in DNA synthesizer (15min coupling time). After the synthesis, DNA was cleaved from the solid support, deprotected, and purified by reverse phase HPLC using a C4 column (BioBasic-4, 200mm x 4.6mm, Thermo Scientific), 100mM triethylamine-acetic acid buffer (TEAA, pH 7.5)-methanol (0-5min, 50-80%; 5-15min, 80-100%) as an eluent. Lipophilic ODNs typically eluted at 13min while unconjugated oligos eluted at 7min. Fluorescein label ODNs were synthesized using 3’-(6-Fluorescein) tagged controlled pore glass. Lipid-conjugated CpG ODN 158525 (Class A CpG: 5’-ggGTTCAACGTTGAggggg-3’), CpG ODN 182626 (Class B CpG: 5’-tcctgacgtttctctagtt-3’), CpG ODN 239527 (Class C CpG: 5’-tcgctgttttcggcgccgcg-3’) (lower case letters indicate phosphorothioate linkage; capital letters refer to phosphodiester linkage) were synthesized using the above method.

2.2.3 DNase I protection assay

To evaluate the role of lipid modification in protecting CpG ODNs, three classes of free CpG ODNs and their lipid-modified counterparts were incubated in the presence of the DNase I (0.5 unit per µg of ODN) at 37 °C. At different time points, aliquots of these samples were collected and were immediately added 2 µL of EDTA (0.5 M) to stop DNase degradation. Samples were stored at -20 °C until subjected to gel electrophoresis analysis. The integrity of the ODNs in each formulation was compared with untreated ODNs as a control. The ODN’s stability was further evaluated in freshly isolated mouse serum. 30 µM free or lipid conjugated CpG A, B, and C (fluorescein labeled at 3’-end)
were incubated with or without 5 µL mouse plasma serum in PBS (1 X) with a final volume of 25 µL at 37 °C for 8 h. After incubation, 2.5 µL aliquot mixture of each sample was aspirated to a vial containing 6.5 µL TBE buffer (1X) and 1 µL glycerol. Electrophoresis was run in 1% agarose gel at 75 V for 10 min, and then the gel was photographed under UV.

2.2.4 Albumin binding assay

We used a gel electrophoresis mobility shift assay\(^{28}\) to detect albumin protein complexes with nucleic acids. Solutions of different concentrations of albumin protein and fluorescein-labeled CpG ODNs in different formulations were combined and incubated in PBS at 37 °C for 2h, the resulting mixtures are subjected to electrophoresis under native conditions through 1% agarose gel. The dissociation constant (Kd) values were estimated by using ImageJ software (NIH) to quantitate the fluorescent signal in each ODN band.\(^{28}\) Background signals from blank regions of the gel were subtracted from the signal intensities obtained from bands. The fraction of ODN bound was determined from the background-subtracted signal intensities using the expression: bound/total ODN. The fraction of ODN bound in each reaction was plotted versus the concentration of albumin. The data were fit with the following binding equation using GraphPad Prism software to perform non-linear regression and obtain a value for Kd.

2.2.5 In vitro DC uptake and confocal imaging

DC2.4 cells were cultured with RPMI-1640 supplemented with 10% FBS and 1% P/S; and were incubated with 1 µM fluorescein-labeled free or lipid-modified CpG A, B, and C at 37 °C for 4 h. After incubation, cells were washed twice with PBS by centrifuge at 800X g for 5 min prior to flow cytometry quantification. To visualize in vitro cellular
uptake, DC2.4 cells were cultured and incubated again under the same experimental conditions and were subjected to confocal imaging by Zeiss LSM 510 microscope.

**2.2.6 In vitro TLR reporter cells stimulation**

HEK-Blue™-mTLR9 and RAW-Blue™-mTLR9 and RAW-Blue™ cells were purchased from InvivoGen and were used to evaluate CpG ODN’s adjuvant activities *in vitro*. 0.5μM CpG formulations were added to InvivoGen HEK-Blue™ murine TLR9 or RAW-Blue™ mouse macrophage reporter cell lines, both of which are engineered with secreted embryonic alkaline phosphatase (SEAP) reporter system. After 24h incubation, SEAP levels were quantified by developing supernatant with QuantiBlue™ substrate for 1h and reading absorption at 620 nm, following the manufacturer’s instructions.

**2.2.7 In vitro Bone Marrow Dendritic Cells stimulation**

Bone marrow-derived dendritic cells (BMDCs) were generated by following the previous protocol, with minor changes. Briefly, bone marrow was flushed from femurs and tibia of C57BL/6 mice (5-6 weeks) and plated at 5×10^5 cells/ml in complete RPMI plus 5ng/ml recombinant murine GM-CSF. On day 3 and day 5, half of the culture volume was replaced with fresh complete RPMI + GM-CSF. On day 8, BMDCs were harvested and were stimulated with 0.5μM immunostimulatory CpG ODNs for 24h. The inflammatory cytokines secreted by BMDCs, including IL-6, IL-12, and TNF-α, were measured by ELISA (enzyme-linked immunosorbent assay).

**2.2.8 Lymph node imaging and cellular uptake**

Groups of C57BL/6 (n=4 Lymph nodes/group) were injected subcutaneously at the tail base with 3nmol of fluorescein-labeled free CpG or lipo-CpG of class A, B, or C. After 24h, animals were sacrificed and inguinal and axillary LNs were excised and imaged
using In-Vivo Xtreme (Bruker) imaging system. LNs were then digested with 1.5mL of freshly prepared enzyme mixture comprised of RPMI-1640 containing 0.8mg/mL Collagenase/Dispase (Roche Diagnostics) and 0.1mg/mL DNase (Roche Diagnostics) and LN cells were stained with antibodies against F4/80 and CD11c versus CpG fluorescence in viable cells. Percentages of CpG+ cells in the LNs were determined by flow cytometry.

2.2.9 *In vivo* immunization with immunostimulatory ODN

C57BL/6 mice (6-8 weeks) were vaccinated by a homologous prime-boost regimen: animals were primed on day 0 and boosted on day 14 (unless stated otherwise) with 10µg Ovalbumin and 1.24nmol CpG (in unmodified or in lipid-modified forms) dissolved in PBS. The volume of all vaccine injections was 100 µL/animal. All injections were performed subcutaneously at the base of the tail.

2.2.10 Tetramer Staining

Seven days after the final immunization, blood samples were collected. Red blood cells were lysed by ACK lysis buffer. Cells were then blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody) and stained with SIINFEKL loaded phycoerythrin-labeled tetramers (Beckman Coulter) and anti-CD8-APC (ebioscience) for 30min at 4oC. Cells were washed twice, resuspended in FACS buffer, and analyzed on Attune Focus flow cytometer. Analysis typically gated on live CD8+, Tetramer positive cells.

2.2.11 ELISA for OVA-Specific IgG

Mice blood samples were collected. Serum anti-OVA IgG levels were determined by ELISA: 96-well plates were coated overnight with 10µg/ml OVA in PBS and blocked
with 1% BSA in PBS. After incubation of serum samples for 1h at a series of dilutions, plates were washed with PBS/1% Tween-20. Goat anti-mouse IgG conjugated to Horseradish peroxidase (HRP) was added at 1 μg/ml for 30min. Plates were washed with PBS/1% Tween-20 and ELISA was developed by (3,3’,5,5’-Tetramethylbenzidine) (TMB, ebioscience). The reaction was stopped by 1M H₂SO₄ and the absorbance was read at 450 nm and 570 nm using a plate reader.

2.2.12 Statistical analysis

All plots show mean values and error bars represent the SEM. Comparisons of the mean values of two groups were performed using unpaired Student’s t-tests. One-way analysis of variance (ANOVA), followed by a Bonferroni post-test was used to compare >2 groups. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant unless otherwise indicated. Statistical analysis was performed using GraphPad Prism software (San Diego, CA).

2.3 Results and discussion

2.3.1 Lipid modification enhances phosphodiester CpG ODN’s stability in vitro

The ability of chemical modification to protect oligonucleotides from nuclease degradation is an important property for efficient immunostimulation. CpG ODN must be protected from nuclease digestion for maximal activity in vivo. To test whether the lipid modification improves the ODN’s stability, all three classes of CpG ODNs were incubated with DNase I at 37 °C. CpG A contains a core sequence with phosphodiester backbone and a few nucleases resistant phosphorothioate (PS) poly-G blocks at both ends. Despite a partial PS backbone stabilization, CpG A ODN is still susceptible to nuclease degradation, as shown by the complete disappearance of the CpG band illustrated in the
gel-electrophoresis 16 hours after incubation (Figure 2-1 (a)). However, 5'-lipid conjugation protects the CpG A ODN from DNase I degradation, showing a nearly intact band after 24h incubation (Figure 2-1 (a)). The stability of full phosphorothioate CpG B and CpG C ODN were unaffected in the presence of DNase I (Figure 2-1 (b) and (c)). Thus, lipid conjugation does not further improve its enzymatic stability.

Figure 2-1. (a-c), CpG A, B, C, and their lipid conjugated formulations were incubated with DNase I and their stability were analyzed using incubation times of 1, 2, 3, 15, and 21 hours. (d-f), Measurement of the albumin binding by gel shift assay. 15 µM of CpG ODNs were incubated with different concentrations of albumin (15, 50, 100, 200, 300, 400, and 600 µM) in PBS for 2 h at 37 °C, sample aliquots were analyzed by agarose gel electrophoresis. The binding of albumin induces a motility shift in CpG ODNs.

Covalent conjugation or noncovalent interaction with albumin has been shown to extend the half-life of shortly-lived oligonucleotides. Albumin functions as the main fatty acid binding protein in extracellular fluids. Thus, lipid conjugation might facilitate
the binding between albumin and CpG ODN and improve their enzymatic stability. We used a gel mobility shift assay to visualize the interaction between three classes of CpG ODNs and albumin protein. As shown in Figure 2-1 (d-f), when different classes of CpG ODNs were titrated with purified albumin protein, unmodified CpG ODN showed sharp trailing bands only at high albumin concentrations. This is most likely due to the low affinity, non-specific interaction between the PS backbone modification and albumin protein.\textsuperscript{32-35} Phosphorothioate modification of inter-nucleoside linkage is known to induce non-specific protein binding. The binding was demonstrated to be sequence- and protein-independent and correlated with the extent (number of PS bonds) of the PS modification. In contrast, lipo CpG ODN bound to albumin protein at low concentration via the lipid-albumin interaction. We estimated the equilibrium dissociation constant (Kd) between 75 µM-100 µM for unmodified CpG ODNs.\textsuperscript{16} These values are consistent with previous reports but are nearly 1000 times higher than the Kd of lipid-modified CpG (ca. 100 nM).\textsuperscript{9}

**Figure 2-2.** CpG's serum stability assayed by agarose gel. 30 µM free or lipid conjugated CpG A, B, and C (fluorescein labeled at 3’-end) were incubated with or without 5 µL mouse plasma serum in PBS (1X) with a final volume of 25 µL at 37 °C for
8h. Samples were subjected to agarose gel electrophoresis and subsequently photographed under UV.

Similar stability results were obtained when CpGs were incubated with freshly isolated mouse serum (Figure 2-2). Taken together, these data showed that lipid modification enhanced the binding between CpG and albumin and improved the CpG's stability in vitro.

2.3.2 Lipid modification on CpG enhances cellular uptake

![Figure 2-3](image)

**Figure 2-3.** Three classes of CpG ODNs and their lipid-modified forms were incubated with DC2.4 cells for 4 hours at 37 °C. Cells were washed and were subjected to laser scanning confocal microscopy imaging for intracellular distribution (a) showing representative class B CpG and flow cytometry analysis for quantification of cellular uptake (b). Scale bar: 10 μm. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 by one-way ANOVA with Bonferroni post-test.

The accumulation of FAM-labeled CpG ODNs and their lipophilic conjugates in DC2.4 cells was measured using flow cytometry and confocal microscopy after 4 hours of incubation with the corresponding CpGs. As expected, DC2.4 cells (a murine dendritic cell line) treated with 1μM lipid-modified CpG showed a 2~5-fold increase than their
unmodified counterpart after 4h incubation, independent of CpG type (Figure 2-3). Flow cytometry analysis suggested that class B and C CpG ODNs exhibited higher cellular fluorescence compared with CpG A (Figure 2-3 (b)), possibly because of their improved enzymatic stability. Confocal microscopy analysis showed similar intracellular distributions of free CpG and lipo CpG for all three classes, most of which were confined in intracellular membrane structures, including endosomes, where TLR9 is expressed. Thus, we confirmed that in vitro, lipid modification greatly enhanced the uptake of CpG ODNs but did not alter their intracellular distributions.

2.3.3 Adjuvant activities of lipid-modified CpG in vitro

**Figure 2-4.** Three classes of CpG ODNs and their lipid-modified forms (1 μM) were incubated with HEK mTLR9 cells, which secrete SEAP upon TLR9 stimulation (a) or Raw-Blue mouse macrophage reporter cells, which secrete SEAP upon multiple TLR, NOD or Dectin-1 stimulation (b), SEAP levels were quantified by incubating supernatant with QuantiBlue substrate for 1 h and reading absorption at 620 nm. (c-e), Bone-marrow-
derived immature dendritic cells were incubated overnight with 0.5 µM of CpG formulations, the inflammatory cytokines secreted by BMDCs, including IL-12 (c), IL-6 (d) and TNF-α (e), were measured by ELISA (enzyme-linked immunosorbent assay). Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

The adjuvant activities of the lipid-modified CpG were first examined in vitro. Murine TLR9 reporter cells, which were co-transfected with murine TLR9 gene and an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene, were incubated with lipid-modified CpG ODNs, using their unmodified counterparts as controls. CpG A induced a low but detectable level of TLR9 stimulation in murine TLR9 cells (Figure 2-4 (a)). Lipid-modified CpG A significantly enhanced the TLR9 stimulation (Figure 2-4 (a)), as shown by increased SEAP production at 500nM CpG for 24h incubation, suggesting lipid conjugation does not compromise the TLR9 stimulation for class A CpG. However, it is believed the accessibility of 5'-terminal of B and C class CpG oligonucleotide is important for receptor recognition and subsequent immune stimulation. Chemical modification on 5’ terminal thus has been shown to abrogate B class CpG’s stimulatory activities in vitro. Consistent with the previous report, the immune stimulatory activities of the lipid-modified class B and C CpG ODNs were significantly reduced when compared with SEAP secretion induced by the unmodified CpGs (Figure 2-4 (a)). The CpG type-dependent immune modulation was also demonstrated in RAW-Blue™ mouse macrophage reporter cells (Figure 2-4 (b)), which secreted SEAP upon multiple TLRs, NOD or Dectin-1 stimulation. TLR stimulation is known to stimulate DC to produce higher levels of multiple cytokines which directly impact
The ability of lipo CpG to induce the production of inflammatory cytokines such as IL-12, IL-6 and TNF-α were evaluated in bone marrow-derived dendritic cells (BMDCs). Following incubation, CpG A-induced an elevated level of the TNF-α but not IL-12 and IL-6 (Figure 2-4 (c-e)). The lipid-modified CpG A potentiated the TNF-α production as compared to unmodified CpG A. However, lipid modification on CpG A had no effect on IL-12 and IL-6 levels (Figure 2-4 (c-e)). Like the TLR reporter cell results, lipid modification on class B and C CpG ODNs reduced the IL-12, IL-6 and TNF-α production by 2-3-fold, though all the cytokines were still detectable (Figure 2-4 (c-e)). Together, these data demonstrated that lipid modification modulated CpG immune activities in a CpG class dependent manner. Lipid modification augmented the adjuvant activity of CpG A but compromised that of class B and C CpG in vitro.

2.3.4 Lipid-modified CpG accumulates in LNs and abrogates CpG-induced toxicity in vivo

Lipid-modified CpG binds to albumin and accumulates in draining lymph nodes after s.c. injection. Such site-specific accumulation also reduces the systemic exposure of CpG. Systemic exposure of CpG ODN is known to generate non-specific immune activation, characterized by lymphocyte proliferation in the spleen and release of proinflammatory cytokines. The lymph node accumulation of all three classes of CpGs and their lipid conjugates were tested in C57BL/6 mice. Mice were injected with fluorescein-labeled CpG in original or in lipid-conjugated forms. 24h post-injection, both inguinal and axillary lymph nodes were isolated and imaged. Both lipo CpG B and lipo CpG C showed dramatically enhanced accumulation in inguinal and axillary nodes (Figure 2-5 (a-b)) when compared with class B and C CpG ODNs, respectively. In
contrast, the overall accumulation of lipo CpG A and CpG A remained low and no statistical difference was observed between these two groups (Figure 2-5 (a-b)). To determine the immune cell uptake of CpG, lymph nodes were digested, and cells were analyzed by flow cytometry after antibodies staining to identify the specific immune cells. Unmodified CpGs exhibited less uptake in CD11c+ DCs and F4/80+ macrophages, the key antigen presentation cells in the lymph nodes. In contrast, lipid modification enhanced the cellular uptake by 2-4 folds, though some fluctuations were observed in the inguinal nodes in mice injected with class A CpG (Figure 2-5 (c-e)). Overall, these results clearly showed lipid modification on CpG ODNs enhanced their lymph node accumulation and immune cell uptake. Among these three types of CpG ODNs we tested, lipo CpG A exhibited a modest enhancement in lymph node retention. This might be explained by relatively low stability of class A CpG in vivo. In contrast, lipid modification dramatically increased the lymph node accumulation of PS backbone-stabilized class B and C CpG.

To test whether lipid modification could reduce the systemic toxicity of CpG, mice received 3 injections of CpG (1.24 nmol on day 0; 2.48 nmol each on day 2 and day 4) and their spleens were excised and analyzed for splenomegaly, a common feature of CpG ODN driven systemic immune activation in mice. Neither CpG A nor lipo CpG A-induced splenomegaly, showing spleen weights indistinguishable from PBS control (Figure 2-5 (f)). Mice injected with CpG B induced a nearly 1.5-fold increase in spleen weight, indicating a systemic exposure of CpG B.2,39 However, lipid modification on class B CpG ODN markedly alleviated the acute splenomegaly in mice, suggesting a minimized systemic exposure of class B CpG ODN after lipid modification (Figure 2-5 (f)). Interestingly, both free CpG C and lipo CpG C treated mice showed no noticeable sign of
splenomegaly when contrasted with the effect of class B CpG ODN (Figure 2-5 (f)), suggesting this type of CpG could be safer than CpG B in mice. This data was consistent with previous findings that lipid conjugated CpG ODNs accumulated in the draining lymph nodes and reduced systemic exposure.²

Figure 2-5. Mice (n=4 lymph nodes/group) were injected s.c. at the tail base with 3.3 nmol fluorescein-labeled class A, B, and C CpG or lipo-CpG. Inguinal LNs (proximal nodes) and axillary LNs (distal nodes) were isolated 24 hours post-injection and CpG accumulation in nodes was imaged (a) and quantified (b) by using In-Vivo Xtreme (Bruker) imaging system. (c-e), lymph nodes were digested, and lymph nodes cells were stained with DAPI and antibodies against F4/80, CD11c. Shown are representative flow cytometry plots of CD11c staining and F4/80 staining (c) versus CpG fluorescence in viable (DAPI-).
cells. Percentages of CpG+ cells in the LNs determined by flow cytometry at 24 hr (d, e). (f), Splenomegaly in mice (n=3/group) assessed on day 6 after 3 injections of CpG (1.24 nmol on d0, 2.48 nmol each on d2 and d4). Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

2.3.5 Lipid modification on class B and C CpG ODNs, but not on class A CpG ODN, dramatically enhance immunogenicity of molecular vaccines

![Graphs](image_url)

**Figure 2-6.** C57Bl/6 mice (n=4/group) were immunized with ovalbumin (10 µg) + CpG (1.24 nmol) on d0 and d14. Six days after the final immunization, mice were bled and antigen-specific T-cell responses in peripheral blood were evaluated by SIINFEKL tetramer assay. Shown are representative flow cytometric dot plots of H-2Kb/SIINFEKL tetramer staining of CD8+ cells (a) and quantification (b). (c), at d 20, serum samples
were collected and assayed by ELISA for anti-OVA IgG production. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

The in vivo activities of lipid-modified CpG ONDs as vaccine adjuvants were evaluated in C57BL/6 mice. CpG ODN has been widely studied to improve both humoral and cellular immune responses in animals.\textsuperscript{10,16,17} Mice were immunized subcutaneously on day 0 and day 14 with 10μg Ovalbumin (OVA) protein antigen adjuvanted by 1.24nmol CpG ODNs in original or in lipid conjugates formulations. OVA was simply mixed with CpG ODNs and was not associated with CpG or lipo-CpG. No OVA-specific CD8\textsuperscript{+} T cells and anti-OVA IgG antibody above the baseline were detected in mice immunized with class A CpG (\textbf{Figure 2-6}).

Despite the enhanced serum stability as well as TLR9 stimulation \textit{in vitro}, lipid-modified CpG A did not enhance the frequency of OVA-specific CD8\textsuperscript{+} T cells in blood, showing an average of 5.4% comparable with those with OVA alone. However, in mice immunized with lipo CpG B or lipo CpG C, nearly 16% and 22% of the blood CD8\textsuperscript{+} T cells were specific for the MHC-I-restricted peptide OVA\textsuperscript{257-264}, respectively (\textbf{Figure 2-6 (a-b)}). These frequencies were markedly improved when compared to mice immunized with unmodified class B and C CpG ODNs. These results were in contrast with the stimulatory activities of lipo CpG B and lipo CpG C \textit{in vitro}, where a 2-3-fold reduction of activities was observed. Furthermore, lipo CpG B and lipo CpG C rapidly potentiated the development of an OVA-specific IgG response as compared to their unmodified counterpart (\textbf{Figure 2-6 (c)}). In mice immunized with OVA together with CpG A or lipo CpG A, no increase in OVA-specific IgG was seen compared with mice immunized with
OVA alone. We thus concluded that lipid modification on CpG B and CpG C, but not on CpG A, efficiently enhanced CD8 T cell and B cell responses to a protein antigen in vivo.

Unmethylated cytosine-phosphate-guanosine (CpG) dinucleotide is recognized by TLR9 and induces immune system activation.\textsuperscript{12-14} Immunostimulatory activities of CpG were first discovered when a DNA from Bacille Calmette-Guerin was found to stimulate the production of type I interferon.\textsuperscript{29} Since then, synthetic immunostimulatory oligonucleotides containing CpG motifs have been widely used as vaccine adjuvant because these ODNs were stable against enzymatic digestion, and were capable of stimulating a wide variety of immune cells.\textsuperscript{10,16,17} To date, however, the in vitro promise of CpG has not been translated to in vivo primarily due to limited therapeutic efficacy and safety concerns.\textsuperscript{17} When used as a vaccine adjuvant, CpG must reach LNs, the anatomic site where all the immune cells interact with each other. Attempt to enhance the lymph node delivery of CpG has included the nanoparticulate materials.\textsuperscript{23,24} However, it remains difficult to design nanocarriers which meet all the criteria for in vivo LNs targeting.\textsuperscript{40,41} Most of the current nanoparticles do not fulfill clinical demands primarily due to requirements for complex designs including surface engineering to reduce non-specific tissue interactions, structural modification to enhance CpG encapsulation and incorporate ligands to maintain immune cells targeting.\textsuperscript{41} Possible stability, toxicological issues also restrict the nanocarrier’s clinical application in the short-term.\textsuperscript{42-44} Thus, a molecular approach would be more attractive.

Previously we developed a lipo CpG conjugation approach which targeted the CpG to the draining LNs via an albumin-mediated mechanism.\textsuperscript{2} Structurally optimized lipo CpG conjugate binds to endogenous albumin and subsequently transports to the draining LNs.
Albumin binding dramatically increased CpG ODN's hydrodynamic size, prevented it from diffusing into blood circulation, and retargeted it to the lymphatics. However, only class B CpG was investigated. CpG ODNs have been divided into three major classes (A, B and C types) based on their sequences, structural characteristics and immunological activities, so it is important to evaluate the immunostimulatory activities of each CpG class after lipid modification. Although additional classes of immunostimulatory ODNs have been described, of great interest are the recently discovered P-type and S-type ODNs which have been recently discovered and not been utilized extensively. The present study describes in vitro and in vivo immunostimulatory activities of three representative sequences derived from different types of CpG ODNs after lipid modification. Lipid modification at 5’-end of all three classes of CpG greatly enhanced their cellular uptake in vitro. Class A CpG, which contains palindromic CpG phosphodiester sequences with phosphorothioate G-rich ends, lacks the stability in vitro and in vivo. Subsequent lipid conjugation enhanced class A CpG’s stability and immunostimulatory activities in vitro. However, the ability of class A CpG to boost the humoral and cellular immune responses in vivo remains limited, as no potent immune responses were observed in both unmodified and lipid conjugated class A CpG ODNs when combined with a protein antigen. Outstanding responses to antigen have been reported in a variety of studies using class B CpG as an adjuvant.  

Our results demonstrated that lipid modification on both class B and C CpG, contrasted with class A CpG, acted as very potent Th-1-promoting adjuvants in mice, although lipid modification appeared to greatly compromise their immunostimulatory activities in vitro. The observed difference between the activities in vitro and in vivo might be explained by increased LN accumulation and APC uptake in
vivo, however, current studies cannot rule out the existence of other mechanisms independent of lymphatic system targeting. One possible explanation could be CpG adjuvant’s kinetic in terms of LN accumulation. Interestingly, despite dramatic increases in antigen-specific CD8+ T cells and antibody responses, repetitive injection of class C CpG into mice did not induce splenomegaly, one of the major side effects associated with systemic immune activation by CpG. Similar abrogation of systemic toxicity was observed in lipid-modified class B and C CpG ODNs, suggesting lipid modification was an effective approach to simultaneously improving the efficacy and safety of CpG in vaccines.

2.4 Conclusions

Overall, we have confirmed that lipid conjugated CpG ODNs are potent adjuvants in vivo. Our findings strongly suggest that in vivo adjuvant properties of CpG do not correlate with activities in vitro, highlighting the importance of lymphatic targeting in vaccines. Our study extends recent work on CpG as an adjuvant by demonstrating that targeting APCs in LN is the key to potentiating adjuvant activities of CpG ODN in vivo.
CHAPTER 3 TARGETING SUPPRESSIVE ODN TO LYMPH NODES INHIBITS TLR9-MEDIATED ACTIVATION OF ADAPTIVE IMMUNITY

3.1 Introduction

TLRs are highly conservative transmembrane receptors which are critical factors for early detection of foreign pathogens and following initiation of adaptive immune responses. TLRs recognize specific PAMPs associated with microbial pathogens, as well as a number of endogenous ligands such as self-protein, lipids, and nucleic acids. Other than pathogen sensing and clearance, much research reveals that endogenous ligand-mediated signaling through TLR is closely related to the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In particular, TLRs sensing intracellular nucleic acid, such as TLR9, play a pivotal role in the activation of autoimmune pathology. For example, under certain conditions, TLR9 recognized and sensed self-DNA, leading to the activation of B- and T-cells and production of proinflammatory cytokines. Such unrestrained infectious or self-pathogen DNA-induced immune activation can cause tissue damage, resulting in the development of autoimmune diseases such as RA. Targeting antagonists which suppress TLR-mediated immune responses to corresponding TLR receptors may be effective in improving the therapeutic efficacy of autoimmune diseases.

It was found that activated immune cells were restrained from secreting pro-inflammatory and Th1-promoting cytokines once they were exposed to telomeric DNA containing repetitive TTAGGG motifs. Synthetic ODNs containing repetitive TTAGGG motifs which mimic mammalian telomeric sequence can neutralize bacterial DNA-driven immune activation. These suppressive ODNs (Sup-ODNs) can act on dendritic cells and macrophages, leading to inhibition of B- and T-cells activation and differentiation.
In several disease models, Sup-ODNs were proved to reduce the level of autoimmune-mediated stimulation and modulate progression of diseases. These beneficial effects of Sup-ODNs are characterized by inhibition of antigen-specific cellular and humoral immune responses as well as downregulation of proinflammatory cytokines. However, immune suppression and therapeutic benefits were achieved at relatively high ODN concentrations. Such high-dose treatment can cause serious side effects which limit clinical application of Sup-ODNs. In chronic autoimmune diseases such as RA, draining LNs are emerging as therapeutic targets for immune modulation. Housing a large portion of APCs and lymphocytes, LNs specialize in antigen presentation, defining the immunological fates of T- and B-cell responses. Since Sup-ODNs act on APCs, we hypothesize that approaches targeting Sup-ODNs to APCs in the draining LNs can intensify the Sup-ODN-mediated immune suppression, enabling possible dose-sparing manner in the treatment of autoimmune diseases.

We have developed an ‘albumin-hitchhiking’ approach which uniquely targets synthetic ODNs to APCs in the LNs. In this approach, ODNs were conjugated to an albumin-binding diacyl lipid at the 5'-terminal and subsequently accumulated in the draining LNs via hitchhiking endogenous albumin after subcutaneous injection. The efficacy of this approach depends on a structural and molecular design that hijacks traffic route of endogenous albumin in the interstitial fluid: following subcutaneous injection, lipo ODNs bind avidly to albumin, and transport to draining LNs via lymphatic capillary, and accumulate considerably in LN-resident APCs. This approach simultaneously improves an immunostimulatory ODN’s adjuvant efficacy and safety by confining the ODN in the draining LNs, restraining it from systemic dissemination. We hypothesize that the
efficacy of immunosuppressive ODNs can be substantially improved by targeting them to LNs where a large portion of APCs reside. Here we evaluated inhibitory properties of a lipid-modified suppressive ODN in vitro and in vivo. Our results revealed that in vitro, lipid-modified Sup-ODN encoding repetitive TTAGGG motif enhanced cellular uptake while showed similar inhibitory efficacy as unmodified ODN in TLR9-mediated activation. In vivo, subcutaneous injection of a low dose of lipo Sup-ODN led to increased accumulation in APCs in draining LNs and dramatically suppressed TLR9 agonist-adjuvanted humoral and cellular immunity. Collectively, our findings indicated that LN-targeting of Sup-ODN via lipid modification may be a promising approach to potentiate immunoinhibitory properties of Sup-ODN and thus might be applicable for the control of TLR9-mediated immune activation.

3.2 Materials and methods

3.2.1 Materials

All reagents for DNA synthesis were purchased from Glen research (Sterling, VA) or Chemgenes (Wilmington, MA) and used following the manufacturer’s instructions. 3’-Fluorescein amidite (FAM) labeled controlled pore glass was purchased from Allele Biotechnology (San Diego, CA). Fatty acid-free BSA was purchased from Sigma-Aldrich. Ovalbumin protein was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Murine MHC class I tetramer was obtained from MBL international Corporation (Woburn, MA). Antibodies were purchased from eBioscience (San Diego, CA) or BD Bioscience (San Jose, CA). All other reagents were from Sigma-Aldrich and used as received except where otherwise noted.

3.2.2 Animals and cells
Animals were housed in the USDA-inspected WSU Animal Facility under federal, state, local and NIH guidelines for animal care. Female C57BL/6 mice (6-8 weeks) were obtained from the Jackson Laboratory. RAW-Blue™ and HEK-Blue™-mTLR9 reporter cell lines were purchased from InvivoGen (San Diego, California). Cells were cultured in complete medium (DMEM, 10% fetal bovine serum (Greiner Bio-one), 100 U/mL penicillin G sodium and 100μg/mL streptomycin (Pen/Strep), DMEM sodium pyruvate (1mM) and 20μM β-mercaptoethanol (β-ME)).

### 3.2.3 Synthesis of diacyl lipid phosphoramidite

The diacyl lipid phosphoramidite was synthesized as previously described. A solution of stearoyl chloride (6.789 g, 22.41 mmol) in 1,2-dichloroethane (50 mL) was added dropwise to a solution of 1,3-diamino-2-dihydroxypropane (1.0 g, 11.10 mmol) in the presence of 1,2-dichloroethane (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 h at 25 °C and then heated at 70 °C overnight. The reaction mixture was then cooled to 25 °C, filtered, and the solid was sequentially washed with CH2Cl2, CH3OH, 5% NaHCO3 and diethyl ether. The product was dried under vacuum to give the intermediate product as a white solid (yield: 90%). 1H NMR (300 MHz, CDCl3, ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.4-3.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3-1.2 (m, 60H), 0.9 (t, 6H). The intermediate product (5.8g, 9.31mmol) and N, N-Diisopropylethylamine (DIPEA, 4.2mL, 18.62mmol) was then suspended in anhydrous CH2Cl2 (100mL). The mixture was cooled on an ice bath and 2-Cyanoethyl N, N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25 °C for 1 h, the solution was heated to 60 °C for 90 min. The solution was washed with 5% NaHCO3 and brine, dried over Na2SO4 and concentrated.
under vacuum. The final product was isolated by precipitation from cold acetone to afford 4 g (55% yield) lipid phosphoramidite as a white solid. 1H NMR (300 MHz, CDCl3): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0-2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3-1.2 (m, 7H), 0.9 (t, 6H). 31P NMR (CDCl3): 154ppm.

3.2.4 Synthesis and purification of oligonucleotides

Both lipid-modified and free Sup-ODN were synthesized on a 1.0 micromole scale using an ABI 394 synthesizer. Diacyl lipid phosphoramidite was conjugated as a final ‘base’ on the 5’ end of oligos. Lipid phosphoramidite was coupled using the DNA synthesizer as previously described. After the synthesis, ODNs were cleaved from the solid support, deprotected, and purified by reverse phase HPLC using a C4 column (BioBasic-4, 200 mm x 4.6 mm, Thermo-Scientific). A gradient of 20-60% (buffer B) in 10 min, was used for the unmodified ODN purification and for lipid-modified ODN, the gradient was set at 50-80% (Buffer B) for 10 min and 80-100% for 5 min. Buffer A: triethylammonium acetate (TEAA, 0.1 M, pH 7.0), buffer B: Methanol. Lipophilic ODNs typically eluted at 12 min while unconjugated oligos eluted at 5 min. Fluorescein label ODNs were synthesized using 3’-(6-Fluorescein) tagged controlled pore glass purchased from Chemgenes. Lipid-conjugated Sup-ODN (ODN A151: 5’-ttagggttagggtaggttaggt-3’)55 and CpG ODN 1826 (CpG B ODN: 5’-tccatgacgttcctgacgt-3’)64 were synthesized by the above method and characterized by Mass Spec. All ODN sequences were modified by phosphothioation to improve stability against nuclease degradation.

3.2.5 In vitro DC uptake and confocal imaging

DC2.4 cells were cultured with RPMI-1640 supplemented with 10% FBS and 1% P/S, and were incubated with 1 µM FAM-labeled free or lipid-modified Sup-ODN at 37 °C
for 12 h. After incubation, cells were washed twice with 1 x PBS by centrifuge at 800X g for 5 min prior to flow cytometry quantification. To visualize in vitro cellular uptake, DC2.4 cells were cultured and incubated again under the same experimental conditions and were subjected to confocal imaging by Zeiss LSM 510 microscope.

3.2.6 in vitro TLR reporter cells stimulation

HEK-Blue™-mTLR9 and RAW-BlueTM cells were purchased from InvivoGen and were used to evaluate Sup-ODN's inhibitory activities in vitro. In a typical procedure, 500 nM CpG ODN 1826 and 1.5 μM Sup-ODN were added to InvivoGen HEK-Blue™ murine TLR9 or RAW-Blue™ mouse macrophage reporter cells, both of which are engineered with secreted embryonic alkaline phosphatase (SEAP) reporter gene. After incubating for 24 h, SEAP levels were quantified by developing supernatants with QuantiBlue™ substrate for 1 h and reading absorption at 620nm, following manufacturer's instructions.

3.2.7. LNs imaging and cellular uptake

Groups of C57BL/6 mice (n=4 LNs/group) were injected subcutaneously at the tail base with 3.3 nmol of FAM-labeled free or lipid-modified Sup-ODN. After 24 h, animals were sacrificed and inguinal and axillary LNs were excised and imaged using In-Vivo Xtreme (Bruker) imaging system. LNs were then digested with 1.5 mL freshly prepared enzyme mixture comprised of RPMI-1640, 0.8 mg/mL Collagenase/Dispase (Roche Diagnostics) and 0.1 mg/mL DNase (Roche Diagnostics). LNs cells were stained with antibodies against F4/80 and CD11c versus ODN fluorescence in viable cells. Percentages of ODN positive cells in the LNs were determined by flow cytometry.

3.2.8 In vivo tolerization with lipo ODN A151
C57BL/6 mice (6-8 weeks; n=3 per group) were tolerized with PBS, 1.24 nmol of free or lipo-Sup ODN on day 0. On day 3, mice were immunized with 3.72 nmol CpG 1862 ODN plus 10 μg OVA. On day 10, blood was collected from mice and OVA-specific immune responses were evaluated. The same mice were tolerized once again as previously on day 14 and three days later, a boost injection of 1.24 nmol lipo-G2-CpG ODN 1826 2 plus 10 μg OVA was injected into each animal. Blood samples were collected on day 24 to analyze the cellular and humoral anti-OVA immune responses. The volume of all vaccine injections was 100 μL/mouse. All injections were performed subcutaneously at the base of the tail.

3.2.9 Tetramer staining

Seven days after challenge, blood samples were collected, and red blood cells were lysed by ACK lysing buffer. Cells were then blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody) and stained with SIINFEKL loaded phycoerythrin-labeled tetramer (Beckman Coulter) and anti-CD8-APC (ebioscience) for 30min at room temperature. Cells were washed twice, resuspended in FACS buffer, and analyzed on Attune Focus flow cytometer. Analysis typically gated on live CD8+, Tetramer positive cells.

3.2.10 Intracellular cytokine staining

Peripheral blood was lysed with ACK buffer and washed with PBS twice. Purified cells were seeded in 96-well round-bottomed plates and pulsed with 10 μg/mL OVA peptide SIINFEKL for 2 h at 37 °C in T-cell media (RPMI-1640, 10% FBS, 50 μM β-mecaptoethanol, 1% P/S), followed by the addition of brefeldin A for 4 hours. Cells were stained with anti-CD8-APC and then fixed using Cytofix following the manufacturer's
instructions. Next, cells were washed and permeabilized. Intracellular staining for anti-IFN-γ-PE and anti-TNF-α-FITC was performed following the manufacturer's instructions and cells were analyzed by Attune Focus flow cytometer.

### 3.2.11 ELISA for OVA-specific IgG

Mice were bled, and blood samples were collected. Serum anti-OVA IgG levels were determined by ELISA: 96-well plates were coated overnight with 10 μg/ml OVA in PBS and blocked with 1% BSA in PBS. After incubation of serum samples for 1h at a series of dilutions, plates were washed with PBS/1% Tween-20. Goat anti-mouse IgG conjugated to Horseradish peroxidase (HRP) was added at 1 μg/ml for 30 min. Plates were washed with PBS/1% Tween-20 and ELISA was developed by (3,3',5,5'-Tetramethylbenzidine) (TMB, ebioscience). The reaction was stopped by 1 M H2SO4 and the absorbance was read at 450 nm and 570 nm using a plate reader.

### 3.2.12 Statistical analysis

All plots represent mean values and error bars represent the standard error of the mean (SEM). Comparisons of the mean values of two groups were performed using unpaired Student's t-tests. One-way analysis of variance (ANOVA), followed by a Bonferroni post-test, was used to compare >2 groups. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 6 software (San Diego, CA).

### 3.3 Results and discussion

#### 3.3.1 Lipid functionalization of ODN A151 enhances the cellular uptake in vitro

Synthetic ODN A151 containing TTAGGG motif repeats has been shown to inhibit TLR9 signaling and attenuate a variety of inflammatory responses. To target ODN A151
to the APCs in LNs through the “albumin-hitchhiking” approach, we functionalized A151 at the 5'-terminal with a diacyl lipid (lipo-A151) as an albumin binding domain. Hydrophobic modification of ODNs such as antisense oligos and siRNA has been shown to be an effective approach to enhance their cellular uptake.\textsuperscript{65,66}

\textbf{Figure 3-1.} (a) DC 2.4 cells were incubated with 1 μM of free A151 or lipo-A151 for 12 h at 37 °C. Cells were washed and then subjected to laser scanning confocal microscopy imaging for intracellular distribution. Representative confocal images of cells treated with A151 (top panels) and lipo-A151 (bottom panels). Scale bar: 20 μm. (b) Flow cytometry analysis for quantification of cellular uptake. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 by unpaired two-tailed t-test.

To test whether lipid modification alters cellular uptake and intracellular distribution of ODN A151, we incubated DC2.4 cells with fluorescein-tagged lipo-A151, using unmodified ODN A151 as a control. DC2.4 cells treated with lipo-A151 exhibited significantly enhanced cellular uptake when compared with unmodified ODN A151 (\textbf{Figure 3-1}), as showed by flow cytometry and confocal microscopy analysis. Like our previous results, lipid-modified ODN preferentially accumulated in intracellular membrane
structures.\textsuperscript{2,66} In vitro, lipid modification promotes quick membrane association and following cellular internalization by a mechanism similar to endocytic pathways. In contrast, unmodified ODN A151 alone demonstrated less cellular uptake in DC2.4 cells, indicating plasma membrane permeability of unmodified ODN A151 was restricted by the negatively charged nature of oligonucleotide following exposure to dendritic cells.

### 3.3.2 Lipid-modified ODN A151 inhibits CpG-induced immune activation \textit{in vitro}

**Figure 3-2.** (a) Free A151 or lipo-A151 (1.5 μM) with or without CpG B ODN (500 nM) were co-incubated for 24 h in HEK-TLR-9 reporter cells. Cell activation was quantified by measuring the SEAP levels in the supernatant. (b) HEK-TLR-9 cells were stimulated with 500 nM CpG for 3 h first, after which 1.5 μM of free A151 or lipo-A151 were added to the cells. Cell activation was determined. (c and d) HEK-TLR-9 cells were incubated with A151, or lipo-A151 for 12 h, after which cells were washed (c), or without a wash (d) before CpG ODN (500 nM) was added for the TLR-9 stimulation. (e and f) A151 and lipo-
A151 were co-incubated with imiquimod (TLR-7 agonist) (e) or lipopolysaccharide (LPS) (TLR-4 agonist) (f) for 24 h in Raw-Blue reporter cells. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

Modification on immunostimulatory ODNs often influences their biological functions. To test whether lipid modification on ODN A151 compromises its inhibitory activity, the immune suppressive properties of both lipo-A151 and unmodified A151 were first evaluated in murine TLR9 (mTLR9) reporter cells. HEK-Blue cells transfected with murine TLR9 and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene were incubated with lipo-A151 or free A151 in the presence of CpG ODN and the activation of NF-κB was quantified by measuring the SEAP concentrations in the supernatant. TLR9 reporter cells were activated when stimulated with a class B CpG ODN (CpG ODN 1826, 500 nM) which specifically bound to mTLR9 (Figure 3-2 (a)). CpG-dependent TLR9 stimulation was inhibited by up to 75% following incubation with free A151 (1.5μM). Residual TLR9 activation was retained in cells treated with free A151, showing a small but statistically significant increase in SEAP production as compared with no treatment group (Figure 3-2 (a)). However, the addition of same concentration of lipo-A151 completely inhibited the CpG ODN induced TLR9 activation in HEK cells (Figure 3-2 (a)), lowering the SEAP production to the basal level that was statistically indistinguishable from control levels (Figure 3-2 (a)). The successful inhibition of CpG-induced TLR9 activation required treatment of TLR9 cells with suppressive ODN before or during the CpG stimulation, as both lipo-A151 and free A151 were unable to reduce the SEAP production when mTLR9 cells were stimulated with CpG prior to addition of
suppressive ODN (Figure 3-2 (b)). However, when mTLR9 cells were treated with suppressive ODN for 3 hours, washed, and then stimulated with CpG ODN, both free A151 and lipo-A151 were partially effective in the suppression of TLR9 activation (Figure 3-2 (c)). In this case, statistically significant inhibition persisted for lipo-A151, showing retention of 50% of its inhibitory capacity compared to unmodified A151 (Figure 3-2 (c-d)). These findings suggest that instead of compromising the suppressive properties, lipid-modification on A151 enhances the inhibitory efficacy of TLR9 stimulation. To test whether lipo-A151 can act broadly to suppress immune activation elicited by other TLR stimulants, Raw-Blue cells were used to replace mTLR9 cells as reporter cells in the above experiments. Raw-Blue cells are stably transfected with SEAP gene and NF-κB inducible by many pattern-recognition receptors, including Toll-like receptors (except TLR5) and NOD-like (nucleotide-binding oligomerization domain-like) receptors. In contrast to inhibition of NF-κB activation by a TLR9 agonist, both lipo-A151 and unmodified ODN A151 showed little suppressive effect on Raw-Blue cells stimulated by imiquimod (TLR7 agonist) and LPS (lipopolysaccharide, TLR4 agonist) (Figure 3-2 (e-f)), respectively, suggesting in these reporter cells, ODN A151 was a TLR9-specific inhibitor.

3.3.3 Lipid-modified ODN A151 accumulates in draining LNs in vivo

Our previous study showed that lipid-modified ODNs accumulated in the LNs following subcutaneous injection by binding and trafficking with endogenous albumin. To test whether lipid modification results in efficient trafficking of ODN A151 to draining LNs, we assessed LN accumulation of ODN A151 following subcutaneous injection. C56BL/6 mice were injected subcutaneously with 3.3nmol FAM-labeled free A151 or lipo-A151 at the tail base and 24 h later, inguinal and axillary LNs were isolated and imaged.
Consistent with our previous findings, lipid-modified A151 exhibited 10- and 18-fold increases in inguinal and axillary nodes than unmodified A151, respectively (Figure 3-3 (a-b)). To identify the cells in the LNs that internalized ODN A151, the LNs were digested and stained with antibodies against F4/80 and CD11c. By flow cytometry, cellular uptake of lipo-A151 in the draining LNs was highest in APCs, with the majority of lipo-A151 accumulated in CD11c+ DCs and F4/80+ macrophages (Figure 3-3 (c-d)). These cells were key APCs that specialized in antigen presentation and immune activation. In accord with the imaging analysis, lipo-A151 showed considerably more uptake in both DCs and macrophages than free A151. Together, these results confirmed that in vivo, lipid functionalized A151 efficiently accumulated in the APCs in the draining LNs.

**Figure 3-3.** Mice (n = 4 LNs/group) were injected s.c. at the tail base with 3.3 nmol FAM-labeled A151 or lipo-A151. Inguinal LNs (proximal nodes) and axillary LNs (distal nodes) were isolated 24 h post injection and were imaged (a) and quantified (b) by using In-Vivo Xtreme (Bruker) imaging system. (c and d) LNs were digested and LNs cells were...
stained with antibodies against F4/80, CD11c. Percentages of A151 ODN positive cells in inguinal nodes (c) and axillary nodes (d) were determined by flow cytometry at 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 by unpaired two-tailed t-test.

3.3.4 Lipid-modified ODN A151 inhibits CpG ODN-induced antigen-specific cellular and humoral responses in vivo

To determine whether LNs targeting suppressive ODN A151 can suppress the CpG-induced immune responses in vivo, the inhibitory effects of lipo-A151 versus unmodified A151 on vaccine-induced T- and B-cell responses were evaluated. Naïve C57BL/6 mice were tolerized with a low dose (1.24nmol) of lipo-A151 or unmodified A151, by subcutaneous injection at the tail base. Three days later, all the animals were challenged with a large dose of CpG (3.72nmol) plus 10μg of ovalbumin (OVA). OVA-specific immune responses were determined in the blood on day 10 (Figure 3-4 (a)). ODN A151 at this dosage had little impact on OVA-specific CD8+ T cells, as similar frequencies of OVA tetramer+ CD8+ T cells were seen in mice tolerized with unmodified A151 and PBS (Figure 3-4 (b-c)). In contrast, lipo-A151 treatment notably reduced the frequency of antigen-specific CD8+ T cells in the blood (Figure 3-4 (b-c)). An identical tolerization injection was administered on day 14 and three days later, mice were challenged with 1.24nmol LN-targeting CpG (lipo CpG) mixed with 10μg OVA. As shown in Figure 3-4 (b) and (d), CD8+ T cell responses after boost were relatively potent in mice tolerized with PBS or unmodified A151, characterized by 17.5% and 14% tetramer-positive CD8+ T cells in the blood, respectively. However, in mice tolerized with lipo-A151, less than 5% of CD8+ T cells in blood were OVA-specific. To our knowledge, this is the
first example where the priming of CD8\(^+\) T cells is suppressed to such a low level without using a global immunosuppressant. Interestingly, at this low (1.24 nM) dose used, both A151 and lipo-A151 significantly reduced Th-1-biased CD4\(^+\) T cells capable of secreting IFN-\(\gamma\) after antigen re-stimulation (Figure 3-4 (e)). Additionally, we measured the levels of OVA-specific IgG in the sera of immunized mice to assess the ability of lipo-A151 to mediate humoral immunity. Sera from immunized mice were collected on day 27 following the initial treatment. ELISA measurements of serum titers of OVA-specific IgG showed a significantly lower level of anti-OVA IgG in mice tolerized with lipo-A151 but not unmodified A151 (Figure 3-4 (f)). Together, these results suggested LN-targeting suppressive ODN A151 dramatically improved immune suppressive activities that modulated both the CD8\(^+\) T cell- and B cell-mediated immunity.

Figure 3-4. (a) Tolerizing model displays experimental protocol. C57Bl/6 mice (n = 3/group) were tolerized with PBS, 1.24 nmol of A151, or lipo-A151 on day 0 and day
14. Mice were challenged with 3.72 nmol CpG ODN plus 10 μg OVA on day 3 and 1.24 nmol lipo-CpG ODN plus 10 μg OVA on day 17. One week after each challenge (day 10 and day 24), blood samples were collected and antigen-specific CD8+ T-cell responses in peripheral blood were evaluated by SIINFEKL tetramer assay. (b) Representative flow cytometric plots of H-2Kb/SIINFEKL tetramer staining of CD8+ cells post-prime (upper panels) and post-boost (lower panels). (c and d) Quantification of SIINFEKL tetramer-positive CD8+ cells in the blood after CpG and OVA antigen prime (c) and boost (d). (e) Stimulation of white blood cells ex vivo for 6 h in the presence of OVA showed a reduced response in CD4+ T cells after tolerization using A151 or lipo-A151 compared with PBS. IFN-γ-secreting CD4+ T cells frequencies were determined by intracellular cytokine staining. (f) On day 24, serum samples were collected and assayed by ELISA for anti-OVA IgG production. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

Enhancing activation of innate immunity and thereby potentiating adaptive immune responses has long been considered in a clinic in the treatment of cancer, allergy, and infections.45,46 In parallel with the discovery of CpG ODN in activating TLR9 and inducing potent antigen-specific immunity, it was found activation of innate immunity through TLR, such as TLR9, could lead to deleterious immune responses that resulted in the destruction of self-tissues.47-53 This has sparked the search for specific inhibitors which could inhibit CpG-mediated activation.47-53 Studies have revealed several structurally distinct ODNs can differentially modulate TLR9-mediated immune stimulation and suppress the biological activity of TLR9 ligation.55,58,60,68 In particular, ODNs containing immunosuppressive motif TTAGGG were recognized for their ability to attenuate
inflammatory responses \textit{in vivo} by blocking TLR9 signal pathways.\textsuperscript{58,68} Administration of A151 (a representative suppressive ODN with TTAGGG motif) has been shown to inhibit dendritic cell activation, suppress T\textsubscript{H}-1 differentiation and support the induction of regulatory T cells, prevent STAT1 and STAT4 phosphorylation, block cytosolic nucleic acid sensing pathways.\textsuperscript{58} However, systemic administration of phosphorothioate ODN is known to induce side effects including platelet activation, which has limited the clinical translation of suppressive ODN in humans.\textsuperscript{61,62}

Emerging evidence supports the contribution of LNs in the generation and perpetuation of autoimmunity resulted from chronic inflammation.\textsuperscript{11,63} During the progression of autoimmune diseases such as RA, antigens and inflammatory signals (e.g., DNA) resulted from the immune destruction drain into the LNs and aggravate the pathogenic inflammation.\textsuperscript{63} Thus, approaches that target and modulate the immune cell cross-talk and reciprocal interactions within the LNs microenvironment hold great promise for the treatment of autoimmune inflammation.

We present here the results of our attempt to design and implement an ‘albumin-hitchhiking’ approach to locally deliver an immune suppressive ODN to draining LNs to suppress the TLR9-activation-induced adaptive immune responses. We previously developed a diacyl lipid-ODN conjugation which uniquely targeted ODNs to APCs in the LNs via an albumin-mediated transportation.\textsuperscript{2} Albumin-binding domain increases the hydrodynamic size of ODN and prevents it from diffusing into blood circulation, re-targeting it to the lymphatics where a large portion of APCs reside. In addition to LNs targeting, lipo-ODN exhibited high affinity toward membrane and accumulated in the endosome.\textsuperscript{2,66} We assessed the \textit{in vitro} and \textit{in vivo} properties of diacyl lipid-modified ODN
A151. *In vitro*, lipo-A151 significantly enhanced the cellular uptake compared with unmodified A151. Our data are consistent with previous observation where hydrophobic conjugation on siRNA resulted in the enhancement of cellular uptake.\textsuperscript{65} We observed that although co-preservation of lipo-A151 and CpG was most effective at inhibiting TLR9 activation in HEK-TLR9 cells, lipo-A151 still resulted in partial inhibition when it was incubated with cells for 3 hours prior to CpG stimulation, showing a significantly better suppression than unmodified A151. Our results suggest that *in vitro*, lipid modification on A151 not only keeps intact its suppressive properties toward TLR9 stimulation but also leads to prolonged suppression when CpG ODN was added later.

*In vivo*, diacyl lipid-modified A151 accumulated in the draining LNs following subcutaneous injection. The LNs enrichment was consistent with our previous observations where lipid-modified ODNs bound endogenous albumin and transported to the LNs.\textsuperscript{2} In contrast, unmodified ODN quickly diffused into blood circulation due to its small molecular size, and exhibited minimal LNs accumulation.\textsuperscript{2,70} Flow cytometry analysis of cells in the LNs revealed that CD11c\textsuperscript{+} dendritic cells and F4/80\textsuperscript{+} macrophages were the major cells responsible for the uptake of lipo-A151. These cells are key APCs in the LNs that actively bind and internalize macromolecules such as albumin.\textsuperscript{71}

Using the well-studied ovalbumin as a model antigen, we also assessed the ability of LNs targeting lipo-A151 to suppress the OVA-specific immunity *in vivo*. Subcutaneously injected OVA combined with TLR9 ligand CpG ODN induced potent CD8\textsuperscript{+} T cell and humoral responses, mimicking the inflammatory responses of autoimmune diseases. Injection of LN-targeting lipo-A151, but not unmodified A151, effectively suppressed OVA-specific CD8\textsuperscript{+} T cell and B cell immune responses,
demonstrating our LNs targeting strategy was superior in the immune inhibition. In a real situation, autoimmune diseases can be triggered by multiple TLR stimulations. In our experiments, the immune suppression by lipo-A151 appeared to be TLR9 specific, as no suppressive effect was observed for lipo-A151 when Raw-Blue cells were stimulated with TLR7 (imiquimod) or TLR4 (lipopolysaccharide, LPS) agonists. However, broad-spectrum ODN-based antagonists have been recently demonstrated. For example, IMO-3100 and IMO-8400 were reported to act as antagonists against multiple TLRs, including TLR7, TLR8, and TLR9. Thus, it is possible to extend our approach to other endosomal TLR antagonists with broad immunosuppressive capacity. Although clinical safety data of phosphorothioate ODN were well documented, long-term toxicity effects are lacking. For example, a recent study has shown the development of antibodies against phosphorothioate ODN in patients. As our approach targets lipo-A151 to the LNs, it is possible that there may exist a potent anti-lipo-A151 antibody response.

We leveraged albumin to target LNs, which accommodates a wide variety of immune cells including APCs, T cells and B cells. Unlike nanoparticles that require sophisticated encapsulation design or modification, this delivery system exhibits a natural, simple and efficient way to target LNs and modulate the immune response. Our results may pave the way to amplify the induction of immunosuppression in the immune modulation that would target autoimmune conditions.

3.4 Conclusions

In summary, the data presented in this study suggest that the targeted delivery of immunosuppressive ODN to LNs by “albumin-hitchhiking” improves its inhibitory activities in vitro and in vivo. This finding emphasizes the potential of targeting immunomodulators
to the LNs in the treatment of autoimmune diseases. Further work is required to assess the effectiveness of suppressing human TLR-driven inflammation by lipid-modified ODN.
CHAPTER 4 STRUCTURE-DEPENDENT STABILITY OF LIPID-BASED POLYMER AMPHIPHILES INSERTED ON ERYTHROCYTES

4.1 Introduction

Cell-based therapy has attracted much research attention and is a rapidly expanding field in treating many diseases, including cancer, neurological diseases, and autoimmune disorders. As the molecular landscape of the plasma membranes dominates interactions of cells with the surrounding environment, therapeutic cells are often engineered to regulate cell functions such as cell survival, adhesion, migration, targeting, cell-cell interactions, or to enable targeted drug transport. Cell surface engineering utilizes engineering, chemistry, and biology techniques to enable modification of cell surface molecules with targeting ligands, biological functionalities, nanoparticles, or synthetic drugs. However, the complex and dynamic nature of cell surface sets a significant barrier for efficient modification without physically compromising fundamental cell functions such as viability, adhesion, migration, and immune recognition.

To date, many approaches to engineering cell surface have been developed. For example, genetic engineering is a versatile and robust methodology to express or suppress specific cell surface molecules. However, technical difficulties and safety concerns such as limitation of cell types and restriction of introducible therapeutic materials have confined its wide applications in the clinical setting. In parallel, another approach that involves covalent chemical modification of cells can be utilized to conjugate a broad range of functional molecules to the cell surface. This approach mainly engineers functional groups including amines, thiols, and carboxyls present in proteins, carbohydrates, or lipids on the cell surface. A major disadvantage of covalent functionalization is a possible abrogation of biological functions of native proteins. Cell
surface functionalization by lipid-based amphiphiles which are capable of spontaneously and exogenously inserting into the plasma membranes is a great alternative approach which can circumvent those limitations. To facilitate spontaneous membrane insertion, these amphiphiles are designed with a functional moiety of interest, which is conjugated to a suitable hydrophobic anchor (e.g., lipid or steroid) via a solubility-enhancing polymeric spacer (e.g., PEG). This approach is simple, allowing rapid and uniform surface functionalization of a wide variety of molecules without compromising the cell viability and functions. We chose to utilize erythrocytes as mature RBCs feature long circulation time (up to 120 days) and reduced endocytosis. Although these lipid-based amphiphiles appear to be stably anchored on the cell surface under cell culture conditions, their stability under mechanical stress (e.g., in the blood flow) in the presence of blood remains uncharacterized. Since many cells used in cell-based therapies need to enter the blood circulation, it is important to evaluate the retention of membrane-anchored amphiphiles in vivo. In this study, we analyzed and compared the effect of the molecular structure of lipid-based amphiphiles on their kinetic stability on the erythrocyte surface under static condition and mechanical stress. Our results revealed that longer diacyl lipid chain offered amphiphiles more stable retention kinetics on RBCs, while shorter PEG spacers were preferred for greatly extended circulation half-life on RBCs in vivo. Besides, we revealed that fluid shear stress and plasma serum promoted the release of amphiphiles anchored on RBCs. More importantly, cationic lipids conjugated amphiphile exhibited dramatically improved the retention kinetics on RBCs in vivo and led to a 40-fold increase in circulation half-life compared to the anionic lipid-modified one. These results emphasized the
significance of structural design in the retention kinetics of membrane-anchored amphiphiles.

4.2 Materials and methods

4.2.1 Materials

DMT hexaethyloxy glycol phosphoramidite and all reagents used on ABS 394 DNA synthesizer were purchased from Glen research or Chemgenes and used following manufacturer’s instructions. Vybrant DiD Cell-Labeling Solution was purchased from Thermo Scientific. 3'-FAM resin was purchased from Allele Biotechnology. DSPE-PEG-NH₂ was bought from Laysan Bio Inc. NHS-FITC and NHS-PEG-FITC 2000 were purchased from Nanocs Inc. All other reagents were purchased from Sigma-Aldrich except where otherwise noted.

4.2.2 Synthesis of diacyl lipid phosphoramidite

The C18 diacyl lipid phosphoramidite was synthesized as previously described.² A solution of stearoyl chloride (6.789 g, 22.41 mmol) in 1,2-dichloroethane (50 mL) was added dropwise to a solution of 1,3-diamino-2-dydroxypropane (1.0 g, 11.10 mmol) in the presence of 1,2-dichloroethane (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 h at 25 °C and then heated at 70 °C overnight. The reaction mixture was then cooled to 25 °C, filtered, and the solid was sequentially washed with CH₂Cl₂, CH₃OH, 5% NaHCO₃, and diethyl ether. The product was dried under vacuum to give the intermediate product as a white solid (yield: 90%). 1H NMR (300 MHz, CDCl₃, ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.4−3.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3−1.2 (m, 60H), 0.9 (t, 6H). The intermediate product (5.8 g, 9.31 mmol) and N, N-diisopropylethylamine (DIPEA, 4.2 mL, 18.62 mmol) was then suspended in anhydrous
CH₂Cl₂ (100 mL). The mixture was cooled on an ice bath and 2-cyanoethyl N, N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25 °C for 1 h, the solution was heated to 60 °C for 90 min. The solution was washed with 5% NaHCO₃ and brine, dried over Na₂SO₄, and concentrated under vacuum. The final product was isolated by precipitation from cold acetone to afford 4 g (55% yield) lipid phosphoramidite as a white solid. 1H NMR (300 MHz, CDCl₃): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0–2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3–1.2 (m, 72H), 0.9 (t, 6H). 31P NMR (CDCl₃): 154 ppm. The C12 diacyl lipid phosphoramidite was synthesized following the same procedures above except that the final product was purified by silica gel column chromatography.

4.2.3 Synthesis and purification of fluorescein-labeled lipo-(EG)n

All anionic lipo-(EG)n were synthesized using an ABI 394 synthesizer on a 1.0 micromole scale. DMT-hexaethyloxy glycol phosphoramidites were conjugated as a ‘base’ initiated from 3’-(6-Fluorescein) labeled controlled pore glass. All lipophilic phosphoramidites were conjugated as a final ‘base’ on the 5’ end of the sequence in the DNA synthesizer. After synthesis, lipo-(EG)n were cleaved from the solid support and deprotected, purified by HPLC using a reverse phase C4 column (BioBasic-4, 200mm X 4.6mm, Thermo Scientific), 100mM triethylamine-acetic acid buffer (TEAA, pH7.5)-Methanol (0-15min, 50-100%) as an eluent. Fluorescein-labeled lipo-(EG)n typically eluted at 14 min detected by the wavelength of 480nm. All other lipo-PEG amphiphiles were purified by HPLC using the same eluting method.

4.2.4 Synthesis of cationic lipid
To a 50 mL round-bottom flask, 0.6 g (3.744 mmol) of NBoc-ethylenediamine and 3.744 g (11.232 mmol) of octadecyl bromide was taken in ethyl acetate (12 mL) and refluxed in the presence of anhydrous potassium carbonate (2.069 g; 14.976 mmol) for 24h. After completion, the mixture was diluted to 100 mL ethyl acetate, and washed with water (2 X 100 mL), dried over anhydrous magnesium sulfate and filtered. Ethyl acetate was removed by rotary evaporation. The residue was purified by silica gel column chromatography to afford 0.5 g intermediate product (10% ethyl acetate in hexane, v/v, as the eluent). MS(LC-MS) calculated: m/z 664.68; found: 665.67. To a 25 mL round-bottom flask, the intermediate product and 0.3 g methyl iodide was taken in 10mL acetone and refluxed for 24 h. The reaction mixture was concentrated under reduced pressure and the residue was washed twice with 50 mL hexane by centrifuge for 10 min at maximum speed to yield 0.3 g white powder. MS(LC-MS) calculated: m/z 679.71; found: 679.55.

Scheme of synthesis of cationic lipid
The white powder obtained (0.3 g, 0.44 mmol) was dissolved in 6 mL of anhydrous dichloromethane (DCM) and 2 mL trifluoroacetic acid (TFA) was added. The reaction mixture was stirred at room temperature for 24 h. After completion, the reaction mixture was mostly concentrated by rotary evaporator and vacuumed by oil pump overnight without further purification. Afterward, 10 mL NaOH (1N) and 10 mL dichloromethane were added the unpurified product and the biphasic mixture was stirred at room temperature (RT) for 2 h. The organic layer was washed with water (20 mL x 3), dried over magnesium sulfate and filtered. The solvent in the filtrate was completely removed by rotary evaporator and then vacuumed oil pump overnight to afford 0.15 g final product. MS(LC-MS) calculated: m/z 579.66; found: 579.56.

4.2.5 Synthesis and purification of fluorescein-labeled cationic lipo PEG.

6 mg (10.51 μmol) of cationic lipid and 13 mg (4.2 μmol) of NHS-PEG-FITC 2000 was dissolved in 1mL dimethylformamide (DMF) and the reaction mixture was stirred at RT overnight in the presence of 1μL of triethylamine (TEA). After completion, the mixture was purified by HPLC as previously described.

4.2.6 Synthesis of fluorescein-labeled DSPE-PEG-2000

5.2 mg of DSPE-PEG-NH2 2000 and 1.7 mg of NHS-FITC was dissolved in 1mL dimethylformamide (DMF) and the reaction mixture was stirred at RT overnight in the presence of 1 μL of triethylamine (TEA). After completion, the mixture was purified by HPLC.

4.2.7 Insertion of amphiphilic cargos on RBC ex vivo

Fresh blood was collected from Balb/c mice.10 μL whole blood was washed twice with PBS(1x) and resuspended in 100 μL PBS. Then fluorescein labeled lipo PEG was
added into RBC suspension at a final concentration of 5 μM and incubated at 37 °C for 1 h. Afterward, samples were washed with PBS (1 X) twice to remove excessive unbound lipo PEG, and the residue was further incubated with whole blood, plasma serum (original concentration in whole blood) or pure RBC suspension (original cell density in whole blood). At designated time points, a small aliquot of the mixture was washed and then assayed by flow cytometry.

4.2.8 In vivo kinetics study

RBC from 100 μL whole blood was treated with fluorescein-labeled lipo PEG as described previously and resuspended in 150 μL PBS (1 X). If necessary, RBC was further treated with DiD dye after amphiphilic cargo insertion. A Balb/c mouse was i.v. injected with 150μL treated RBC suspension at the tail and was bled at the tail tip for flow cytometry analysis at the designated time point.

4.2.9 Exertion of shear stress on RBCs

RBC was treated as previously described. 5 μL treated RBC was mixed with 100 μL whole blood or RBC suspension or plasm serum (diluted to physiological concentration with 1 X PBS). After mixing, samples were then loaded onto ARES rheometer plate and sheared at 3000 1/s for 15 min at room temperature. When shearing was completed, 1 μL fresh sample was collected immediately for flow cytometry analysis. Besides shear stress exerted by rheometer, a syringe pump with a flow rate of 0.30 mL/min was set to shear samples mixed with whole blood for 12 min.

4.2.10 Hemolysis of RBC

The freshly collected blood was washed with PBS (1X) and then was resuspended in PBS (1X) with 50 times dilution. Each well 200 μL of RBCs suspension was incubated
at 37 °C for 1 h with 5 µM amphiphilic cargoes, 1% Triton-X 100 (v/v) or without treatment in a round-bottomed 96-well plate. For each group, samples were loaded in quadruplicate. After incubation, the plate was centrifuged for 5 min at 500 x g to pellet intact RBCs. 100 µL of supernatant from each well was transferred into a flat-bottomed 96-well plate. The absorbance of hemoglobin was measured by UV-vis plate reader at 540 nm. After background subtraction, the hemolysis percentage of each group was normalized to positive control Triton-X 100 group which represented 100% hemolysis.

4.3 Results and discussion

4.3.1 The effect of the length of lipid and PEG spacer on membrane retention time

To mimic the complex biological environment after injection, RBCs (isolated from 10 µL mouse whole blood) were incubated with 5 µM of different dye-labeled amphiphiles (Figure 4-1 (a)) in PBS buffer, washed, and mixed with 100 µL whole blood at 37 °C for 1 h. To reduce the competitive binding from serum proteins, RBCs were separated from serum and resuspended in PBS buffer (1 X). The retention of amphiphiles on the RBC surface was quantified by flow cytometry over time. Like previous studies,2 amphiphilic polymers with short lipid tail (12 carbons) rapidly dissociated from RBC surface when mixed with whole blood. 15 minutes after incubation, no populations with fluorescent signal was detectable in both C12-EG6 and C12-EG48 treated RBCs, suggesting a rapid and complete release of short lipid tail (C12-PEG) from RBC surface when mixed with blood (Figure 4-1 (b-c)). By contrast, the frequency of FITC-positive cells remained constant in RBCs loaded with long lipid (C18)-conjugated amphiphiles for more than 3 hours (Figure 4-1 (b-c)). These results suggested that sufficient hydrophobicity was required to firmly anchor the payload on the RBC surface in the presence of blood.
Interestingly, we also observed the amphiphiles with a long PEG block were released at a faster rate than those with a shorter PEG spacer, as shown in Figure 4-1 (d). Additionally, we noticed that RBCs, but not plasma serum, played a vital role in burst release of amphiphiles at the instance when amphiphiles-loaded RBCs were mixed with whole blood (Figure 4-1 (e-f)). It is worth pointing out that neither PEGs modified with C18 lipids caused hemolysis to RBCs (Figure 4-1 (g)). The reductions in fluorescence intensity were caused by the release of amphiphiles from cells into the culture medium. This could be due to the increased interaction between high molecular weight PEG with serum protein.

**Figure 4-1.** (a) Structures of C12 and C18 lipid-modified PEG amphiphiles. (b) Representative flow cytometry plots of FAM-labeled lipo PEG-loaded RBCs. (c) The frequency of RBC inserted by C12 or C18 lipid conjugated (EG)₆ or (EG)₄₈ over 200 min. (d) The relative release percentage of C18 lipid conjugated (EG)₆ or (EG)₄₈ over 200 min.
(e) The relative burst release percentage of C18 lipid conjugated (EG)$_6$ or (EG)$_{48}$ at different time points over 30 min. The release of FAM lipo-(EG)$_n$ on RBC before mixing with untreated RBC suspension was set to 0%. (f) Mean Fluorescence Intensity of FAM-labeled lipo-(EG)$_n$ on RBC before and after mixing with plasm serum diluent (1X). (g) The viability of RBCs treated with lipo PEG amphiphiles. Each specimen was assayed with three replicates. Error bars represent the SEM.

### 4.3.2 The effect of shear stress on the stability of membrane-anchored amphiphiles

Having demonstrated the structure-based stability of amphiphiles under static conditions, we set out to evaluate the mechanical impact to the amphiphiles loaded on the RBCs surface. Unlike static cell culture conditions, cells are exposed to various levels of mechanical stresses in the circulatory system under physiological conditions. Thus, the stability of membrane-anchored amphiphiles under mechanical stress is expected to be different when compared to that under static conditions.

![Flow Cytometry Plots](image)

**Figure 4-2.** (a) Representative flow cytometry plots of C18 lipo (EG)$_6$- and lipo (EG)$_{48}$-inserted RBCs treated with or without plate shearing stress. The percentage of
relative fluorescence decrease in C18 lipo (EG)$_6$- and lipo (EG)$_{48}$-inserted RBC after plating shearing (b), or after syringe pumping (c). Each specimen was assayed with three replicates. Error bars represent the SEM. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by using unpaired Student’s t-tests.

To test this hypothesis, lipid-conjugated PEGs loaded RBCs were subjected to in vitro shear conditions. A stress-controlled commercial rheometer was used to administer shear stress to amphiphiles-loaded RBCs. As shown in Figure 4-2 (a-b), under mechanical stress, the amphiphiles were released significantly faster than under static conditions. After being sheared at a rate of 3000 1/s for 15 min, 7.5% C18-(EG)$_6$ and 29.2% C18-(EG)$_{48}$ were released from RBC surfaces, as compared to 4.3% and 6.9% under static conditions. In both dynamic and static situations, long PEG-linked amphiphile was released at a higher rate than short PEG conjugated one, suggesting the length of PEG spacer was an important factor that controlled the membrane stability after insertion. In our experiment, amphiphiles loaded RBCs were mixed with freshly isolated whole blood. It was possible that the release was caused by competitive association from unlabeled RBCs or the serum proteins. To investigate the role of different blood components in the observed release, amphiphiles-loaded RBCs were sheared after mixing with separated erythrocytes or serum respectively. As shown in Figure 4-2 (b), it appeared that serum was the dominant factor responsible for the amphiphile release. To simulate the physiological conditions of blood flow-induced shear stress, amphiphiles-loaded RBCs were mixed with whole blood and pumped through a capillary tube (0.1 mm in diameter). The pressure and flow rate were controlled by regulating the pump speed to 0.30 mL/min.
for 12 min. Similar release profiles were obtained by this extracorporeal model (Figure 4-2 (c)). These two \textit{in vitro} models demonstrated the mechanical stress accelerated the release of lipid-based amphiphiles on RBC surface.

\textbf{4.3.3 \textit{In vivo} stability of membrane-anchored amphiphiles}

\textbf{Figure 4-3.} (a) Representative flow cytometry plots of \textit{in vivo} kinetics of FAM-tagged lipo-(EG)$_6$, lipo (EG)$_{48}$ and DSPE-PEG 2000 loaded RBC, (b) The relative frequency decrease of RBCs loaded with FAM-tagged C18 lipo (EG)$_6$, lipo (EG)$_{48}$ and DSPE-PEG 2000 at different time points over 24h, (c) The relative release percentage of
C18 lipo (EG)$_6$, lipo (EG)$_{48}$ and DSPE-PEG 2000 at different time points over 24h. Each specimen was assayed with three replicates. Error bars represent the SEM.

To investigate \textit{in vivo} stability of amphiphiles loaded on RBCs, RBCs loaded with C18 lipid-conjugated amphiphiles were injected intravenously into mice. Blood samples were analyzed at different time points. As shown in Figure 4-3, the release rate of amphiphiles constructed with long PEG (C18-(EG)$_{48}$) was significantly faster when compared to that at static cultures. Under static conditions, the frequencies of RBCs treated with C18 lipid-modified amphiphiles remained constant over 3 hours’ incubation. However, 1 hour after injection, the fluorescence positive RBCs, carrying amphiphiles linked with long PEG, dropped to an undetectable level. Surprisingly, the release rate of amphiphiles conjugated with short PEG \textit{in vivo} was comparable to that under static cell culture conditions, suggesting that \textit{in vivo} conditions affected longer PEG more profoundly than short PEG in terms of the stability.

4.3.4 Cationic lipid conjugation on amphiphiles prolongs membrane retention \textit{in vivo}.

The fast dissociation rate of long PEG-conjugated amphiphiles from the RBCs surface \textit{in vivo} raises the question of how to improve it. Fluorescent lipophilic dyes (e.g., long-chain dicarbocyanine) are widely used to label the plasma membrane. For long-term dye retention on cells, these dyes share some common design characteristics such as fluorophore directly conjugated to long aliphatic tail to ensure strong hydrophobic interactions with surrounding lipids. However, due to the relatively short hydrophilic fluorescent head, in salt-containing buffers, these dyes rapidly form micelles or aggregate and require special diluent for optimal staining.
Figure 4-4. (a) Structure of fluorescein-tagged cationic DSPE-PEG-2000, (b) The fluorescence release profiles of C18 lipo (EG)$_6$, lipo (EG)$_{48}$ and cationic DSPE-PEG-2000 under static conditions, (c) Representative flow cytometry plots of in vivo kinetics of cationic amphiphile-loaded RBCs, (d) The relative frequency decrease of RBCs loaded with cationic amphiphiles at different time points over 72 h, (e) The relative release percentage of cationic amphiphiles at different time points over 72 h. Each specimen was assayed with three replicates. Error bars represent the SEM. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.
In many cases, a long PEG linker is preferred for the retention of cellular functions.\textsuperscript{86,87} However, the fast dissociation rate of C18 lipo (EG)\textsubscript{48} from RBCs surface \textit{in vivo} suggests that hydrophobic interaction alone is insufficient for the retention of payload with the long PEG linker. Since the plasma membranes are negatively charged, we postulate that a positively-charged lipid tail would offer electrostatic attraction besides hydrophobic interaction and thus prolong the retention time of long PEG conjugated amphiphiles on the plasma membranes. We synthesized a cationic C18 lipid and conjugated it with fluorescein-labeled long PEG spacer (PEG 2000 averagely equals to 45 EG units) (\textbf{Figure 4-4 (a)}). The retention kinetics of cationic C18 lipo (EG)\textsubscript{45} was dramatically improved under \textit{ex vivo} static conditions (\textbf{Figure 4-4 (b)}). More importantly, cationic lipid significantly prolonged circulation half-life of amphiphiles conjugated with long PEG spacer \textit{in vivo} as compared to the original phospholipid-modified analogs, characterized by a 40-fold improvement in retention half-life on RBCs. (\textbf{Figure 4-4 (c-e))}. 24 hours after injection, only 24.3 \% of cationic amphiphile was released from RBCs, as compared to 72.2\% of anionic counterpart. There were a considerable amount of cargos remaining inserted on RBCs even after 48 hours of circulation. We concluded that a cationic lipid design facilitated the long-term retention of amphiphiles anchored on RBCs during circulation.

\textbf{4.4 Conclusions}

In summary, we studied the structural factors that affected the retention kinetics of lipid-based amphiphiles anchored on RBCs. The results revealed that longer diacyl lipid contributed to more stable retention kinetics of amphiphiles, while shorter PEG spacers had more profound impact on the stability of amphiphiles anchored on RBCs \textit{in vitro} and
in vivo. In addition, we identified that fluid shear stress accelerated the release of membrane-anchored amphiphiles from RBCs and serum albumin was a dominant factor that controlled the retention kinetics of amphiphiles loaded on RBCs as well. Furthermore, in contrast to an anionic lipid modification, a cationic lipid conjugation to long PEG block led to a 40-fold extension in circulation half-life on RBCs in vivo, indicating that a cationic structure design of amphiphile was preferred for long-term retention on RBCs. This study underscored the rational structural design in the retention kinetics of membrane-anchored amphiphiles during systemic circulation, and these findings might be of great interest and significance for the advancement of cell-based drug delivery.
CHAPTER 5 LACE MOLECULAR VACCINE WITH TLR7/8 ADJUVANT BOOSTER TO ENHANCE IMMUNOGENICITY

5.1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease where the host immune system wrongly mounts immune attacks against a large part of healthy tissues. In SLE patients, elevated serum levels of interferon-α (IFN-α) are closely related to disease activity and severity. The increased levels of IFN-α can be induced by DNA and RNA viruses and immune complexes (ICs) that consist of autoantibodies specific to endogenous DNA- and RNA-derived self-antigens. In innate immunity, TLR7 and TLR9 signaling can respectively sense mammalian RNA and DNA in the forms of ICs via pathogen-associated molecular patterns (PAMPs) and thus induce IFN-α production in plasmacytoid dendritic cells (pDC). Accordingly, inhibitors specific to TLR7 and TLR9 can be utilized to suppress IFN-α production and thus treat SLE patients. To date, a series of oligonucleotide-based inhibitors of TLR signaling have been developed. To leverage the “albumin-hitchhiking” delivery approach, we evaluated the therapeutic efficacy of lipid-modified ODN A151 which showed improved immunosuppressive activity in inhibiting TLR9-mediated immune activation when compared to the unmodified counterpart.

To further expand the pool of lipo suppressive ODNs, we set out to investigate the therapeutic benefits of lipid-modified TLR7 inhibitor. TLR7 is an endosomal receptor which binds viral single-stranded RNA such as RNA of hepatitis B virus, or synthetic guanine-rich RNA base analogs such as R848 and imiquimod (IMQ). As one of the lead compounds of TLR7 agonists, IMQ has been approved by FDA for topical application, which is effective in treating several major skin tumors and cutaneous metastases.
To evaluate the efficacy of lipid-modified immunoregulatory sequence 954 (lipo IRS 954), a dual inhibitor of TLR7 and TLR9 signaling,\(^{50}\) we used IMQ as a primary ligand to induce TLR7-mediated immune activation.

Surprisingly, we found that lipo IRS 954 enhanced TLR7-mediated activation instead of showing TLR7-specific immunosuppression. The immune enhancement was also observed in another potent TLR7-specific antagonist, 20-mer thymidine oligodeoxynucleotide (T20),\(^{90}\) showing that lipid-modified T20 (lipo T20) also significantly improved immune activation of IMQ. Interestingly, in the absence of serum albumin, lipo T20 markedly inhibited IMQ-induced activation in Raw-Blue cells, suggesting an indispensable role of albumin in the reversal of biological properties of lipo T20. Moreover, bovine serum albumin (BSA)-T20 conjugate, which was obtained via covalently conjugating T20 to BSA, exhibited potent immunosuppression in TLR7-mediated immune activation. Therefore, lipid modification converted the oligonucleotide-based TLR7 inhibitors into TLR7 adjuvant boosters in the presence of albumin. Because of its small molecular size, unformulated IMQ injected subcutaneously showed little LN accumulation. Instead, IMQ tends to diffuse into the bloodstream and activate the immune system nonspecifically. We reasoned that lipo T20, with intrinsic LN-targeting capability via “albumin-hitchhiking”, might be able to enhance the adjuvant activities of IMQ, allowing dose-sparing and reducing toxicity. As expected, subcutaneous administration of “IMQ/protein antigen” vaccine laced with lipo T20 in mice resulted in a five-fold increase in T-cell priming and enhanced antibody response in comparison to the parent vaccine. Accordingly, lacing vaccine formula with the adjuvant booster is a novel and simple approach to enhance the immunogenicity of molecular vaccines.
5.2 Materials and methods

5.2.1 Materials

All reagents and commercially available compounds for DNA synthesis were purchased from Glen research (Sterling, VA) or Chemgenes (Wilmington, MA) and used following the manufacturer's instructions. 3’-Fluorescein amidite (FAM) labeled controlled pore glass was purchased from Allele Biotechnology (San Diego, CA). Fatty acid-free BSA was purchased from Sigma-Aldrich. Ovalbumin protein was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Murine MHC class I tetramer was obtained from MBL international Corporation (Woburn, MA). Antibodies were purchased from eBioscience (San Diego, CA) or BD Bioscience (San Jose, CA). Imiquimod, resiquimod, gardiquimod were purchased from Cayman Chemical Company (Ann Arbor, MI). 1-(4-(Aminomethyl)benzyl)-2-butyl-1Himidazo[4,5-c]quinolin-4amine dihydrochloride was bought from Synnovator, Inc (Durham, NC). CL075, TLR8-506, poly I:C and all cell lines were purchased from InvivoGen (San Diego, CA). Cy5-labeled T20 was purchased from IDT (Coralville, Iowa). All other reagents were from Sigma-Aldrich and used as received except where otherwise noted.

5.2.2 Synthesis of diacyl lipid phosphoramidite

The diacyl lipid phosphoramidite was synthesized as previously described. A solution of stearoyl chloride (6.789 g, 22.41 mmol) in 1,2-dichloroethane (50 mL) was added dropwise to a solution of 1,3-diamino-2-dydroxypropane (1.0 g, 11.10 mmol) in the presence of 1,2-dichloroethane (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 hours at 25 °C and then heated at 70 °C overnight. The reaction mixture was then cooled to 25 °C, filtered, and the solid was sequentially washed.
with CH$_2$Cl$_2$, CH$_3$OH, 5% NaHCO$_3$ and diethyl ether. The product was dried under vacuum to give the intermediate product as a white solid (yield: 90%). 1H NMR (300 MHz, CDCl$_3$, ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.4-3.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3-1.2 (m, 60H), 0.9 (t, 6H). The intermediate product (5.8g, 9.31mmol) and N, N-Diisopropylethylamine (DIPEA, 4.2mL, 18.62mmol) was then suspended in anhydrous CH$_2$Cl$_2$ (100 mL). The mixture was cooled on an ice bath and 2-Cyanoethyl N, N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25 °C for 1 hour, the solution was heated to 60 °C for 90 min. The solution was washed with 5% NaHCO$_3$ and brine, dried over Na$_2$SO$_4$ and concentrated under vacuum. The final product was isolated by precipitation from cold acetone to afford 4 g (55% yield) lipid phosphoramidite as a white solid. 1H NMR (300 MHz, CDCl$_3$): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0-2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3-1.2 (m, 72H), 0.9 (t, 6H). 31P NMR (CDCl$_3$): 154 ppm.

**5.2.3 Synthesis and purification of oligonucleotides**

All ODN sequences were synthesized using an ABI 394 DNA synthesizer on a 1.0 micromole scale. All lipophilic phosphoramidites were conjugated as a final ‘base’ on the 5’ end of oligos. Lipophilic phosphoramidite was dissolved in dichloromethane and coupled to oligos in DNA synthesizer (15 min coupling time). After the synthesis, DNA was cleaved from the solid support, deprotected, and purified by reverse phase HPLC using a C4 column (BioBasic-4, 200mm x 4.6mm, Thermo Scientific), 100mM triethylamine-acetic acid buffer (TEAA, pH 7.5)-methanol (0-5 min, 50-80%; 5-15 min, 80-100%) as an eluent. Lipophilic ODNs typically eluted at 13 min while unconjugated oligos eluted at 7 min. Fluorescein-labeled ODNs were synthesized using 3’-(6-Fluorescein)
tagged controlled pore glass. Lipid-conjugated IRS 954 (5’-tgctctctggagggggtgt-3’), T20 (5’-ttttttttttttttttttt-3’), ODN 2087 (5’-tcctgagcttgag-3’) CpG ODN 1826 (5’-tcctagcgtcctagtt-3’) (phosphorothioate linkage) were synthesized using the above method.

5.2.4 In vitro TLR reporter cells stimulation

HEK-Blue™-mTLR7, mTLR8, hTLR7, hTLR8, and RAW-Blue™ cells were purchased from InvivoGen and were used to evaluate bioactivities of lipo ODNs and TLR 7/8 ligands in vitro. All these cell lines were cultured with DMEM supplemented with 10% FBS, 1% P/S and 100 μg/mL Normocin at 37 °C with 5% CO2. In a typical procedure, 2 μM IMQ and 1 μM unmodified T20 or lipo T20 (or lipo IRS 954 or lipo ODN 2087) were added to InvivoGen HEK-Blue™ murine or human TLR7/8 or RAW-Blue™ mouse macrophage reporter cells, both of which are engineered with secreted embryonic alkaline phosphatase (SEAP) reporter gene. After incubating for 24 h, SEAP levels were quantified by developing supernatants with QuantiBlue™ substrate for 1 h and reading absorption at 620 nm, following manufacturer’s instructions.

5.2.5 In vitro cellular uptake and confocal imaging

DC 2.4 cells were cultured with RPMI-1640 supplemented with 10% FBS and 1% P/S; Raw-Blue cells were cultured as previously described. 1 μM FAM-labeled unmodified T20 or lipo T20 at 37 °C for 12 h. After incubation, cells were washed twice with 1 x PBS by centrifuge at 800X g for 5 min prior to flow cytometry quantification. To visualize in vitro cellular uptake, DC 2.4 cells or Raw-Blue cells were cultured and incubated under the same experimental conditions and were subjected to confocal imaging by Zeiss LSM 510 microscope. To investigate endocytosis pathways, DC 2.4 cells seeded in 12-well plates
(5 X 105 cells/well) were pretreated with endocytic inhibitors at 37 °C for 30 min in Opti-MEM and then treated with 1 μM Alexa-647 BSA or 1 μM FAM-labeled T20 and Lipo T20 at 37 °C for 2 h. Inhibitors: 5 μg/mL Filipin; 50 μM EIPA; 300mM Sucrose were tested.

5.2.6 Albumin-ODN conjugates

Thiol-terminated T20 or CpG was synthesized by solid-phase coupling of fluorescein-labeled T20 or CpG with C6 S-S CE phosphoramidite (Thiol-Modifier C6 S-S, ChemGenes) at the 5’ end of the oligo. 27 mg Bovine serum albumin (BSA, 50 mg/μL in PBS) was co-dissolved with 0.32 mg N-(β-Maleimidopropoxyloxy) succinimide ester (BMPS, 30mg/ml in DMSO, Aldrich) and the mixture was agitated at RT for 2h. Extra BMPS was removed by diluting in PBS solution and passing the mixture through a PD MidiTrap G-25 desalting column (GE Healthcare) and concentrated and further purified by centrifuge filter tube (30K MWCO). 125 nmol of 5’-disulfide-modified fluorescein-T20 or CpG was pre-activated by 1000 nmol TCEP (3,3’,3”-Phosphanetriyltripropionic acid, 100mM in deionized water) and then purified through centrifuge filter tube (30K MWCO). Subsequently, activated T20 or CpG was added to the modified-BSA solution. The mixture was agitated overnight at RT and unconjugated T20 or CpG was removed by configuring filter tube (30K MWCO); free T20 or CpG removal was confirmed by size-exclusion chromatography.

5.2.7 Preparation of murine splenocytes

Spleens were freshly collected from mice and were cut into small pieces. A plunger end of a syringe was used to further disassemble spleen tissues into single cells. The cell suspension was subsequently filtered and washed. Lysing buffer was utilized to remove excessive red blood cells. After twice washes, 95% viability of splenocytes was confirmed.
by trypan blue. Splenocytes were resuspended in RPMI-1640 supplemented with 10% FBS and 1% P/S. Splenocytes were seeded at 1 x 10⁶ cells/ml in 200μL in a 96-well plate. Splenocytes were treated with 2 μM IMQ alone or plus 1 μM T20 or lipo T20 for 24 h under culturing conditions.

5.2.8 Preparation of primary human cells

Whole blood was obtained from Red Cross where healthy volunteers had provided informed consent to donate blood. PBMC were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). The mononuclear cells were washed twice with cold PBS (1X) and resuspended in completed RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. PBMC were seeded at 2 x 10⁶/ml in 200 μL in a 96-well plate. Plasmacytoid DC (pDC) monocytes were isolated from PBMC by immunomagnetic bead positive selection using BDCA-4 according to manufacturer’s instructions. Briefly, PBMC were first blocked with human FcR blocking reagent and then incubated with immunomagnetic MACS microbeads, and the labeled cells were collected with Miltenyi columns. The positively selected cells were resuspended in complete RPMI and seeded at 1 x 10⁶ cells/ml in 200μL in a 96-well plate. pDC purity and viability from positively isolated cells and populations of cells were determined by flow cytometry.

5.2.9 Determination of secreted cytokines

Supernatants collected from splenocytes suspension or human PBMC suspension after incubation were separately analyzed for IL-6, IL-12p40, TNF-α and IFN-α by ELISA following manufacturer’s instructions.

5.2.10 In vivo immunization
C57BL/6 mice (6-8 weeks) were immunized by a homologous prime-boost regimen: animals were primed on day 0 and boosted on day 14 with 50 µg Ovalbumin and 15 µg IMQ mixed with or without 6.2 nmol lipo T20 dissolved in PBS. The volume of all vaccine injections was 100 µL/animal. All injections were performed subcutaneously at the base of the tail.

5.2.11 Tetramer Staining

Seven days after the final immunization, blood samples were collected. Red blood cells were lysed by ACK lysing buffer. Cells were then blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody) and stained with SIINFEKL loaded phycoerythrin-labeled tetramers (Beckman Coulter) and anti-CD8-APC (ebioscience) for 30 min at 4 °C. Cells were washed twice, resuspended in FACS buffer, and analyzed on Attune Focus flow cytometer. Analysis typically gated on live CD8⁺, Tetramer positive cells.

5.2.12 Intracellular cytokine staining

Peripheral blood was lysed with ACK buffer and washed with PBS twice. Purified cells were seeded in 96-well round-bottomed plates and pulsed with 10 µg/mL OVA peptide SIINFEKL for 2 h at 37 °C in T-cell media (RPMI-1640, 10% FBS, 50 µM β-mecaptoethanol, 1% P/S), followed by the addition of brefeldin A for 4 hours. Cells were stained with anti-CD8-APC and then fixed using Cytofix following the manufacturer’s instructions. Next, cells were washed and permeabilized. Intracellular staining for anti-IFN-γ-PE and anti-TNF-α-FITC was performed following the manufacturer’s instructions and cells were analyzed by Attune Focus flow cytometer.

5.2.13 ELISA for OVA-specific IgG
Mice were bled, and blood samples were collected. Serum anti-OVA IgG levels were determined by ELISA: 96-well plates were coated overnight with 10 μg/ml OVA in PBS and blocked with 1% BSA in PBS. After incubation of serum samples for 1h at a series of dilutions, plates were washed with PBS/1% Tween-20. Goat anti-mouse IgG conjugated to Horseradish peroxidase (HRP) was added at 1 μg/ml for 30min. Plates were washed with PBS/1% Tween-20 and ELISA was developed by (3,3’,5,5’-Tetramethylbenzidine) (TMB, ebioscience). The reaction was stopped by 1M H2SO4 and the absorbance was read at 450 nm and 570 nm using a plate reader.

5.2.14 Statistical analysis

All plots show mean values and error bars represent the SEM. One-way analysis of variance (ANOVA), followed by a Bonferroni post-test, was used to compare >2 groups. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant unless otherwise indicated. Statistical analysis was performed using GraphPad Prism software (San Diego, CA).

5.3 Results and discussion

5.3.1 Lipid conjugation specifically reverses oligonucleotide-based TLR7 inhibitors

Our previous finding demonstrated that lipid-modified oligonucleotide-based TLR9 inhibitor was more potent and efficacious than unmodified oligo in targeting LNs and suppressing CpG-induced immune activation. Inspired by this study, we aimed to take advantage of lipid functionalization in other oligonucleotide-based TLR inhibitors to further expand the pool of lipo suppressive ODNs. We set out to investigate the therapeutic efficacy of lipid-modified IRS 945 in suppressing TLR7-mediated immune activation and alleviating the severity of SLE. As shown in Figure 5-1 (a), we first used imiquimod (IMQ)
to stimulate TLR7-mediated immune activation in Raw-Blue reporter cells and determined immunosuppressive capability of unmodified or lipo IRS 954 by quantifying the level of TLR7-induced NF-κB activation. However, the results showed that lipo IRS 954 markedly enhanced TLR7-mediated activation, while the unmodified IRS 954 itself remained immunosuppressive toward TLR7 signaling (Figure 5-1 (b)).

**Figure 5-1.** (a) Structure of imiquimod (IMQ) and single-stranded DNA sequence of IRS 945 and lipo IRS 954, (b) Raw-Blue cells were stimulated with 2 μM IMQ alone or IMQ + 1 μM IRS 954 or lipo IRS 954 for 24 h, (c) Raw-Blue cells were stimulated with 2 μM IMQ alone or plus 1 μM T20 or lipo T20 for 24 h, (d) Raw-Blue cells were stimulated with 10 μg/ml Poly I:C, 2 μM IMQ and 100 nM CpG ODN 1826 alone, or plus 1 μM lipo T20 respectively for 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

Based on these compelling results, we hypothesized that lipid modification might be used to reverse the immunological properties of oligonucleotide-based TLR7 inhibitors.
To test this hypothesis, we conjugated the C18 lipid tail to another classic oligonucleotide-based TLR7 inhibitor, 20-mer thymidine oligodeoxynucleotide, to generate lipo T20 and then tested it following previous experimental settings. Similar to lipo IRS 954, lipo T20 enhanced the immunostimulatory activities of IMQ by 5 folds. In contrast, unmodified T20 was suppressive toward IMQ stimulation, lowering the NF-κB activation by 2 folds (Figure 5-1 (c)). The enhancement of NF-κB activation by lipo T20 appeared to be TLR7 specific, as no enhancement was observed when Raw-Blue cells were stimulated by CpG ODN (a TLR9 ligand) and Poly I:C (a TLR3 ligand) (Figure 5-1 (d)). Collectively, we found that lipid conjugation could specifically reverse the inhibitory effect of oligonucleotide-based TLR7 inhibitors.

5.3.2 The role of albumin in the mechanism of action

![Figure 5-2](image)

**Figure 5-2.** (a) Raw-Blue cells were stimulated with 2 μM IMQ alone or plus 1 μM lipo T20 or lipo (EG)48 for 24 h. (b) Flow cytometry analysis of DC 2.4 cells treated with 1 μM of FAM-T20 or FAM-lipo T20 for 12 h or 24 h at 37 °C. (c) Raw-Blue cells were stimulated with 2 μM IMQ alone or plus 1 μM lipo T20 or 5 μM T20 for 24 h. (d)
Representative confocal images of DC 2.4 cells treated with 1 μM Cy5-labeled T20 and FAM-labeled lipo T20 simultaneously. Scale bar: 10 μm. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test or unpaired Students’ t-test.

Structurally, lipo T20 might be stimulatory due to the lipid moiety. To test whether lipid tail can stimulate cells, we conjugated the same diacyl lipid to oligo-mimicking “hexaethyleneglycol” blocks via DNA synthesizer. The amphiphilic polymer lipo (EG)$_{48}$ has a length comparable to lipo T20. Lipid tail itself did not affect IMQ-induced activation (Figure 5-2 (a)). According to our previous studies, lipid conjugation typically resulted in increased cellular uptake. The results here also showed that DC 2.4 cells preferentially internalized more lipo T20 than unmodified T20 over 24 h incubation (Figure 5-2 (b)). We then speculated that quantitatively increased cellular uptake of T20 might contribute to the qualitative reversal of lipo T20’s immunosuppressive properties. However, high-dose T20, five times more concentrated than lipo T20, exhibited significantly enhanced inhibition in IMQ-elicited activation (Figure 5-2 (c)), suggesting an enhanced uptake was not responsible for the improved stimulation. In addition, lipid modification did not alter cellular localization of T20, as demonstrated in confocal imaging of DC 2.4 cells treated with 1 μM Cy5-labeled T20 and FAM-labeled lipo T20 simultaneously (Figure 5-2 (d)).

A previous work reported that poly-thymidine could interact with TLR7 ligands like IMQ.$^{90}$ Therefore, we postulated that lipo T20, with uptake-facilitating lipid tail, might promote cellular uptake of TLR7 ligands via ODN-drug intercalation and subsequently enhanced IMQ-induced activation. To verify this possibility, we used a functionalizable TLR7 analog of IMQ, “1-(4-(Aminomethyl) benzyl)-2-butyl-1Himidazo[4,5-c]quinolin-
4amine” (IMDQ), for quantification of cellular uptake and fluorescence imaging. Flow cytometry data indicated that neither T20 nor lipo T20 increased cellular uptake of FITC-labeled IMDQ (Figure 5-3 (a)). Besides, confocal imaging indicated that IMDQ was primarily in the endosomal-lysosomal compartment in the presence of T20 or lipo T20 (Figure 5-3 (b)). Taken together, the data we showed here demonstrated that lipid conjugated T20 was a TLR7 adjuvant enhancer which dramatically improved the immunostimulatory effect of IMQ.

**Figure 5-3.** (a) Structure of FITC labeled-IMDQ and flow cytometry analysis of Raw-Blue reporter cells treated with 2 μM FITC-IMDQ alone or in combination with 1 μM T20 or lipo T20, (b) Representative confocal images of DC 2.4 cells which were treated with 2 μM Cy5-labeled IMDQ alone or in combination with 1 μM T20 or lipo T20 for 24 h and then washed and stained with 50 nM LysoTracker Green and 300 nM DAPI (4', 6-diamidino-2-phenylindole) following manufacturer’s instructions. Scale bar: 10 μm. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.
Figure 5-4. (a) Flow cytometry analysis of DC 2.4 cells treated with endocytosis inhibitors for 30 min, followed by 1.5 h incubation with 1 μM FAM-labeled T20 or lipo T20 or 1 μM Alexa-647 BSA respectively, (b) Raw-Blue reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM T20 or lipo T20 under FBS-free and normal culture conditions respectively for 24 h, (c) Flow cytometry analysis of Raw-Blue reporter cells which were cultured with complete or FBS-free medium separately for 24 h and then stained with Annexin V and PI for cell apoptosis assay, (d) Raw-Blue reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM lipo T20 pre-complexed with BSA at 1:1 and 1:2 ratio respectively under FBS-free (O/F) condition for 24h; Meanwhile, Raw-Blue reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM lipo T20 under FBS-supplemented (W/F) condition for 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.
As lipo T20 contained a lipophilic domain which enabled avid “albumin-binding”, it was reasonable to ask the question of whether albumin was playing a role in enhancing IMQ stimulation by lipo T20. In fact, the culture medium was normally supplemented with fetal bovine serum (FBS) containing excessive albumin relative to the quantity of lipo T20 utilized, so it was highly possible that lipo T20 would bind to serum albumin (FBS) once added to culture medium. As part of the proof to support this hypothesis, we observed that endocytosis pathway of lipo T20 exactly mimicked that of BSA, which was internalized mainly via macropinocytosis (EIPA) and clathrin-mediated endocytosis (Sugar) in DC 2.4 cells (Figure 5-4 (a)). Our observation agreed with previous studies on endocytosis of lipid-modified ODN conjugates and albumin. To further determine the role of albumin in the enhanced stimulation of IMQ, Raw-Blue cells were seeded overnight and then stimulated as previously described using the serum-free medium. Interestingly, lipo T20 completely suppressed IMQ-induced NF-κB activation under FBS-free culture conditions, indicating that albumin played an important role in the effect of lipo T20 on TLR 7 stimulation (Figure 5-4 (b)). Meanwhile, we ruled out the cell viability as a potential factor for downregulated NF-κB stimulation by confirming that the survival rate of Raw-Blue cells was unchanged under the FBS-free culture condition (Figure 5-4 (c)).

Since lipo ODN is able to hijack albumin and subsequently accumulates in APCs residing in LNs, forming stable albumin-lipo ODN complexes in physiological condition is a prerequisite for survival on their thorny way to LNs and lymphoid APCs. Similarly, lipo T20 should be able to complex with albumin under FBS-supplemented culture condition before being transported into cells. Besides, our research into erythrocytes insertion revealed that amphiphilic polymer was unable to massively insert into the plasma
membrane in the presence of blood serum. Collectively, these reasonings pointed to a “fact” that lipo T20 was transferred into cells in the form of lipo T20-albumin complex. To verify our postulation, we pre-incubated lipo T20 with BSA at a ratio of 1:1 and 1:2 for 1h and then treated pre-seeded Raw-Blue cells with 1 μM BSA-complexed lipo T20 under FBS-free condition. The results clearly showed that lipo T20’s capability of suppressing IMQ-induced activation was progressively abrogated as the ratio of pre-complexed BSA increased (Figure 5-4 (d)). Hence, we confirmed that the reversal of lipo T20’s biological capability of inhibiting TLR7 signaling primarily resulted from albumin which formed albumin-lipo T20 complex via noncovalent interaction.

**Figure 5-5.** (a) Representative confocal images of Raw-Blue reporter cells treated with 1 μM FAM-labeled T20 or FAM-labeled lipo T20 under FBS-supplemented condition, or (b) under FBS-free condition, and then washed and stained with 50 nM LysoTracker...
Red following manufacturer’s instructions. Scale bar: 10 μm. (c) Raw-Blue cells were stimulated with 2 μM IMQ alone or in combination with 1 μM disulfide bond-linked T20 or lipo T20 or BSA-T20 conjugate under FBS-supplemented condition, (d) under FBS-free condition, for 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

Additionally, we also illustrated that both T20 and lipo T20 colocalized with lysosomes regardless of the presence of FBS, suggesting that albumin did not significantly affect lipo T20’s intracellular destination (Figure 5-5 (a-b)). However, a covalently-synthesized BSA-T20 conjugate failed to reverse the intrinsic properties of T20 under both FBS-supplemented and FBS-free culture conditions as it greatly suppressed IMQ-induced activation (Figure 5-5 (c-d)). Accordingly, the reversal of lipo T20’s immunological property was more than due to the endocytosis pathway via hijacking albumin and was not due to quantitatively increased cellular uptake.

5.3.3 Optimization of TLR7 adjuvant booster
Figure 5-6. (a) Structure of C18 diacyl lipid conjugated poly-oligo(dT) and C14 diacyl lipid conjugated T20, (b) Raw-Blue reporter cells were stimulated with 2 μM IMQ alone or with 1 μM lipo T10, lipo T20, lipo T25 and lipo T50 for 24 h, (c) Raw-Blue reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM C14 diacyl lipid-modified T20 or C18 diacyl lipid-modified T20 for 24 h, (d) Raw-Blue cells were stimulated with 2 μM IMQ alone or in combination with 1 μM lipo T10, lipo T20, lipo T25 and lipo T50 for 24 h, (e) Structure of 3’- and 5’-modified Cholesterol T20, (f) HEK mTLR7 reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM 3’- and 5’-modified Cholesterol T20 for 24 h, (g) HEK mTLR8 reporter cells were stimulated with 2 μM R848 alone or in combination with 1 μM 3’- and 5’- modified Cholesterol T20 for 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

To optimize TLR7 stimulation enhancing efficacy, we synthesized a series of lipo poly-oligo-deoxythymidine (poly-Oligo(dT)) to treat Raw-Blue cells along with IMQ (Figure 5-6 (a)). As shown in Figure 5-6 (b), lipo T20 or lipo T25 mixed with IMQ induced the highest level of NF-κB stimulation. In contrast, lipo T10 showed little effect on IMQ-mediated activation, while lipo T50 slightly suppressed TLR7 signaling. ODNs conjugated with short diacyl lipids, such as C12 and C14 lipid chains, were less effective in targeting LNs due to relatively weak hydrophobic interaction between lipid tails and albumin.² However, we observed that C14 lipid-modified T20 gave a comparable level of immune activation when mixed with IMQ in vitro (Figure 5-6 (c)). In addition, we found that lipo T20 was unable to enhance IMQ-induced activation once the ratio of lipo T20 to IMQ was less 1:10, while no further potency-enhancing effect was observed when this ratio was
greater than 5:10 (Figure 5-6 (d)). Although most lipo ODNs were modified at 5’ terminal of ODN due to simplicity and cost of synthesis, 3’-terminal might give different levels of effects, as reported on the modification of CpG oligonucleotides. To test whether lipid modification at 3’ terminal of T20 would affect immune modulation, we synthesized 5’- and 3’-conjugated lipo T20 by using a 5’-cholesterol phosphoramidite and a 3’-cholesterol beads respectively (Figure 5-6 (e)). Since T20 itself was a TLR8 booster as well as a TLR7 inhibitor, we used murine TLR7 and TLR8 reporter cells separately for NF-κB stimulation assay. The results revealed that both 5’- and 3’-modified T20s were able to significantly enhance IMQ-induced stimulation in murine TLR7 cells, while both were functionally compromised after modification and unable to activate resiquimod (R848)-elicited murine TLR8 signaling (Figure 5-6 (f-g)). Together, these data demonstrated that lipid poly-oligo(dT) with 20~25-mer was the optimal structure to induce the highest level of TLR7-mediated NF-κB activation, and T20 modified with less hydrophobic lipid tail, compared to C18 lipid chain, at either 5’ or 3’ terminal was able to reverse the TLR7-specific suppression.

5.3.4 Immune modulation on TLR7 and TLR8 reporter cells

To investigate differential modulation of lipo T20 on TLR7 and TLR8, we evaluated half-maximal effective concentration (EC50) and potency of several classic TLR7/8 ligands in murine TLR7 and TLR8 reporter cells. The results implied that lipo T20 significantly lowered EC50 and improved the potency of IMQ, CL075, and R848, while T20 functioned in an opposed trend in murine TLR7 reporter cells (Figure 5-7 (a-c)). On the contrary, T20 was able to activate murine TLR8 only if mixed with a high dose of TLR7/8 ligands, while lipo T20 showed no effect (Figure 5-7 (d-f)). To determine the
levels of overall stimulation, we repeated the titration experiments in Raw-Blue reporter cells. Similar to that in murine TLR7 reporter cells, lipo T20 improved the potency and EC50 of these ligands and T20 suppressed their NF-κB stimulation in Raw-Blue reporter cells, indicating that although lipo T20 and T20 exhibited opposite stimulation toward TLR7 and TLR8, lipo T20 enhanced the overall NF-κB stimulation. These data clearly demonstrated lipo T20, but not T20, could act as an adjuvant enhancer to improve the immune stimulation of TLR7 (Figure 5-7 (g-i)).

**Figure 5-7.** (a-c) HEK mTLR7 reporter cells were stimulated with IMQ, CL075, R848 at indicated concentrations alone or in combination with 1 μM T20 or lipo T20 for 24 h, (d-f) HEK mTLR8 reporter cells were stimulated with IMQ, CL075, R848 respectively at indicated concentrations alone or in combination with 1 μM T20 or lipo T20 for 24 h,
Raw-Blue reporter cells were stimulated with IMQ, CL075, R848 respectively at indicated concentrations alone or in combination with 1 μM T20 or lipo T20 for 24 h.

Human and murine TLR7/8 shared many similarities in genetic sequence and function. To determine whether the above results could be translated to human cells, we stimulated human TLR7 reporter cells with several lipid-modified oligonucleotides. The results showed that besides lipo T20 and lipo IRS 954, lipid-modified ODN 2087, originally a human TLR7 and TLR8 inhibitor, also remarkably enhanced IMQ-induced NF-κB stimulation (Figure 5-8 (a)). In addition, lipo T20 exhibited significantly better stimulation than other lipid-modified oligonucleotides, showing an improved EC50 and potency of IMQ in human TLR7 reporter cells (Figure 5-8 (b)). As for human TLR8 signaling, lipo T20 did not affect the stimulatory potency of TLR8-506, a human TLR8 ligand. However, lipo ODN 2087 suppressed TLR8-506 stimulation. In contrast, T20 remained efficacious in potentiating TLR8 signaling in human TLR8 reporter cells (Figure 5-8 (c-d)), especially at high TLR8-506 concentrations. Overall, these results suggested the feasibility of applying lipo T20 and analogs to human models.

Figure 5-8. (a) HEK hTLR7 reporter cells were stimulated with 2 μM IMQ alone or in combination with 1 μM T20 or lipo T20, 1 μM IRS 954 or lipo IRS 954, 1 μM ODN 2087
or lipo ODN 2087 respectively for 24 h, (b) HEK hTLR7 reporter cells were stimulated with IMQ at indicated concentrations alone or in combination with 1 μM T20 or lipo T20 for 24 h, (c) HEK hTLR8 reporter cells were stimulated with 1 μg/ml TLR8-506 alone or in combination with 1 μM T20 or lipo T20, 1 μM ODN 2087 or lipo ODN 2087 respectively for 24 h, (d) HEK hTLR8 reporter cells were stimulated with TLR8-506 at indicated concentrations alone or in combination with 1 μM T20 or lipo T20 for 24 h. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

5.3.5 Ex vivo evaluation of adjuvant booster

![Graphs illustrating cytokine production](image)

**Figure 5-9.** (a-d) Murine splenocytes were stimulated with 2 μM IMQ alone or in combination with 1 μM T20 or lipo T20 for 24 h, and the inflammatory cytokines and type I IFN secreted by splenocytes including IL-6 (a), IL-12p40 (b), TNF-α (c), IFN-α (d) were measured by ELISA, (e) Representative flow cytometry plots of pDC enriched from
human PBMC, (f) human pDC cells were stimulated with 2 μM IMQ alone or in combination with 1 μM T20 or lipo T20 for 24 h, and IFN-α secreted by pDC cells was determined by ELISA, (g) Human PBMC cells were stimulated with 60 nM TLR8-506 alone or in combination with 1 μM T20 or lipo T20 for 24 h, and TNF-α secreted by PBMC cells was determined by ELISA. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

To evaluate the adjuvant enhancing activities of lipo T20 in primary immune cells, we isolated splenocytes from mice spleens and stimulated them with IMQ as previously described in TLR7/8 reporter cells. Consistent with our previous findings in the reporter cells, lipo T20 markedly potentiated the potency of IMQ by upregulating the production of NF-κB-signaled pro-inflammatory cytokines including IL-6, IL-12p40, and TNF-α, while IMQ mixed with T20 led to a significantly lower level of pro-inflammatory cytokines compared with IMQ alone (Figure 5-9 (a-c)). Interestingly, we observed that both T20 and lipo T20 decreased the production of IFN-α induced by IMQ (Figure 5-9 (d)). As secretion of IFN-α from pDC was mainly regulated through IRF7 signaling but independent of NF-κB activation,99 the results suggested that lipo T20 primarily intensified TLR7-signaled NF-κB activation but was still able to inhibit TLR7-mediated production of IFN-α.

Using human pDC enriched from human PBMC with 55.7% purity (Figure 5-9 (e)), we further confirmed that lipo T20 partially conserved T20’s capability of downregulating TLR7-mediated activation, evidenced by almost no secretion of IFN-α from pDC treated with IMQ and lipo T20 (Figure 5-9 (f)). Interestingly, we observed that lipo T20 was able
to significantly increase the production of TNF-α when mixed with TLR8-506 to treat human PBMC (Figure 5-9 (g)). However, we demonstrated that lipo T20 had no impact on TLR8-mediated NF-κB activation which regulated the production of pro-inflammatory cytokines including TNF-α. Although underlying mechanisms remained unknown, one possible interpretation was that lipo T20 mixed with TLR8 ligand might simultaneously activate various immune cells in PBMC, which led to TLR8-mediated NF-κB activation. These data emphasized specific innate immune activation pathways that lipo T20 possibly acted on, and it also suggested that lipo T20 could be a dual-booster for TLR7 and TLR8 adjuvants, which might be applied in clinical practice.

5.3.6 Adjuvant booster enhances immune responses of molecular vaccines in vivo

![Figure 5-10. (a) Representative flow cytometry plots of H-2K\(^b\)/SIINFEKL tetramer staining of CD8\(^+\) cells, (b) Quantification of H-2K\(^b\)/SIINFEKL tetramer staining of CD8\(^+\) cells, (c) Quantification of IFN-γ-secreting CD8\(^+\) T cell determined by intracellular...](image-url)
cytokine staining, (d) Quantification of TNF-α-secreting CD8+ T cell determined by intracellular cytokine staining, (e) On day 20, serum samples were collected and assayed by ELISA for anti-OVA IgG production. Data show the mean values ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant by one-way ANOVA with Bonferroni post-test.

We revealed that lipo T20 improved the stimulatory potency and efficacy of IMQ in the presence of albumin. In fact, interstitial space in the body harbors considerable serum albumin which circulates through lymphatic system back to bloodstreams. Therefore, lipo T20 theoretically was able to hijack interstitial albumin and then leverage it for intensifying the potency of TLR7/8 ligands in the LNs. Previous studies reported that unmodified IMQ showed little accumulation in the LNs while lipo T20 was able to target APCs in the LN via ‘albumin-hitchhiking’. Besides, our data showed that lipo T20 amplified IMQ-induced immune activation in TLR reporter cells. Thus, we hypothesized that subcutaneous administration of lipo T20 would improve the adjuvant effect of IMQ and immune activation. To test this hypothesis, we immunized mice with model antigen ovalbumin adjuvanted with a low dose of IMQ in the presence or absence of 6.2 nmol lipo T20. Unformulated IMQ + OVA did not elicit detectable antigen-specific CD8+ T cells in blood, presumably due to the insufficient LN draining of IMQ. However, the addition of lipo T20 resulted in an approximate five-fold increase in OVA-specific CD8+ T cells and up to a four-fold expansion of IFN-γ-secreting and TNF-α-secreting CD8+ T cells, as well as significantly improved OVA-specific IgG titers in mice (Figure 5-10).

These results demonstrated that lipo T20 could function as a potent TLR7/8 adjuvant booster to potentiate immune responses induced by the molecular vaccine in
Thus, lacing vaccine formulation with the adjuvant booster represents a novel and simple approach to amplify the immunogenicity of subunit vaccines. The concept of adjuvant enhancers can be immediately applied to current molecular vaccines adjuvanted by TLR7/8 ligands, providing a plug and play approach to catalyze the development of next-generation cancer vaccines.

5.4 Conclusions

In summary, we found that lipid-modified oligonucleotide-based TLR7/8 inhibitors were able to improve the potency and efficacy of TLR7/8 ligands in the presence of albumin. We found that these lipo ODNs could tolerate a wide range of sequences, but the structurally-optimized lipo poly (dT) (20-25 nucleotides) showed maximal enhancing effects. In addition, we found that lipo T20 was also applicable to human cells and was able to enhance human TLR7- and TLR8-mediated NF-κB stimulation. However, lipo T20 still conserved the immunosuppressive capability of reducing TLR7-mediated production of IFN-α in both murine and human cells. More significantly, lipo T20 was proved to be a powerful adjuvant enhancer in enhancing the immune responses to molecular vaccines, as lacing subunit vaccine formulation with lipo T20 led to markedly improved cellular and humoral responses in mice. This finding might be broadly applicable in many current vaccines, where both efficacy and safety are needed.
CHAPTER 6 SUMMARY AND OUTLOOK

The major focus of this dissertation is to expand the research scope of the “albumin-hitchhiking” approach. We mainly studied the therapeutic efficacy and immunological properties of a variety of lipid-modified ODNs that were utilized for immune modulation through LN-targeting delivery. For stimulatory ODNs, we showed that lipid modification compromised the immunological activities of CpG ODNs in vitro, while lipid-modified class B and C CpG ODNs were able to markedly enhance the immunogenicity of subunit vaccines in mice. In addition, lipid modification dramatically increased the accumulation of CpG ODNs in LNs and reduced CpG-associated systemic toxicity. Collectively, this study highlights the significance of targeted delivery to local LNs and suggests that the “albumin-hitchhiking” approach holds great promise in optimizing therapeutic efficacy of cancer vaccines while minimizing adjuvants-related side effects.

On the other hand, lipid modification on suppressive ODN A151 resulted in the greater inhibitory capability of suppressing TLR9-mediated immune activation and enhanced cellular uptake. Besides, compared to unmodified ODN A151, lipid-conjugated ODN A151 showed increased LN accumulation and demonstrated markedly improved therapeutic efficacy in suppressing immune responses induced by CpG-adjuvanted vaccines in vivo. These findings indicate that targeting suppressive ODN to LNs via hitchhiking endogenous albumin may be a promising approach to potentiate immunoinhibitory properties of suppressive ODN. In future works, we look forward to targeting other immune modulators to local LNs to manipulate immune responses for the treatment of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.
Based on the intrinsic membrane-insertion properties of amphiphilic molecules, we developed cell-based delivery strategies for systemic administration of therapeutics. Specifically, we studied the structure-dependent stability of lipid-based amphiphiles anchored on RBCs. The data revealed that longer diacyl lipid chain and short PEG spacer contributed to more stable retention kinetics of amphiphiles loaded on RBCs \textit{in vivo}. More importantly, we observed that cationic lipid-modified amphiphiles exhibited exponentially prolonged circulation half-life on RBCs \textit{in vivo} relative to anionic lipid-conjugated analogs. This research demonstrated a simple yet efficient cellular-hitchhiking delivery strategy and emphasized the importance of rational structure design in \textit{in vivo} stability of amphiphiles anchored on RBCs. Many research directions may be developed based on these findings. For example, loading amphiphilic chemotherapeutic drugs on RBCs through spontaneous insertion may significantly extend their circulation half-life \textit{in vivo} and circumvent rapid immune clearance, and thus it may lead to sustained tumor killing and achieve remarkable therapeutic efficacy in cancer treatment. In parallel, by leveraging immunological properties of RBCs, amphiphilic peptides that hitchhike RBCs may evade immune surveillance and stimulation and thus induce systemic tolerance towards self-antigens that trigger autoimmunity. Hence, the “RBC-hitchhiking” approach may hold great promise in treating autoimmune disorders as well.

More importantly, we discovered a novel class of lipid-modified oligonucleotide-based adjuvant booster which could enhance the adjuvant potency of TLR7/8 ligands \textit{in vitro} and \textit{in vivo}. We explored the underlying mechanism by which lipid modification converted oligonucleotide-based TLR7 inhibitors into TLR7 adjuvant boosters. The data revealed that albumin was playing a pivotal role in the functional reversal of lipid-modified
TLR7 inhibitors. Furthermore, we showed that lipid-modified oligonucleotide-based adjuvant booster was applicable to human cells and could enhance human TLR7- and TLR8-mediated NF-κB stimulation. More strikingly, the results demonstrated that subcutaneous administration of imiquimod-adjuvanted vaccine laced with the adjuvant booster in mice markedly enhanced antigen-specific cellular and humoral responses, compared to the same vaccine without the adjuvant booster. Current strategies that are employed to enhance the immunogenicity and safety of vaccines mainly focus on refining the delivery efficiency of therapeutics to manipulate immune responses while minimizing systemic toxicity. The concept of adjuvant booster-laced “molecular vaccine cocktail” features a simple, novel and effective approach to considerably boosting the potency of vaccine adjuvant and subsequently improving the immunogenicity of therapeutic vaccines. The future work may further explore the molecular and structural mechanism by which adjuvant boosters act on TLR7/8-mediated immune activation. By studying and understanding the constitutive mechanisms, we may be able to design and engineer biomolecules in a more rational way to modulate immune activation and achieve optimal efficacy of cancer vaccines for clinical application. Another future research topic that may be derived from this study may focus on the application of adjuvant booster in some representative tumor models combined with current cancer vaccines, with the goal to reverse tumor-associated immunosuppression and lead to tumor regression. Accordingly, we will set out to investigate the efficacy of adjuvant booster in maximizing the immunogenicity of cancer vaccines while minimizing systemic inflammatory reactions induced by vaccine adjuvants in tumor models.
REFERENCES


17 Scheiermann, J. & Klinman, D. M. Clinical evaluation of CpG oligonucleotides as
adjuvants for vaccines targeting infectious diseases and cancer. Vaccine 32,

18 Klinman, D. M. Therapeutic applications of CpG-containing
oligodeoxynucleotides. Antisense & nucleic acid drug development 8, 181-184


20 Speiser, D. E. et al. Rapid and strong human CD8+ T cell responses to
vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest

21 COOPER, C. L. et al. CPG 7909, an Immunostimulatory TLR9 Agonist
Oligodeoxynucleotide, as Adjuvant to Engerix-B® HBV Vaccine in Healthy
Adults: A Double-Blind Phase I/II Study. Journal of Clinical Immunology 24, 693-

22 Halperin, S. A. et al. A phase I study of the safety and immunogenicity of
recombinant hepatitis B surface antigen co-administered with an
immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 21,
2461-2467 (2003).

23 Bourquin, C. et al. Targeting CpG oligonucleotides to the lymph node by
nanoparticles elicits efficient antitumoral immunity. Journal of immunology


Holden, C. A., Yuan, Q., Yeudall, W. A., Lebman, D. A. & Yang, H. Surface engineering of macrophages with nanoparticles to generate a cell-nanoparticle


96 Ugarte-Uribe, B. et al. Lipid-modified oligonucleotide conjugates: Insights into gene silencing, interaction with model membranes and cellular uptake


ABSTRACT

IMMUNE MODULATION BY AMPHIPHILIC OLIGONUCLEOTIDES

by

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May 2019

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Immunotherapy is emerging as one of the most promising therapeutic strategies for cancer treatment in clinical practice. Immunotherapy leverages the host’s immune system to attack and kill tumor cells. Rationally-designed cancer vaccines are emerging as one of the powerful immunotherapies primarily because of the promise of inducing potent anti-tumor immunity and eradicating tumors. To improve the immunogenicity of cancer vaccines, vaccine antigens are administered with vaccine adjuvants which can robustly activate the innate immune system and subsequently lead to adaptive immune responses. However, the major challenge of achieving significant therapeutic efficacy is the lack of efficient targeted delivery of vaccine components. By taking advantage of endogenous albumin, lipid-modified oligonucleotides-based adjuvants can be efficiently transported to draining lymph nodes (dLNs), where a variety of immune cells reside, and subsequently activate the adaptive immune system. This “albumin-hitchhiking” strategy represents a novel and efficient way for LN-targeted delivery of biologics, improving the therapeutic efficacy of cancer vaccines.
The aims of this dissertation are to further expand the scope of the “albumin-hitchhiking” approach in immune modulation. We first evaluated the efficacy of three representative classes of lipid-modified CpG ODNs as vaccine adjuvants. Lipo CpG ODNs considerably drained to LNs more than unmodified CpG ODNs, while they appeared to be functionally compromised in *in vitro* immune activation. However, administration of lipo CpG class B and C ODNs, not lipo CpG class A ODN, with a protein antigen in mice led to improved antigen-specific CD8+ T cell responses and humoral responses relative to their parent compounds. We then evaluated the therapeutic efficacy of immunosuppressive ODN A151 and its lipid-modified form in inhibiting TLR9-mediated immune activation. The data showed that lipo ODN A151 was preferentially internalized by cells and accumulated in LNs in a larger magnitude compared to unmodified ODN A151. More importantly, prophylactic administration of lipo ODN A151, but not unmodified ODN A151, resulted in profound inhibition of immune responses challenged and induced by CpG-adjuvanted vaccines in mice. Next, we investigated the structure-dependent stability of lipid-modified amphiphilic polymers on red blood cells (RBCs). We revealed that longer lipid chain was correlated with more stable insertion on RBCs surface *ex vivo*, while shorter polyethylene glycerol (PEG) spacer favored amphiphiles with more lasting retention on RBCs *in vivo*. Furthermore, cationic amphiphiles constructed with cationic lipids demonstrated significantly improved retention kinetics on RBCs *in vivo*, evidenced by a 40-fold increase in circulation half-life relative to anionic counterparts. Finally, we discovered a novel adjuvant enhancer which potentiated the adjuvant activities of TLR7/8 ligands *in vitro* and *in vivo*. The results showed that certain amphiphilic oligonucleotides could improve TLR7 ligand-induced NF-κB activation in the presence of albumin protein.
In vivo, administration of imiquimod-adjuvanted vaccine mixed with the structurally-optimized amphiphilic oligonucleotide-based adjuvant booster induced a five-fold increase in the frequency of antigen-specific CD8+ T cells and the production of antigen-specific IgG in mice, compared to the same vaccine without adjuvant booster. Accordingly, lipid-functionalized oligonucleotide-based adjuvant booster could dramatically improve the immunogenicity of subunit vaccines. Collectively, lipid-functionalized oligonucleotides not only accumulate in LNs by hitchhiking endogenous albumin but also versatilely modulate the immune responses. Amphiphilic oligonucleotides may be broadly employed for the treatment of diseases involving the immune system, where the immune modulation is needed.
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