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Lipopeptide immunogens targeting the membrane proximal region of HIV-1 gp41

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Lipopeptide immunogens targeting the membrane proximal region of HIV-1 gp41

by

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DISSERTATION

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To my wife, Misty:

Our adventures are the great joys of my life.

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Chapter 4 is co-authored by Virginia Platt. V. Platt assisted in designing experiments, preparation of polyhistidinylated OVA, preparation of liposome formulations, liposome-protein binding studies, and revising the manuscript.

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Ecclesiastes 9:11

Abstract

Lipopeptide Immunogens Targeting the Membrane Proximal Region of HIV-1 gp41

Douglas Stuart Watson

The membrane proximal region (MPR) of HIV-1 gp41 is a desirable candidate for development of a vaccine that elicits neutralizing antibodies since it is targeted by three of the most potent broadly neutralizing monoclonal antibodies isolated from infected patients. Structural studies of these antibodies and their epitopes suggest that MPR immunogens may be presented in a lipid membrane environment. In this thesis, I report the synthesis and evaluation of MPR lipid-peptide conjugates for membrane presentation of the MPR and elicitation of neutralizing antibodies to HIV-1.

In **Chapter 2**, I hypothesized that covalent attachment of lipid anchors would enhance the humoral immune response to MPR-derived peptides presented in liposomes. In a comparison of eight lipids conjugated to MPR peptides, cholesterol hemisuccinate promoted the strongest anti-peptide titers in mice. Lipid conjugation was employed to manipulate the biophysical properties and antibody inducing capability of MPR peptides, and this lipopeptide toolkit will be useful for interrogating the role of structure in the immune response to the MPR.

In **Chapter 3**, MPR lipopeptide immunogens were further utilized to study the cause of weak immune responses to the MPR. We initially hypothesized that the antibody response against the MPR is restricted by immunologic tolerance, but a comparative assessment of antibody responses to MPR lipopeptides in two mouse models of

defective immune tolerance indicated that tolerance mechanisms are insufficient to explain the poor antibody responses to the MPR.

I also hypothesized that MPR immunogenicity could be increased through phosphorylation of amino acid side chains. Evaluation of modified MPR immunogens revealed a modification that increased anti-MPR antibodies by an order of magnitude. Importantly, the sera of rabbits immunized with these modified constructs neutralized a laboratory-adapted HIV-1 strain *in vitro*, providing a foundation for further work on this strategy.

Finally, supplementary studies were conducted involving delivery systems and novel molecular adjuvants for MPR peptide immunogens. In **Chapter 4**, metal chelation through lipid-anchored multivalent nitrilotriacetic acid was investigated as a non-covalent strategy to attach peptide and protein antigens to liposomal vaccine carriers. Also, in **Chapter 5** retinoids were explored as candidate liposomal adjuvants for enhancing the antibody response to MPR immunogens. These studies may lead to improved formulations for delivery of MPR peptide antigens to elicit neutralizing antibodies against HIV-1.

Table of Contents

Preliminary pages

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 1

Antibody-mediated neutralization in preventative vaccination against HIV-1

1.1 Overview

This dissertation concerns the design and evaluation of lipopeptide immunogens for elicitation of neutralizing antibodies to the membrane proximal region of HIV. These constructs are employed in the assessment of the role of tolerance in restricting the antibody response to the MPR, as well as in evaluation of candidate delivery systems and adjuvants for improved immune responses to the MPR. In Chapter 1, a brief introduction of the significance of the HIV/AIDS epidemic is presented and approaches to HIV vaccine design are reviewed. In Chapter 2, the synthesis and biophysical characterization of a library of covalent lipid-peptide MPR conjugates are reported. The anti-MPR antibody responses to these constructs are extensively evaluated in mice and the critical factors contributing to antibody induction are discussed. In Chapter 3, the hypothesis that antibody responses to the MPR are restricted by tolerance mechanisms is tested in mice through a two-pronged approach involving murine models of defective immune tolerance and specific side chain modifications intended to overcome tolerance.

Chapters 4 and 5 describe investigations of delivery systems and molecular adjuvants for enhancement of the humoral immune response to the MPR. In Chapter 4, a method for non-covalent attachment of polyhistidine-tagged recombinant MPR antigens to particulate delivery systems is characterized. Two model antigens are non-covalently attached to liposomes containing metal-chelating nitrilotriacetic acid derivatives and antibody responses are compared to covalent lipid conjugates in mice. In Chapter 5, molecular adjuvants derived from Vitamin A are investigated for enhancement of antibody responses to MPR lipopeptides. Finally, salient findings of the work, including *in*

vitro neutralization assays, are discussed in Chapter 6 in the context of future prospects for development of a preventative vaccine for HIV.

1.2 Background

Since the initial recognition of the Acquired Immune Deficiency Syndrome (AIDS) epidemic in the United States in 1981, over 550,000 Americans have died from the disease [1]. At present, over one million Americans are estimated to be living with HIV/AIDS and approximately 40,000 new infections are reported annually. Worldwide, approximately 400,000 new infections are reported annually as the estimated total of individuals living with HIV/AIDS approaches 40 million [2]. Scientific evidence overwhelmingly indicates that AIDS is caused by infection with human immunodeficiency (HIV) viruses [3].

HIV type 1 (HIV-1), the more virulent and infectious of the two HIV viruses, is believed to have transferred from primates to humans in the early to mid 20^{th} century [4]. HIV primarily infects CD4+ T lymphocytes but can also establish latent infection in diverse cell types of hematopoietic and neurologic origin, among others [5, 6]. The progressive destruction of CD4+ T cells decimates the immune system and causes AIDS, clinically defined as a CD4+ T cell count lower than 200 cells per μL of blood [7]. This clinical state results in increased susceptibility to opportunistic infections and cancers that eventually result in death.

HIV-1 is a single stranded, positive sense, enveloped RNA virus of the genus Lentivirus (**Figure 1-1**) [3]. The HIV-1 genome consists of approximately 10 kb, containing 46 translated open reading frames that are translated into 16 proteins. A single full-length mRNA transcript encodes the major enzymatic and structural proteins, and is translated

into two large precursor proteins – Gag-Pol and Env. Gag-Pol is cleaved by viral proteases to generate the viral enzymes (protease, reverse transcriptase, integrase) and structural proteins (matrix, capsid). The Env precursor is cleaved by cellular proteases into gp120, an external surface glycoprotein, and gp41, a transmembrane protein. The Env proteins gp120 and gp41 form a heterotrimer consisting of three subunits of each protein. This complex comprises the viral envelope spike responsible for mediating binding and uptake by target cells, and thus it is a primary target for HIV vaccine development.

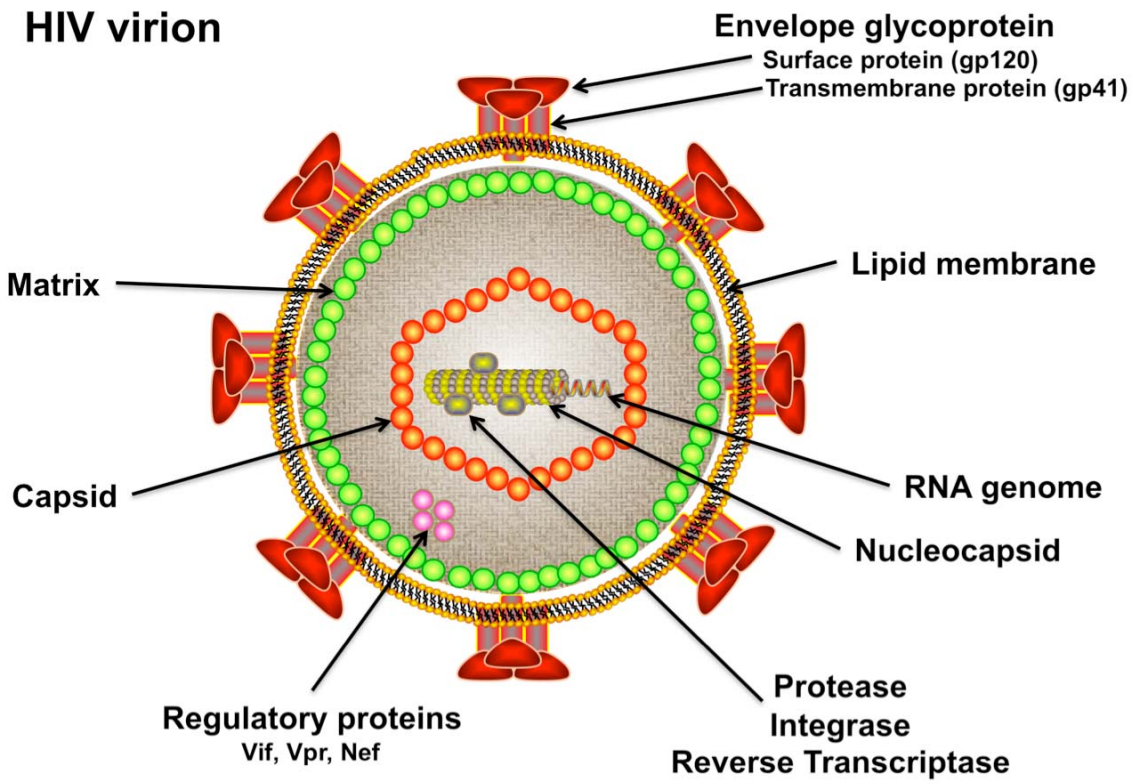

Figure 1-1. The HIV-1 virion.

HIV is primarily spread through sexual contact, contaminated blood and from mother to child [8]. Although the public often associates HIV with the homosexual community, heterosexual activity accounts for the majority of infections globally [9]. Upon infection,

acute mononucleosis-like symptoms are manifested for approximately 1 to 4 weeks in up to 90% of patients [10]. Virus is first detected in blood approximately 2 weeks post-infection, peaks at 3 weeks and reaches a steady state level within 5 months [11, 12]. Adaptive immunity to HIV first appears during acute infection and reaches plateau in 3 to 5 months concomitant with the plateau in virus load [3]. CD8+ cytotoxic T lymphocytes (CTLs) appear within 3 weeks post infection and CD8+ T cell responses (both cytotoxic and non-cytotoxic) are correlated with reduced viral load, which in turn is predictive of a more prolonged asymptomatic clinical course [13-15]. Anti-HIV antibodies appear approximately 4 weeks post-infection and are not correlated with control of virus replication [16]. Neutralizing antibodies do not typically appear until 10 to 12 weeks post-infection [17].

HIV infected persons progress to AIDS within 2 to 17 years after seroconversion, with a median progression time of approximately 10 years [3, 18]. Median survival following progression to AIDS is approximately 9.2 months [19]. Fortunately, the development of anti-retroviral therapies, particularly triple therapy (Highly Active Anti-Retroviral Therapy; HAART), has reduced disease severity and improved survival by 4 to 12 years [20, 21]. HAART regimens consist of a combination of reverse transcriptase inhibitors and protease inhibitors, although an inhibitor of virus-cell fusion has also been approved and [22] is an alternative treatment for drug-resistant strains [23]. Although HAART has improved survival and quality of life of infected persons, multi-drug resistance continues to increase [24]. Thus, control of viral transmission is critical to the effort to eliminate new infections and ultimately defeat the epidemic. An effective vaccine would be an essential tool in the fight to prevent transmission.

1.3 Preventative vaccines against HIV-1

1.3.1 Challenges to HIV-1 vaccine development

While an ideal HIV vaccine would provide sterilizing immunity and prevent infection, a vaccine capable of preventing disease progression would be of enormous clinical benefit. Even a vaccine that reduces viral load would be useful because lower viral loads result in decreased rates of transmission [9]. Many hurdles must be addressed in the development of an effective vaccine, and recently an increasing appreciation has been realized of the need for better understanding of the basic biology of HIV and of host immune responses to infection [25]. Features of HIV that must be paid particular attention in vaccine development are summarized in **Table 1-1**.

HIV Characteristic	Implication for Vaccination
Infection and destruction of CD4+ T cells	Compromised immune function
Genomic integration of viral DNA	Establishment of latent virus reservoirs
Tremendous sequence variation among strains	Difficulty developing a vaccine with broad coverage
Poor fidelity of reverse transcriptase and rapid virus mutation	Escape from protection upon vaccine-induced evolutionary pressure
Direct cell-cell transfer of virus	Virus masked from immune recognition and clearance

Table 1-1. Features of HIV and implications for vaccine development.

There is also a need for greater understanding of the correlates of protection against infection and disease [26]. Potential correlates include innate immunity, CTL responses,

neutralizing antibodies, and non-neutralizing antibody-mediated responses such as antibody-dependent cellular cytotoxicity. Of these, CTL activity is best correlated with reduced viral load and more favorable clinical course. Although neutralizing antibodies do not appear to be involved in control of infection, a number of studies in primates indicate that the presence of neutralizing antibodies in the blood is sufficient to prevent mucosal transmission of the virus [27]. Conversely, vaccines that elicit only T cell responses have not prevented infection in primates, but have significantly reduced virus load and severity of disease [13, 28]. Taken together, these observations suggest that antibodies play a role in preventing initial infection, while T cell responses control viral replication after infection. Thus, a general consensus has arisen that a successful preventative HIV vaccine will elicit both humoral and cellular immune responses [29]. Mucosal immunity is also widely thought to be important since most transmission events occur via the mucosae [30].

1.3.2 Overview of vaccine strategies

In the last 25 years a multitude of HIV vaccine strategies have been pursued [3]. These approaches have included whole killed virus [31-33], attenuated live virus [34-36], recombinant viral vectors [37-39], protein subunit vaccines [40-42], DNA vaccines [43, 44], and others. Killed virus preparations have elicited neutralizing antibodies in primates, but limited T cell responses are elicited because protein expression within the host is generally required to achieve robust cellular responses [31, 45, 46]. Live attenuated vaccines elicit robust cellular and antibody responses and have demonstrated protection from disease, if not infection, in a number of primate studies [47]. However, observations of reversion to virulence and induction of AIDS in vaccinated animals has severely limited the impetus for advancement of live attenuated vaccine candidates [48, 49]. To date, recombinant live virus vectors, such as

adenoviruses or poxviruses, expressing HIV proteins offer the greatest compromise of efficacy and safety [50]. These constructs are often administered in tandem with recombinant envelope glycoproteins, which boost neutralizing antibody responses [51]. Nucleic acid-based vaccines have also been explored as an alternative to live virus vectors, as they provide transgene expression within host cells and elicit cellular immunity [52]. However, highly promising small animal studies have not translated to success in primates or in man because DNA does not induce vigorous production of viral proteins, prohibitively large DNA doses are required, and robust immune responses have not been observed [53].

1.3.3 Clinical Trials

In 20 years of human trials of preventative HIV vaccines, more than 50 vaccine candidates have been evaluated in at least 114 separate clinical trials [54]. These have included many of the modalities discussed above. Of these, only 3 have progressed to large-scale Phase III efficacy studies. Prompted by the observation of antibody-mediated virus neutralization in the sera of infected patients, early vaccine efforts focused on eliciting neutralizing antibodies to the envelope glycoproteins. Although two phase III trials of recombinant envelope glycoprotein gp120 raised neutralizing antibodies, the vaccines failed to protect against infection [55, 56]. As discussed below, it is now appreciated that the most susceptible neutralizing antibody targets are masked from immune recognition in the native envelope proteins [57]. More recently, the field shifted to focus on vaccines that elicit cell-mediated immunity. A phase II trial of a recombinant poxvirus vector elicited both cell-mediated and humoral responses, but a phase III trial was halted after the vaccine failed to elicit significant immune responses [58, 59]. An additional phase III canarypox prime – gp120 boost is currently underway in Thailand [60].

In recent years, a great deal of attention has been paid to a phase IIb trial of a live, replication-incompetent adenovirus type 5 (Ad5) vector expressing HIV proteins gag, pol and nef (the Step trial) [61]. Similar constructs elicited potent cellular responses and produced extremely promising results in rhesus monkey protection studies [62]. Unfortunately, the Step study was halted in November 2007 after the data indicated that in some cases the vaccine actually increased the risk of HIV infection in a subpopulation of recipients [63]. Increased risk of infection was associated with pre-existing humoral immunity to the adenoviral vaccine vector, and a subsequent *in vitro* study suggested that preexisting anti-Ad5 antibodies increase activation of dendritic cells and Ad5-specific T lymphocytes, perhaps establishing a more permissive environment for HIV infection [64, 65]. Second generation vaccine candidates constructed from more rare adenovirus serotypes 26 and 35, against which pre-existing vector immunity is uncommon, have demonstrated promising results in primates and early stage clinical trials are underway [66]. Nonetheless, these studies have re-emphasized the potential importance of neutralizing antibody responses in preventing infection.

1.4 Antibody-mediated neutralization of HIV-1

HIV has developed potent evolutionary defenses to prevent recognition by neutralizing antibodies [57, 67, 68]. The variable loops of glycoprotein 120 are accessible and immunogenic, but these regions are poorly conserved and the breadth of protection they generate is extremely limited [69]. Additionally, extensive glycosylation of the envelope glycoproteins renders the underlying protein largely inaccessible [70]. Furthermore, the trimeric native conformation of the envelope spike prevents access to segments that are available on monomers [71]. Moreover, some of the most susceptible targets are only exposed for a brief window during the conformational changes involved in receptor

binding and membrane fusion [72]. Finally, humoral vaccine candidates must avoid the risk of antibody-dependent enhancement, in which non-neutralizing antibodies actually promote infection via cellular uptake mediated by Fc receptors [73, 74].

1.4.1 Mechanisms

Antibody-mediated virus neutralization occurs when an antibody interacts with either a virus or a host cell in a manner that prevents infection of the host cell (**Figure 1-2**) [75, 76]. Primary mechanisms of virus neutralization involve blocking host cell receptor engagement by saturating occupancy of viral surface proteins (**A**) or host cell receptors (**B**). Alternatively, antibodies may block conformational changes in viral proteins necessary for fusion to the cell membrane (**C**) or release from the endosome (**D**). Many of these mechanisms have been observed in the various neutralizing antibodies discussed below.

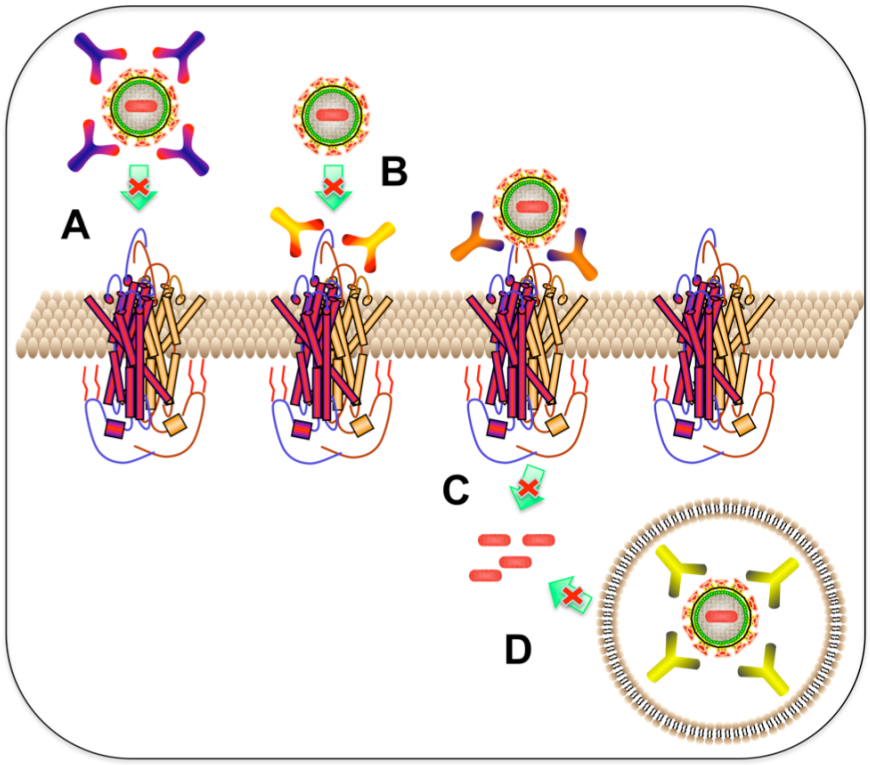

Figure 1-2. Mechanisms of antibody-mediated neutralization of HIV.

1.4.2 Human monoclonal antibodies

The design of immunogens that target the envelope glycoprotein subunits gp120 and gp41 has been driven by structural studies of broadly neutralizing human antibodies isolated from chronically infected patients (**Figure 1-3**). [77]. Anti-gp120 neutralizing antibodies generally recognize the CD4 binding site (IgGb12, F105) [78, 79], the co-receptor binding site (17b, 412D, X5) [80-82], the V3 loop (447-52D) [83], or glycans on the heavily glycosylated V4/V5 regions (2G12) [84]. Of these antibodies, IgGb12 and 2G12 have displayed the greatest breadth and potency of neutralization [77]. However, more work is needed to understand how to elicit antibodies with similar potency and specificity.

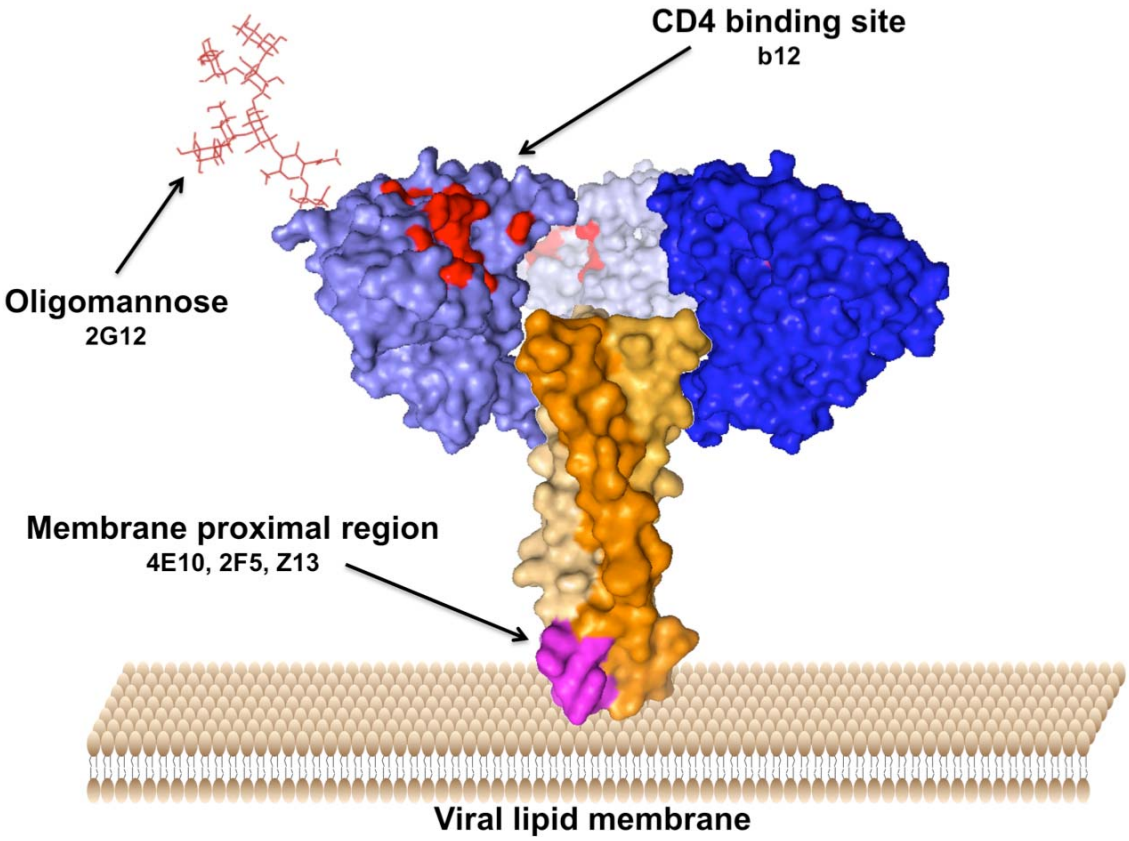

Figure 1-3. Key neutralization targets of the HIV-1 envelope glycoprotein.

1.4.3 Envelope glycoprotein 41 membrane proximal region antibodies

Perhaps the most promising neutralizing antibody target is the highly conserved membrane proximal region (MPR) of gp41, the segment comprised of approximately 25 residues at the N terminus of the transmembrane domain that extends out of the viral membrane. Human monoclonal antibodies against this region (4E10, 2F5, Z13) exhibit greater breadth of neutralization than the antibodies discussed above [77, 85, 86]. These antibodies inhibit viral membrane fusion by binding to the exposed MPR in a conformational intermediate that is exposed during fusion [87]. The intermediate conformation is only accessible during the minutes-long fusion event, which may help explain the scarcity of natural anti-MPR antibodies in infected patients [72].

MPR-targeted immunogen design has not yet yielded vaccine candidates capable of eliciting broadly neutralizing antibodies. Most strategies have consisted of synthetic peptides conjugated to carrier proteins or MPR sequences grafted into recombinant constructs (summarized in **Table 1-2**). In the following section, three specific strategies for rational design of MPR-targeted vaccines are discussed that may improve upon these approaches. In one recent report a library of rhinovirus chimeras were constructed with grafted sequences derived from the 2F5 epitope based upon computational predictions of conformations that could bind 2F5 [88]. This approach generated immunogens that elicited antibodies with broad albeit modest neutralization capability, and represents a promising paradigm for future improvements.

Epitope	Vector	Orientation	Route	Species	NAb Titer	Reference
2F5	Influenza virus	Site B of HA	i.n.; s.c.	Mouse	40-320	[89]
2F5	Potato virus X	N terminus of coat protein	i.p.; i.n.	Mouse	180-200	[90]
2F5	Rhinovirus	Loop 2 of the VP2 “puff” of HRV14	s.c.	Guinea pig	25-250	[88]
2F5	DNA	HIV-1 gp140 variable loops	i.m.	Guinea pig	None	[91]
2F5	Escherichia coli	Maltose-binding protein (multiple sites)	i.p.	Mice	None	[92]
2F5	KLH	Constrained synthetic β-turn peptide	i.m.	Guinea pig	None	[93]
2F5	KLH	Constrained synthetic α-helical peptide	i.m.	Guinea pig	None	[94]
4E10	DNA	HIV-1 gp120 variable loops	i.m.	Rabbit	None*	[95]
MPR	Liposome	Lipid-conjugated constrained peptide	i.p.	Rabbit	30-110	[96]
MPR	Hepatitis B VLP	Surface antigen	i.d.	Rabbit	None	[97]
MPR	Liposome	Trimeric C terminal gp41	i.p.	Mice	None	[98]
MPR	Cholera toxin	Recombinant fusion protein	i.p.; i.n.	Mice	N.D.	[99]
MPR	Virus-like particle	Constrained pre-fusion C terminal gp41	i.d.; i.m.	Guinea pig	None	[100]

Table 1-2. Selected approaches to MPR immunogen design. Adapted from Montero et al [101]. Abbreviations: i.n. intranasal; s.c. subcutaneous; i.p. intraperitoneal; i.m. intramuscular; i.d. intradermal; N.D. Not Determined. *MPR-specific NAb not detected

1.5 Emerging strategies to elicit neutralizing antibodies against the MPR of gp41

1.5.1 Structure-based immunogen design

Structure-based design of humoral vaccine antigens arises from an evolving appreciation of the fact that antibodies recognize three-dimensional electrostatic surfaces and not linear peptides or even contiguous sequences [102]. In this regard, MPR immunogen design has been driven by structural studies of the MPR-targeted neutralizing antibodies 4E10, 2F5 and Z13 [57, 103, 104]. Structures of these antibodies bound to their respective MPR epitopes have been determined by x-ray crystallography and NMR [105-108]. These data have revealed that the epitope of the 4E10 antibody adopts an α-helical conformation; the 2F5 epitope adopts an extended conformation with a β turn. Unfortunately, first generation strategies to reconstruct these structural features, either in synthetic peptides or grafted into recombinant proteins, have not elicited neutralizing antibodies (**Table 1-2**).

A more recent strategy has integrated this structural information with a high-throughput approach by which many recombinant constructs are generated and screened for binding affinity with neutralizing antibodies [25, 57]. Indeed, several early reports have been promising and this tactic may ultimately be successful [87, 109]. However, the approach is resource-intensive and may be restricted to for-profit companies and large institutions. Moreover, it does not overcome circumstances under which the structure of interest is underrepresented in the antibody repertoire due to autoantigen mimicry, as is hypothesized in the case of the MPR (see below). Regardless, these efforts are likely to generate promising candidate immunogens in the near future.

1.5.2 Elicitation of polyspecific autoreactivity

It has been observed that both the 4E10 and the 2F5 antibodies exhibit broad cross-reactivity with phospholipids and affinity for lipid membranes [110, 111]. In a comparison of neutralizing and non-neutralizing anti-gp41 antibodies, this phenomenon was not observed in the non-neutralizing antibodies. Given the close proximity of the MPR to the viral membrane, it has been suggested that interaction with the membrane may facilitate neutralization through added stability and/or affinity of binding [112, 113].

Structural studies of the 4E10 and 2F5 antibodies have revealed unusually long, hydrophobic CDR3 regions immediately adjacent to the epitope binding site [105, 107]. These CDR3 regions may explain the observed lipid reactivity and membrane affinity. Notably, unusually long CDR3 regions are implicated in lipid-reactive autoantibodies, and a natural concern arises that immungens that elicit this type of reponse will induce autoimmunity [114]. However, 4E10 and 2F5 antibodies were investigated for treatment of infected patients and found to be completely safe and to reduce viral loads [115-118]. Taken together, these observations have led to the hypothesis that peptide or protein antigens from the MPR must be presented in a lipid bilayer context to raise antibodies with similar properties. An alternative explanation is that 4E10- and 2F5-like antibodies with long, hydrophobic CDR3 regions may not be elicited during vaccination or infection because they are eliminated from the antibody repertoire by tolerance mechanisms [112].

1.5.3 Breaking tolerance with post-translational modifications

In autoimmunity and cancer, considerable evidence exists to suggest that post-translational events generate “neoepitopes” that generate altered antibody responses and can exacerbate or ameliorate disease [119]. These modifications include

phosphorylation, nitration, deamidation, citrullination, cysteinylation and others. Changes in immune responses may be induced through altered affinity for MHC molecules and B cell receptors, and a recent report also suggests that modified proteins may be inherently immunostimulatory [120]. It is possible that post-translational modifications, arising from inflammation and cell dysregulation during infection or vaccination, stochastically create HIV neoantigens that alter the quality or magnitude of the immune response.

The MPR contains several labile residues that could be post-translationally modified, although these modifications have not been demonstrated *in vivo*. If this phenomenon is found to contribute to overcoming immune tolerance to the MPR, it would provide a potential explanation for the rarity of MPR antibodies during HIV infection. Moreover, oxidative inflammatory environments conducive to such modifications have been reported in HIV-1 infection [121] and other viral infections [122, 123], as well as in immunization with common vaccine adjuvants [124, 125]. Modified epitopes have been observed in such disease agents as influenza virus, rabies virus and African trypanosomiasis [126-128]. If immunogens incorporating these modifications elicit 4E10-like antibodies, this approach would represent an important advance with broad applicability for vaccine design.

1.6 Proposed strategy to elicit neutralizing antibodies against HIV-1

I sought to elicit 4E10- and 2F5-like neutralizing antibodies by presenting minimal MPR peptides in the context of lipid bilayers. Although structurally constrained recombinant proteins have been designed that mimic the conformational intermediate that exposes the MPR, these approaches may raise off-target antibodies directed at other parts of the protein that sterically hinder binding of neutralizing antibodies [129]. Furthermore,

despite significant focus on design of structurally constrained immunogens that mimic the native gp41 conformation, studies have demonstrated that anti-peptide antibodies retain some ability to bind to the parent protein [130, 131].

As a novel approach to raising neutralizing antibodies against HIV-1, I synthesized the peptide epitopes of 4E10 and 2F5 and attached them to a series of lipids. I designed immunogens such that the lipid moieties have sufficient flexibility and length to access the hydrophobic CDR3 regions observed in the crystal structures of 4E10 and 2F5 [107]. These lipids, selected based on their proximity to the virus-cell interface and their affinity for monoclonal antibodies, included phosphatidylcholine, sphingomyelin, phosphatidylglycerol, phosphatidylethanolamine, cholesterol, and others.

The lipopeptide conjugates were inserted into synthetic phospholipid bilayer vaccine carriers called liposomes, which provide the lipid bilayer context that may be necessary for raising lipid-reactive antibodies. By varying the lipid anchor of the peptide epitope, as well as the lipid composition of the liposomal carrier, I sought to determine what lipid components are most effective for eliciting potent and potentially neutralizing antibodies. Liposomes have been safe and efficacious as humoral vaccine carriers in a variety of systems [132-134]. They are efficiently internalized by antigen presenting cells in subcutaneous sites and also accumulate in lymphatic drainage sites [135, 136]. Moreover, liposomes adjuvanted with monophosphoryl lipid A (MPL) elicit anti-lipid antibodies in small animals [113, 137]. For these reasons, I hypothesized that liposomal vaccines containing membrane-anchored, lipid-conjugated MPR peptide segments would elicit antibodies that cross-react with both peptide epitopes and membrane lipids. I further hypothesized that cross-reactive antibodies would neutralize HIV strains *in vitro*.

If successful, this approach would constitute a significant step toward the implementation of an effective vaccine against HIV.

Additionally, I tested the hypothesis that antibody responses to the MPR can be increased through derivatization of amino acid side chains with phosphate or nitrate groups. Similar modifications, which occur as a result of inflammation or aberrant post-translational modification, lead to the generation of “neoepitopes” with altered immunity in cancer and autoimmunity [119]. Similar inflammatory environments occur during HIV infection, and if neutralizing antibodies are generated as a result of these modifications it would explain the rarity of such antibodies. Finally, I sought to examine the utility of MPR lipopeptides as tools for probing immune responses to the MPR and for evaluating novel adjuvants and delivery systems for enhancing anti-MPR antibody responses.

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Chapter 2

Role of lipid structure in the humoral immune response in mice to covalent lipid-peptides from the membrane proximal region of HIV-1 gp41

2.1. Abstract

The membrane proximal region (MPR) of HIV-1 gp41 is a desirable target for development of a vaccine that elicits neutralizing antibodies since the patient-derived monoclonal antibodies, 2F5 and 4E10, bind to MPR and neutralize primary HIV isolates. The 2F5 and 4E10 antibodies cross-react with lipids and structural studies suggest that MPR immunogens may be presented in a membrane environment. We hypothesized that covalent attachment of lipid anchors would enhance the humoral immune response to MPR-derived peptides presented in liposomal bilayers. In a comparison of eight different lipids conjugated to an extended 2F5 epitope peptide, a sterol, cholesterol hemisuccinate (CHEMS), was found to promote the strongest anti-peptide titers (5.3×10^4) in BALB/C mice. Two lipid anchors, palmitic acid and phosphatidylcholine, failed to elicit a detectable anti-peptide response. No single factor, such as position of the lipid anchor, peptide helical content, lipopeptide partition coefficient, or presence of phosphate on the anchor determined the ability of a lipopeptide to elicit anti-peptide antibodies. Conjugation to CHEMS also rendered a 4E10 epitope peptide immunogenic (5.6×10^2 titer). Finally, attachment of CHEMS to a peptide spanning both the 2F5 and 4E10 epitopes elicited antibodies that bound to each of the individual epitopes as well as to recombinant gp140, which consists of gp120 plus the ectodomain of gp41. Further research into the mechanism of how structure influences the immune response to the MPR may lead to immunogens that could be useful in prime-boost regimens for focusing the immune response in an HIV vaccine.

2.2 Introduction

Despite extensive research, attempts to elicit broadly neutralizing antibodies (bnAb) to HIV have not yet succeeded. Vaccines targeting the envelope glycoprotein must cope with sequence variation, extensive glycosylation and rapid conformational changes that expose target epitopes before a robust and effective vaccine can be available [4]. Rational design of immunogens to overcome these defenses has been guided by a small number of bnAb isolated from HIV-infected patients. Three of these bnAb (2F5, 4E10 and Z13) target the membrane proximal region (MPR) of gp41, a segment comprised of approximately 35 amino acids N terminal to the transmembrane domain (**Figure 2-1**). The MPR is a desirable vaccine target because it is well conserved across viral clades and is essential for virus-cell fusion [6, 7]. However, efforts to date have not succeeded in eliciting antibodies with the breadth or potency of patient-derived bnAb [8].

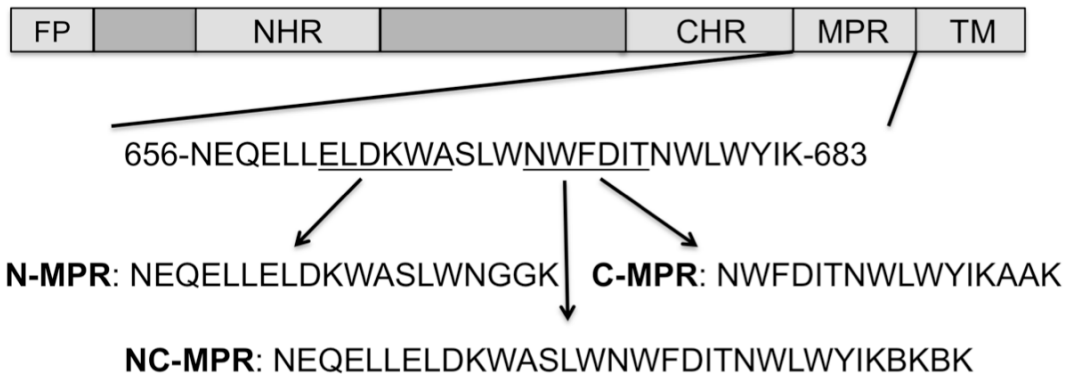


Figure 2-1. MPR peptides from the ectodomain of gp41.
N-MPR and C-MPR contained the nominal epitopes of monoclonal antibodies 2F5 and 4E10, respectively, with additional flanking sequences previously reported to improve binding [2, 3]. The C terminus is amended with a two residue linker and a lysine for on-resin lipid conjugation. NC-MPR contained both the 2F5 and the 4E10 epitopes with helix-promoting constraints at the C terminus [5]. 'B' indicates aminoisobutyric acid.

A lack of consensus regarding secondary structure has impeded MPR immunogen design. NMR, crystallography, and biophysical studies suggest that the MPR is an α-helix [9, 10]. Crystal structures of 4E10 bound to its peptide epitope corroborate this helical conformation; however, structures of 2F5 bound to its peptide epitope show the peptide adopting an extended conformation with a β-turn [2, 3]. Regardless, attempts to present structurally constrained epitopes, either conjugated to carrier proteins or grafted on recombinant constructs, have not elicited neutralizing antibodies [11-13]. Strategies to graft the antibody interacting surface onto a generic scaffold represent a promising approach, but neutralizing titers have not been published as of yet [14]. In addition to a lack of consensus regarding the epitope structure, the relatively weak immunogenicity of the MPR may result in immune responses to recombinant envelope immunogens directed toward immunodominant regions, such as the gp120 variable loops, or toward determinants on gp41 that mask the MPR from antibody recognition [15, 16]. Law and coworkers grafted a helical 4E10 epitope peptide into the highly immunogenic V3 loop of gp120 but the construct did not elicit 4E10-specific antibodies [12]. Moreover, although MPR antibodies have been associated with neutralizing activity of patient sera in some cohort studies, they are relatively uncommon, further supporting the assertion that poor immunogenicity may be a barrier to the success of MPR-targeted vaccines [17]. Thus, the structure and immunogenicity of the MPR remain poorly defined, hampering vaccine efforts.

The lipid reactivities of bnAb 2F5 and 4E10 have been a topic of intense study. Both antibodies have unusually long, hydrophobic CDRH3 regions and cross-react with phospholipids and other autoantigens [18-21]. Moreover, biophysical models suggest that the MPR intercalates into the membrane in native virions [22]. These observations have led to suggestions that MPR immunogens may be presented optimally in a lipid

bilayer environment. The majority of strategies to insert the epitopes in a lipid environment have involved chimeric viruses or liposomal formulations of recombinant constructs with transmembrane peptide domains [2, 23-25]. Additionally, variations in lipid membrane composition appear to alter MPR peptide accessibility [26], and modulation of the peptide anchoring mechanism may exert similar effects.

We hypothesized that covalent attachment of lipid anchors would enhance the humoral immune response to MPR-derived peptides presented in liposomal bilayers. Three peptides were selected, corresponding to the 2F5 epitope (N-MPR), the 4E10 epitope (C-MPR) and a helically constrained peptide spanning both epitopes (NC-MPR; summarized in **Figure 2-1**). We systematically examined the effects of the lipid anchors on the humoral response in mice immunized with the lipopeptides in liposomes. Of the lipid anchors tested, cholesterol hemisuccinate (CHEMS) attached to the C terminus of the peptide induced the highest titer antibodies against the 2F5 epitope and also elicited antibodies against the 4E10 epitope. These CHEMS-peptide conjugates may be useful in prime-boost regimens for focusing the immune response or as analytical tools for probing the immunogenicity of the MPR.

2.3 Materials and Methods

2.3.1 Materials

Amino acid building blocks, resins and coupling agents were obtained from Novabiochem (Darmstadt, Germany), Anaspec (San Jose, CA) or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), oxidized phosphatidylcholine (PC; #870601), brain sphingomyelin (SM; #860082) and tetramyristoylcardiolipin (CL; #710332) were obtained from Avanti Polar Lipids (Alabaster, AL). Dipalmitoylphosphatidylethanolamine

(PE; #LP-R4-019) and dipalmitoylglycerol (DPG; #LP-R4-028) were obtained from Genzyme Pharmaceuticals (Cambridge, MA). Palmitic acid (PA; #P5585) and 5-cholenic acid-3β-ol (CHOL; #C2650) were obtained from Sigma-Aldrich (St. Louis, MO). Anhydrous solvents of 99.8% or greater purity were obtained from Acros Organics (Geel, Belgium). Monophosphoryl lipid A derived from Escherichia coli (MPL; #L6638) was obtained from Sigma-Aldrich. 2F5 and 4E10 monoclonal antibodies were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Hermann Katinger. Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich.

2.3.2 Lipopeptide synthesis

Peptides were synthesized on NovaPEG resin in an automated solid phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA) with standard fluorenylmethyloxycarbonyl/o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate/n-hydroxybenzotriazole (FMOC/HBTU/HOBT) protocols. When appropriate, an orthogonally protected lysine (Fmoc-Lys(1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl)-OH; Fmoc-Lys(ivDde)-OH) was incorporated at the C terminus for on-resin conjugation of lipids or biotin. The N terminus was generally Boc-protected unless the peptide was intended for N terminal modification, in which case Fmoc protection was utilized. Removal of the ivDde group was accomplished by 3 x 15 minute treatments of the peptidyl resin with 2% hydrazine hydrate in dimethylformamide (DMF; 10 mL per g resin). The resin was washed in DMF (3 x 10 mL) and dichloromethane (DCM; 3 x 10 mL) and dried under vacuum.

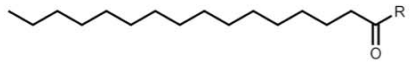
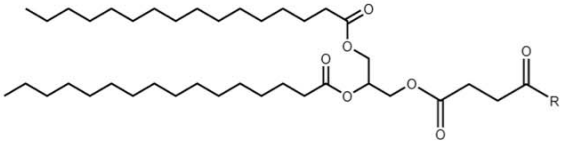
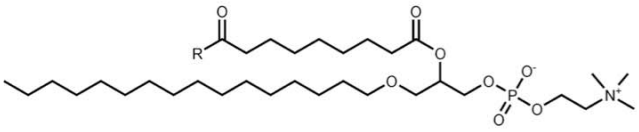
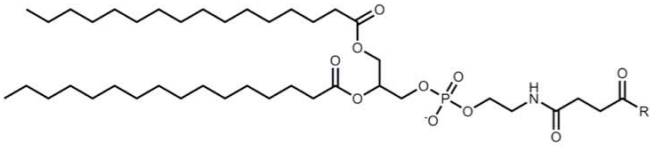
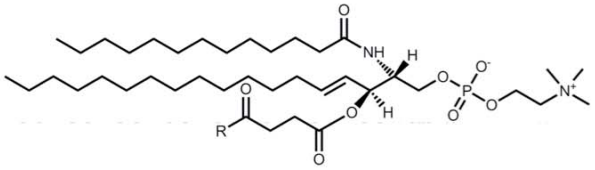
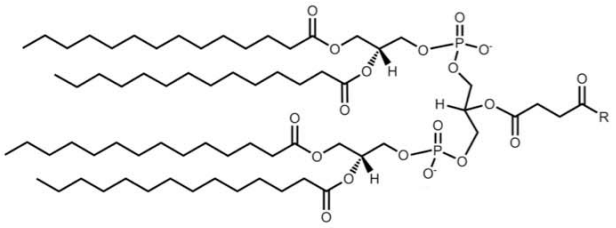
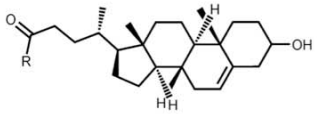
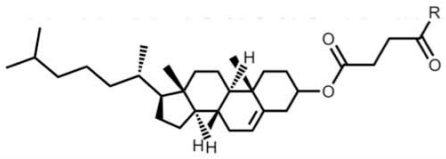
Lipid structure	Nomenclature
	PA
	DPG
	PC
	PE
	SM
	CL
	CHOL
	CHEMS

Table 2-1. Structures and nomenclature of lipids.
Abbreviations: PA – palmitic acid; DPG – dipalmitoylglycerol; PC – phosphatidylcholine; PE – phosphatidylethanolamine; SM – sphingomyelin; CL – cardiolipin; CHOL – cholenic acid; CHEMS – cholesteryl hemisuccinate.

Nomenclature and structures of lipids used in this study are summarized in **Table 2-1**. Lipid conjugation was accomplished via amidation of a carboxylated lipid and a deprotected lysine ε-amine at the C terminus. For N terminal conjugation, lipids were attached directly to the deprotected N terminus. Several of the lipids contained carboxyl groups. In the case of DPG, PE, SM, and CL, a carboxyl group was introduced via reaction of an available alcohol (DPG, SM, CL) or amine (PE) with succinic anhydride. For DPG-Suc, 1.8 mmol DPG was dissolved in 5 mL anhydrous DCM and combined with 3.6 mmol succinic anhydride in 10 mL anhydrous pyridine. The mixture was refluxed at 60 ^{o}C overnight. For PE-Suc, 1.5 mmol PE was combined with 3 mmol succinic anhydride and 6 mmol triethylamine in 50 mL anhydrous chloroform ($CHCl_3$). The mixture was stirred at room temperature overnight. For CL-Suc, 80 μmol CL was combined with 400 μmol succinic anhydride and 400 μmol triethylamine in 5 mL anhydrous $CHCl_3$. The mixture was refluxed at 60 ^{o}C overnight. For SM-Suc, 136 μmol SM was combined with 684 μmol succinic anhydride and 684 μmol triethylamine in 5 mL anhydrous $CHCl_3$. The mixture was refluxed at 60 ^{o}C overnight. Reactions were continued to completion as monitored by thin layer chromatography (TLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS; Voyager DE, Applied Biosystems, Foster City, CA) in para-nitroaniline matrix. Products were washed twice with 1M hydrochloric acid (HCl), dried over sodium sulfate and stored dry until use. Carboxylated lipids were obtained in approximately 90-100% yield. Molecular weights and TLC R_F values were as follows: DPG-Suc, 668.19 Da, R_F 0.71 in 20:1 DCM:acetone; PE-Suc, 790.02 Da, R_F 0.81 in 65:25:4 $CHCl_3$:MeOH:NH_4OH; CL-Suc, 1335.90 Da, R_F 0.24 in 65:25:4 $CHCl_3$:MeOH:NH_4OH; SM-Suc, 767.83 Da, 826.62 Da, 853.02 Da, 910.78 Da, R_F 0.66-0.79 in 65:25:4 $CHCl_3$:MeOH:NH_4OH. SM-Suc gave a

series of peaks because the starting material was a natural product with a distribution of aliphatic chain lengths.

Lipidation was accomplished by activation of 270 μmol carboxylated lipid with 270 μmol each of HBTU, HOBT and diisopropylethylamine (DIEA) in anhydrous DMF/DCM (DCM as needed for lipid solubilization) for 30 min at room temperature followed by addition of 67.5 μmol resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with DMF (4 x 10 mL) and DCM (4 x 10 mL) to remove unreacted lipids and dried under vacuum. Peptides were cleaved from the resin by treatment with trifluoroacetic acid containing 2.5% water, 2.5% ethanedithiol and 1% triisopropylsilane for 4 hours under argon. Cleaved peptides were precipitated into cold ethyl ether. The precipitate was pelleted by centrifugation at 3000 rpm (RT6000, Sorvall, Waltham, MA) and washed once with cold ethyl ether. The ether was poured off and the pellet was re-dissolved in methanol (MeOH), transferred to a round bottom flask, dried by rotary evaporation under reduced pressure and further dried under high vacuum. Lipopeptides were further separated from unconjugated peptide by reverse phase high pressure liquid chromatography (RP-HPLC; DX 500, Dionex, Sunnyvale, CA) on a semi-preparative C4 column (214TP510, Grace Vydac, Deerfield, IL) until unconjugated peptide was no longer detectable by MALDI-MS. Lipopeptide fractions were identified by MALDI-MS in 2,5-dihydroxybenzoic acid matrix, pooled and lyophilized. Stock lipopeptide solutions were prepared in MeOH or MeOH/$CHCl_3$ and stored at -20 ^{o}C. Final yields were approximately 5-10%.

Biotinylated peptides were prepared for use in ELISA by an analogous method. Biotin was attached to the deprotected C terminal amine by activation of 500 μmol D-biotin with

500 μmol HBTU/HOBT/DIEA in 1.65 mL anhydrous 1:1 DMF/dimethylsulfoxide (DMSO) for 30 min followed by addition of resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with 1:1 DMF/DMSO (3 x 10 mL), DMF (3 x 10 mL) and DCM (3 x 10 mL) and dried under vacuum. Biotinylated peptides were cleaved and purified as described above. Biotin content was quantified by 4´-hydroxyazobenzene-2-carboxylic acid dye exclusion (Sigma #H2153) according to the manufacturer's instructions.

2.3.3 Liposome preparation

Lipopeptides were formulated in liposomes composed of 15:2:3:0.3 DMPC:DMPG:Cholesterol:MPL [27]. Prior to use, glassware was rinsed with MeOH and $CHCl_3$ and dried for at least 90 min at 150 ^{o}C to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. Films were further dried under high vacuum overnight. Lipids were hydrated in sterile PBS (UCSF Cell Culture Facility) by intermittent vortexing and bath sonication under argon for a brief period (approximately 15 seconds) to disperse the lipids into the buffer. Defined diameter vesicles were formed by extrusion 11 times through 400 nm polycarbonate membranes using a hand-held extruder (Avestin, Ottowa, Canada) [28]. To prevent contamination, the extruder was disassembled and thoroughly cleaned with MeOH and sterile PBS between samples. The final formulation contained 1 mg/mL lipopeptide and 0.5 mg/mL monophosphoryl lipid A in 20 mM carrier lipid. Vesicle size was characterized by dynamic light scattering (Zetasizer 3000, Malvern, New Bedford, MA). Liposomes were stored at 4 ^{o}C under argon until use.

2.3.4 Circular dichroism

Liposomal lipopeptide samples were prepared as described above with the following modifications. Stock liposome solutions containing 5 mM carrier lipid and 500 μM lipopeptide were prepared in 10 mM phosphate, pH 7.4. To minimize light scattering, liposomes were prepared by bath sonication under argon until a size of less than 100 nm was obtained. For analysis, samples were diluted to 5 μM lipopeptide in 10 mM phosphate buffer containing 1 mM carrier lipid. Spectra were obtained with a J-715 spectrapolarimeter (Jasco, Easton, MD) and data were processed using Jasco software. Data were acquired in continuous scanning mode with a pathlength of 1 cm, 0.1 nm interval and scan speed of 1 nm/s. Each spectrum represents an average of two scans. A background spectrum of "empty" liposomes in buffer was subtracted from each sample spectrum. Percent helicity was estimated from θ_{222} according to the method of Taylor and Kaiser [1].

2.3.5 Tryptophan fluorescence

Lipopeptide membrane partitioning was characterized by measurement of tryptophan fluorescence intensity as described with modifications [29]. Briefly, DMPC:DMPG:Cholesterol liposomes were prepared in phosphate-buffered saline as described above. Lipopeptide stock solutions were prepared in MeOH. 12 nmol lipopeptide was injected via glass syringe (Hamilton, Reno, NV) into 1.2 mL buffer containing diluted liposomes (10-150 μM lipid). The samples were mixed by inversion and allowed to equilibrate in the dark at room temperature overnight. Fluorescence emission spectra were obtained on a SPEX Fluorolog spectrophotometer (Horiba Jobin Yvon, Edison, NJ) with 1 cm pathlength, 2.5 mm excitation slit, 5.0 mm emission slit, 1 s integration time and 1 nm interval. For each liposome concentration, a background

spectrum of “empty” liposomes in buffer was subtracted from the sample spectrum. Fluorescence intensity was determined by integration of the tryptophan fluorescence peak and data were normalized to the highest intensity in each sample series. Partition coefficients were calculated from the double reciprocal plot of normalized fluorescence intensity versus lipid concentration, according to the equation $F = (F_0*L*K_p)/(55.6 + K_p*L)$ [30].

2.3.6 Animal immunizations

All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. 8 week-old female BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF specific pathogen-free barrier facility. Animals received subcutaneous immunizations in alternating hind hocks on Days 0 and 14 as described [31]. Each injection contained 50 μg lipopeptide, 25 μg MPL and 1 μmol lipid vehicle in 50 μL sterile phosphate-buffered saline. On Day 28 blood was collected from the submandibular vein for characterization of antibody responses. Cells were removed by centrifugation at 14,000 rpm for 15 min (5415C, Eppendorf, Westbury, NY) and sera were stored at -80 ^{o}C until use.

2.3.7 ELISA

ELISAs were developed to quantify binding of immune sera to peptides, lipids, and recombinant gp140. Peptide ELISAs were conducted using MPR peptides biotinylated as described above and captured on 96 well streptavidin-coated plates (#15120, Pierce, Rockford, IL). Assays were performed according to the manufacturer’s instructions with modifications. Biotinylated peptides were added to wells in PBS containing 0.1% Tween-20 (PBS-T) and incubated for 2 hr at 37 ^{o}C. Following a wash step, sera were serially diluted in PBS containing 0.1% casein (C7078, Sigma-Aldrich) (PBS-C), added to wells

and incubated for 30 min at 37 ^{o}C. After reconstitution, horseradish peroxidase-conjugated secondary antibodies (IgG, IgG1, IgG2a; Jackson Immunoresearch, West Grove, PA) were diluted 1:1 in glycerol for long-term storage at -20 ^{o}C and further diluted 1:1000 in PBS-C immediately prior to use. Following a wash step, secondary antibodies were added to wells and incubated for 30 min at 37 ^{o}C. Following a final wash step, a tetramethylbenzidine substrate solution (#T0440, Sigma-Aldrich) was added to wells and incubated for 30 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 and the yellow product was monitored at 450 nm (Optimax, Molecular Devices, Sunnyvale, CA). All incubations were done in 100 μL volumes and wells were washed 6 times with PBS-T between each step. Titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 preimmune sera after subtraction of background wells lacking serum. IgG1/IgG2a ratios were calculated as an average of optical density quotients measured at 3 dilutions after subtraction of background values. All samples were assayed in duplicate.

Lipid ELISAs were performed as described with modifications [32]. Lipids were diluted to 0.2 mg/mL in EtOH and 50 μL per well were added to flat-bottomed untreated polystyrene plates (Fisher) and allowed to dry overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were diluted 1:200 in 10% fetal bovine serum in PBS and incubated in wells for 1 hr. Wells were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 1 hr. Following a wash step, plates were then read as indicated above. All incubations were done in 100 μL volumes at room temperature and wells were washed 6 times with PBS between each step.

Recombinant gp140 ELISAs were performed follows: Ba-1 gp140 (Immune Technology Corp, New York, NY) was diluted to 5 μg/mL in 50 mM sodium carbonate, pH 9.6 and 100 μL per well were added to flat-bottomed high capacity immunoassay plates (Costar). Plates were sealed with parafilm and incubated at 4 ^{o}C overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were diluted 1:50 in PBS-C and incubated in wells for 1 hr. Wells were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 30 min. Following a wash step, plates were then developed and read as indicated above. All incubations were done in 100 μL volumes at 37 ^{o}C and wells were washed 6 times with PBS-T between each step.

2.3.8 Statistical analysis

Statistical significance was assessed by analysis of variance and two-tailed Student's t test. Differences were considered significant if they exhibited p values < 0.05 in the Student's t test. Data analyses were performed using Microsoft Excel and SigmaPlot.

2.4 Results

2.4.1 Preparation of lipopeptides and liposomes

This study sought to address the role of lipid structure in the humoral immune response to MPR lipopeptides formulated in liposomes. Three peptides were selected for lipid modification, corresponding to the 2F5 epitope (N-MPR), the 4E10 epitope (C-MPR) and an extended peptide spanning both epitopes (NC-MPR; summarized in **Figure 2-1**). The sequences of N-MPR and C-MPR included flanking residues that were found to maximize binding affinities for their respective antibodies *in vitro* [2, 33]. Two helix-promoting isobutyric acid residues were incorporated into NC-MPR, as previously implemented in the design of a helically constrained 4E10 epitope peptide [5]. The N

terminus of NC-MPR was extended to include the full 2F5 epitope. An orthogonally protected lysine was included for lipid conjugation at the C terminus to mimic the native structure, in which the C terminus is anchored to the membrane.

Lipid anchors were selected to represent several basic lipid types: fatty acids, diacylglycerols, phospholipids and sterols (**Table 2-1**). Additionally, some are implicated in cross-reactivity with 4E10 and 2F5 (cardiolipin) or in virus-cell fusion (virion lipid phosphatidylethanolamine; raft lipids sphingomyelin and cholesterol) [19, 34, 35]. Consideration was also given to lipid anchors that may facilitate elicitation of antibodies binding to both peptide and lipid moieties. Specifically, lipids lacking a phosphate (palmitic acid and diacylglycerol) were selected for comparison to phosphate-containing lipids because the phosphate and head group moieties are important in recognition by anti-phospholipid antibodies [36]. Cholenic acid (CHOL) was chosen in addition to

Name	MW (exp)	MW (obs)
N-MPR-PA	2467.9	2468.0
N-MPR-DPG	2880.0	2903.7 (Na^+)
N-MPR-PC	2863.4	2865.1
N-MPR-PE	3003.5	3002.7
N-MPR-SM	2979.2, 3038.0, 3064.4*	3049.3, 3146.0, 3186.33*
N-MPR-CL	3587.2	3587.2
N-MPR-CHOL	2586.1	2588.2
N-MPR-CHEMS	2698.3	2718.2 (Na^+)
C-MPR-PA	2306.8	2326.3 (Na^+)
C-MPR-DPG	2749.4	2748.0
C-MPR-PC	2702.3	2703.4
C-MPR-CHOL	2425.0	2427.5
C-MPR-CHEMS	2537.1	2539.6
NC-MPR-DPG	4859.4	4861.7
NC-MPR-CHEMS	4677.6	4696.8 (Na^+)
N-MPR-biotin	2455.6	2455.0
C-MPR-biotin	2294.7	2296.8
NC-MPR-biotin	4435.2	4433.3

Table 2-2. Molecular weights of lipid- and biotin-modified peptides determined by MALDI.

cholesterol hemisuccinate (CHEMS) due to work indicating that the 3β-hydroxyl is a primary moiety responsible for recognition of cholesterol by anti-cholesterol antibodies [37, 38]. Additional lipids, such as galatosyl ceramide, which may serve as a receptor for MPR binding, would be of interest in future studies but were not included here [39].

For those lipids lacking a carboxyl group, one was introduced by reaction with succinic anhydride (TLC and MW data in **Methods**). For peptide modification, the on-resin lipidation strategy allowed complete removal of unreacted lipid via extensive washing of the resin prior to cleavage. The remaining contaminant, unreacted peptide, was removed by RP-HPLC. Molecular weights of lipid- and biotin-modified peptides are reported in **Table 2-2**. Modified peptides were obtained in approximately 5-10% yield; steric

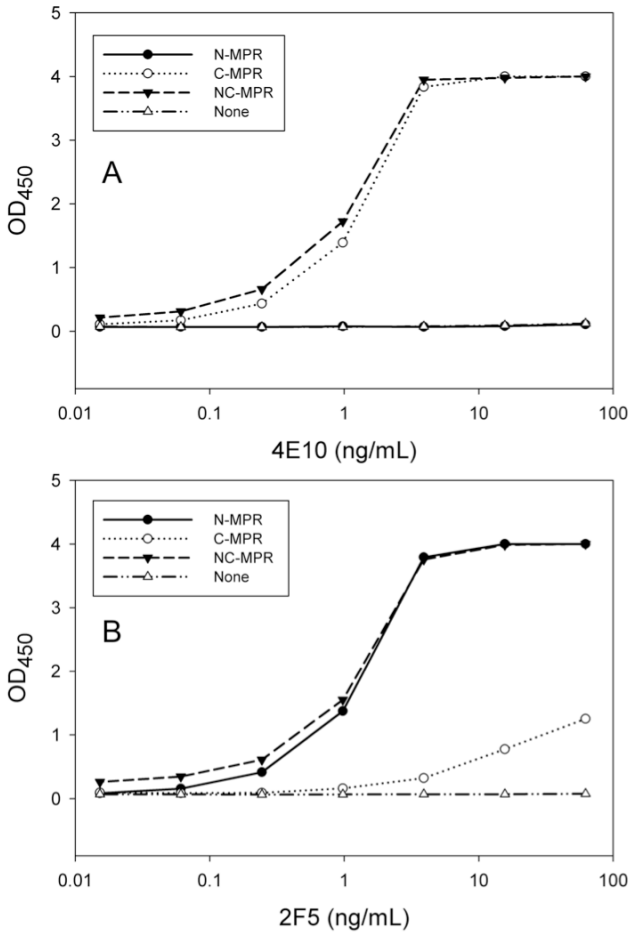


Figure 2-2. Binding of human monoclonal antibodies 2F5 and 4E10 to MPR peptides attached to the surface of ELISA plates.

Panel A:
4E10 mAb bound to biotinylated peptides containing the 'NFWDIT' epitope (C-MPR and NC-MPR) but not a peptide containing only the 'ELDKWA' epitope (N-MPR). Data representing 4E10 binding to 'N-MPR' and 'None' overlap in the figure.

Panel B:
2F5 mAb bound to biotinylated peptides containing the 'ELDKWA' epitope, with very weak binding to the 'NWFDIT' peptide. Data are representative of two independent experiments.

hinderance in modification of the C terminal lysyl ε-amine and loss upon RP-HPLC purification may have contributed to the relatively poor yield. Human monoclonal antibodies 2F5 and 4E10 bound strongly to biotinylated MPR peptides containing their epitopes (N-MPR and C-MPR, respectively) by ELISA (**Figure 2-2**). The cause for weak binding of 2F5 to C-MPR is uncertain but may be attributed to partial overlap in the peptide sequences (**Figure 2-1**). Regardless, sera of mice immunized with N-MPR lipopeptides did not bind to C-MPR by ELISA and vice versa (data not shown). Liposomal formulation of MPR lipopeptides resulted in vesicles approximately 175-250 nm in diameter (**Table 2-3**). Addition of peptide or lipopeptide did not appreciably affect vesicle size, with the exception of N-MPR-PE liposomes, which were slightly smaller than the others.

Formulation	Vesicle Diameter (nm)	Standard Deviation (nm)
Empty	214.9	2.2
N-MPR	185.6	3.7
N-MPR-PA	175.5	9.1
N-MPR-DPG	218.5	4.4
N-MPR-PC	212.6	17.1
N-MPR-PE	118.5	9.6
N-MPR-SM	193.7	2.5
N-MPR-CL	241.1	6.5
N-MPR-CHOL	153.2	8.9
N-MPR-CHEMS	192.9	6.8
C-MPR-PA	213.4	1.3
C-MPR-DPG	216.6	2.1
C-MPR-PC	246.2	1.8
C-MPR-CHOL	211.7	0.2
C-MPR-CHEMS	220.7	9.0
NC-MPR-DPG	235.7	1.1
NC-MPR-CHEMS	249.5	5.7
CHEMS-NC-MPR	288.7	13.5
CHEMS-NC-MPR-CHEMS	225.9	9.6

Table 2-3. Vesicle sizes of lipopeptide liposomes determined by dynamic light scattering.

2.4.2 The attached lipid moiety alters N-MPR lipopeptide structure and behavior in membrane vesicles

When formulated in liposomes, N-MPR secondary structure was greatly altered by the attached lipid moiety (**Figure 2-3a**). Whereas attachment of CHEMS to N-MPR resulted in a modest increase in helicity (26.5% versus 20.7%), attachment of DPG substantially

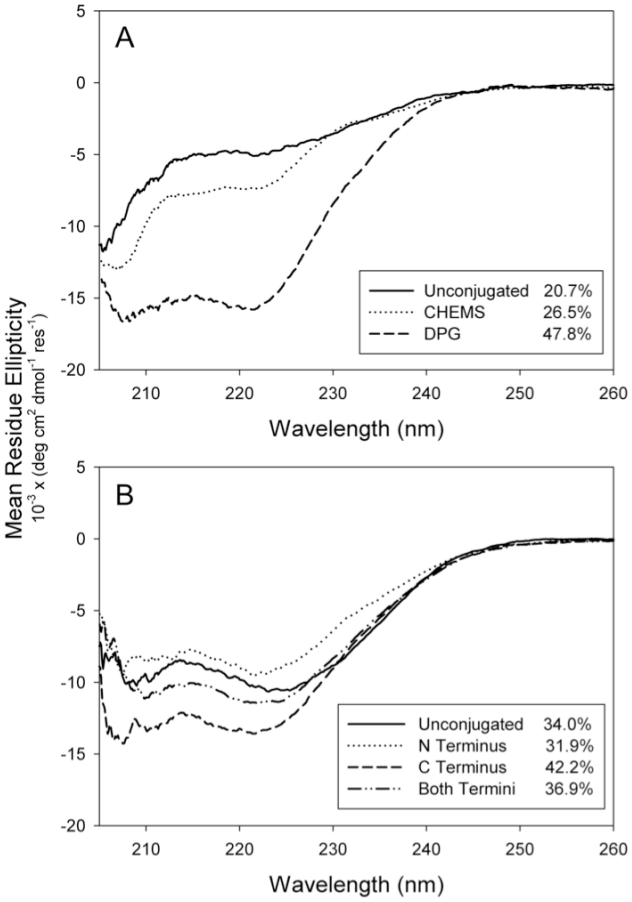


Figure 2-3. Effect of lipid and attachment site on the circular dichroism spectra of MPR lipopeptides.

Panel A:
Attachment of DPG to N-MPR promoted helix formation more strongly than did attachment of CHEMS to N-MPR.

Panel B:
NC-MPR-CHEMS conjugates exhibited helical character regardless of CHEMS attachment site. Data are normalized for concentration and peptide length. *Inset:* Percent helicity estimated by the method of Taylor and Kaiser [1]. Spectra represent averages of two scans.

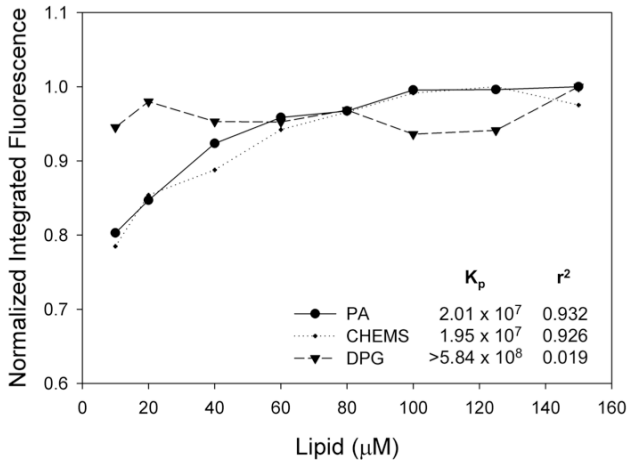


Figure 2-4. Partitioning of N-MPR lipopeptides into DMPC:DMPG:Cholesterol vesicles.

PA and CHEMS conjugates exhibited comparable partitioning, whereas DPG conjugates appeared maximally partitioned at the lowest lipid concentrations measured. K_p values for PA and CHEMS conjugates are calculated as described in Methods. The invariant fluorescence intensity of N-MPR-DPG yielded a poor curve fit and the calculated K_p was estimated as a lower bound of the true value. Data are representative of two independent experiments.

increased helical content (47.8% versus 20.7%). In contrast, attachment of CHEMS to NC-MPR only modestly affected its already helical conformation (**Figure 2-3b**). For NC-MPR, the data suggest a trend in which C terminal attachment promotes helicity (8% and 5% respective increases when comparing 'C Terminus' versus 'Unconjugated' and 'Both Termini' versus 'N Terminus'), whereas N terminal attachment decreases helicity (2% and 5% respective decreases when comparing 'N Terminus versus 'Unconjugated' and 'Both Termini' versus 'C Terminus'). The NC-MPR spectra are in agreement with those reported for 4E10 epitope peptides with nearly identical C terminal helix restraints [5]. By comparison, the lower overall helicity of NC-MPR may be attributed to the contribution of the extended N terminal segment not present in the peptide synthesized by Cardoso and coworkers [5].

Tryptophan fluorescence experiments revealed that the attached lipid moiety also affects partitioning of N-MPR into lipid bilayers (**Figure 2-4**). Both PA and CHEMS conjugates exhibited incremental differences in tryptophan fluorescence as a function of liposome concentration. This indicates that as the concentration of liposomes is increased, additional lipopeptides partition into the membrane. However, tryptophan fluorescence of N-MPR-DPG was unaffected by increasing lipid concentration over the range measured. The K_p of N-MPR-DPG was estimated to be at least an order of magnitude greater than that of N-MPR-PA or N-MPR-CHEMS (5.84×10^8 versus 2.01×10^7 and 1.95×10^7, respectively). This observation suggests that N-MPR-DPG partitions more strongly into bilayer membranes than the other conjugates. Alternatively, the possibility that DPG promotes self-aggregation cannot be excluded. As hydrophobic bilayer environments are known to promote helicity of peptides [40], the increased helicity of N-MPR-DPG (**Figure 2-3a**) relative to N-MPR-CHEMS may correspond to increased membrane partitioning.

Taken together, these data indicate that the attached lipid alters both the peptide's secondary structure and its behavior in bilayer vesicles.

2.4.3 CHEMS conjugation elicits the greatest anti-peptide immune response to N-MPR and induces an anti-peptide response to C-MPR in BALB/C mice

N-MPR lipid conjugates exhibited considerable differences in their ability to induce anti-

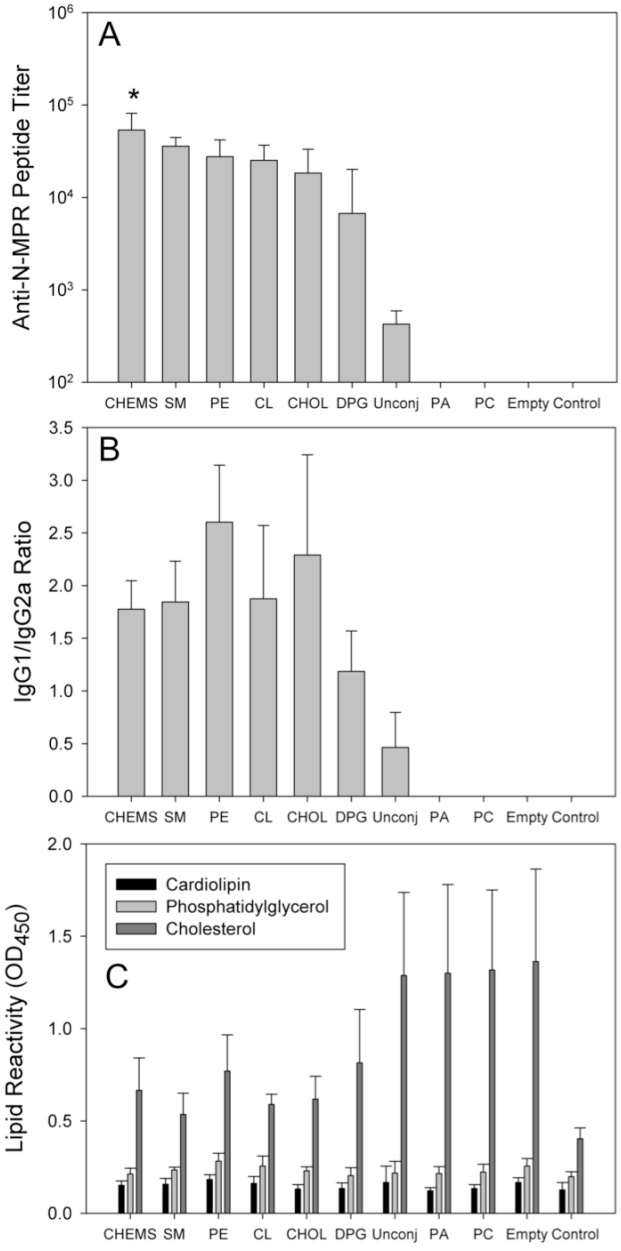


Figure 2-5. Anti-peptide total IgG titer, IgG1/IgG2a ratio and lipid reactivity of sera of mice immunized with N-MPR lipopeptides.

Panel A:
N-MPR lipopeptides elicited anti-peptide total IgG titers in the range of 10^4 to 10^5. Antibodies were detected in 2 of 5 mice that received liposomes containing peptide not conjugated to lipid ('Unconj'). In all other responding groups, antibodies were detected in every mouse. Antibodies were not detected in mice receiving PC or PA conjugates. 'Empty' denotes animals receiving liposomes lacking a peptide immunogen. 'Control' animals received no injection. Titers are expressed as geometric means.

Panel B:
IgG responses exhibited a balanced IgG1/IgG2a ratio with a slight preponderance of IgG1.

Panel C:
Immune sera contained detectable antibodies to cholesterol but not cardiolipin or phosphatidylglycerol. Anti-cholesterol antibodies were negatively correlated with N-MPR peptide titers (Spearman rank order correlation R = -0.853, p = 0.0000002). Each group consisted of at least n = 5 animals. Error bars represent standard deviations. * p = 0.033 vs CHOL, 0.001 vs DPG, 0.039 vs Unconjugated.

peptide antibodies when administered to BALB/C mice (**Figure 2-5**). Sterols and lipids containing two or more acyl chains generally elicited anti-peptide titers in the range of 10^4 to 10^5. These lipopeptides elicited balanced IgG1/IgG2a responses, suggesting a balanced T helper response, with a slight preponderance of IgG1 [41]. Anti-peptide IgA responses were not detected in serum (data not shown). Unconjugated peptide formulated in liposomes induced a greater anti-peptide response (detected in 2 of 5 mice) than either palmitic acid or PC conjugates, both of which failed to elicit a detectable response. N-MPR-PC, in which the peptide was attached to the distal end of an acyl chain, may have functioned more as a single chain due to the distribution of polar groups (peptide and head group) throughout the molecule. Conjugation to CHEMS, but not DPG or CHOL, also elicited a weak response against the C-MPR peptide (**Figure 2-6**).

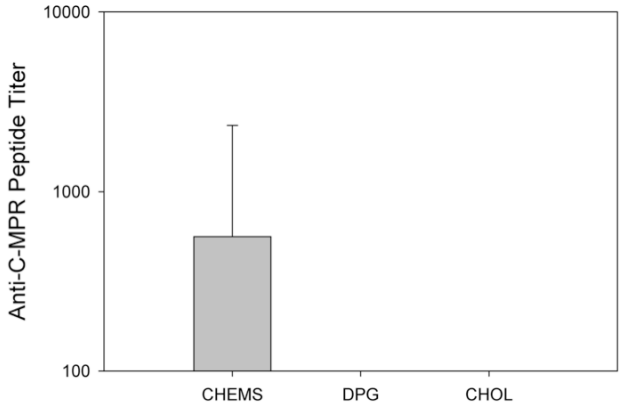


Figure 2-6. Humoral immune response to C-MPR lipopeptides.
Conjugation of C-MPR to CHEMS, but not DPG or CHOL, elicited anti-peptide antibodies in 4 of 5 animals. Each group consisted of n = 5 animals. Error bar represents the standard deviation.

Lipid reactivity of murine antisera was assayed because cross-reactivity of 2F5 and 4E10 with anionic phospholipids is thought to be important in their ability to neutralize HIV [42]. The lipopeptide formulations did not elicit antibodies against either cardiolipin or phosphatidylglycerol but did evoke a weak response against cholesterol, which was negatively correlated with anti-peptide titers (Spearman rank order correlation R = -0.853, p=0.0000002). No difference in anti-cholesterol antibodies was detected between sera of mice that received the CHOL

lipopeptide, in which the 3β-hydroxyl is available, and the CHEMS lipopeptide, in which the 3β-hydroxyl is masked. Cholesterol antibodies were likely generated by the unmodified cholesterol in the carrier formulation in addition to the lipopeptide itself. These assays were repeated with Tris-buffered saline to address concerns that the presence of soluble phosphate in the assay buffer may have inhibited anti-phospholipid antibody binding [43]. However, phospholipid reactivity was also not detected in these assays (data not shown).

2.4.4 Alteration of the CHEMS attachment site modulates the anti-peptide humoral response to the N terminus but not the C terminus of NC-MPR

To further probe the utility of CHEMS conjugation for promoting the immunogenicity of

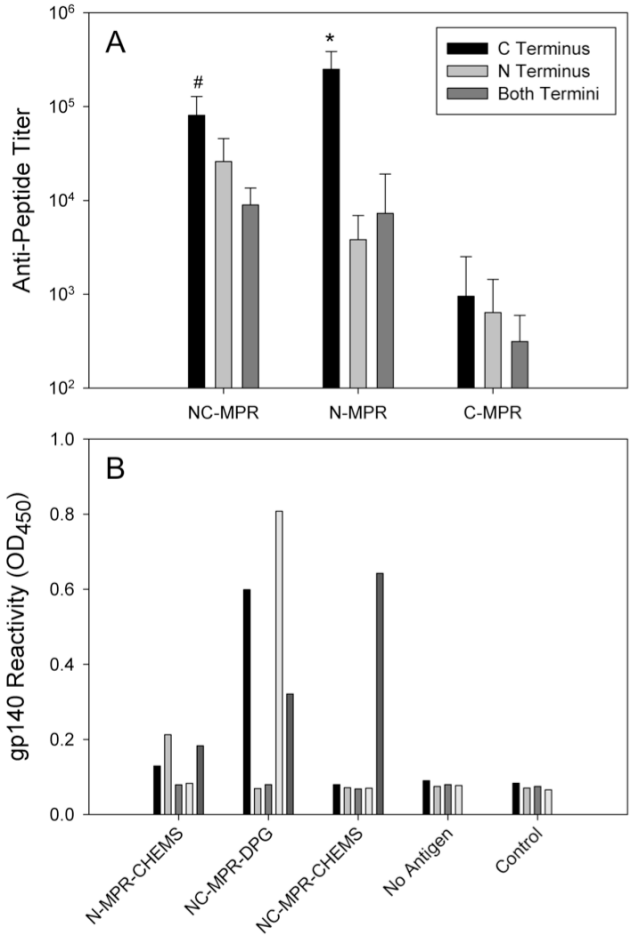


Figure 2-7. Anti-peptide titers of sera from mice immunized with NC-MPR-CHEMS lipopeptides with CHEMS conjugated at the N terminus, C terminus, or both termini assayed against N-MPR, C-MPR or NC-MPR peptides.

Panel A:
CHEMS attachment site exerted a dramatic effect on anti-peptide responses to NC-MPR lipopeptides. Attachment to the N terminus dramatically reduced antibodies directed against the N terminal peptide (N-MPR), whereas attachment site did not significantly alter levels of antibody directed against the C terminal peptide (C-MPR). Each group consisted of n = 5 animals. Error bars represent standard deviations.

Panel B:
Sera of individual mice immunized with NC-MPR lipopeptides bound weakly to recombinant gp140. Each bar represents an individual animal. * p = 0.003 vs N Terminus, 0.004 vs Both Termini. # p = 0.044 vs N Terminus, 0.010 vs Both Termini.

the MPR, lipopeptides were synthesized in which CHEMS was attached to the C terminus, the N terminus, or both (**Figure 2-7a**). All three molecules elicited antibodies that bound to the individual 2F5 and 4E10 epitopes (represented by N-MPR and C-MPR). Notably, the NC-MPR-CHEMS C terminal conjugate elicited a stronger response to N-MPR than to itself. The other two conjugates elicited significantly lower antibodies to N-MPR ($p < 0.004$), suggesting that attachment of CHEMS to the N terminus diminished the antibody response to the N terminal segment of the peptide. However, conjugation to the C terminus exerted no detectable effect on the antibody response to the C terminal segment. None of the conjugates elicited detectable antibodies to cardiolipin or phosphatidylglycerol (data not shown).

Finally, we sought to determine if these conjugates could elicit antibodies that bind to recombinant gp140 (**Figure 2-7b**). The gp140 construct used (Clade B, Strain Ba-1) differed from the MPR consensus sequence by only one residue (N677E). In control experiments, bnAb 2F5 and bnAb 4E10 bound strongly to this gp140 at 1 μg/mL (data not shown). Several immune sera bound weakly to gp140, but only at a very low dilution (1:50), suggesting that the majority of antibodies recognize structures other than that of the native protein. Although NC-MPR-DPG elicited greater reactivity to gp140 than NC-MPR-CHEMS (3/5 responders versus 1/5 responders), the reactivity is low and it is unclear if this difference is meaningful. Since the sequence of interest is positioned at the end of the C terminus of the recombinant construct, there was concern that adsorption on the ELISA plate may alter the structure and interfere with binding. However, binding was not stronger when the recombinant construct was attached to hexahistidine-binding plates via a hexahistidine tag (data not shown).

2.5 Discussion

The discovery of broadly neutralizing monoclonal antibodies reactive with the MPR region of gp41 from patient-derived cells raised the hope for an HIV vaccine against the epitopes recognized by these antibodies [4, 8, 13, 42]. Numerous studies of MPR-specific neutralizing antibodies suggest that presentation of MPR immunogens in a membrane environment could facilitate elicitation of neutralizing responses [8]. However, recombinant viruses and MPR-transmembrane fusion constructs in lipid vesicles have not elicited high titer neutralizing antibodies [2, 23-25, 44-47].

We hypothesized that covalent attachment of lipid anchors to MPR segments would improve upon these approaches by increasing anti-peptide antibody titers, altering epitope structure within the membrane, or eliciting neutralizing antibodies. We compared sterols, fatty acids and phospholipids for promoting humoral responses to covalently attached antigens. The key finding of this study is that the structure of the lipid anchor exerts significant influence on the anti-peptide titer. Unexpectedly, cholesterol hemisuccinate (CHEMS) promoted the greatest antibody response to an attached peptide, although the differences in immunogenicity were relatively small amongst the more potent anchors (**Figure 2-5**). CHEMS elicited significantly greater anti-peptide responses than cholenic acid (CHOL), a similar molecule (geometric mean titers of 5.3×10^4 and 1.8×10^4, respectively; $p = 0.033$). Conjugation of CHEMS to the C terminus of the MPR promoted significantly greater anti-peptide responses than did conjugation of CHEMS to the N terminus ($p < 0.05$). The two lipid-anchored NC-MPR peptides tested also elicited antibodies that bound weakly to gp140 by ELISA.

No single factor, such as position of the lipid anchor, peptide helical content, lipopeptide partition coefficient, or presence of phosphate on the anchor determined the ability of a

lipopeptide to elicit anti-peptide antibodies. However, the N terminal portion of the MPR (containing the 2F5 epitope) was considerably more immunogenic in BALB/C mice than the C terminal segment (containing the 4E10 epitope). For unstructured peptides, lipid conjugation may be used to manipulate secondary structure of peptides within membranes. Thus, these lipids augment the toolbox available to HIV-1 vaccine researchers for probing MPR immunogenicity and designing MPR-targeted vaccines.

Our strategy is analogous to that reported by Giannecchini and colleagues, in which octadecanoic acid was attached to the C terminus of MPR of feline immunodeficiency virus [48]. However, this immunogen elicited only weak anti-peptide antibodies (ELISA OD < 1.0 at 1:100 serum dilution) in cats. Thus, there is a need for immunogens that not only target the appropriate antigenic structure, but also elicit high titer antibodies. Coutant and coworkers also recently derivatized an MPR peptide with phosphatidylethanolamine to probe its physiological structure within membranes [49], but did not report antibody titers. Our findings suggest that lipid-anchored MPR peptides are highly immunogenic in mice; the titers are an order of magnitude higher than those reported by Lenz and colleagues in BALB/C mice immunized with liposome-anchored trimeric gp41 [23].

The use of liposomes containing monophosphoryl lipid A (MPL) for induction of antibody and cytotoxic T lymphocyte responses against liposome-associated peptides and proteins has been pioneered by Alving and colleagues [27, 36, 50-53]. Adjuvant mechanisms attributed to liposomes containing MPL include enhanced uptake, processing and presentation by antigen presenting cells [52, 54], prolonged persistence at the injection site [55] and activation of innate immunity through ligation of Toll-like receptor 4 [56]. Incorporation of MPL into liposomes also reduces reactogenicity while

maintaining adjuvant activity [52, 57]. Moreover, several studies have demonstrated that covalent attachment of peptides to liposomes enhances humoral immune responses to liposome-associated peptides and proteins [27, 53, 58-60]. As compared to non-covalent encapsulation, White and colleagues demonstrated increased antibody responses to a peptide derived from the V3 loop of gp120 when the peptide was acylated at the N terminus prior to liposome formulation or attached via a reversible disulfide bond to liposomes containing a thiolated cholesterol derivative [27]. Liposomes adjuvanted with MPL have also been used to elicit anti-lipid antibodies of diverse specificities [36]. A murine monoclonal antibody to phosphatidylinositol phosphate with no known HIV-1 binding specificity has also been shown to neutralize primary isolates, suggesting that membrane binding alone may be sufficient for neutralization [61].

The failure to elicit anti-phospholipid antibodies in the present study is at odds with a recent report in which immunization of BALB/C mice with a liposome-associated peptide adjuvanted by MPL elicited dual specificity, low titer (O.D. ~1.0 at 1:100 serum dilution) antibodies that recognized both peptide and lipid determinants [51, 62]. In these studies the MPR sequence was modified with a universal T helper epitope from tetanus toxin but did not contain a covalent lipid. As induction of anti-lipid antibodies by liposomes is affected by a number of factors, including formulation and injection route, modulation of these parameters in future studies may enable MPR lipopeptides presented here to elicit lipid cross-reactive antibodies [63, 64].

It is unclear to us why a sterol-anchored peptide would be more immunogenic than a peptide anchored by aliphatic chains. The mechanism does not appear to arise from induced changes in secondary structure; N-MPR-CHEMS, which differed little from free N-MPR peptide by circular dichroism, elicited nearly an order of magnitude higher

geometric mean titer (GMT) than N-MPR-DPG (5.3×10^4 and 6.7×10^3, respectively; **Figure 2-5**), which exhibited considerably greater helical content (26.5% and 47.8%, respectively; **Figure 2-3**). Membrane partitioning does not explain the disparity in anti-peptide titers either, as N-MPR-DPG partitioned much more strongly into liposomes than N-MPR-CHEMS ($K_p > 5.84 \times 10^8$ and $K_p = 1.95 \times 10^7$; **Figure 2-4**). Moreover, although N-MPR-CHEMS and N-MPR-PA exhibited very similar partitioning behavior, N-MPR-PA failed to elicit any detectable peptide antibodies (**Figure 2-5**). Thus, the adjuvant activity of CHEMS conjugates arises from some other mechanism. In the study of White and coworkers using the V3 loop of gp120, peptide exposure on the surface of liposomes was critical in elicitation of anti-V3 antibodies [27]. In light of this report, CHEMS conjugates may adopt a more highly exposed surface structure than CHOL, DPG, or other less immunogenic lipopeptides. However, efforts to quantitate liposome surface accessibility of lipid-modified MPR peptides are complicated by the ability of the 2F5 and 4E10 antibodies to intercalate into the membrane and "extract" their epitopes [22]. Alternatively, the lipid moiety may alter the processing of associated T helper epitopes or facilitate membrane transfer to cells that provide more efficient presentation to B lymphocytes [65, 66]. The lipid composition of the carrier vesicle may also be important, as several groups have shown that the MPR structure in membranes is modulated by membrane composition [26, 49]. Regardless, further studies with additional peptide antigens will be needed to determine if the findings presented here are broadly applicable or can only be applied to MPR antigens.

Several of the findings reported here may prove useful in studies of the MPR as a target for design of immunogens that elicit neutralizing antibodies. First, the data bolster the assertion that the immunogenicity of the MPR arises predominantly from the N terminal portion. This fact was borne out through immunization studies with peptides containing

only a single bnAb epitope (N-MPR and C-MPR) or both epitopes (NC-MPR). N-MPR-CHEMS elicited an anti-N-MPR GMT of 5.3 x 10^4 whereas C-MPR-CHEMS elicited anti-C-MPR titers of less than 6 x 10^2. Additionally, mice immunized with NC-MPR derivatized with CHEMS at the C terminus generated extremely high titers (GMT 2.5 x 10^5) against the N terminal region of the peptide but only low titers against the C terminal segment (GMT 9 x 10^2). The poor immunogenicity of the 4E10 epitope may arise from masking of the epitope within the membrane, as is predicted to occur in native envelope spikes [22]. However, other studies indicate that the peptide sequence itself is poorly immunogenic [12]. If this is due to autoantigen mimicry, more potent adjuvants may be needed to circumvent a peripheral tolerance barrier [42].

The lipopeptide immunogens described here may be useful in a prime-boost immunization regimen for focusing the immune response to the MPR, similar to those proposed by others [2, 8]. First, the immune system would be primed with highly immunogenic, membrane-bound peptides that induce antibody responses targeted to MPR peptides in the context of membrane, minimizing antibodies directed against other immunodominant, non-neutralizing envelope determinants. Second, the immune system would be boosted with a recombinant construct in which the MPR is constrained in the appropriate structural confirmation [14, 67]. Thus, only MPR-reactive antibodies of the appropriate confirmation would be boosted, minimizing antibodies directed against irrelevant MPR structures.

An important observation is that the α-helicity of unstructured MPR peptides can be modulated through alteration of the attached lipid moiety. Additionally, attachment of the lipid anchor to the C terminus produced a more potent immunogen than did attachment

of the anchor to the N terminus. Finally, the results indicate that cholesterol hemisuccinate is a simple but effective lipid anchor for creating lipopeptide immunogens.

2.6 Acknowledgements

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Chapter 3

Lipopeptide immunogens derived from the membrane proximal region of HIV-1 gp41: Antibody responses in mouse models of defective immune tolerance and enhancement of antibody responses to the 4E10 epitope through side chain phosphorylation

3.1 Abstract

Weak antibody responses to the membrane proximal region (MPR) of HIV-1 gp41 have hampered efforts to target the MPR for development of a vaccine that elicits neutralizing antibodies. In the present study, we sought to determine whether antibody responses to the MPR can be enhanced through improved immunogen design or if the lack of response is due to tolerance mechanisms. Our group recently reported the synthesis of MPR-derived lipopeptide immunogens that elicit high titers of anti-MPR antibodies in mice. Here, these conjugates are utilized to further probe the capability of the MPR to induce antibody responses. We hypothesized that the antibody response against the MPR, particularly the epitope of the broadly neutralizing monoclonal antibody 4E10, is restricted by immune tolerance. This hypothesis was tested through a comparative assessment of antibody responses to MPR lipopeptides in two mouse models of defective immune tolerance – (New Zealand Black x New Zealand White)F1 (NZBxW/F1) and non-obese diabetic (NOD). As compared to BALB/C, serum anti-MPR IgG responses were modestly increased in NZBxW/F1 but not in NOD mice. However, antibody responses to the 4E10 epitope remained weak (serum anti-peptide titers less than 10^3) relative to the rest of the sequence, suggesting that tolerance mechanisms are insufficient to explain the poor antibody responses to the 4E10 epitope in these mouse models.

We further hypothesized that anti-MPR antibody responses could be increased through derivatization of amino acid side chains with phosphate or nitrate groups. These modifications arise as a result of inflammation or cell dysregulation in cancer and autoimmunity, generating “neoepitopes” that induce altered immune responses to self. We have proposed that similar modifications may occur during HIV infection and may contribute to the heterogeneity of the anti-MPR antibody response. Evaluation of six MPR immunogens containing these modifications revealed one modification ($S668PO_3$) that increased IgG responses to the 4E10 epitope by an order of magnitude (GMTs of 7.3×10^3 and 6.8×10^2, $p = 0.011$). Taken together, these studies suggest that antibody responses to MPR immunogens may not be exclusively limited by tolerance and may be enhanced through immunogen design.

3.2 Introduction

An effective HIV vaccine will likely require both humoral and cellular responses to prevent infection [1]. While insights garnered from the failed Merck trial of a recombinant adenoviral vaccine have led to recent progress in T cell-mediated vaccines [2], efforts to elicit broadly neutralizing antibodies (bnAb) to HIV have been less successful. Thus far, a small number of bnAb isolated from HIV-infected patients have guided the rational design of envelope glycoprotein immunogens to overcome the robust evolutionary defenses of HIV [3]. Three of the most potent and broadly neutralizing bnAb (2F5, 4E10, and Z13) are directed to the membrane proximal region (MPR) of gp41, comprised of approximately 35 amino acids N terminal to the transmembrane domain (**Figure 3-1**) [4]. The MPR is well conserved across viral clades; it is also essential for virus-cell fusion and in some cases MPR escape mutants have exhibited decreased infectivity [5-7]. However, with the possible exception of one recent report [8], MPR immunogens have

not succeeded in eliciting antibodies with the breadth or potency of patient-derived bnAb [4].

The lack of structural definition of the MPR is a primary concern in development of immunogens that target this sequence [4]. However, weak antibody responses to the MPR have also limited immunogen design. MPR-specific antibodies are rare in infected patients and highly immunogenic scaffolds grafted with MPR sequences have failed to elicit detectable MPR reactivity in animals [9-11]. The cause of the poor antibody responses to the MPR remains unresolved.

Haynes and others have suggested that antibody responses to the MPR are inherently limited by tolerance mechanisms [12, 13]. This hypothesis is supported by data demonstrating that MPR-targeted bnAb 2F5 and 4E10 cross-react with phospholipids and other self antigens [14, 15]. Moreover, these antibodies exhibit a rare characteristic: they contain unusually long, hydrophobic heavy chain complementarity determining region 3 (CDRH3) sequences. In humans, antibodies with long CDRH3 segments are typically deleted in the bone marrow due to their autoreactive character, which could explain the rarity of 2F5-like and 4E10-like bnAb after infection or vaccination [16]. Alternatively, the rarity of MPR antibodies may be explained by immunodominance of the gp120 variable loops or other segments [17], the rapidity of conformational changes that expose the MPR for immune recognition [18], masking by non-neutralizing cluster II epitopes [19] or a bias in the germline antibody repertoire. The cause of weak antibody responses to the MPR is important because if antibody responses to this sequence are inherently limited by tolerance mechanisms, the MPR may not be a viable target for HIV vaccine design.

Given the lipid reactivity of anti-MPR bnAb 2F5 and 4E10, coupled with the putative importance of lipid membrane binding in neutralization of these bnAb, several groups have suggested that MPR immunogens may be presented in a membrane bilayer context, which might mimic the environment of gp41 in the viral envelope [4, 12, 20]. To this end, our laboratory recently reported the synthesis of MPR-derived lipopeptide immunogens for presentation of MPR peptides in lipid bilayer vesicles [21]. We observed that lipid conjugation could be used to modulate the biophysical properties of MPR lipopeptides and the antibody responses to their epitopes. The MPR lipopeptides provide a useful toolkit for structural and antigenic studies of the MPR. In the present report, these conjugates were utilized to further characterize antibody responses to the MPR in mice.

We tested the MPR tolerance hypothesis in mice through a comparative assessment of antibody responses to MPR lipopeptides in two models of defective immune tolerance – (New Zealand Black x New Zealand White)F1 (NZBxW/F1) and non-obese diabetic (NOD) [22, 23]. Important differences between species must be considered when conducting immunological studies in rodents and extrapolating the results to primates or men. In particular, mice have shorter CDRH3 regions and may be less likely to generate 2F5-like and 4E10-like bnAb in response to vaccination [24]. However, NZBxW/F1 and NOD mice have been shown to develop antibodies to phospholipids and other autoantigens in response to immunization, which led us to select these mice as model strains for tolerance studies [25, 26]. Our results indicate that antibody responses to the 4E10 epitope remained very weak relative to the rest of the sequence in these mouse strains, suggesting that tolerance mechanisms are insufficient to explain poor antibody responses to this epitope.

In search of an explanation for the generation of anti-MPR bnAb in infected patients, we postulated that the inflammatory milieu in an HIV infection may lead to post-translational modifications within the MPR that generate altered anti-MPR antibody responses. If anti-MPR bnAb are elicited in humans by MPR sequences containing extremely rare post-translational modifications, it would provide an explanation for the rarity of bnAb that target this sequence. Although these modifications have not been observed *in vivo*, several labile residues exist within the MPR sequence and similar modifications have been shown to promote antibody responses to weak antigens in cancer and autoimmunity [27, 28]. Thus, we hypothesized that anti-MPR antibody responses could be increased through derivatization of amino acid side chains with phosphate or nitrate groups that mimic these modifications. Evaluation of MPR immunogens containing phosphorylated or nitrosylated side chains revealed one modification ($S668PO_3$) that increased antibody responses to the 4E10 epitope by an order of magnitude (serum anti-peptide titers of 7.3×10^3 and 6.8×10^2, $p < 0.011$). Taken together, these data suggest that antibody responses to the MPR are not exclusively limited by immune tolerance and can be enhanced through immunogen design.

3.3 Materials and Methods

3.3.1 Materials

Amino acid building blocks, resins and coupling agents were obtained from Novabiochem (Darmstadt, Germany), Anaspec (San Jose, CA) or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS; #C6512) and monophosphoryl lipid A derived from Escherichia coli (MPL; #L6638) were obtained from Sigma-Aldrich (St. Louis, MO). Anhydrous solvents of 99.8% or greater purity were obtained from Acros

Organics (Geel, Belgium). Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich.

3.3.2 Lipopeptide synthesis

Peptides were synthesized on NovaPEG resin in an automated solid phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA) with standard fluorenylmethyloxycarbonyl/o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate/n-hydroxybenzotriazole (FMOC/HBTU/HOBT) protocols as described [21]. Phosphorylated residues were incorporated by addition of amino acid building blocks with side chains derivatized with O-benzyl-protected phosphate. Nitrosylated tyrosine was incorporated by addition of Fmoc-3-nitro-tyrosine-OH (Bachem, Torrence, CA). An orthogonally protected lysine (Fmoc-Lys(1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl)-OH; Fmoc-Lys(ivDde)-OH) was incorporated at the C terminus for on-resin conjugation of lipids or biotin. The N terminus was Boc-protected. Removal of the ivDde group was accomplished by 3 x 15 minute treatments of the peptidyl resin with 2% hydrazine hydrate in dimethylformamide (DMF; 10 mL per g resin). The resin was washed in DMF (3 x 10 mL) and dichloromethane (DCM; 3 x 10 mL) and dried under vacuum.

CHEMS conjugation was accomplished via amidation of the CHEMS carboxyl group and a deprotected lysine ε-amine at the C terminus. Essentially, 270 μmol CHEMS was activated with 270 μmol each of HBTU, HOBT and followed by addition of 67.5 μmol resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with DMF (4 x 10 mL) and DCM (4 x 10 mL) to remove unreacted lipid and dried under vacuum. Peptides were cleaved from the resin by treatment with trifluoroacetic acid containing 2.5% water, 2.5% ethanedithiol and 1%

triisopropylsilane for 4 hours under argon. Cleaved peptides were precipitated into cold ethyl ether. The precipitate was pelleted by centrifugation at 3000 rpm (RT6000, Sorvall, Waltham, MA) and washed once with cold ethyl ether. The ether was poured off and the pellet was re-dissolved in methanol (MeOH), transferred to a round bottom flask, dried by rotary evaporation under reduced pressure and further dried under high vacuum. Lipopeptides were further separated from unconjugated peptide by reverse phase high pressure liquid chromatography (RP-HPLC; DX 500, Dionex, Sunnyvale, CA) on a semi-preparative C4 column (214TP510, Grace Vydac, Deerfield, IL) until unconjugated peptide was no longer detectable by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS; Voyager DE, Applied Biosystems, Foster City, CA). Lipopeptide fractions were identified by MALDI-MS in 2,5-dihydroxybenzoic acid matrix, pooled and lyophilized. Stock lipopeptide solutions were prepared in MeOH or MeOH/$CHCl_3$ and stored at -20 ^{o}C. Final yields were approximately 5-10%. Nomenclature, sequences and molecular weights of lipopeptides used in this study are summarized in **Table 3-1**.

Biotinylated peptides were prepared for use in ELISA by an analogous method. Biotin was attached to the deprotected C terminal amine by activation of 500 μmol D-biotin with 500 μmol HBTU/HOBT/DIEA in 1.65 mL anhydrous 1:1 DMF/dimethylsulfoxide (DMSO) for 30 min followed by addition of resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with 1:1 DMF/DMSO (3 x 10 mL), DMF (3 x 10 mL) and DCM (3 x 10 mL) and dried under vacuum. Biotinylated peptides were cleaved and purified as described above. Biotin content was quantified by 4´-hydroxyazobenzene-2-carboxylic acid dye exclusion (Sigma #H2153) according to the manufacturer's instructions.

3.3.3 Liposome preparation

Lipopeptides were formulated in liposomes composed of 15:2:3:0.3 DMPC:DMPG:Cholesterol:MPL [29, 30]. Prior to use, glassware was rinsed with MeOH and $CHCl_3$ and dried for at least 90 min at 150 ^oC to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. Films were further dried under high vacuum overnight. Lipids were hydrated in sterile PBS (UCSF Cell Culture Facility) by intermittent vortexing and bath sonication under argon for a brief period (approximately 15 seconds) to disperse the lipids into the buffer. Defined diameter vesicles were formed by extrusion 11 times through 400 nm polycarbonate membranes using a hand-held extruder (Avestin, Ottowa, Canada). To prevent contamination, the extruder was disassembled and thoroughly cleaned with MeOH and sterile PBS between samples. The final formulation contained 1 mg/mL lipopeptide and 0.5 mg/mL monophosphoryl lipid A in 20 mM carrier lipid. Vesicle size was characterized by dynamic light scattering (Zetasizer 3000, Malvern, New Bedford, MA). Liposomes were stored at 4 ^oC under argon until use.

3.3.4 Animal immunizations

All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. 8 week-old female mice (BALB/C, NOD, NZBxW/F1; Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF specific pathogen-free barrier facility. Animals received subcutaneous immunizations on Days 0 and 14 in alternating hind hocks as described [31]. Each injection contained 50 μg lipopeptide, 25 μg MPL and 1 μmol lipid vehicle in 50 μL sterile phosphate-buffered saline. On Day 28 blood was collected from the submandibular vein for characterization of antibody responses. Cells were removed by centrifugation at

14,000 rpm for 15 min (5415C, Eppendorf, Westbury, NY) and sera were stored at -80 ^{o}C until use.

3.3.5 ELISA

ELISAs were developed to quantify binding of immune sera to peptides and lipids. Peptide ELISAs were conducted using MPR peptides, lacking any side chain modifications, biotinylated as described above and captured on 96 well streptavidin-coated plates (#15120, Pierce, Rockford, IL). Assays were performed according to the manufacturer's instructions with modifications. Biotinylated peptides were added to wells in PBS containing 0.1% Tween-20 (PBS-T) and incubated for 2 hr at 37 ^{o}C. Following a wash step, sera were serially diluted in PBS containing 0.1% casein (C7078, Sigma-Aldrich) (PBS-C), added to wells and incubated for 30 min at 37 ^{o}C. After reconstitution, horseradish peroxidase-conjugated secondary antibodies (IgG, IgG1, IgG2a; Jackson Immunoresearch, West Grove, PA) were diluted 1:1 in glycerol for long-term storage at -20 ^{o}C and further diluted 1:1000 in PBS-C immediately prior to use. Following a wash step, secondary antibodies were added to wells and incubated for 30 min at 37 ^{o}C. Following a final wash step, a tetramethylbenzidine substrate solution (#T0440, Sigma-Aldrich) was added to wells and incubated for 30 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 and the yellow product was monitored at 450 nm (Optimax, Molecular Devices, Sunnyvale, CA). All incubations were done in 100 μL volumes and wells were washed 6 times with PBS-T between each step. Titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 preimmune sera after subtraction of background wells lacking serum. All samples were assayed in duplicate.

Lipid ELISAs were performed as described with modifications [32]. Lipids were diluted to 0.2 mg/mL in EtOH and 50 μL per well were added to flat-bottomed untreated polystyrene plates (Fisher) and allowed to dry overnight. For liposome ELISAs, 33 nmol total lipids of pre-formed liposomes composed of 15:2:3:0.3 DMPC:DMPG:Chol:MPL in PBS were added to wells in 50 μL and allowed to dry overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were diluted 1:200 in 10% fetal bovine serum in PBS and incubated in wells for 1 hr. Wells were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 1 hr. Following a wash step, plates were then read as indicated above. All incubations were done in 100 μL volumes at room temperature and wells were washed 6 times with PBS between each step. Buffers for lipid ELISAs did not contain Tween 20.

3.3.6 Statistical analysis

Statistical significance was assessed by analysis of variance and two-tailed Student's t test. Differences were considered significant if they exhibited p values < 0.05 in the Student's t test. Data analyses were performed using Microsoft Excel and SigmaPlot.

3.4 Results

3.4.1 Preparation of lipopeptides and liposomes

This study sought to determine if antibody responses to MPR lipopeptides are restricted by tolerance in mice, and furthermore to determine if side chain modifications can be exploited to enhance anti-MPR responses. The peptides employed in this study correspond to the 2F5 epitope (N-MPR), the 4E10 epitope (C-MPR) and a larger peptide spanning both epitopes (NC-MPR; summarized in **Figure 3-1a**). For both antibody epitopes, side chain modifications were incorporated at each available labile residue (serine, threonine, tyrosine). Phosphorylation and nitrosylation were accomplished by

incorporation of pre-modified amino acid building blocks during synthesis. Threonine was not modified in the 4E10 epitope peptide (C-MPR) because it is essential for recognition by the cognate bnAb [33, 34], but may be considered in future studies for generation of anti-MPR antibodies with alternative binding specificities.

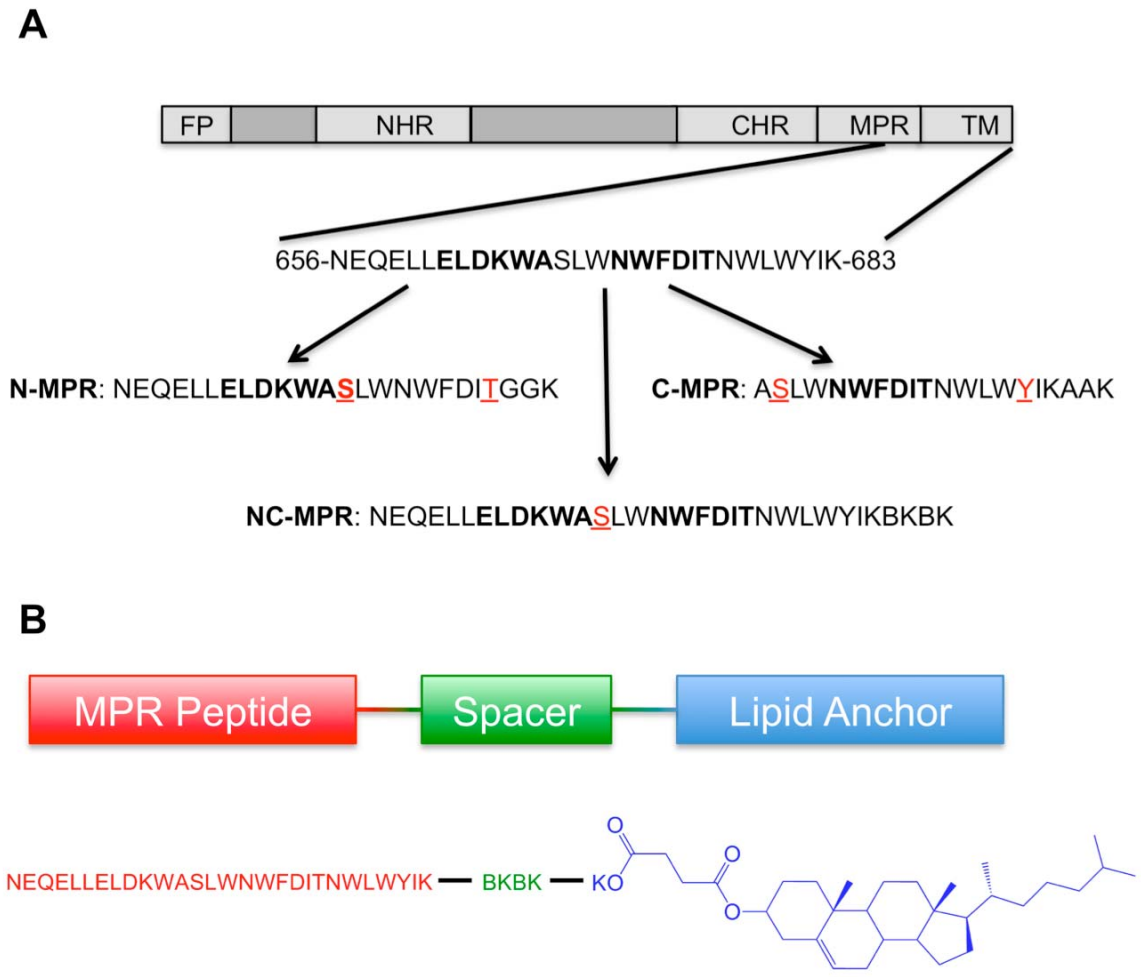

Figure 3-1. MPR peptides and MPR-CHEMS lipopeptides. N-MPR and C-MPR contained the nominal epitopes of monoclonal antibodies 2F5 and 4E10, respectively, with additional flanking sequences previously reported to improve binding [20, 33]. The C terminus is amended with a two residue linker and a lysine for on-resin lipid conjugation. NC-MPR contained both the 2F5 and the 4E10 epitopes with helix-promoting constraints at the C terminus [35]. 'B' indicates aminoisobutyric acid. Residues that were modified with phospho or nitro groups are indicated in red.

For immunization, these peptides were attached to a lipid, cholesteryl hemisuccinate, which was previously found to greatly enhance antibody response to liposomal MPR peptides in mice [21]. The lipid was attached to the peptidyl C terminus via an orthogonally protected lysine to recapitulate the native orientation of the MPR, in which the C terminus is adjacent to the viral membrane and the N terminus extends outward (**Figure 3-1b**). A summary of nomenclature and molecular weight data of the lipid-modified MPR peptides is reported in **Table 3-1**. Liposomal formulation of MPR lipopeptides resulted in vesicles approximately 175-250 nm in diameter and the addition of lipopeptide did not appreciably affect vesicle size (data not shown).

Name	Sequence	Linker	Modification	MW (exp)	MW (obs)
C-MPR	671-683	AA	None	2537.1	2539.6
C-MPR Ext	667-683	AA	None	2994.6	2993.9
C-MPR(S-PO_3)	667-683	AA	S-PO_3	3077.6	3075.2
C-MPR(Y-PO_3)	671-683	AA	Y-PO_3	2615.1	2617.0
C-MPR(Y-NO_2)	671-683	AA	Y-NO_2	2585.2	2583.2
N-MPR	656-671	GG	None	2698.3	2718.2 (Na^+)
N-MPR(S-PO_3)	656-671	GG	S-PO_3	2776.2	2776.7
N-MPR(T-PO_3)	656-677	GG	T-PO_3	3554.0	3556.3
NC-MPR	656-683	BKBK	None	4677.6	4696.8 (Na^+)
NC-MPR(S-PO_3)	656-683	BKBK	S-PO_3	4755.6	4758.9

Table 3-1. Nomenclature and molecular weights of lipopeptides.

3.4.2 Comparative immunogenicity of MPR lipopeptides in BALB/C, NOD and NZBxW/F1 mice

To assess the effects of immune tolerance on MPR immunogenicity in mice, antibody responses to MPR lipopeptides were compared in mouse strains of defective tolerance. Non-obese diabetic (NOD) mice exhibit multiple deficiencies in central and peripheral tolerance, including defective thymic selection and deficient regulatory T cell populations

[22]. The New Zealand Black x White F1 (NZBxW/F1) mouse, a model of spontaneous systemic lupus erythematosus, manifests thymic deficiencies in addition to B cell abnormalities that induce spontaneous polyclonal expansion and hyper-reactivity to antigenic stimuli [23, 36]. MPR lipopeptide formulations were administered at 8 and 10 weeks of age, before the spontaneous development of autoantibodies [25, 26], to avoid skewing the results through a pre-existing bias in the response. Indeed, mice exhibited little anti-lipid antibody reactivity at the conclusion of the study as measured by ELISA (**Figure 3-2**). As described in **Methods**, a combination of anti-lipid and anti-liposome ELISAs was employed to detect antibodies with potentially different binding characteristics: one to detect antibodies to individual lipids without any aggregate structure (anti-lipid), and one to detect antibodies to pre-formed lipid membranes (anti-liposome). Immunization with liposomal formulations elicited a measurable, but modest, anti-lipid antibody response.

Following immunization of mice with liposomal formulations containing NC-MPR-CHEMS lipopeptide, antibody responses in serum to the N terminal MPR segment (N-MPR), C terminal segment (C-MPR) and entire MPR (NC-MPR) were measured by ELISA. Responses to NC-MPR and N-MPR were modestly reduced in NOD mice as compared to BALB/C (**Figure 3-3a**). However, antibody responses to NC-MPR and N-MPR in NZBxW/F1 mice were increased by an order of magnitude over BALB/C (Anti-NC-MPR GMT: 7.5×10^5 vs. 7.8×10^4, $p = 0.0003$; Anti-N-MPR GMT: 1.1×10^6 vs. 2.3×10^5, $p = 0.0004$). Responses to C-MPR were also modestly increased in NZBxW/F1 as compared to BALB/C, although the difference was not significant (GMT 3.8×10^3 vs. 4.7×10^2; $p = 0.177$). In mice immunized with liposomes containing C-MPR-CHEMS, anti-C-MPR responses were weak and no differences were observed amongst groups (**Figure**

3-3b). Thus, the data indicated that antibody responses to the N terminal portion of the MPR were significantly increased in NZBxW/F1 mice as compared to BALB/C, but antibody responses to the C terminus were consistently weak. Notably, a prior study found that the attachment site of CHEMS to NC-MPR exerts a significant effect on the magnitude of antibody responses to the N terminus of the sequence [21]. Thus, alternative lipid conjugation sites may influence the outcome of these studies as well.

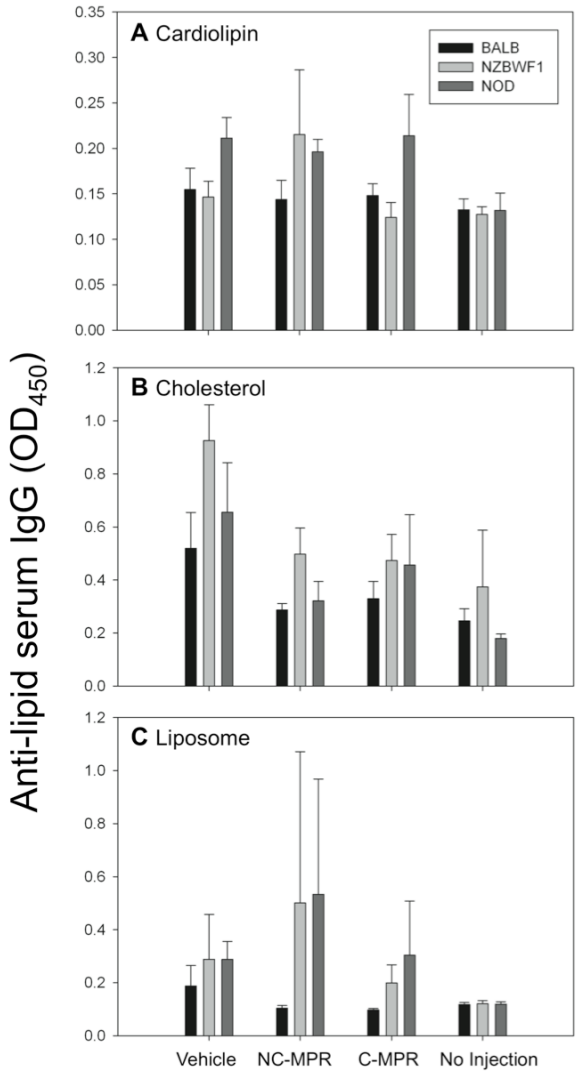

Figure 3-2. Lipid reactivity in sera of BALB/C, NOD and NZBxW/F1 mice immunized with NC-MPR-CHEMS. Anti-lipid antibody levels were not significantly increased by immunization with liposomal formulations containing monophosphoryl lipid A. The liposome capture antigen in *Panel C* was composed of 15:2:3:0.3 DMPC:DMPG:Chol:MPL. Sera were assayed at 1:200 dilution.

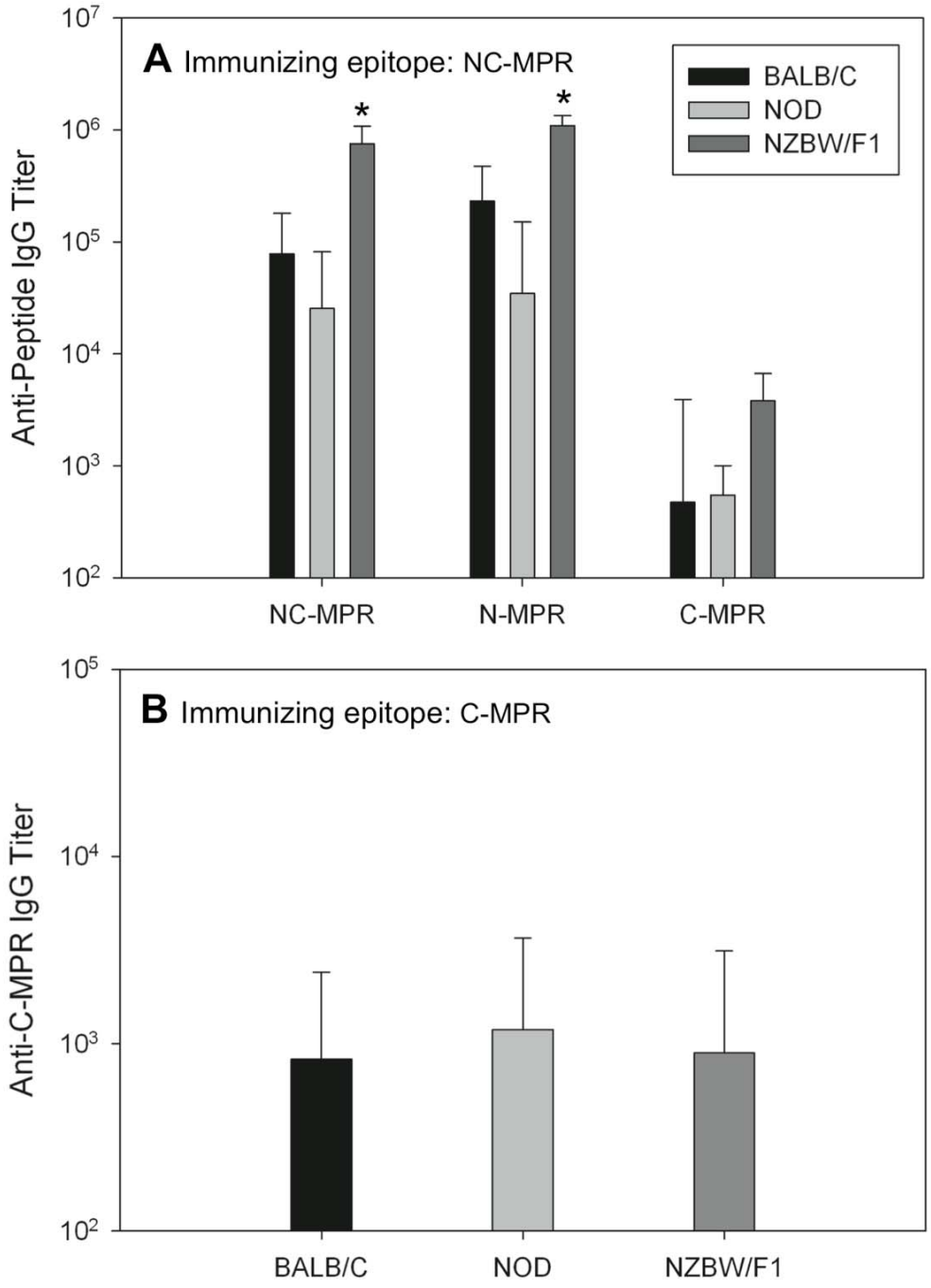

Figure 3-3. Anti-MPR IgG titers in sera of BALB/C, NOD and NZBxW/F1 mice immunized with NC-MPR-CHEMS and C-MPR-CHEMS. *Panel A:* Antibody responses to NC-MPR, N-MPR and C-MPR peptides elicited by immunization with NC-MPR-CHEMS. Anti-C-MPR antibodies were consistently weak, but anti-NC-MPR and anti-N-MPR titers were significantly enhanced in NZBxW/F1 mice. * $p < 0.0005$ *Panel B:* Antibody responses to C-MPR elicited by immunization with C-MPR-CHEMS. Anti-C-MPR antibodies were consistently weak, and no differences amongst groups were observed.

3.4.3 Effect of side chain modification on antibody responses to MPR lipopeptides in BALB/C mice

We sought to determine if antibody responses to the MPR could be modulated through specific modification of MPR amino acid side chains. Phosphorylation, nitrosylation and other modifications have been shown to enhance antibody responses to weak self antigens in autoimmunity and cancer [28], and we hypothesized that these modifications may exert similar effects with the MPR. Indeed, a comparison of modified C-MPR-CHEMS lipopeptides revealed multiple effects on antibody responses in mice (**Figure 3-4a**). Importantly, modification of Y681 with either a phosphate or a nitro group completely blunted the antibody response (0/5 responders to C-MPR(Y-PO_3); 1/5 responders to C-MPR(Y-NO_2). This indicates that Y681 may be important in immune recognition of the 4E10 epitope. Although an alanine scan did not identify this residue as important for binding of synthetic MPR peptides to bnAb 4E10 [34], the added negative charge density of a phosphate or nitrate group may alter immune recognition by altering MPR structure within the membrane.

Conversely, phosphorylation of S668 enhanced antibody responses to the original C-MPR peptide by an order of magnitude (GMT 7.3 x 10^3 vs. 6.8 x 10^2, p = 0.011). Since C-MPR(S-PO_3) was extended at the N terminus, an additional control lipopeptide with the identical amino acid sequence was synthesized (C-MPR Ext). Antibody responses to this control conjugate were modestly, but not significantly, enhanced as compared to the original C-MPR immunogen (GMT 2.7 x 10^3). Thus, the increased peptide length may contribute to the augmented antibody responses to C-MPR(S-PO_3) but is insufficient to completely explain it. By contrast, antibody responses to the N terminus of the MPR (N-MPR), which were already two orders of magnitude greater than those to the C terminus, were not significantly affected by phosphorylation of S668 or T686 (**Figure 3-4b**).

Phosphorylation of S668 did not significantly alter the antibody response to a full-length MPR lipopeptide either (NC-MPR(S-PO_3)-CHEMS; data not shown). Thus, the data collectively suggest that antibody responses to MPR segments that are poor inducers of antibody, particularly the C terminus, can be manipulated through chemical modification of amino acid side chains. If this phenomenon occurs *in vivo*, it may represent a source of heterogeneity in the antibody response to the MPR.

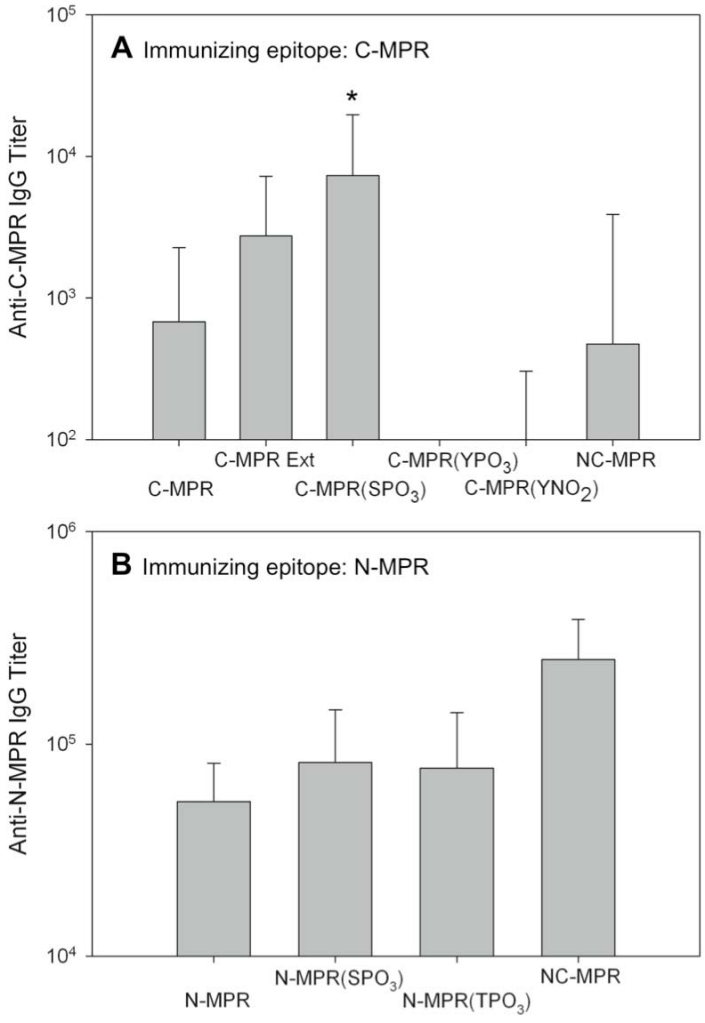

Figure 3-4. Anti-MPR IgG titers in BALB/C mice immunized with side chain-modified MPR lipopeptides. *Panel A:* Antibody responses to C-MPR elicited by immunization with modified C-MPR lipopeptides. S668 phosphorylation significantly increased the response ($p = 0.011$), while phosphorylation or nitrosylation of Y681 abrogated the response. *Panel B:* Antibody responses to N-MPR elicited by modified N-MPR lipopeptides. No significant differences were observed.

3.5 Discussion

The membrane proximal region of gp41 has been extensively investigated as a potential target for development of a vaccine that elicits neutralizing antibodies [4]. However, antibody responses to MPR immunogens, particularly the C terminus, have been generally weak and very few studies have reported elicitation of neutralizing antibodies with MPR immunogens [8, 37, 38]. Before the MPR can be fully exploited as a vaccine target, the cause of poor antibody responses to the MPR must be determined. Some investigators have hypothesized that antibody responses to the MPR are inherently limited by immune tolerance [12]. If this is correct, more potent adjuvants or alternate immunization schedules may be required to break tolerance. Otherwise, the MPR may not be a suitable target for vaccine design.

In the present study, we sought to characterize the contribution of immune tolerance in the antibody response to the MPR in mice. Liposomal MPR lipopeptides, previously developed in our laboratory [21], were used as model immunogens to present MPR sequences in a highly immunostimulatory membrane-bound conformation. Antibody responses to these constructs were compared in BALB/C mice and two mouse strains, NOD and NZBxW/F1, which exhibit defective tolerance and an autoimmune phenotype [22, 23]. In mice immunized with NC-MPR-CHEMS, the response to the N terminal region was significantly increased in NZBxW/F1 mice, but not NOD mice, as compared to BALB/C (**Figure 3-3**). While this may suggest a role for tolerance in shaping the antibody response to the N terminus of the MPR, the titer of the response was already quite high in BALB/C (GMT 10^5-10^6). Thus, the implications of this particular observation for vaccine design may not be significant.

In contrast, antibody responses to the C terminus remained weak in all mice immunized with either the full sequence (NC-MPR-CHEMS) or the C terminal sequence only (C-MPR-CHEMS). From these data, we conclude that tolerance mechanisms are not sufficient to explain the weak antibody responses to the C terminus of the MPR (the 4E10 epitope) in these strains of mice. An important caveat of this study is that antibody responses often vary amongst mouse strains, partly because of discrepancies in MHC restriction and thus T cell help. However, previous studies comparing antibody responses to exogenous protein antigens in BALB/C and NZBxW/F1 mice have not reported substantial differences [39-41]. Additionally, although it is possible that other mouse strains or other species will behave differently, the 4E10 epitope is also a weak immunogen in other species, including rabbits [10, 42], guinea pigs [43], and hens (unpublished observations).

Importantly, this study does not address additional critical features of the 4E10 and 2F5 bnAb, such as the unusually long, hydrophobic CDRH3 regions (4E10 = 18 residues, 2F5 = 22 residues) that may mediate lipid cross-reactivity and neutralizing capability [44]. Murine CDRH3 segments average only 8-10 residues in length, whereas human CDRH3 regions average 10-14 residues and can be longer than 20 residues [24]. Thus, mice may be less likely to generate 2F5-like and 4E10-like bnAb in response to vaccination, and it is possible that the improved MPR immunogenicity will not be sufficient to generate neutralizing responses in mice because autoreactive antibodies with hydrophobic CDRH3 regions will not be among the elicited anti-MPR repertoire. We selected NZBxW/F1 and NOD mice as models for study because they have been shown to develop antibodies to phospholipids and other autoantigens in response to immunization [25, 26]. However, the CDRH3 lengths of antibodies in these mice have not been extensively investigated, although anti-dsDNA autoantibodies from NZBxW/F1

mice were found to have longer CDRH3 regions as compared to the NZBxW/F1 antibody repertoire as a whole (10.5 residues vs. 8.5 residues) [45]. Studies in larger animals (e.g. rabbits, CDRH3 10-14 residues), humanized mice, or transgenic mice with fixed CDRH3 segments would be of interest to address this point.

Upon observing that the antibody response to the 4E10 epitope was not significantly increased in mice with defective immune tolerance, we pursued chemical modifications of MPR residue side chains as a strategy to enhance the antibody response. In autoimmunity and cancer, there are numerous reports of aberrant side chain modifications, resulting from inflammation or cell dysregulation, which generate 'neoepitopes' that elicit an enhanced immune response to the original sequence [28]. This phenomenon may occur in infectious disease as well [46]. Thus, we hypothesized that modification of labile residues with phosphate or nitro groups would alter the response in a similar manner. Selective modification of amino acid side chains in the C terminal sequence (4E10 epitope) resulted in enhancement ($S668PO_3$) or abrogation ($Y681PO_3$ or $Y681NO_2$) of the response (**Figure 3-4**). Modification of residues within the N terminal sequence (N-MPR; 2F5 epitope) or the full-length sequence (NC-MPR), which are more potent immunogens, did not significantly alter antibody responses.

In the context of this study, it appears that weak immunogens are more susceptible to alteration of antibody responses by side chain modification as compared to potent immunogens. It remains to be determined if this observation applies to other antigenic models, although it is consistent with the observed role of post-translational modifications in breaking tolerance against self antigens in autoimmunity and cancer [28]. Importantly, there are no published reports of post-translational MPR modifications in HIV infection, and available computational tools did not predict any sulfation or

phosphorylation sites within the sequence (ExPASy Sulfinator, www.ca.expasy.org/tools/sulfinator; NetPhos, www.cbs.dtu.uk/services/netphos). However, if these events do occur with extremely low frequency, they could contribute to the heterogeneity of anti-MPR responses. Proteomic tools would be helpful in parsing out a potential role of post-translational modifications in eliciting immunity to the MPR.

Altered immunity to post-translationally modified MPR lipopeptides may arise from changes in binding affinity of helper T cell epitopes within the MPR sequence to MHC molecules or T cell receptors, as has been shown in other systems [28, 47]. This explanation is consistent with our observation that post-translational modifications can have divergent effects on the antibody response: a gain of affinity can increase T cell help and thus enhance responses, whereas decreased affinity can diminish responses. Though no BALB/C helper T cell epitopes within the MPR have been defined, an epitope prediction algorithm identified two putative BALB/C MHC II epitopes (haplotype H-2^d, allele I-A^d) with binding IC50 less than 100 nM, one of which contained serine at anchor position P4 (KWASLWNWF, IC50 7.19 nM; WFDITNWLW, IC50 23.77 nM; www.jenner.ac.uk/MHCPred/). Additionally, the presence of IgG2a in serum following immunization with MPR lipopeptides suggests the involvement of helper T cell-dependent immunoglobulin class switching in the antibody response [48].

Post-translational modification could also modulate affinity for B cell receptors [49]. In this study, only antibody responses against the unmodified sequences were measured; however, analysis of antibody reactivity to post-translationally modified peptides would assist in determining the role of antibody binding specificity in the enhanced response to S668PO_3. For example, if mice immunized with C-MPR(SPO_3)-CHEMS generate antibodies specific for C-MPR(SPO_3) that also bind to C-MPR but more weakly, that

would indicate a significant contribution of S668PO_3 to the antibody binding specificity. Ouchterloney double gel diffusion assays and competition ELISAs may provide additional information in this respect [50].

An alternative mechanism for the effect of side chain modification on the antibody response to the MPR may involve alterations in peptide structure. This explanation is consistent with the divergent effects we have observed, since structural changes could either mask or expose critical residues for immune recognition. Moreover, phosphorylation has been shown to disrupt secondary structure of peptides, including α-helices [51]. Circular dichroism and solution NMR would be useful in clarifying any structural changes that occur as a result of post-translational modification of the MPR. Modified MPR peptide structure could also result in altered exposure of the epitope on the liposome surface; addition of a large negatively charged group such as a phosphate may prevent peptide insertion into the bilayer, thus exposing the sequence for immune recognition. Studies by Alving and colleagues have demonstrated that peptide exposure on the liposome surface is critical in determining the magnitude of the antibody response to liposomal peptides [29]. Tryptophan fluorescence experiments could determine whether post-translational modification of MPR lipopeptides alters membrane partitioning behavior.

A second alternative explanation could involve the existence of an innate immune sensor that stimulates or suppresses the antibody response upon detecting specific phosphorylation or nitration signatures. A recent study reported that genetic introduction of nitrophenylalanine is capable of breaking tolerance to self proteins, and an earlier report demonstrated that nitrosylation of IgG tyrosine residues can overcome tolerance to self IgG in mice [52, 53]. Modification in T cell epitope – MHC affinity was suggested

as an explanation for these effects, but the possibility of an as-of-yet unidentified innate sensor of modified proteins has not been excluded. However, it is unclear how such a sensor would discriminate between normal and abnormal post-translational modifications. Additionally, to be consistent with our data, an innate sensor may require the capability to both enhance (S668) and diminish (Y681) the response.

In summary, the key findings of these studies are two-fold. First, antibody responses to the 4E10 epitope of the MPR were poor in two mouse models of defective immune tolerance. This supports the conclusion that induction of MPR antibodies is limited by a mechanism other than immune tolerance in the models studied. Second, specific chemical modification of epitope amino acid side chains can modulate (enhance or abrogate) the antibody response to the 4E10 epitope. This suggests that a more extensive examination of side chain modifications could result in identification of more potent modifications. Thus, we believe these approaches may be integrated with structure-based designs to optimize the magnitude of the anti-MPR antibody response.

3.6 Acknowledgements

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expressed herein do not necessarily reflect the policies and views of DHS, DOE, or ORISE.

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Chapter 4

Liposomal nitrilotriacetic acid for delivery of polyhistidine-tagged peptide and protein antigens

*****Chapter 4 co-authored by Virginia M. Platt, see Acknowledgements for author contributions.

4.1 Abstract

Particulate delivery systems enhance antibody responses to protein subunit vaccines. In this report, we test the hypothesis that increased avidity of non-covalent antigen-particulate interactions will result in enhanced antibody responses to model antigens in mice. Formulations in which the antigen is physically associated with the particle have been shown to elicit higher antibody titers than admixed preparations; however, chemical and physical association methods can disrupt protein structure and damage vital epitopes. Metal chelation via nitrilotriacetic acid (NTA) is an alternative approach for non-destructive association of polyhistidine-tagged protein antigens to particulate delivery systems. Moreover, lipid-anchored NTA molecules may be useful for presentation of membrane protein immunogens within a lipid bilayer context. Recently, we and others reported the synthesis of multivalent NTA lipids with substantially higher affinity for polyhistidine than monovalent NTA (K_D 5-20 nM vs. 10 μM). The effect of the antigen-liposome linkage on antibody responses to two model antigens, ovalbumin (OVA) and a peptide derived from the membrane proximal region of HIV-1 gp41 (N-MPR), was determined in mice. Polyhistidinylated antigens were attached to liposomes via monovalent NTA, trivalent NTA or a covalent linkage and administered to BALB/C mice. When N-MPR-His_6 was attached to liposomes via an NTA linkage, anti-N-MPR IgG was detected in sera of 4 of 4 mice, whereas an unattached N-MPR-His_6 admixed with liposomes lacking NTA failed to generate a response (0 of 4 mice). However, a

trivalent NTA linkage did not confer an enhancement of antibody response to either N-MPR or OVA as compared to a monovalent NTA linkage. In both cases, covalently attached antigens elicited significantly stronger antibody responses than NTA-anchored antigens (OVA: Titer $3.4x10^6$ vs. $1.4\text{-}1.6x10^6$, $p < 0.001$; N-MPR: Titer $4.4x10^4$ vs. $5.5\text{-}7.6x10^2$, $p < 0.003$). The data indicate that as compared to admixed formulations, NTA linkages may be useful to increase antibody titers of weak antigens (e.g. N-MPR) but may not be suitable for delivery of larger, more potent antigens (e.g. OVA).

4.2 Introduction

Preventative vaccination plays a critical role in reducing the global impact of infectious diseases. In recent years, recombinant vaccines have been developed as alternatives to traditional vaccine modalities, such as killed or inactivated microorganisms [1]. Of these recombinant approaches, purified protein subunit vaccines are attractive because their composition can be precisely controlled and they offer superior safety profiles [2]. Currently available vaccines against hepatitis B virus and human papillomavirus are two examples of successful protein subunit vaccines [3, 4]. However, protein subunit preparations elicit weak antibody and T lymphocyte responses when administered without adjuvants [5].

Vaccine adjuvants that enhance humoral and cellular immunity to co-delivered antigens are generally parsed into two overlapping functional categories: molecular immunopotentiators and particulate delivery systems [6]. Molecular immunopotentiators, such as cytokines, co-stimulatory molecules and toll-like receptor ligands, activate discrete signaling pathways to promote activation or function of immune cells. Particulates, including emulsions, gels, liposomes, and microparticles, facilitate delivery to antigen presenting cells, provide prolonged antigen presentation through a “depot

effect," and in some cases generate pro-inflammatory "danger" signals. The potency of these systems generally requires that the subunit antigen be chemically or physically associated with the particulate [1]. Precipitation or adsorption onto aluminum salts is the traditional approach and alum remains the only vaccine adjuvant approved for use in the United States [7, 8]. Alternatively, proteins can be associated with lipidic or polymeric particulates via encapsulation or chemical conjugation [9-11]. However, these strategies present significant challenges – for example, encapsulation techniques can result in protein denaturation through exposure to harsh emulsification processes or organic solvents [12]. Covalent conjugation relies on chemical modification of the protein surface and can alter or destroy vital epitopes [13, 14]. Adsorption to solid particles, such as poly(lactide-co-glycolide) (PLG) microparticles, represents an improvement over these methods but does not allow precise control of antigen orientation and display [15, 16].

Non-covalent chemical attachment methodologies have been proposed to address these issues. One promising approach to non-covalent antigen conjugation involves metal chelation, in which polyhistidine-tagged proteins are attached to nitrilotriacetic acid-containing liposomes and microparticles with micromolar affinity [17, 18]. Since NTA-Ni(II)-His binding is site-specific, the physical orientation of the antigen on the particulate surface can be controlled. This is of particular importance for delivery of membrane protein antigens such as HIV-1 gp41 and other viral envelope glycoproteins, where presentation of key neutralizing determinants in their native orientation within a membrane context is desired [19]. A recent study reported the use of lipid-anchored NTA for attachment of polyhistidine-tagged HIV-1 Gag p24 antigen to wax nanoparticles [20]. These formulations elicited superior anti-p24 antibody and T lymphocyte responses as compared to p24 admixed with nanoparticles lacking Ni(II) or p24 adsorbed onto alum. However, the NTA-conjugated preparation was not compared to a formulation in which

the antigen was covalently attached to the nanoparticles. Additionally, concerns have been raised regarding the stability of the NTA-Ni(II)-His interaction in biological fluids [21].

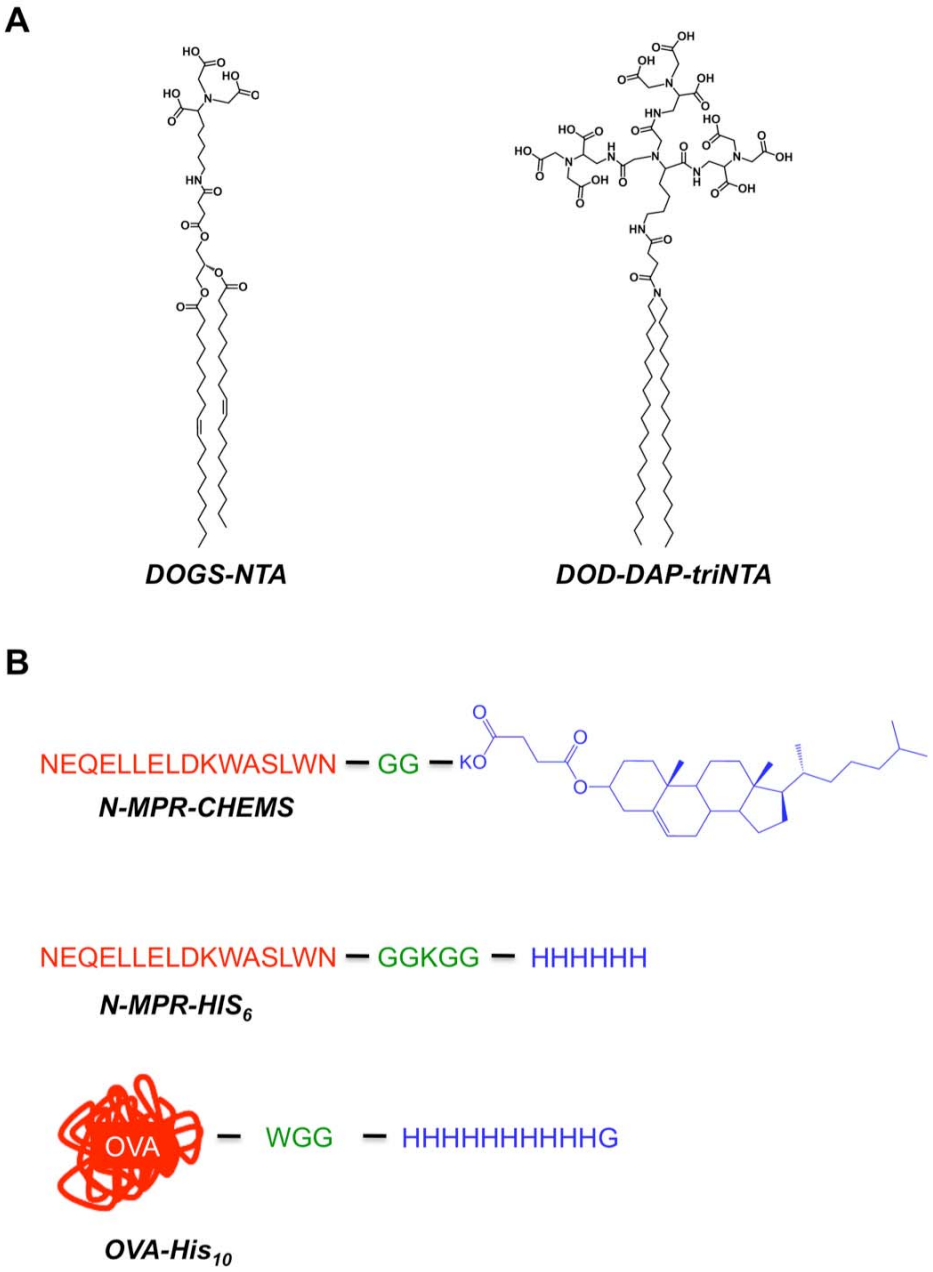

Figure 4-1. Structures of NTA lipids and polyhistidine-tagged antigens. DOGS-NTA, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt); DOD-DAP-triNTA, dioctadecylamine-(diaminoproprionic acid)-tri(nitrilotriacetic acid); N-MPR-CHEMS, N terminal peptide of the membrane proximal region of HIV-1 gp41 conjugated to cholestryl hemisuccinate; N-MPR-HIS_6, N-MPR conjugated to hexahistidine; OVA-His_{10}, Ovalbumin conjugated to decahistidine.

Recently, we and others reported the synthesis of multivalent nitrilotriacetic acid adaptors with nanomolar affinities for polyhistidine-tagged proteins (**Figure 4-1**) [22-24]. We hypothesized that the increased affinity of the antigen-particulate interaction would result in enhanced antibody responses as compared to a monovalent NTA linkage. In this study, we assessed the effect of the antigen-liposome linkage on antibody responses to two model antigens, ovalbumin (OVA) and a peptide derived from the membrane proximal region of HIV-1 gp41 (N-MPR). Polyhistidinylated antigens were attached to liposomes via monovalent NTA, trivalent NTA or a covalent linkage and administered to BALB/C mice. When N-MPR-His_6 was attached to liposomes via an NTA linkage, anti-N-MPR IgG was detected in sera of 4 of 4 mice, whereas an unattached N-MPR-His_6 admixed with liposomes lacking NTA failed to generate a response (0 of 4 mice). In contrast, NTA linkages did not enhance antibody responses as compared to OVA-His_{10} admixed with control liposomes. In both cases, trivalent NTA did not confer a significant enhancement of antibody response over monovalent NTA, and covalently attached antigens elicited significantly stronger antibody responses than NTA-anchored antigens. The data indicate that NTA linkages may be useful for delivery of weak antigens (e.g. N-MPR) but offer little advantage for delivery of higher molecular weight, more potent antigens (e.g. OVA).

4.3 Materials and Methods

4.3.1 Materials

Amino acid building blocks, resins and coupling agents were obtained from Novabiochem (Darmstadt, Germany), Anaspec (San Jose, CA) or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DOGS-NTA; monoNTA)

and 1,2-Dioleoyl-sn-Glycero-3-Phosphoehtanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS) and maleimidoproprionic acid (MPA) were obtained from Sigma-Aldrich. Anhydrous solvents of 99.8% or greater purity were obtained from Acros Organics (Geel, Belgium). Monophosphoryl lipid A derived from Escherichia coli (MPL; #L6638), aluminum hydroxide gel (Alum; #A8222) and ovalbumin (Grade V; #A5503) were obtained from Sigma-Aldrich. Endotoxin-free buffers were obtained from the UCSF Cell Culture Facility. Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich.

4.3.2 Synthesis of peptides and lipids

Peptides were synthesized on Rink Amide MBHA or NovaPEG resin in an automated solid phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA) with standard fluorenylmethyloxycarbonyl/o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate/n-hydroxybenzotriazole (FMOC/HBTU/HOBT) protocols. Peptides containing N-MPR were synthesized on NovaPEG resin with an orthogonally protected lysine (Fmoc-Lys(1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl)-OH; Fmoc-Lys(ivDde)-OH) incorporated at the C terminus for on-resin conjugation of lipid, polyhistidine or biotin. For N-MPR peptides, the N terminus was Boc-protected; for other peptides N terminal Fmoc protection was utilized. Removal of the orthogonal ivDde group was accomplished by 3 x 15 minute treatments of the peptidyl resin with 2% hydrazine hydrate in dimethylformamide (DMF; 10 mL per g resin). The resin was washed in DMF (3 x 10 mL) and dichloromethane (DCM; 3 x 10 mL) and dried under vacuum.

Attachment of CHEMS to N-MPR was accomplished via amidation of a carboxylated lipid and a deprotected lysyl ε amine at the C terminus. CHEMS (270 μmol) was activated with 270 μmol each of HBTU, HOBT and diisopropylethylamine (DIEA) in anhydrous DMF/DCM (DCM as needed for lipid solubilization) for 30 min at room temperature followed by addition of 67.5 μmol resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with DMF (4 x 10 mL) and DCM (4 x 10 mL) to remove unreacted lipids and dried under vacuum. N-MPR-CHEMS were cleaved from the resin by treatment with trifluoroacetic acid containing 2.5% water, 2.5% ethanedithiol and 1% triisopropylsilane for 4 hours under argon. Cleaved peptides were precipitated into cold ethyl ether. The precipitate was pelleted by centrifugation at 3000 rpm (RT6000, Sorvall, Waltham, MA) and washed once with cold ethyl ether. The ether was poured off and the pellet was re-dissolved in methanol (MeOH), transferred to a round bottom flask, dried by rotary evaporation under reduced pressure and further dried under high vacuum. The lipopeptide was further separated from unconjugated peptide by reverse phase high pressure liquid chromatography (RP-HPLC; DX 500, Dionex, Sunnyvale, CA) on a semi-preparative C4 column (214TP510, Grace Vydac, Deerfield, IL) until unconjugated peptide was no longer detectable by MALDI-MS. Lipopeptide fractions were identified by MALDI-MS in 2,5-dihydroxybenzoic acid matrix, pooled and lyophilized. Stock lipopeptide solutions were prepared in MeOH or MeOH/$CHCl_3$ and stored at -20 ^{o}C. Final yields were approximately 5-10%.

Biotinylated N-MPR was prepared for use in ELISA by an analogous method. Biotin was attached to the deprotected C terminal amine by activation of 500 μmol D-biotin with 500 μmol HBTU/HOBT/DIEA in 1.65 mL anhydrous 1:1 DMF/dimethylsulfoxide (DMSO) for

30 min followed by addition of resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with 1:1 DMF/DMSO (3 x 10 mL), DMF (3 x 10 mL) and DCM (3 x 10 mL) and dried under vacuum. Biotinylated peptides were cleaved and purified as described above. Biotin content was quantified by 4´-hydroxyazobenzene-2-carboxylic acid dye exclusion (Sigma #H2153) according to the manufacturer's instructions.

Synthesis of N-MPR-His_6 was accomplished by appending the C terminus of N-MPR with a hexahistidine tag. The orthogonally protected lysyl ε amine was deprotected and appended by automated solid phase synthesis with Fmoc protection as described above. The peptide was cleaved and purified as described, and stock solution was prepared in sterile water and stored at -20 ^{o}C.

Maleimide functionalized decahistidine (His_{10}-maleimide) was prepared by on-resin modification of the deprotected peptidyl N terminus with maleimidoproprionic acid (MPA). MPA (290 μmol) was activated with 290 μmol HBTU, 290 μmol HOBT and 580 μmol DIEA in 3 mL anhydrous DMF for 30 mins at room temperature under argon. Peptidyl resin (74 μmol) was added and shaken at room temperature under argon for 2.5 hr. The resin was washed with DMF (4x) and DCM (4x) and dried under high vacuum overnight. The modified peptide was cleaved and purified as described above, except the cleavage cocktail did not contain ethanedithiol. Molecular weights of all peptides were confirmed by MALDI-MS in dihydroxybenzoic acid matrix. Peptide concentration of stock solutions was confirmed by A280 of tryptophan residues with extinction coefficient calculated by the method of Pace [25]. Nomenclature, sequences and molecular weights of peptides used in this study are summarized in **Table 4-1**.

Name	Peptide Sequence	MW (exp)	MW (obs)
N-MPR-CHEMS	NEQELLELDKWASLWNGGK-CHEMS	2698.3	2718.2 (Na^+)
N-MPR-His_6	NEQELLELDKWASLWNGGKGGHHHHHH	3166.5	3163.0
N-MPR-biotin	NEQELLELDKWASLWNGGK-biotin	2455.6	2455.0
His_{10}-maleimide	WGGHHHHHHHHHHG	1896.9	1901.0

Table 4-1. Sequences and molecular weights of peptide antigens.

DOD-DAP-triNTA (triNTA) was synthesized and characterized as described in detail elsewhere ([23] and Huang et al, manuscript in preparation). Prior to liposome formation, DOD-DAP-triNTA was loaded with Ni(II) by incubation with a 2.85:1 molar excess of $NiCl_2$ at 60 °C for 15 min and cooled to room temperature.

4.3.3 Preparation of polyhistidinylated ovalbumin

Aside from endotoxin removal, ovalbumin polyhistidinylation was done by Virginia Platt. Endotoxin contamination of ovalbumin was minimized by centrifugal filtration of a 10 mg/mL solution in PBS through 100KDa membrane (Amicon Ultra, Millipore, Billerica, MA) to remove aggregates followed by passage down a polymixin B endotoxin removal column (Detoxi-Gel, Pierce, Rockford, IL). Prior to polyhistidinylation, ovalbumin (73 nmol) was thiolated by treatment with 0.6 mM 2-iminothiolane (1.6 μmol) in sodium phosphate buffer (0.1 M $NaPO_4$, 50 mM NaCl, pH 7.53) for 1.5 hours at room temperature. Thiolated ovalbumin (OVA-SH) was separated from excess 2-iminothiolane by passage down a desalting column (PD-10, GE Healthcare, Piscataway, NJ).

Concentration of purified OVA-SH was determined by the Bradford method (Bio-rad, Hercules, CA) and preparations were stored at 4 ^{o}C until use.

Polyhistidinylation of OVA-SH (100 nmol) was accomplished by reaction with three-fold molar excess of His_{10}-maleimide (300 nmol) in sodium phosphate buffer overnight at room temperature. To purify polyhistidinylated OVA (OVA-His_{10}) from unreacted OVA-SH, imidazole was added to a final concentration of 20 mM and the protein was loaded onto a 1 mL Ni^{2+}-NTA column (HisTrapFF, GE Healthcare). The column was washed with 10 mL sodium phosphate buffer containing 20 mM imidazole and OVA-His_{10} was eluted in sodium phosphate buffer containing 500 mM imidazole. To remove imidazole and excess His-maleimide, the solution was dialyzed at a sample to dialysate volume ratio of 1:150 overnight in sterile PBS with 3 buffer changes (Slide-A-Lyzer, 10,000 MWCO, Pierce). Protein stability and extent of modification were monitored by sodium dodecylsulfate polyacrylamide gel electrophoresis. Protein concentration was determined by the Bradford method and preparations were stored at 4 ^{o}C until use.

A far-red fluorescent protein (mKat) was used as a surrogate protein for characterization of polyhistidine-tagged protein binding to NTA liposomes. mKat was expressed and purified as described (Platt et al, manuscript in preparation).

4.3.4 Liposome preparation

Peptide and protein antigens were formulated in liposomes comprised of 15:2:3:0.3 DMPC:DMPG:Cholesterol:MPL [26]. NTA-containing liposomes were formulated with addition of either DOD-DAP-triNTA or DOGS-NTA at a molar ratio of 15:2:3:0.3:0.3. Maleimide-containing liposomes were formulated with addition of MPB-PE at a molar

ratio of 15:2:3:0.3:0.3. Prior to use, glassware was rinsed with MeOH and $CHCl_3$ and dried for at least 90 min at 150 ^oC to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. For liposomes containing N-MPR-CHEMS, lipopeptide in MeOH was added to the lipid solution prior to drying. Films were further dried under high vacuum overnight. Lipids were hydrated in sterile PBS (UCSF Cell Culture Facility) by intermittent vortexing and liposomes were prepared by bath sonication for 10 mins at room temperature under argon. N-MPR-His_6 and OVA-His_{10} were associated with NTA-containing liposomes for 1 hr at room temperature immediately prior to injection. Covalent attachment of OVA to liposomes was accomplished by addition of OVA-SH to liposomes containing MPB-PE immediately after liposome formation and continued reaction overnight at 4 ^oC. The final formulations contained 0.1 mg/mL OVA derivative or 0.5 mg/mL N-MPR derivative and 0.5 mg/mL monophosphoryl lipid A in 20 mM carrier lipid. Vesicle size was characterized by dynamic light scattering (Zetasizer 3000, Malvern, New Bedford, MA). Liposomes were stored at 4 ^oC under argon until use. As a control, OVA-His_{10} was adsorbed onto Alum according to the manufacturer's instructions.

4.3.5 Liposome-antigen association in vitro

Association of OVA-His_{10} with NTA-containing liposomes was characterized by size exclusion chromatography by Virginia Platt. Liposomes were prepared as described above and passed down a 1 x 20 cm sepharose 4B-CL column under gravity flow. Liposomes eluted in the void volume and were assayed for the presence of OVA by the Bradford method. Controls included liposomes containing covalently bound OVA, liposomes lacking NTA and liposomes lacking protein.

The dissociation of polyhistidine-tagged protein from liposomes in the presence of serum was monitored using a surrogate fluorescent protein, mKat, by Virginia Platt. Dissociation from liposomes was measured by challenging pre-associated mKat with refiltered fetal calf serum (FCS, UCSF Cell Culture Facility). Liposomal mKat was mixed with FCS at a 1:1 volume ratio and incubated at 37 ºC for the indicated time. After incubation, samples were passed down a 1 x 20 cm sepharose 4B-CL column under gravity flow to separate free from liposome-associated mKat. Liposomes eluted in the void volume and were assayed for the presence of mKat by fluorescence (ex/em 544/590; Fluostar 403, BMG LabTechnologies GmbH, Durham, NC). Free mKat was also monitored without liposomes for fluorescence degradation over time and fluorescence intensity was unchanged after 24 hours (data not shown).

4.3.6 Animal immunizations

All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. 10 week-old female BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF specific pathogen-free barrier facility. Animals received subcutaneous immunizations in alternating hind hocks on Days 0 and 14 as described [27]. Each injection contained 5 μg OVA derivative or 25 μg N-MPR derivative, 25 μg MPL and 1 μmol lipid vehicle in 50 μL sterile phosphate-buffered saline. OVA/alum injections contained 5 μg OVA adsorbed onto 325 μg alum (6.5 mg/mL). On Day 28 blood was collected from the submandibular vein for characterization of antibody responses. Cells and clotted material were removed by centrifugation at 14,000 rpm for 15 min (5415C, Eppendorf, Westbury, NY) and sera were stored at -80 ^{o}C until use.

4.3.8 ELISA

ELISAs were developed to quantify binding of immune sera to N-MPR or OVA. Peptide ELISAs were conducted using N-MPR biotinylated as described above and captured on 96 well streptavidin-coated plates (#15120, Pierce, Rockford, IL). Assays were performed according to the manufacturer's instructions with modifications. Biotinylated peptide was added to wells in PBS containing 0.1% Tween-20 (PBS-T) and incubated for 2 hr at 37 ^{o}C. Following a wash step, sera were serially diluted in PBS containing 0.1% casein (C7078, Sigma-Aldrich) (PBS-C), added to wells and incubated for 30 min at 37 ^{o}C. After reconstitution, horseradish peroxidase-conjugated IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) was diluted 1:1 in glycerol for long-term storage at -20 ^{o}C and further diluted 1:1000 in PBS-C immediately prior to use. Following a wash step, secondary antibodies were added to wells and incubated for 30 min at 37 ^{o}C. Following a final wash step, a tetramethylbenzidine substrate solution (#T0440, Sigma-Aldrich) was added to wells and incubated for 30 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 and the yellow product was monitored at 450 nm (Optimax, Molecular Devices, Sunnyvale, CA). All incubations were done in 100 μL volumes and wells were washed 6 times with PBS-T between each step. Titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 preimmune sera after subtraction of background wells lacking serum. All samples were assayed in duplicate.

OVA ELISAs were performed as follows: ovalbumin was diluted from a 5 mg/mL PBS stock solution to 0.1 mg/mL in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). 10 μg OVA (100 μL) per well was added to flat-bottomed high capacity immunoassay plates (Costar). Plates were sealed with parafilm and incubated at 4 ^{o}C overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were serially diluted in PBS-C and incubated in wells for 30 mins. Wells

were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 30 min. Following a wash step, plates were then developed and read as indicated above. All incubations were done in 100 μL volumes at 37 ^{o}C and wells were washed 6 times with PBS-T between each step. Titer was defined as described above and all samples were assayed in duplicate.

4.3.9 Statistical analysis

Statistical significance was assessed by analysis of variance and two-tailed Student's t test. Differences were considered significant if they exhibited p values < 0.05 in the Student's t test. Data analyses were performed using Microsoft Excel and SigmaPlot.

4.4 Results

4.4.1 Preparation of antigens and liposomes

Nitrilotriacetic acid-mediated attachment has garnered interest as a means for tethering polyhistidine-tagged peptide and protein antigens to particulate vaccine carriers [20]. The goal of this investigation was to determine the role of NTA-Ni(II)-His affinity on antibody responses to two antigens in mice. Liposomes were an ideal delivery system for this study because they deliver associated antigens efficiently to antigen presenting cells [28], stimulate potent immune responses when adjuvanted with monophosphoryl lipid a (MPL) [9], and lipid-anchored NTA molecules can be readily incorporated into the formulation [17, 23].

The N terminal peptide of the membrane proximal region of HIV-1 gp41 (N-MPR) was selected because this sequence is a key target for development of vaccines that elicit neutralizing antibodies [29]. Since the N-MPR peptide is smaller than OVA and contains fewer antigenic determinants, we believed it would provide insight into the use of NTA

linkages for delivery of less potent antigens. Moreover, broadly neutralizing antibodies that target the MPR exhibit lipid binding activity, and it has been postulated that elicitation of similar antibodies will require presentation of MPR immunogens in a lipid bilayer environment [30]. A site-specific NTA-mediated tether could also allow control over directional orientation and presentation of the structure in its native orientation.

A hexahistidine-tagged N-MPR peptide was synthesized in which the polyhistidine tag was attached at the C terminus via an orthogonal protecting group (**Figure 4-1**). This was done for two reasons: first, it allowed orientation of the peptide in a manner that mimics the native sequence, wherein the C terminus of the sequence is tethered to the membrane and the N terminus extends outward [19]. Second, attachment via the ε lysyl amine permitted the structure to most closely resemble that of a previously synthesized lipid-anchored control peptide found to elicit high anti-peptide titers when administered in liposomal formulations to BALB/C mice [31]. Vesicle sizes of N-MPR-containing formulations were not reported because liposomes visibly precipitated within 5 mins following addition of N-MPR antigens and thus the structures presented to the immune system were likely not vesicles of defined diameter. Interestingly, in a previous study in which the N-MPR-CHEMS-containing formulations were extruded through polycarbonate membranes following sonication, rapid aggregation was not observed [31].

Ovalbumin was selected because it is widely used as a model antigen for assessment of humoral and cellular responses [32]. The protein was modified with decahistidine in a two step reaction in which free amines were first modified by 2-iminothiolane to generate free thiol groups [33]. These groups were then reacted with a maleimide-functionalized decahistidine peptide to generate the final conjugate. The conjugate was assured to be free of unmodified OVA because it was purified using a NTA-Ni(II) affinity column. SDS-

PAGE analysis revealed a slight increase in molecular weight upon polyhistidinylation, corresponding to 1-2 decahistidine peptides per protein molecule. It is unclear why the proteins ran in the 37-40 kDa molecular weight range, which was less than the expected molecular weight of 45 kDa (**Figure 4-2**). Liposomes prepared with OVA-His_{10} exhibited vesicle diameters consistently in the 130-200 nm range regardless of introduction of NTA- or maleimide-functionalized lipids into the formulation (**Table 4-2**). Moreover, vesicle sizes were qualitatively consistent over time and no visible aggregation was observed following the addition of protein.

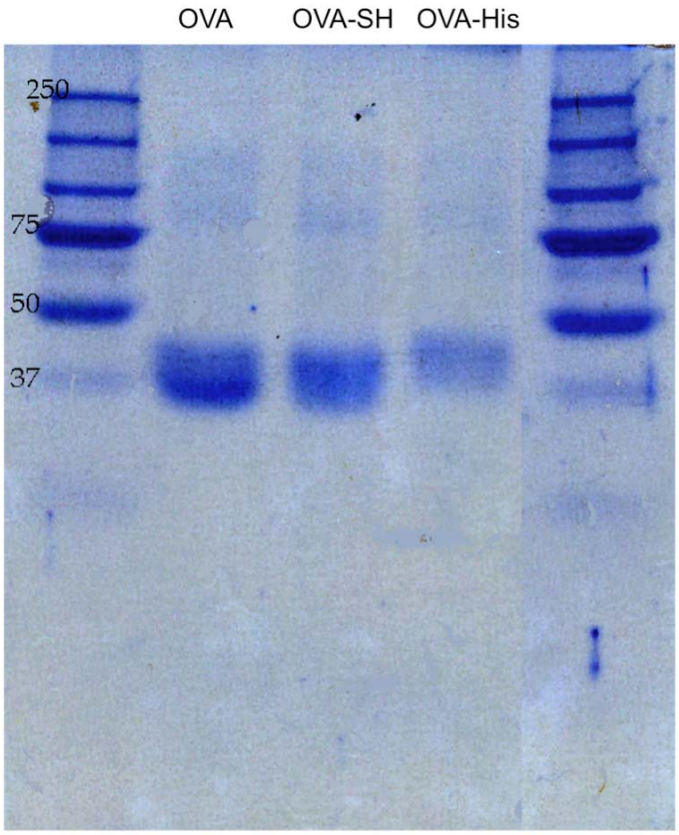

Figure 4-2. SDS-PAGE of polyhistidinylated ovalbumin. OVA-His_{10} ran slightly larger than OVA-SH or unmodified OVA, corresponding to the addition of 1-2 decahistidine tags to the total molecular weight.

Formulation	Vesicle Diameter (nm)	Standard Deviation (nm)
OVA-His_{10} mixed-lipo	131.5	1.3
OVA-SH covalent-lipo	157.4	3.1
OVA-His_{10} + monoNTA-lipo	135.5	0.7
OVA-His_{10} + triNTA-lipo	168.2	14.8
OVA-His_{10} + triNTA-lipo (no MPL)	194.3	1.6

Table 4-2. Vesicle sizes of ovalbumin-containing liposome preparations.

4.4.2 Liposome-antigen association

Interest in multivalent NTA adaptors arose in part due to concerns that the micromolar affinity of monovalent NTA-Ni(II)-His may be too unstable for *in vivo* applications [21, 23]. To address this question, we determined the effect of serum on the stability of binding between liposomes containing DOGS-NTA or DOD-DAP-triNTA and a surrogate hexahistidine-tagged fluorescent protein, mKat. In this experiment, protein was added to pre-formed NTA liposomes and allowed to associate prior to addition of fetal calf serum. 1 hr after addition of serum, liposome-associated protein was separated from free protein by size exlusion chromatography. Under the conditions studied, both monoNTA and triNTA liposomes initially bound 100% of the protein added (**Figure 4-3**). However, over time protein dissociated from monoNTA liposomes more quickly, with less than 50% of the protein remaining associated after 4 hrs, whereas at the same time point approximately 80% remained associated with triNTA liposomes.

The association of polyhystidinylated OVA to NTA liposomes or control liposomes in PBS was determined by a size exclusion chromatography method with detection by the Bradford assay. In formulations containing DOD-DAP-triNTA, the protein was entirely

retained in the liposome fraction (**Figure 4-4**). MonoNTA- and maleimide-functionalized liposomes exhibited an intermediate level of protein retention, whereas control liposomes ('OVA-His_{10}') did not exhibit any protein binding above the background signal of empty liposomes. When the background contribution was subtracted and peak areas

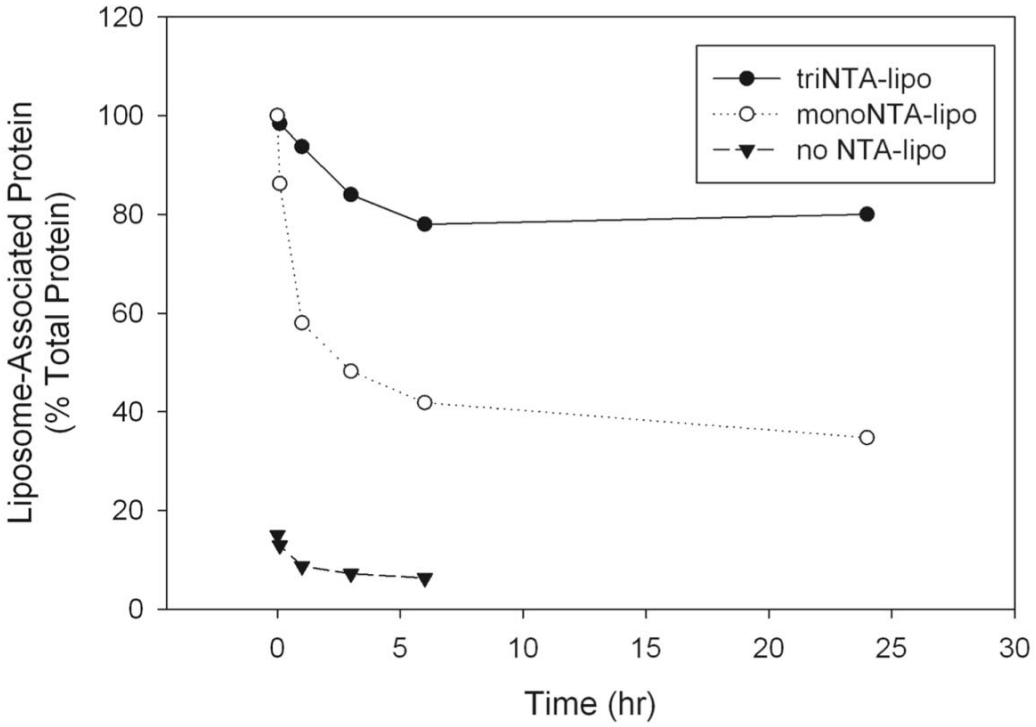

Figure 4-3. Effect of serum on association of a fluorescent hexahistidine-tagged protein with liposomes containing NTA lipids. The dissociation of polyhistidine-tagged protein from liposomes in the presence of serum was monitored using a surrogate fluorescent protein, mKat, by challenging liposome-associated mKat with refiltered fetal calf serum and incubating at 37 ºC for the indicated time. After incubation, free and liposome-associated mKat were separated by size exclusion. Liposomes containing triNTA retained nearly double the protein of monoNTA liposomes by 4hr. Results are representative of two independent experiments.

were integrated, triNTA liposomes bound 77.7-83.0% of the added OVA, whereas monoNTA- and maleimide-functionalized liposomes bound 34.4-38.6% of the protein (**Table 4-3**). These results underscore the differences in protein binding between monoNTA- and triNTA-containing formulations, while also revealing a considerable amount of unconjugated protein in the maleimide-functionalized liposomes. This could

be caused by saturation of available binding sites or steric restraints at the liposome surface.

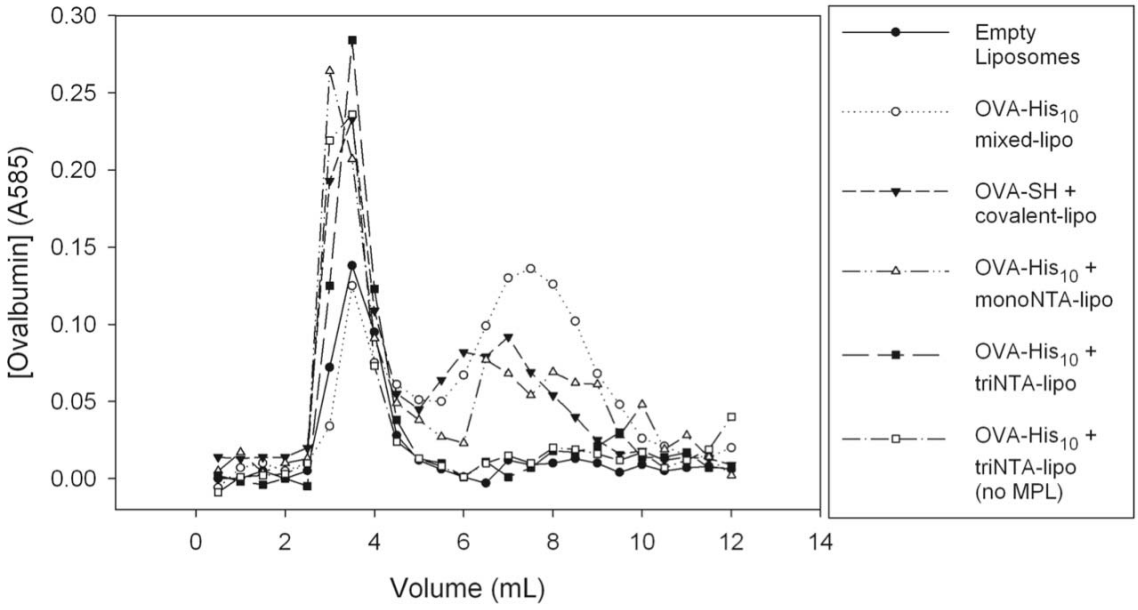

Figure 4-4. Association of ovalbumin with liposomes containing NTA lipids. Association of OVA-His_{10} with NTA-containing liposomes was characterized by size exclusion. Liposomes containing triNTA were found to completely retain OVA-His_{10} under the conditions studied, whereas monoNTA liposomes and maleimide liposomes exhibited intermediate retention. Control liposomes lacking NTA did not exhibit protein-liposome binding greater than background. Results are representative of two independent liposome preparations.

Formulation	Liposome-Associated Protein (% of total)
OVA-His_{10} mixed-lipo	0.0
OVA-SH covalent-lipo	34.4
OVA-His_{10} + monoNTA-lipo	38.6
OVA-His_{10} + triNTA-lipo	77.7
OVA-His_{10} + triNTA-lipo (no MPL)	83.0

Table 4-3. Association of ovalbumin with liposomes containing nitrilotriacetic acid-conjugated lipids. The association of polyhystidinylated OVA to NTA liposomes or control liposomes in PBS was determined by a size exclusion chromatography method with detection by the Bradford assay. Integrated peak areas were calculated following subtraction of the background contribution of liposomes lacking protein. Percent association was calculated as Associated/(Associated + Free) x 100. Results are representative of two independent preparations.

4.4.3 Humoral immune responses in mice

Antibody responses to liposome-associated OVA and N-MPR were assessed in BALB/C mice. Liposomes containing N-MPR-His_6 attached via either a monoNTA or a triNTA linkage elicited anti-peptide IgG in sera of 4 of 4 mice in each group. However, N-MPR-His_6 admixed with control liposomes lacking NTA failed to elicit a detectable anti-N-MPR antibody response.

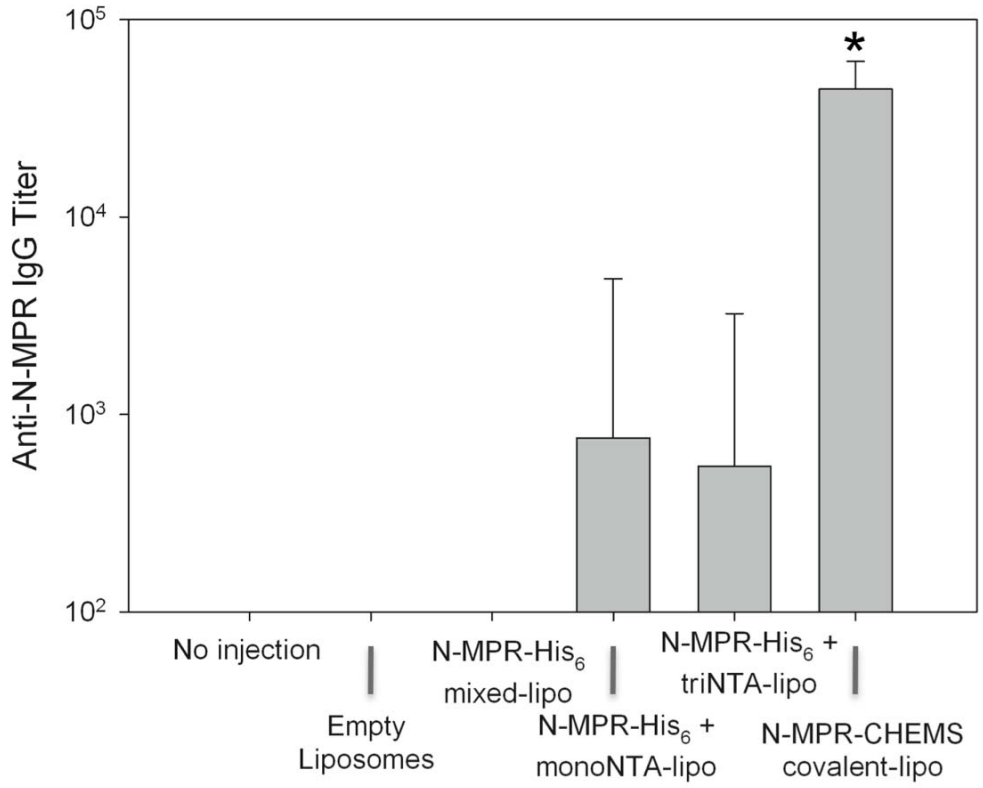

Figure 4-5. Effect of N-MPR-liposome linkage on anti-N-MPR IgG response in mice. Covalent attachment of N-MPR to liposomes via a cholesteryl hemisuccinate anchor was superior to NTA-mediated conjugation for elicitation of antibody responses to N-MPR ($p = 0.002$ vs. monoNTA, $p = 0.002$ vs. triNTA). However, polyhistidinylated N-MPR admixed with control liposomes failed to elicit a detectable anti-N-MPR antibody response. No significant difference was observed when comparing antibody responses elicited by monoNTA and triNTA linkages ($p = 0.67$).

This is consistent with a previous study by our group in which liposomal N-MPR peptide required a lipid anchor to generate a detectable antibody response in BALB/C mice [31]. No significant difference was observed when comparing antibody responses elicited by

monoNTA and triNTA linkages (p = 0.67). Importantly, covalent attachment of N-MPR to liposomes via a cholesteryl hemisuccinate anchor was superior to NTA-mediated conjugation for elicitation of antibody responses to N-MPR (GMT 4.4 x 10^4 vs. 5.5-7.6 x 10^2; p = 0.002 vs. monoNTA, p = 0.002 vs. triNTA; **Figure 4-5**).

When OVA-His_{10} was the immunizing antigen, DOGS-NTA-mediated attachment elicited significantly greater antibody responses than adsorption on aluminum hydroxide gel, as reported by Patel and coworkers (GMT 1.6 x 10^6 vs. 4.4 x 10^5, p = 0.0002; **Figure 4-6**) [20]. Omission of MPL from the NTA formulation resulted in an order of magnitude decrease in anti-OVA titers (GMT 1.6 x 10^5, p = 0.003). As seen in the case of N-MPR-His_6, linkage via triNTA did not confer any advantage over conjugation via monoNTA (p = 0.53). Moreover, both monoNTA and triNTA formulations were inferior to covalently conjugated OVA (GMT 1.4-1.6 x 10^6 vs. 3.4x10^6, p < 0.001). Surprisingly, control liposomes in which the protein was unattached also elicited significantly greater anti-OVA titers than NTA liposomes (GMT 2.6 x 10^6; p = 0.006 vs MonoNTA, p = 0.01 vs TriNTA). Statistical comparisons between groups, as determined by two-tailed Student's t test, are summarized in **Table 4-4**.

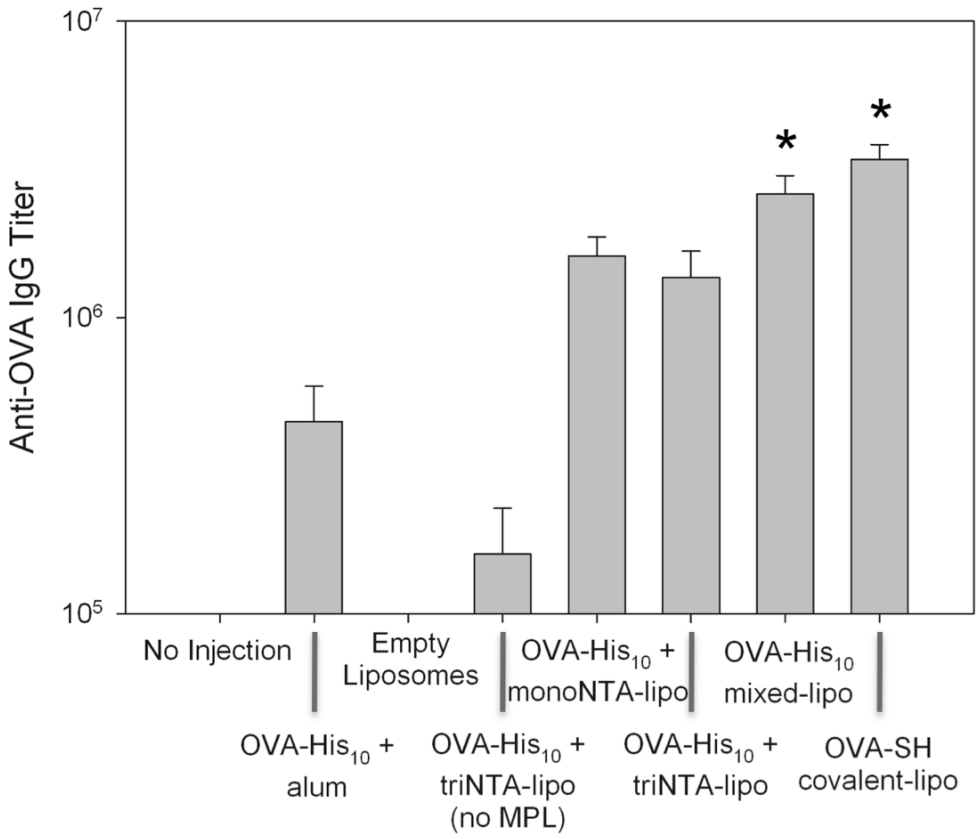

Figure 4-6. Effect of ovalbumin-liposome linkage on anti-ovalbumin IgG response in mice. Antibody responses to liposome-associated OVA were assessed in BALB/C mice. DOGS-NTA-mediated attachment elicited significantly greater antibody responses than adsorption on aluminum hydroxide gel ($p = 0.0002$). However, both monoNTA and triNTA formulations were inferior to covalently conjugated OVA (* = $p < 0.001$). Control liposomes in which the protein was unattached also elicited significantly greater anti-OVA titers than NTA liposomes ($p = 0.006$ vs MonoNTA, $p = 0.01$ vs TriNTA). No responses were observed in control groups ('No Injection' and 'Empty Liposomes'). Statistical comparisons between groups, as determined by two-tailed Student's t test, are summarized in **Table 4-4**.

	OVA-SH covalent-lipo	OVA-His_{10} + monoNTA-lipo	OVA-His_{10}- + triNTA-lipo	OVA-His_{10} + triNTA-lipo (no MPL)	OVA-His_{10} + alum
OVA-His_{10} mixed-lipo	3.08E-02	5.61E-03	1.02E-02	1.98E-05	5.36E-05
OVA-SH covalent-lipo		2.87E-04	8.53E-04	4.11E-06	9.26E-06
OVA-His_{10} + monoNTA-lipo			5.28E-01	3.26E-05	2.09E-04
OVA-His_{10} + triNTA-lipo				2.57E-03	1.02E-02
OVA-His_{10} + triNTA-lipo (no MPL)					9.33E-03

Table 4-4. Statistical significance of differences in anti-ovalbumin IgG titer amongst groups of mice immunized with ovalbumin-containing liposome formulations. Analyses were performed using two-tailed Student's t test assuming equal variances and differences were considered significant if p values were less than 0.05. All comparisons were significant except that of monoNTA-lipo vs. triNTA-lipo (indicated in red).

In summary, NTA-mediated attachment was more effective than simply admixing antigen with liposomes lacking NTA for elicitation of serum IgG to N-MPR-His_6 but not to OVA-His_{10}. NTA-mediated attachment was also more effective than adsorption on alum for elicitation of serum anti-OVA IgG responses, as reported previously [20]. Importantly, the triNTA anchor did not provide any enhancement as compared to the monoNTA anchor for induction of antibody to either OVA or N-MPR. Lastly, covalent conjugation elicited greater serum antibody titers to both OVA and N-MPR as compared to NTA attachment.

4.5 Discussion

Particulate delivery systems are required to elicit robust immune responses to subunit protein antigens, and more potent alternatives are needed to replace traditional aluminum salts [1, 5]. Liposomes, polymeric particles, emulsions, and other microscale and nanoscale carriers have been developed to deliver payloads efficiently to antigen

presenting cells [2, 6]. However, methodologies for association of antigen with these systems, including entrapment, adsorption or chemical conjugation, can damage proteins and present epitopes in an uncontrolled orientation [12-14]. Metal chelation via NTA-Ni(II)-His has been suggested as a site-specific, non-destructive approach to particulate delivery of polyhistidine-tagged antigens [20]. However, concerns have arisen that the micromolar affinity of monovalent NTA for hexahistidine may be too weak for *in vivo* applications [21-23].

This study sought to characterize the importance of NTA-Ni(II)-His affinity in promoting antibody responses to polyhistidine-tagged antigens formulated with particulate carriers. We hypothesized that the increased affinity of trivalent NTA for polyhistidine would translate to increased liposome association and enhanced antibody titers. Antibody responses to liposomal preparations of two model proteins wherein the antigen was attached via a trivalent NTA lipid anchor, a commercially available monovalent NTA anchor, or a covalent linkage were assessed in BALB/C mice.

The key findings of the study are three-fold. First, attachment of N-MPR-His_6 but not OVA-His_{10} to liposomes via an NTA lipid elicited stronger antibody responses in mice as compared to a formulation in which the antigen was simply admixed with control liposomes lacking NTA. This difference may arise from the greater antigenic diversity of OVA, a large protein with many B and T cell epitopes, as compared to N-MPR, a peptide that contains only a few epitopes. Further studies will be needed to parse out the precise guidelines that determine whether NTA-liposome delivery will be useful for promoting antibody responses to a particular antigen.

The second key finding of the study was that antigen attachment via multivalent NTA linkages with greater affinity did not result in enhanced antibody responses as compared to monovalent NTA linkages despite association of a greater fraction of the antigen with triNTA liposomes (**Figures 4-5 and 4-6**). Although triNTA liposomes bound more than twice the protein of monoNTA liposomes (**Figure 4-4**), no enhancement of antibody responses was observed. Although the extent of binding may be substantially altered *in vivo*, similar differences in binding of OVA-His_{10} to monoNTA and triNTA were also observed in the presence of serum (**Figure 4-3**). Importantly, a weaker antigen (N-MPR) seemed to require association with the carrier, via either NTA or a covalent linkage, to elicit a response (**Figure 4-5).** However, the extent of peptide binding was not quantified in this case due to the destabilization of the vesicles in the presence of peptide.

Third, NTA linkages were inferior to covalent conjugation for elicitation of antibody responses to liposomal formulations of OVA and N-MPR (**Figures 4-5 and 4-6**). Surprisingly, antibody titers did not correspond with extent of protein-liposome binding as assessed by size exclusion chromatography; control liposomes in which the protein was completely unassociated elicited significantly greater anti-OVA responses than liposomes containing NTA-anchored OVA (**Figure 4-4 and Table 4-3**). These findings conflict with the work of Patel and coworkers showing that NTA-Ni(II) wax nanoparticles elicited greater responses to polyhistidine-tagged p24 antigen than control nanoparticles lacking Ni(II) [20]. This discrepancy may be attributed to the greater immunostimulatory capacity of the MPL-adjuvanted liposomes as compared to the nanoparticles studies by Patel and coworkers [34]. Indeed, unassociated OVA with MPL-adjuvanted liposomes also elicited significantly greater antibody responses than OVA alone in PBS (GMT 3.1 x 10^4, $p = 0.0001$), indicating a key role of the liposomes in activating the response, perhaps through toll-like receptor 4 engagement or through other inflammatory

mechanisms. It should be noted that thiolation of OVA could have altered the response in the current experiment; however, thiolated bovine serum albumin was not found to elicit antibody responses when admixed with control liposomes in A/J mice in a similar experiment [35]. Moreover, analogous modifications to other proteins generally reduce, rather than increase, immune responses [36]. Thus, we surmise that for potent antigens or highly immunostimulatory delivery systems, NTA linkage may not confer any benefit.

In summary, association with an MPL-adjuvanted liposomal carrier was required to elicit antibody responses to a weak antigen (N-MPR) but not a potent antigen (OVA). In both cases, no differences were observed between monovalent and trivalent NTA linkages. Finally, covalent attachment to the carrier was superior to NTA-mediated attachment for elicitation of antibody responses. Thus further improvements of the NTA-mediated conjugation strategy are required before it will be an effective method to attach antigens to particulate vaccine carriers and elicit high titer antibody responses.

4.6 Acknowledgements

Author contributions were as follows: D.W. and V.P. designed experiments. D.W. synthesized peptides and lipopeptides. V.P. prepared polyhistidinylated OVA. D.W. and V.P. prepared liposome formulations. V.P. performed liposome-protein binding studies. D.W. performed ELISAs and data analysis. D.W. wrote the manuscript except portions of the Methods which describe the research contributions of V.P., which were written by V.P. We are grateful to Katherine Jerger for assistance with animal studies. We thank Dr. Zhaohua Huang for assistance with synthesis of the DOD-DAP-triNTA lipid and Dr. Limin Cao for assistance with cloning and expression of mKat. This work was supported by NIH R01 GM061851 and by a grant from the National Institutes of Health, University

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Chapter 5

All-*trans* retinoic acid potentiates the antibody response in mice to a lipopeptide antigen adjuvanted with liposomal lipid A

5.1. Abstract

Retinoic acid (RA), the bioactive metabolite of retinol, is essential for robust humoral immunity in animals and humans. Recent interest in RA as a vaccine adjuvant has been encouraged by reports demonstrating cooperative enhancement of antibody responses to tetanus toxoid by all-*trans* RA and a Toll-like receptor 3 (TLR3) agonist in rodents. We hypothesized that retinoic acid would augment the antibody response to a lipopeptide immunogen derived from the membrane proximal region (MPR) of HIV-1 gp41. The MPR generates weak antibody responses and could benefit from potent new humoral adjuvants. RA alone did not elicit antibodies to an MPR-derived lipopeptide co-formulated in liposomes and administered to BALB/C mice. However, addition of all-*trans*, but not 13-*cis*, RA to a liposomal formulation containing the TLR4 agonist monophosphoryl lipid A resulted in a four-fold enhancement of antibody titers as compared to a formulation containing lipid A alone ($p = 0.00039$). This result indicates that *all-trans* RA warrants further study as a vaccine adjuvant.

5.2. Introduction

Dietary retinol is essential for the development and maintenance of a healthy immune system[1]. Retinol is also required to mount robust antibody responses to T cell-dependent and type 2 T cell-independent antigens in animals and humans [2, 3]. Retinoic acid (RA), the bioactive metabolite of retinol, exerts a multitude of immunomodulatory effects *in vitro* and *in vivo* [1, 4] (**Figure 5-1**). Recently, Ross and colleagues have demonstrated cooperative enhancement of antibody responses to

tetanus toxoid by *all-trans* RA (ATRA) and polyriboinosinic:polyribocitidylic acid, a Toll-like receptor (TLR) 3 agonist, in mice and rats [5, 6]. These studies have aroused interest in the utility of ATRA as a vaccine adjuvant.

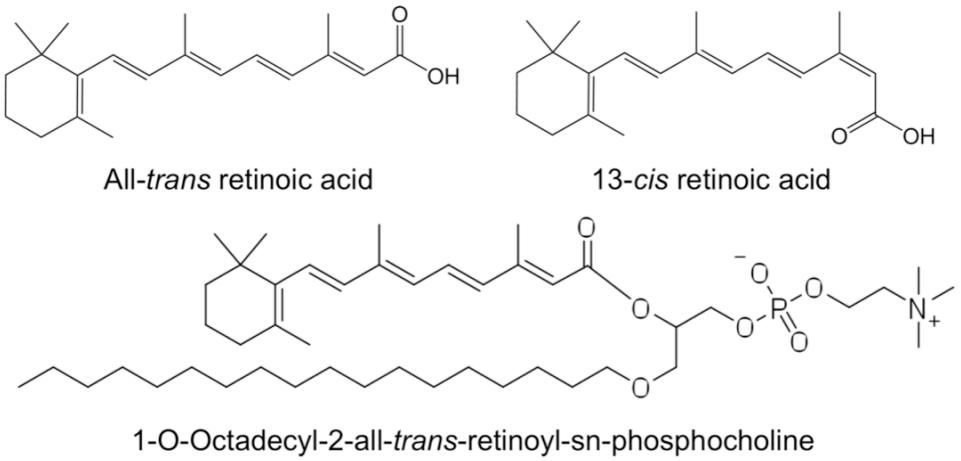


Figure 5-1. Structures of all-*trans* retinoic acid, 13-*cis* retinoic acid, and lipid-anchored all-*trans* retinoic acid (RAL).

We sought to determine if ATRA could promote antibody responses to a co-delivered antigen in mice. A peptide derived from the membrane proximal region of HIV-1 gp41 was selected for study because it is a key neutralizing antibody target for HIV vaccination [7]. This peptide (N-MPR) consisted of the epitope of the broadly neutralizing human monoclonal antibody 2F5 with flanking residues shown to enhance binding to 2F5 *in vitro* [8]. As this antigen is thought to be best presented in a membrane environment, the peptide was covalently attached to a lipid and formulated in lipid bilayer vesicles [9]. We immunized BALB/C mice with liposomes containing lipid-anchored N-MPR and either ATRA or monophosphoryl lipid A (MPL), a TLR4 agonist and potent liposomal vaccine adjuvant [10, 11]. The results indicate that ATRA potentiates the adjuvant effect of MPL in BALB/C mice, supporting further investigation of ATRA as a humoral vaccine adjuvant.

5.3. Materials and Methods

5.3.1. Materials

Amino acid building blocks, resins and coupling agents were obtained from Novabiochem (Darmstadt, Germany), Anaspec (San Jose, CA) or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Distearoylglycerol (DSG; LP-R4-029) and dipalmitoylglycerol (DPG; #LP-R4-028) were obtained from Genzyme Pharmaceuticals (Cambridge, MA). Anhydrous solvents of 99.8% or greater purity were obtained from Acros Organics (Geel, Belgium). Monophosphoryl lipid A derived from Escherichia coli (MPL; #L6638), all-*trans* retinoic acid (ATRA; #R2625) and 13-*cis* retinoic acid (13-cis RA; #R3255) were obtained from Sigma-Aldrich. Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich.

5.3.2. Lipopeptide synthesis

N-MPR peptide (NEQELLELDKWASLNGGK) was synthesized on NovaPEG resin in an automated solid phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA) with standard fluorenylmethyloxycarbonyl/o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate/n-hydroxybenzotriazole (FMOC/HBTU/HOBT) protocols. An orthogonally protected lysine (Fmoc-Lys(1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl)-OH; Fmoc-Lys(ivDde)-OH) was incorporated at the C terminus for on-resin conjugation of lipids or biotin. The N terminus was Boc-protected. Removal of the ivDde group was accomplished by 3 x 15 minute treatments of the peptidyl resin with 2% hydrazine hydrate in dimethylformamide (DMF; 10 mL per g resin). The resin was washed in DMF (3 x 10 mL) and dichloromethane (DCM; 3 x 10 mL) and dried under vacuum.

Lipid conjugation was accomplished via amidation of a carboxylated lipid and a deprotected lysine ε-amine at the C terminus. A carboxyl group was introduced to DPG and DSG via reaction of an available alcohol with succinic anhydride. Briefly, 1.8 mmol DPG or DSG was dissolved in 5 mL anhydrous DCM and combined with 3.6 mmol succinic anhydride in 10 mL anhydrous pyridine. The mixture was refluxed at 60 ^{o}C overnight. Reactions were continued to completion as monitored by thin layer chromatography (TLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS; Voyager DE, Applied Biosystems, Foster City, CA) in para-nitroaniline matrix. Products were washed twice with 1M hydrochloric acid (HCl), dried over sodium sulfate and stored dry until use. Carboxylated lipids were obtained in approximately 90-100% yield. Molecular weights and TLC R_F values were as follows: DPG-Suc, 668.19 Da, R_F 0.29 (DCM/acetone, 20/1); DSG-Suc, 749.6 (+ Mg^{2+}) Da, R_F 0.08 (DCM/acetone, 20/1).

Lipidation was accomplished by activation of 270 μmol carboxylated lipid with 270 μmol each of HBTU, HOBT and diisopropylethylamine (DIEA) in anhydrous DMF/DCM (DCM as needed for lipid solubilization) for 30 min at room temperature followed by addition of 67.5 μmol resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with DMF (4 x 10 mL) and DCM (4 x 10 mL) to remove unreacted lipids and dried under vacuum. Peptides were cleaved from the resin by treatment with trifluoroacetic acid containing 2.5% water, 2.5% ethanedithiol and 1% triisopropylsilane for 4 hours under argon. Cleaved peptides were precipitated into cold ethyl ether. The precipitate was pelleted by centrifugation at 3000 rpm (RT6000, Sorvall, Waltham, MA) and washed once with cold ethyl ether. The ether was poured off and the pellet was re-dissolved in methanol (MeOH), transferred to a round

bottom flask, dried by rotary evaporation under reduced pressure and further dried under high vacuum. In the case of N-MPR-DPG, lipopeptide was further separated from unconjugated peptide by reverse phase high pressure liquid chromatography (RP-HPLC; DX 500, Dionex, Sunnyvale, CA) on a semi-preparative C4 column (214TP510, Grace Vydac, Deerfield, IL) until unconjugated peptide was no longer detectable by MALDI-MS. Lipopeptide fractions were identified by MALDI-MS in 2,5-dihydroxybenzoic acid matrix, pooled and lyophilized. Stock lipopeptide solutions were prepared in MeOH or MeOH/$CHCl_3$ and stored at -20 ^{o}C. Final yields were approximately 5-10%. Molecular weights were as follows: N-MPR-DSG 2963.2 Da; N-MPR-DPG 2903.7 (+ Na^{+}) Da.

Biotinylated N-MPR peptide was prepared for use in ELISA by an analogous method. Biotin was attached to the deprotected C terminal amine by activation of 500 μmol D-biotin with 500 μmol HBTU/HOBT/DIEA in 1.65 mL anhydrous 1:1 DMF/dimethylsulfoxide (DMSO) for 30 min followed by addition of resin and continued reaction under argon for 24h at room temperature. Following the reaction, the resin was washed with 1:1 DMF/DMSO (3 x 10 mL), DMF (3 x 10 mL) and DCM (3 x 10 mL) and dried under vacuum. Biotinylated peptides were cleaved and purified as described above. Molecular weight of N-MPR-biotion was 2455.1 Da as determined by MALDI-MS. Biotin content was quantified by 4´-hydroxyazobenzene-2-carboxylic acid dye exclusion (Sigma #H2153) according to the manufacturer's instructions.

5.3.3. RAL Synthesis

The synthesis of all-*trans* retinoic acid phospholipid (RAL; **Figure 5-2**) was done by Dr. Zhaohua Huang. First, 1-O-octadecyl-2-O-benzyl-sn-glycerol was converted to phosphocholine by phosphorylation with phosphorus oxychloride and coupling to the choline tetraphenyl borate salt. Then the benzyl group was removed by catalytic transfer

hydrogenation. Finally, all-*trans* retinoic acid was attached to the 2-hydroxy group with the typical DCC/DMAP method [12].

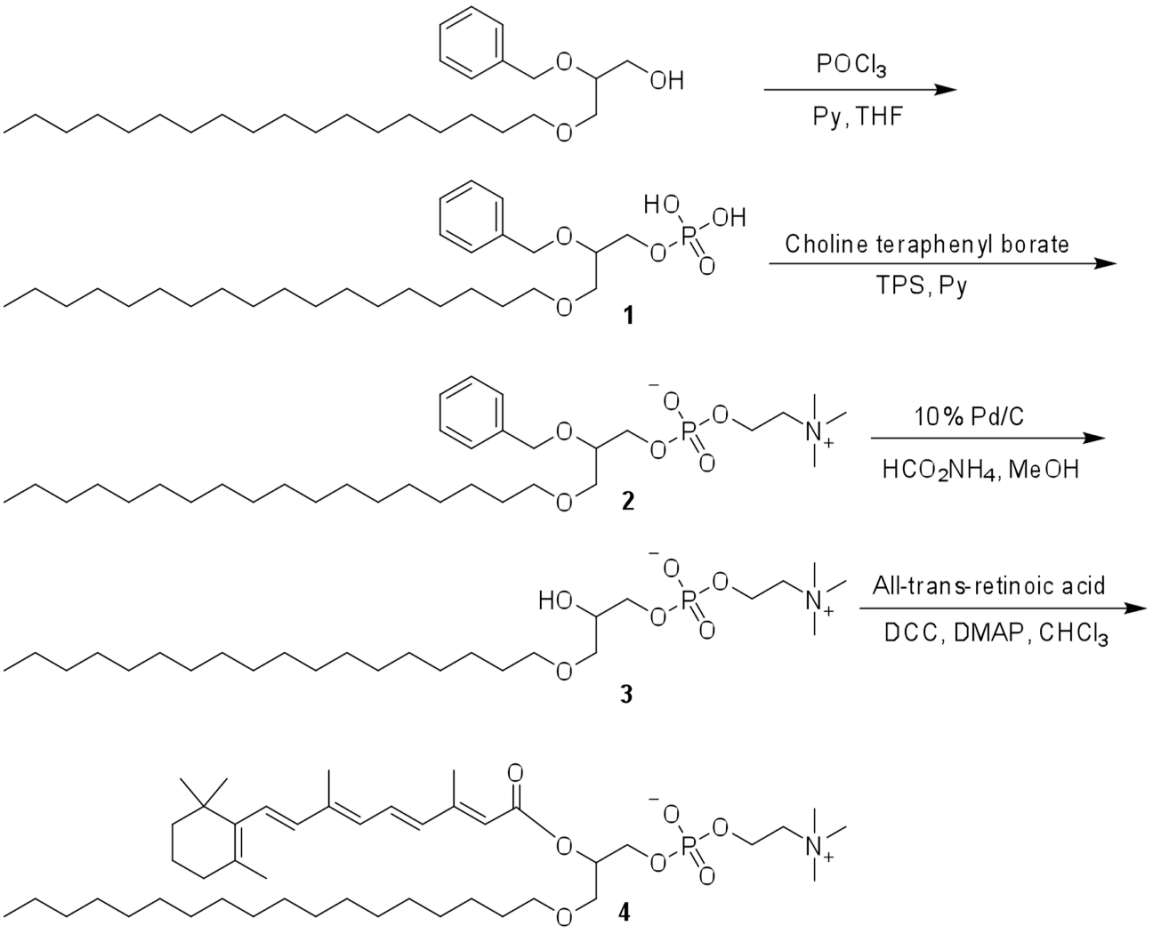


Figure 5-2. Synthesis of 1-O-Octadecyl-2-all-*trans*-retinoyl-sn-phosphocholine.

1-O-octadecyl-2-O-benzyl-sn-glycerol was from BACHEM (Torrance, CA). Other reagents were from Aldrich (Milwaukee, WI). TLC analyses were performed on 0.25-mm silica gel F_{254} plates using a variety of developing systems. High performance flash chromatography (HPFC) was carried out on a Biotage (Charlottesville, VA) Horizon™ HPFC™ system with pre-packed silica gel columns (60 Å, 40-63 μm). Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volumes. ^{1}H NMR spectra were acquired on a Varian 400 MHz instrument. Chemical shifts are expressed as parts per million using tetramethylsilane as internal standard. *J*

values are in Hertz. MALDI-TOF mass spectra were obtained at the Mass Spectrometry Facility, University of California San Francisco.

1-O-octadecyl-2-O-benzyl-sn-glycero-phosphate (1): A solution of 1-O-octadecyl-2-O-benzyl-sn-glycerol (10 g, 23 mmol) and anhydrous pyridine (3.72 mL, 2 equiv.) in anhydrous tetrahydrofuran (40 mL) was added dropwise to the freshly distilled phosphorus oxychloride (2.36 mL, 1.1 equiv.) in tetrahydrofuran (20 mL) with stirring at 0 °C. Stirring was continued for 3 h at 0 °C. Then 10% sodium bicarbonate (60 mL) was added, and the mixture was stirred at 0 °C for 15 min. The solution was then poured on ice water (200 mL), acidified with conc. HCl (pH ca. 2), and extracted with diethyl ether (400 mL × 2). The ether extracts were combined, dried over anhydrous sodium sulfate, evaporated under reduced pressure, azeotropically dried with toluene twice, and used directly for next step reaction. Yield: 11.8 g, 100%. TLC: R_f = 0.05 ($CHCl_3$/MeOH/NH_4OH, 65/25/4).

1-O-octadecyl-2-O-benzyl-sn-glycero-phosphocholine (2): Compound **1**(5.14 g, 10 mmol), choline tetraphenyl borate (8.46 g, 2 equiv.) and 2,4,6-triisoproylbenzene sulfonyl chloride (6.04 g, 2 equiv.) were dissolved in anhydrous pyridine (100 mL) with heating. The reaction mixture was stirred at 70 °C for 1h, then 3 h at room temperature. After the addition of water (10 mL), the solvents were removed by rotary evaporation. The residue was extracted with diethyl ether (250 mL × 2). The extracts were combined and evaporated. The crude product was purified by HPFC using a solvent gradient of 20% - 35% MeOH-H_2O (25/4) in chloroform. Yield: 5.8 g, 96.7%. TLC: R_f = 0.17 ($CHCl_3$/MeOH/H_2O, 65/25/4). ^{1}H NMR ($CDCl_3$-CD_3OD), δ 0.87 (t, J = 7.2, 3H); 1.27 (br, 30H); 1.57 (m, 2H); 3.15 (s, 9H); 3.36-3.60 (m, 7H); 3.83 (m, 1H); 4.04 (m, 1H); 4.16 (m,

2H); 4.68 (m, 2H); 7.31-7.45 (m, 5H). MALDI-MS calculated for $C_{33}H_{63}NO_6P^+$ $[M + H]^+$ 600.45, found 600.54.

1-O-octadecyl-2-hydroxy-sn-glycero-phosphocholine (3): To a solution of compound **2**(5.77 g, 9.6 mmol) in 70 mL methanol, were added 10% Palladium on activated carbon (2 g) and ammonium formate (3.03 g, 48 mmol) under nitrogen atmosphere. The mixture was stirred vigorously under nitrogen for 10 h at 60 °C. The mixture was cooled to room temperature, filtered through Celite 545. The filtrate was concentrated, and applied to a flash40+M column, purified with the HPFC system using an elution gradient of 30% -50% MeOH-H_2O (25/4) in chloroform. Yield: 3.3 g, 67%. TLC: R_f = 0.05 ($CHCl_3$/MeOH/H_2O, 65/25/4). ^{1}H NMR ($CDCl_3$-TFA, 10:1), δ 0.88 (t, J = 6.9, 3H); 1.27 (br, 30H); 1.63 (m, 2H); 3.25 (s, 9H); 3.64-3.85 (m, 6H); 4.30 (m, 1H); 4.58 (m, 1H); 4.68 (m, 2H); 5.03 (m, 1H). MALDI-MS calculated for $C_{26}H_{57}NO_6P^+$ $[M + H]^+$ 510.40, found 510.62.

1-O-octadecyl-2-all-trans-retinoyl-sn-glycero-phosphocholine (4; RAL): To a solution of compound 3 (0.6 g, 1.1 mmol) and all-trans retinoic acid (0.73 g, 2 equiv.) in dry ethanol-free chloroform, were added dimethylaminopyridine (0.15 g) and dicyclohexylcarbodiimide (0.5 g, 2.1 equiv.). The reaction mixture was kept in dark with stirring for 36 h. The precipitate was filtered and the filtrate was concentrated, applied to flash 25+M column, purified with the HPFC using a elution gradient of 10%-30% MeOH/28% NH_4OH (25/4) in chloroform. Yield: 626 mg, 72%. TLC: R_f = 0.52 ($CHCl_3$/MeOH/NH_4OH, 65/25/4). ^{1}H NMR ($CDCl_3$), δ 0.89 (t, J = 6.9, 3H); 1.04 (s, 6H); 1.26 (br, 30H); 1.45-1.53 (m, 4H); 1.63 (m, 2H); 1.72 (s, 3H); 2.00 (s, 3H); 2.04 (m, 2H); 2.34 (s, 3H); 3.32 (s, 9H); 3.41 (m, 2H); 3.58 (m, 2H); 3.78 (m, 2H); 4.00 (m, 2H); 4.30

(m, 2H); 5.17 (m, 1H); 5.79 (m, 1H); 6.12-18 (m, 2H); 6.28 (m, 2H); 7.01 (m, 1H). MALDI-MS calculated for $C_{46}H_{83}NO_7P^+$ $[M + H]^+$ 792.60, found 792.84.

5.3.4. RAL digestion by phospholipase A_2

The cleavage of retinoic acid from RAL by phospholipase A_2 (PLA_2) was examined *in vitro* by Kevin Park, an undergraduate summer student, under the direction of Dr. Zhaohua Huang. 10 μmol RAL were dried to a thin film in a borosilicate glass tube by rotary evaporation under reduced pressure and following by further drying under high vacuum overnight. The film was hydrated in 2 mL containing 10 mM HEPES, 25 mM KCl, 10 mM $CaCl_2$ and 20 mM Triton-X, pH 8.8 and heated at 45 ^oC for 20 min. Fifty μL of a 100 U/mL solution of snake venom PLA_2 (Sigma #P7778) were added to 250 μL of the RAL solution and incubated for 30 min. When analyzed by TLC, incubation with PLA_2 resulted in total loss of the original RAL spot (R_f = 0.38) and appearance of two new spots, corresponding to free retinoic acid (R_f = 0.28) and lysolipid (R_f = 0.18; $CHCl_3$/MeOH/NH_4OH, 65/25/4).

5.3.5. Liposome preparation

Lipopeptides were formulated in liposomes composed of 15:2:3 DMPC:DMPG:Cholesterol with MPL, RA and lipopeptide as indicated [13, 14]. Prior to use, glassware was rinsed with MeOH and $CHCl_3$ and dried for at least 90 min at 150 ^oC to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. Films were further dried under high vacuum overnight. Lipids were hydrated in sterile PBS (UCSF Cell Culture Facility) by intermittent vortexing and bath sonication under argon for a brief period (approximately 15 seconds) to disperse the lipids into the buffer. Defined diameter

vesicles were formed by extrusion 11 times through 400 nm polycarbonate membranes using a hand-held extruder (Avestin, Ottowa, Canada). To prevent contamination, the extruder was disassembled and thoroughly cleaned with MeOH and sterile PBS between samples. Vesicle size was characterized by dynamic light scattering (Zetasizer 3000, Malvern, New Bedford, MA). Liposomes were stored at 4 ^{o}C under argon until use.

5.3.6. Animal immunizations

All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. Eight week-old female BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF specific pathogen-free barrier facility. Animals received subcutaneous immunizations in alternating hind hocks on Days 0 and 14 as described [15]. Each injection contained 50 μg lipopeptide, 25 μg MPL or 25 μg RA (for RAL, a molar equivalent to RA was used) and 1 μmol lipid vehicle in 50 μL sterile phosphate-buffered saline. On Day 28 blood was collected from the submandibular vein for characterization of antibody responses.

5.3.7. ELISA

Peptide ELISAs were conducted using MPR peptides biotinylated and captured on 96 well streptavidin-coated plates (Pierce, Rockford, IL). Assays were performed according to the manufacturer's instructions. Titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 pre-immune sera after subtraction of background wells lacking serum. IgG1/IgG2a ratios were calculated as an average of optical density quotients measured at 3 dilutions after subtraction of background values. All samples were assayed in duplicate.

5.3.8. Statistical analysis

Statistical significance was assessed by analysis of variance and two-tailed Student's t test using Microsoft Excel and SigmaPlot. Differences were considered significant if they exhibited p values < 0.05 in the Student's t test.

5.4. Results and Discussion

This study sought to address the ability of ATRA to promote the antibody response to a co-delivered peptide antigen. A liposomal delivery system was desirable because liposomes effectively co-deliver associated antigens and adjuvants to immune cells *in vivo* [10, 16]. N-MPR was derivatized with diacylglycerol because covalent attachment of lipid anchors was previously found to substantially enhance the antibody response to MPR peptides and other antigens formulated in liposomes [13]. Incorporation of ATRA, 13-*cis* RA, or MPL into liposomes containing N-MPR-DSG (N-MPR-succinyldistearoylglycerol) did not appreciably affect vesicle size (**Table 5-1**).

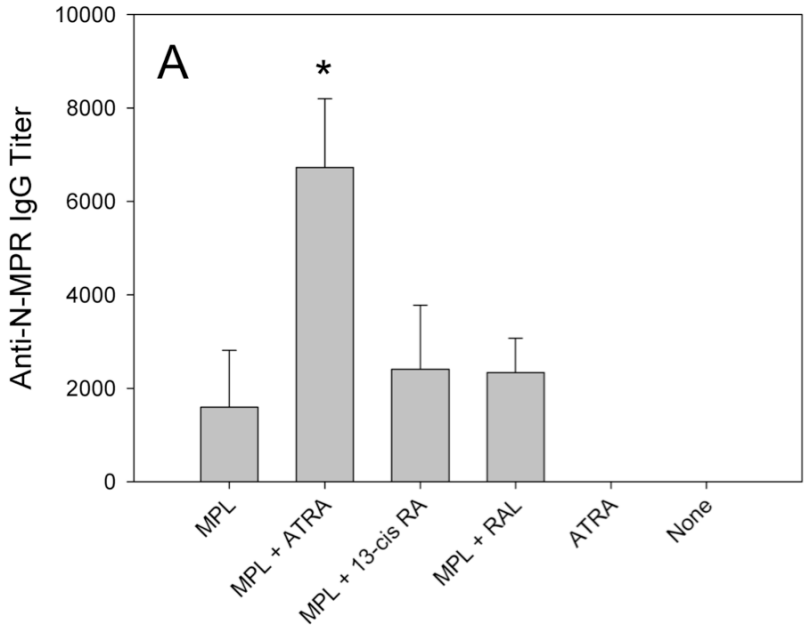
Formulation	Vesicle Diameter (nm)	Standard Deviation (nm)
MPL	244.3	2.0
MPL + ATRA	262.6	5.2
MPL + 13-cis RA	335.1	15.8
MPL + RAL	236.0	4.0
ATRA	246.6	5.3
None	259.6	5.7

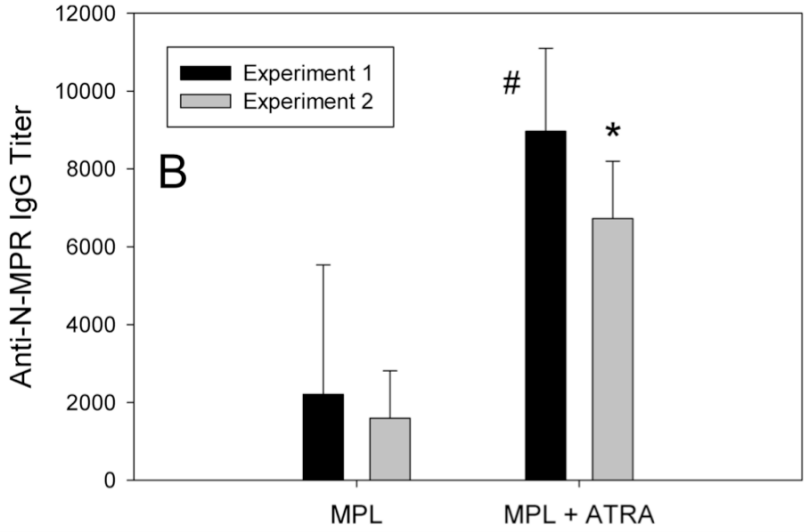
Table 5-1. Vesicle sizes of lipopeptide liposomes determined by dynamic light scattering.

ATRA alone did not stimulate production of antibodies to a co-delivered lipopeptide antigen, N-MPR-DSG. However, addition of ATRA to a liposomal formulation containing MPL resulted in a reproducible four-fold enhancement of antibody titers to an MPR-

derived lipopeptide in BALB/C mice (p = 0.00039; **Figure 5-3**). The trend persisted 7 months after the final immunization, although the difference was not significant at the later time point (titers of 470 and 3250 for 'MPL' + 'MPL + ATRA'). A similar enhancement was also observed with a lipopeptide antigen containing shorter acyl chains, N-MPR-DPG (N-MPR-succinyldipalmitoylglycerol; data not shown). The failure of *13-cis* RA to modulate anti-N-MPR antibody responses suggests that the effect of ATRA is biological, as the two compounds differ by only a single bond orientation (**Figure 5-1**), which is not expected to alter the biophysical properties of the formulation. Additionally, it was hypothesized that attaching ATRA to a lipid anchor (**Figure 5-1**) would afford greater retention of ATRA in the liposomal bilayer, assuring delivery of a higher fraction of the dose to immune cells. However, lipid-anchored retinoic acid (RAL) failed to promote anti-N-MPR antibody responses *in vivo,* raising the question of whether this prodrug approach can deliver retinoic acid to the correct compartment to enhance the immune response.

The magnitude of enhancement mediated by ATRA is comparable to the benefit observed in mice and rabbits when liposomes containing MPL and a recombinant malaria antigen were adsorbed onto aluminum hydroxide, the only adjuvant currently approved for use in the United States [17, 18]. However, several previously reported immunomodulatory effects of ATRA were not observed in the present study. Despite reports showing that ATRA can promote class switching and IgA production [19], anti-N-MPR IgA antibodies were not detected in sera of mice from any group (data not shown). Additionally, the IgG1/IgG2a ratio was not significantly altered by incorporation of ATRA in the formulation (**Figure 5-3c**), suggesting that the T helper profile of the response was unaffected. Although this finding conflicts with prior studies reporting that ATRA





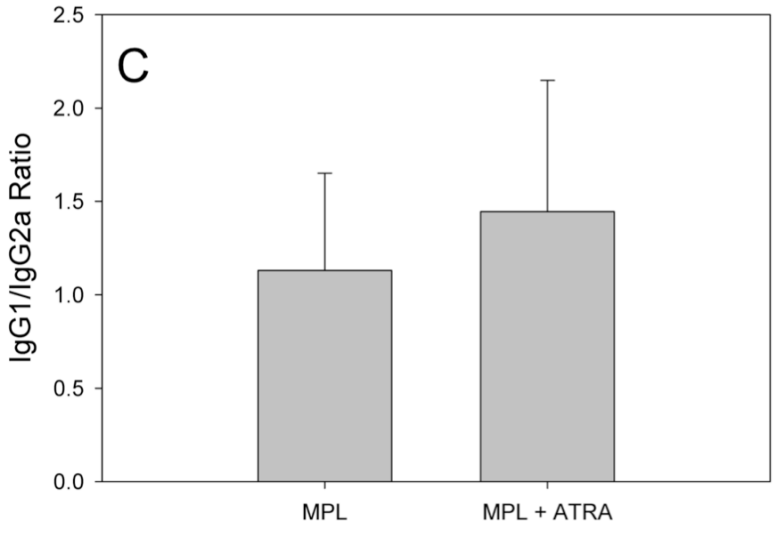


Figure 5-3. Effect of ATRA on total IgG anti-N-MPR antibodies to a lipopeptide antigen adjuvanted with lipid A.

Panel A:
ATRA, but not 13-cis RA, potentiates the MPL-mediated IgG response to N-MPR. Each group represents 5 animals except 'MPL + RAL', which consisted of 4 animals. Titers are expressed as geometric means and error bars represent standard deviations. * p = 0.00039 vs 'MPL', 0.0017 vs 'MPL + 13-cis RA', 0.00095 vs 'MPL + RAL'.

Panel B:
Reproducibility of ATRA enhancement. Experiments 1 and 2 consisted of 4 and 5 animals per group, respectively. Titers are expressed as geometric means and error bars represent standard deviations. # p = 0.026 in Experiment 1; * p = 0.00039 in Experiment 2.

Panel C:
ATRA does not significantly alter the IgG1/IgG2a balance of anti-N-MPR antibody responses. Each group represents 4 animals and error bars represent standard deviations.

supplementation promotes a Th2 phenotype [3], it may be explained by the dominant effect of MPL. Alternatively, it may be due to the nature of the antigen used; prior studies utilized large protein antigens with many B and T cell epitopes, whereas this study employed a small peptide with only a few epitopes.

Further study is needed to determine the mechanistic basis of the interaction between ATRA and MPL. One possibility is the activation of MAP kinases such as ERK, JNK and p38 by ATRA in antigen presenting cells [4]. These MAP kinases also play a role in MyD88-dependent immune activation upon MPL/TLR4 engagement [20]. Alternatively, ATRA is known to promote IL-2 signaling in CD4 T cells, which may increase T cell help to B cells [21]. Both of these effects are mediated by engagement of the retinoic acid receptor (RAR). As 13-*cis* RA binds RAR more weakly than ATRA, an RAR-dependent mechanism would be consistent with the lack of effect of 13-*cis* RA observed in this study [22]. In summary, the data presented here indicate an interaction between ATRA and MPL in the antibody response to a peptide antigen, warranting further investigation of ATRA as a humoral vaccine adjuvant.

5.5. Acknowledgements

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Chapter 6

Outlook: A role for MPR lipopeptides in eliciting neutralizing antibodies against HIV-1

6.1 Abstract

This dissertation explored the utility of MPR lipopeptides in eliciting neutralizing antibodies to HIV-1. **Chapters 2-5** described a series of experiments in which MPR lipopeptides were designed, characterized and employed in the evaluation of candidate vaccine adjuvants and delivery systems. In this final chapter, the ability of these immunogens to elicit neutralizing antibodies in mice, rabbits and hens was assessed. A limited number of animals and birds were immunized with MPR lipopeptides and *in vitro* neutralization activity of MPR-reactive sera and IgY was determined. The results indicate that MPR lipopeptides may elicit weak neutralization activity in rabbits, warranting further study of this approach.

In the concluding pages, the key findings of the dissertation are summarized in the context of the original hypothesis that membrane-bound MPR immunogens would elicit antibodies that cross-react with phospholipids and neutralize HIV. Subsequently, “next steps” toward MPR immunogen design are recommended and a possible role for MPR lipopeptides as one component of a preventative vaccine against HIV-1 is suggested. Lastly, the near-term outlook for development of an effective HIV vaccine is discussed.

6.2 Materials and methods

6.2.1 Materials

Cholesterol, dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Monophosphoryl lipid A derived from Escherichia coli (MPL; #L6638) was obtained from Sigma-Aldrich. Unless

otherwise specified, all other reagents were obtained from Sigma-Aldrich.

6.2.2 Liposome preparation

Liposomes were prepared essentially as described in **Chapters 2-5**. In brief, lipopeptides were formulated in liposomes composed of 15:2:3:0.3 DMPC:DMPG:Cholesterol:MPL [1]. Prior to use, glassware was rinsed with MeOH and $CHCl_3$ and dried for at least 90 min at 150 ^oC to destroy pyrogens. Lipid solutions were combined in borosilicate glass tubes and dried to a thin film by rotary evaporation under reduced pressure. Films were further dried under high vacuum overnight. Lipids were hydrated in a phosphate buffer containing 5% (w/v) sucrose as a cryoprotectant due to concern regarding long-term stability of formulations at 4 ^oC. In control experiments liposomes prepared in 5% sucrose did not exhibit significant change in vesicle size after freezing at 80 ^oC and thawing to room temperature (data not shown).

The hydration solution was buffered with sodium phosphate, with total salts adjusted to maintain physiological osmolarity (300 mOsm). The final buffer contained 5% (w/v) sucrose, 20 mM $NaPO_4$, 57 mM NaCl (pH 7.4). Lipid films were hydrated in this buffer by intermittent vortexing and bath sonication under argon for a brief period (approximately 15 seconds) to disperse the lipids into the buffer. Defined diameter vesicles were formed by extrusion 11 times through 400 nm polycarbonate membranes using a hand-held extruder (Avestin, Ottowa, Canada) [2]. To prevent contamination, the extruder was disassembled and thoroughly cleaned with MeOH and sterile PBS between samples. The final formulation contained 1.0 mg/mL lipopeptides and 0.5 mg/mL monophosphoryl lipid A in 20 mM carrier lipid. Vesicle size was characterized by dynamic light scattering (Zetasizer 3000, Malvern, New Bedford, MA). Liposomes were stored at -20 ^oC under argon until use.

6.2.3 Animal immunizations

Rabbit immunizations were conducted by Covance, Inc. in accordance with the guidelines of their internal Institutional Animal Care and Use Committee. Liposome preparations were divided into three aliquots per animal and shipped frozen on dry ice to Covance. Rabbits received 1 mg total lipopeptide immunogen of the composition reported in **Table 6-1** in three doses on Days 0 (250 μg), 21 (125 μg) and 49 (125 μg).

Rabbit ID	Hen ID	Immunogen	Dose (% of total)
4207	6027	C-MPR-DSG	16.7
		N-MPR-DSG	33.3
		N-MPR-CHOL	33.3
		N-MPR-PC	16.7
4208	6028	C-MPR-DSG	16.7
		N-MPR-DSG	33.3
		N-MPR-CHOL	33.3
		N-MPR-PC	16.7
4462	6151	C-MPR-CHEMS(SPO_3)	50
		C-MPR-CHEMS(YNO_2)	50
4463	6152	C-MPR-CHEMS(SPO_3)	50
		C-MPR-CHEMS(YNO_2)	50
4464	6153	N-MPR-CHEMS(TPO_3)	50
		N-MPR-CHEMS(SPO_3)	50
4465	6154	N-MPR-CHEMS(TPO_3)	50
		N-MPR-CHEMS(SPO_3)	50

Table 6-1. Lipopeptide composition of rabbit and hen immunizations

Formulations were emulsified in Freund's complete (first dose) or incomplete (second and third doses) adjuvant at a 1:1 volume ratio prior to injection. On Day 59, serum was collected via a terminal bleed. Serum was received frozen from Covance and stored at -20 ^{o}C until use.

Hen immunizations were conducted by Aves Labs, Inc. in accordance with the guidelines of their internal Institutional Animal Care and Use Committee. Liposome preparations were divided into three aliquots per animal and shipped frozen on dry ice to Aves Labs. Hens received 2 mg total lipopeptide immunogen of the composition reported in **Table 6-1** in three doses on Days 0 (1000 μg), 21 (500 μg) and 49 (500 μg). Formulations were emulsified in Freund's complete (first dose) or incomplete (second and third doses) adjuvant at a 1:1 volume ratio prior to injection. On Day 65, immunoglobulin Y (IgY) was affinity-purified from collected eggs. Purified IgY in PBS was received at 4 ^{o}C from Aves Labs and stored at 4 ^{o}C until use.

6.2.4 ELISAs

ELISAs were employed to detect antibodies to MPR peptides, recombinant gp140 and lipids as described in **Chapters 2-5**. Peptide ELISAs were conducted using MPR peptides biotinylated and captured on 96 well streptavidin-coated plates (#15120, Pierce, Rockford, IL). Assays were performed according to the manufacturer's instructions with modifications. Biotinylated peptides were added to wells in PBS containing 0.1% Tween-20 (PBS-T) and incubated for 2 hr at 37 ^{o}C. Following a wash step, sera were serially diluted in PBS containing 0.1% casein (C7078, Sigma-Aldrich) (PBS-C), added to wells and incubated for 30 min at 37 ^{o}C. After reconstitution, horseradish peroxidase-conjugated secondary antibodies (IgG, IgG1, IgG2a; Jackson Immunoresearch, West Grove, PA) were diluted 1:1 in glycerol for long-term storage at -

20 ^{o}C and further diluted 1:1000 in PBS-C immediately prior to use. Following a wash step, secondary antibodies were added to wells and incubated for 30 min at 37 ^{o}C. Following a final wash step, a tetramethylbenzidine substrate solution (#T0440, Sigma-Aldrich) was added to wells and incubated for 30 min at room temperature. The reaction was stopped with 0.5M H_2SO_4 and the yellow product was monitored at 450 nm (Optimax, Molecular Devices, Sunnyvale, CA). All incubations were done in 100 μL volumes and wells were washed 6 times with PBS-T between each step. All samples were assayed in duplicate.

Lipid ELISAs were performed as described with modifications [3]. For lipid ELISAs, 33 nmol total lipids of the DMPC:DMPG:Chol:MPL composition described above were diluted to 0.2 mg/mL in EtOH and 50 μL per well were added to flat-bottomed untreated polystyrene plates (Fisher) and allowed to dry overnight. For liposome ELISAs, 33 nmol total lipids of pre-formed liposomes composed of DMPC:DMPG:Chol:MPL in PBS were added to wells in 50 μL and allowed to dry overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were diluted 1:200 in 10% fetal bovine serum in PBS and incubated in wells for 1 hr. Wells were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 1 hr. Following a wash step, plates were then read as indicated above. All incubations were done in 100 μL volumes at room temperature and wells were washed 6 times with PBS between each step.

Recombinant gp140 ELISAs were performed follows: Ba-1 gp140 (Immune Technology Corp, New York, NY) was diluted to 5 μg/mL in 50 mM sodium carbonate, pH 9.6 and 100 μL per well were added to flat-bottomed high capacity immunoassay plates (Costar).

Plates were sealed with parafilm and incubated at 4 ^{o}C overnight. Plates were blocked with 0.5% casein for 2 hr. After a wash step, immune sera were diluted 1:100 in PBS-C and incubated in wells for 1 hr. Wells were washed and peroxidase-conjugated anti-mouse IgG was diluted 1:1000 in PBS-C and added to wells for 30 min. Following a wash step, plates were then developed and read as indicated above. All incubations were done in 100 μL volumes at 37 ^{o}C and wells were washed 6 times with PBS-T between each step.

6.2.5 In vitro neutralization assays

Sera and chicken egg immunoglobulin samples from contracted immunization experiments were sent to Duke university for evaluation of neutralization activity under the direction of Dr. David Montefiori according to a standardized procedure [4, 5]. Samples were thawed, sterile-filtered, aliquotted into sterile eppendorf tubes and shipped overnight to Duke University at 4 ^{o}C on refrigerant blocks. Serum samples from mice immunized with N-MPR lipopeptides were pooled by group and also sent for assessment of neutralization activity. Chicken pre-immune IgY samples contained preservative (sodium azide or thimerosal) and were dialyzed at 1:300 against PBS with three buffer changes to remove the preservative prior to shipment. Samples were assayed for neutralization activity against several laboratory-adapted virus strains known to be sensitive to MPR-directed neutralization [4]. In this assay, CD4-expressing target cells are stably transfected to express luciferase inducibly upon infection by HIV. Reduction of luciferase is measured as a surrogate of decreased infection and thus neutralization (**Figure 6-1**). Data are expressed as ID50 values, where the number represents the dilution required to achieve a 50% reduction in luciferase expression by target cells.

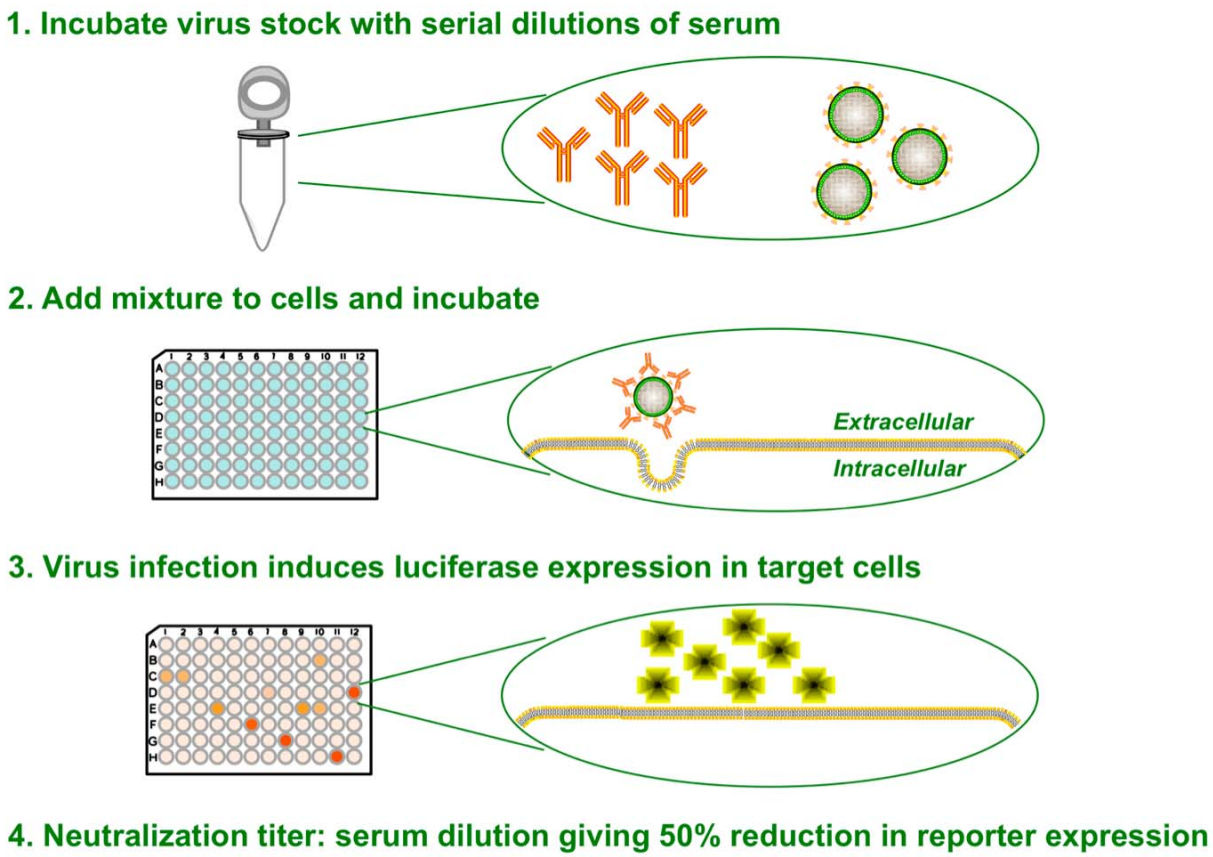

Figure 6-1. Schematic of *in vitro* virus neutralization assay.

6.3 Results

We sought to determine if the lipopeptide immunogens described in **Chapters 2-5** could elicit antibodies capable of neutralizing MPR-sensitive HIV-1 strains *in vitro*. We first evaluated the neutralization activity of pooled sera of mice immunized with N-MPR lipopeptides (**Table 6-2**). The results revealed sporadic weak neutralization of two laboratory strains of HIV-1. However, similar levels of neutralization activity were also detected against a control virus, murine leukemia virus, indicating that the neutralization was not specific to HIV-1. This is consistent with a number of reports demonstrating that mice are not an ideal species for neutralization experiments due to non-specific neutralization activity often detected in murine sera [4].

	ID50 in TZM-bl cells[1]		
Immunogen	**HIV-1 SF162.LS**	**HIV-1 SC422661.8**	**Control MLV**
Empty liposomes	47	80	81
N-MPR peptide	29	62	49
N-MPR-PA	47	69	58
N-MPR-PC	40	72	48
N-MPR-CHOL	73	103	113
N-MPR-DPG	<20	42	40
N-MPR-CL	20	52	47
N-MPR-PE	<20	46	28
N-MPR-SM	20	44	40
N-MPR-CHEMS	<20	36	23
2F5 mAb	1.7 μg/ml	0.9 μg/ml	>25 μg/ml
4E10 mAb	5.1 μg/ml	1.7 μg/ml	>25 μg/ml

Table 6-2. Neutralization of HIV-1 viruses by serum of mice immunized with N-MPR lipopeptides.

Thus, a limited number of rabbits and hens were immunized with liposomes containing pooled lipopeptide antigens as described in **Table 6-1**. Study design was due to financial constraints of the contracted immunizations as well as limitations on the amount of available lipopeptide material. Rabbits were selected because they are a common species for experiments of this nature [6]. Hens were selected because antibody diversity is generated by different mechanisms in hens as compared to in mammals and the use of hens has been suggested for production of antibodies that are normally not made due to self tolerance [7-9]. Hens also have CDRH3 sequences of up to 19 amino acids, longer than those found in mice [10]. All animals and hens developed significant antibodies to MPR peptides (**Figure 6-2a-b** and data not shown). Most hens also

developed detectable antibodies to recombinant gp140, whereas rabbits did not (**Figure 6-2c-d**). Unfortunately, initial experiments revealed no neutralization activity in samples derived from either species (**Tables 6-3 and 6-4**).

Given the substantial peptide reactivity detected by ELISA (anti-N-MPR titers 10^5-10^6; anti-C-MPR titers 10^3-10^5), additional assays were conducted in an alternative target cell line that is more sensitive to MPR-mediated neutralization [4]. As seen from control experiments done with bnAb 2F5 and 4E10, viruses are 5-10 times more sensitive to MPR-mediated neutralization in these cells. Indeed, neutralization activity was detected in each of the four rabbits tested, all of which received N-MPR-CHEMS or C-MPR-CHEMS lipopeptides with side chain modifications (**Table 6-1** and **Table 6-3**). No such activity was detected in these cells in hen samples (**Table 6-4**). Thus, the totality of the data indicates that MPR lipopeptides elicit detectable but modest neutralizing activity in the serum of immunized rabbits. The lipopeptide sequences that elicited neutralizing activity in rabbits are summarized in **Table 6-5**.

One key aspect of the hypothesis presented in **Chapter 1** involved the ability of liposomal MPR lipopeptides to elicit anti-lipid antibodies. In **Chapters 2 and 3**, it was demonstrated that these formulations did not elicit anti-lipid antibodies in mice under the conditions studied. To determine if similar formulations elicited anti-lipid responses in rabbits, anti-lipid and anti-liposome ELISAs were performed. As described in **Methods**, these were intended to detect antibodies with potentially different binding characteristics: one to detect antibodies to individual lipids without any aggregate structure (anti-lipid), and one to detect antibodies to pre-formed lipid membranes (anti-liposome).

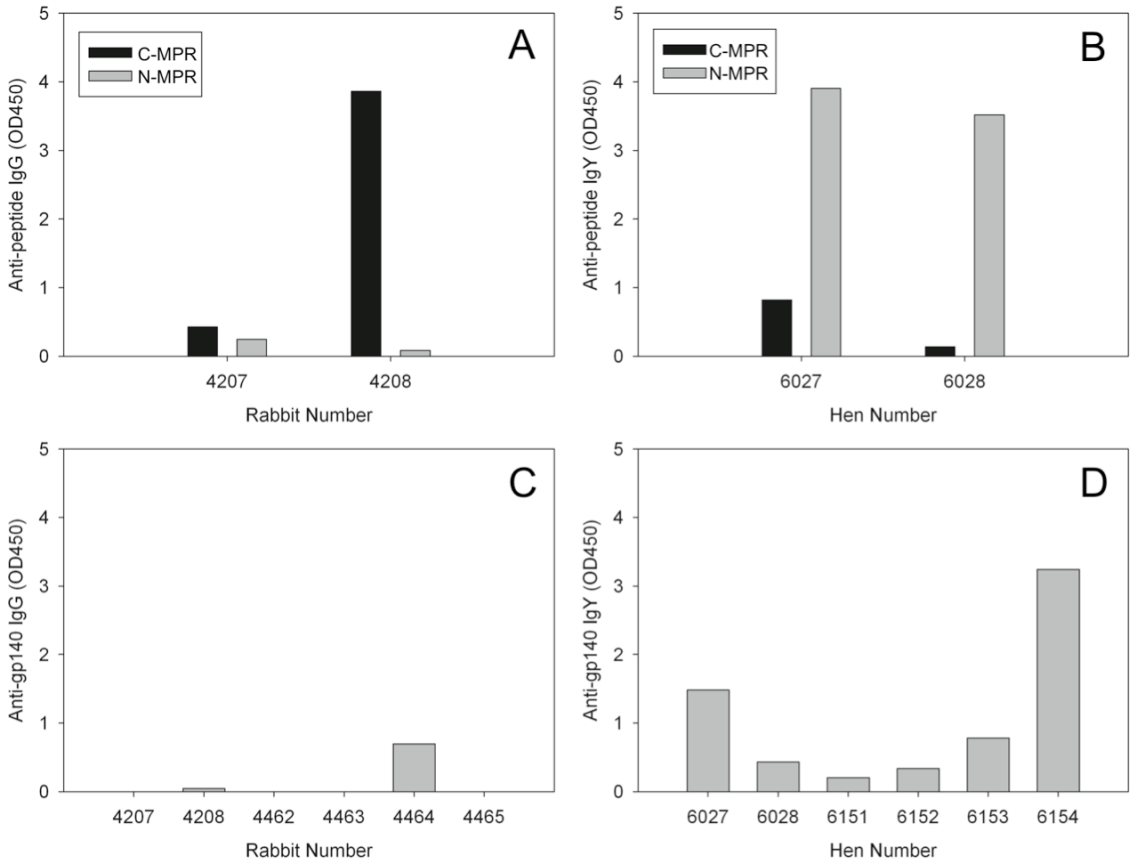

Figure 6-2. Anti-MPR (A, B) and anti-gp140 (C, D) antibodies in sera of immunized rabbits (A, C) and egg IgY of immunized hens (B, D). See **Table 6-1** for information regarding immunization protocols.

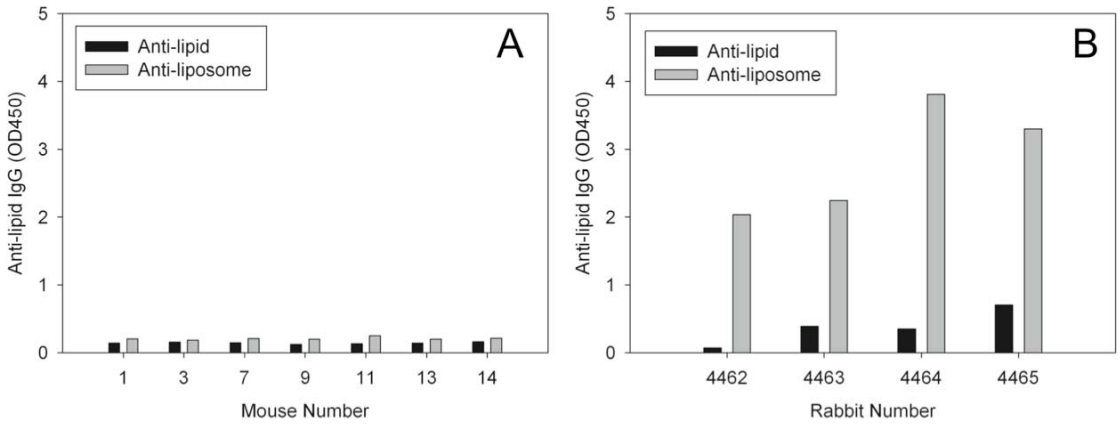

Figure 6-3. Anti-lipid and anti-liposome antibodies in sera of mice (A) and rabbits (B) immunized with MPR lipopeptides.

ID50 in TZM-bl cells				
Rabbit ID	**Time point**	**HIV-1 SF162.LS**	**HIV-1 SC422661.8**	**Control MLV**
4207	Preimmune	<20	<20	<20
	Immunized	<20	25	<20
4208	Preimmune	<20	22	<20
	Immunized	<20	<20	<20
4462	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
4463	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
4464	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
4465	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
2F5 mAb		1.7 μg/ml	0.9 μg/ml	>25 μg/ml
4E10 mAb		5.1 μg/ml	1.7 μg/ml	>25 μg/ml
ID50 in M7-Luc cells				
Rabbit ID	**Time point**	**HIV-1 SF162.LucR.T2A.ecto**	**HIV-1 Bal.LucR.T2A.ecto**	
4462	Preimmune	66	<20	
	Immunized	92	<20	
4463	Preimmune	32	67	
	Immunized	118	72	
4464	Preimmune	<20	<20	
	Immunized	55	<20	
4465	Preimmune	<20	<20	
	Immunized	92	31	
2F5 mAb		0.2 μg/ml	0.3 μg/ml	
4E10 mAb		0.9 μg/ml	0.7 μg/ml	

Table 6-3. Neutralization of HIV-1 viruses by sera of rabbits immunized with N-MPR lipopeptides.

ID50 in TZM-bl cells				
Hen ID	**Time point**	**HIV-1 SF162.LS**	**HIV-1 SC422661.8**	**Control MLV**
6027	Preimmune	<20	<20	<20
	Immunized	51	65	42
6028	Preimmune	<20	<20	<20
	Immunized	45	47	36
6151	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
6152	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
6153	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
6154	Preimmune	<20	<20	<20
	Immunized	<20	<20	<20
2F5 mAb		1.7 μg/ml	0.9 μg/ml	>25 μg/ml
4E10 mAb		5.1 μg/ml	1.7 μg/ml	>25 μg/ml
ID50 in M7-Luc cells				
Hen ID	**Time Point**	**HIV-1 SF162.LucR.T2A.ecto**	**HIV-1 Bal.LucR.T2A.ecto**	
6151	Preimmune	<20	<20	
	Immunized	<20	<20	
6152	Preimmune	<20	<20	
	Immunized	<20	<20	
6153	Preimmune	<20	<20	
	Immunized	<20	<20	
6154	Preimmune	<20	<20	
	Immunized	<20	<20	
2F5 mAb		0.2 μg/ml	0.3 μg/ml	
4E10 mAb		0.9 μg/ml	0.7 μg/ml	

Table 6-4. Neutralization of HIV-1 viruses by egg IgY of hens immunized with N-MPR lipopeptides.

It was postulated that differences would be observed between these two assays. However, sera of immunized mice exhibited binding in neither assay (**Figure 6-3**). In contrast, sera of immunized rabbits did not bind to lipids but bound strongly to dried lipid membranes. These antibodies may have resulted from the more potent adjuvant (Freund's) in which the immunogen was administered.

Immunogen	Sequence	Neutralizing Titer SF162.LucR.T2A.ecto
C-MPR(SPO_3)-CHEMS	AS(PO_3)LWNWFDITNWLWYIKAAK-CHEMS	92, 118
C-MPR(YNO_2)-CHEMS	NWFDITNWLWY(NO_2)IKAAK-CHEMS	92, 118
N-MPR(TPO_3)-CHEMS	NEQELLELDKWASLWNWFDIT(PO_3)GGK-CHEMS	55, 92
N-MPR(SPO_3)-CHEMS	NEQELLELDKWAS(PO_3)LWNGGK-CHEMS	55, 92

Table 6-5. Sequences of lipopeptide immunogens that elicited neutralizing antibodies in rabbits.

6.4 Discussion

6.4.1 Summary of key findings

The membrane proximal region (MPR) of HIV-1 gp41 is a desirable target for development of a vaccine that elicits neutralizing antibodies since the patient-derived monoclonal antibodies, 2F5 and 4E10, bind to MPR and neutralize primary HIV isolates. The 2F5 and 4E10 antibodies cross-react with lipids and structural studies suggest that MPR immunogens may be presented in a membrane environment. This dissertation

tested the hypothesis that 4E10- and 2F5-like neutralizing antibodies can be elicited by presenting minimal MPR peptides in the context of lipid bilayers.

In **Chapter 2** we tested the hypothesis that liposomal vaccines containing membrane-anchored, lipid-conjugated MPR peptide segments would elicit antibodies that cross-react with both peptide epitopes and membrane lipids. We believed that cross-reactivity would contribute to HIV neutralization potency. To address this question, we synthesized the peptide epitopes of 4E10 and 2F5 and attached them to a series of lipids. These conjugates were formulated in liposomes and their biophysical and immunologic properties were characterized. In a comparison of eight different lipids conjugated to an extended 2F5 epitope peptide, cholesterol hemisuccinate (CHEMS), was found to promote the strongest anti-peptide titers in BALB/C mice. Conjugation to CHEMS also rendered a 4E10 epitope peptide immunogenic, whereas none of the other lipids tested were able to generate a response. In an analysis of the biophysical contributions to the antibody response, it was found that no single factor, such as position of the lipid anchor, peptide helical content, lipopeptide partition coefficient, or presence of phosphate on the anchor determined the ability of a lipopeptide to elicit anti-peptide antibodies. These conjugates could be useful for studying the effects of peptide structure within membranes on the antibody response to vaccine candidates. The formulations elicited high titers of anti-peptide antibodies but did not elicit antibodies that cross-reacted with lipids or neutralized HIV, suggesting that further work was needed to realize the full potential of the strategy.

In **Chapter 3**, we tested the hypothesis that tolerance mechanisms contribute to the weak antibody responses to the MPR that occur following vaccination or infection. This question is critical because it may determine whether antibody responses to the MPR

are inherently limited by tolerance mechanisms or can be enhanced through improved immunogen design. The MPR lipopeptides prepared in **Chapter 2** were utilized to address this issue. We initially hypothesized that the antibody response against the MPR, particularly the epitope of the broadly neutralizing monoclonal antibody 4E10, is restricted by immunologic tolerance. This hypothesis was tested through comparative assessment of antibody responses to MPR lipopeptides in two mouse models of defective immune tolerance – NZBxW/F1 and NOD. However, antibody responses to the 4E10 epitope remained very weak in these models, suggesting that tolerance mechanisms are insufficient to explain the poor antibody responses to the 4E10 epitope in these mice.

Building upon these findings, we postulated that anti-MPR antibody responses could be increased through derivatization of amino acid side chains with phosphate or nitrate groups. This hypothesis arose from observations that similar modifications, which occur as a result of inflammation or aberrant post-translational modification, lead to the generation of “neoepitopes” with altered immunity in cancer and autoimmunity. Similar inflammatory environments occur during HIV infection, and if neutralizing antibodies are generated as a result of these modifications it would explain the rarity of such antibodies. Indeed, an evaluation of MPR immunogens containing these modifications revealed a single modification ($S668PO_3$) that increased IgG responses to the 4E10 epitope by an order of magnitude. When interpreted in the context of the data generated from immunization of mice with defective tolerance, these results suggest that antibody responses to the MPR are not inherently limited and can be enhanced through immunogen design.

In **Chapter 4**, MPR lipopeptides were employed as model immunogens to evaluate the utility of metal chelation via nitrilotriacetic acid (NTA) as a method for association of polyhistidine-tagged protein antigens to particulate delivery systems. It has been demonstrated that physical or chemical association of the antigen to the particulate is required for optimal antibody responses to subunit antigens; however, traditional association methods can disrupt protein structure and damage vital epitopes. Attachment to liposomes via lipid-anchored NTA molecules has been proposed by Mumper and Altin as a non-destructive alternative [11, 12]. Importantly, the site-specific nature of NTA-Ni(II)-His attachment allows precise control of antigen orientation on the particulate surface, which is of particular interest for delivery of membrane protein antigens such as the MPR. We hypothesized that increased affinity of the antigen for the particulate carrier, mediated by recently developed multivalent NTA molecules, would correspond to enhanced antibody responses. To test this hypothesis, the effect of the antigen-liposome linkage on antibody responses to two model antigens, ovalbumin and N-MPR, was determined in mice. The data indicated that NTA-mediated association with an MPL-adjuvanted liposomal carrier elicited increased antibody responses to a weak antigen (N-MPR) but not a strong antigen (ovalbumin) as compared to admixed formulations. In both cases, covalently attached antigens elicited significantly stronger antibody responses than NTA-anchored antigens. Additionally, trivalent NTA did not confer an enhancement of antibody response over monovalent NTA. Thus further improvements of the NTA-mediated conjugation strategy are required before it will be an effective method to attach antigens to particulate vaccine carriers and elicit high titer antibody responses.

In **Chapter 5**, the ability of a novel liposomal adjuvant, retinoic acid (RA), to promote antibody responses to the MPR was determined in mice. The MPR elicits low titer

antibodies after vaccination and infection, and HIV vaccine development would benefit from novel adjuvants that can enhance the magnitude or quality of the response. Recent reports of synergy of RA with other vaccine adjuvants led us to hypothesize that this molecule would be a potent liposomal adjuvant. When tested in BALB/C mice, RA alone did not elicit antibodies to an MPR-derived lipopeptide co-formulated in liposomes. However, addition of all-*trans*, but not 13-*cis*, RA to a liposomal formulation containing the TLR4 agonist monophosphoryl lipid A resulted in a four-fold enhancement of antibody titers as compared to a formulation containing monophosphoryl lipid A alone. This result indicates that *all-trans* RA warrants further study as a vaccine adjuvant.

6.4.2 Outlook for MPR lipopeptides in eliciting neutralizing antibodies to HIV-1

The molecules designed and synthesized in this work will be useful tools for interrogating the nature of immune responses to MPR, as well as for evaluating candidate adjuvants for enhancing the response. Importantly, the chief goal of this dissertation was to determine if immunization with MPR lipopeptides can generate antibodies that neutralize HIV, and indeed these immunogens appear to elicit weak but detectable neutralizing activity in rabbits (**Table 6-3**). However, several key challenges remain before this can become a viable approach for vaccination against HIV.

First, it was consistently observed that these lipopeptides elicit weak and variable reactivity with recombinant gp140 protein. This underscores the key issue of designing immunogens with accurate structural features. Importantly, there is no clear consensus regarding these features. Thus, MPR lipopeptides could be presenting the correct structure to the immune system, but those structures may not exist in the recombinant gp140 used as the ELISA capture antigen in this work. However, numerous biophysical studies have concluded that MPR peptide sequences naturally adopt their native 2F5-

and 4E10-reactive conformation in lipid bilayers [6]. Regardless, further work is undoubtedly needed to elucide an unambiguous structure of the gp41 in a restrained conformation that elicits 2F5-like and 4E10-like antibodies.

A European company, Mymetics, has initiated human trials to evaluate a MPR-targeted vaccine candidate essentially identical to that which is presented here – a full-length synthetic MPR peptide (NC-MPR) conjugated at its C terminus to phosphatidylethanolamine and formulated in bilayer vesicles [13]. Immunization data have not been published, but Mymetics' patent filings and Securities and Exchange Commission reports claim that systemic immunization with this formulation elicits neutralizing antibodies in serum and protects primates against a mucosal virus challenge. If this is correct, one interpretation of this dissertation is that their vaccine candidate could be further improved by switching the lipid anchor from phosphatidylethanolamine to cholesteryl hemisuccinate. Conversely, if the composition of the liposomal carrier is of critical importance, it is possible that Mymetics has identified a bilayer composition that facilitates neutralizing antibody generation.

Another important question involves whether membrane presentation is required to elicit neutralizing antibodies against the MPR. The findings presented in this dissertation do not support the conclusion that membrane presentation is *sufficient* to generate neutralization. However, it still may be *necessary*. The most promising MPR vaccine candidates thus far have been comprised of recombinant enveloped viruses with grafted MPR segments, in which those segments were in some way selected for binding to 2F5 and/or 4E10 [14-16]. These constructs have membrane elements, but the proximity and orientation of the MPR is variable, so conclusions regarding this issue are difficult to draw from these studies.

A related question concerns the role of tolerance in antibody responses to the MPR. The studies presented in **Chapter 3** indicate that in mice, antibody responses are not limited *exclusively* by tolerance and can be increased through chemical modification of the epitopes. These findings should encourage broader application of these modifications in antigen design. In humans, however, it is known that antibodies like 2F5 and 4E10 are extremely rare in the natural repertoire due to their long, hydrophobic CDRH3 regions [17]. What is not known is whether this characteristic is vital to the ability of MPR antibodies to neutralize HIV.

Another unexplored area involves the lipid composition of the liposomal vehicle itself. A number of liposome parameters can influence the immune response to associated antigens, including membrane rigidity, fatty acid chain length and lipid composition [18, 19]. Moreover, it seems that different compositions or routes of administration can influence the generation of anti-lipid antibodies [20-22]. Thus, it is reasonable that an examination of these parameters could reveal optimal conditions that elicit lipid-reactive, HIV-neutralizing antibodies.

Perhaps the most promising use for MPR lipopeptides may be as a boosting immunogen for focusing the immune response to the MPR (**Figure 6-4**). In this modality, a recombinant protein would be constructed in which the MPR is constrained in the correct configuration for generating 2F5- and 4E10-like antibodies. The subject would be immunized first with this protein, which would prime the immune system to recognize MPR structures of a particular conformation. The subject would then be boosted with MPR lipopeptides, which are flexible and could theoretically adopt the correct conformation to interact with B cells generated after the protein prime. Since MPR

lipopeptides are capable of eliciting high titers, with the potential for chemical modifications and other improvements to further improve the response, they may be able to direct responses toward the MPR and away from other non-neutralizing determinants

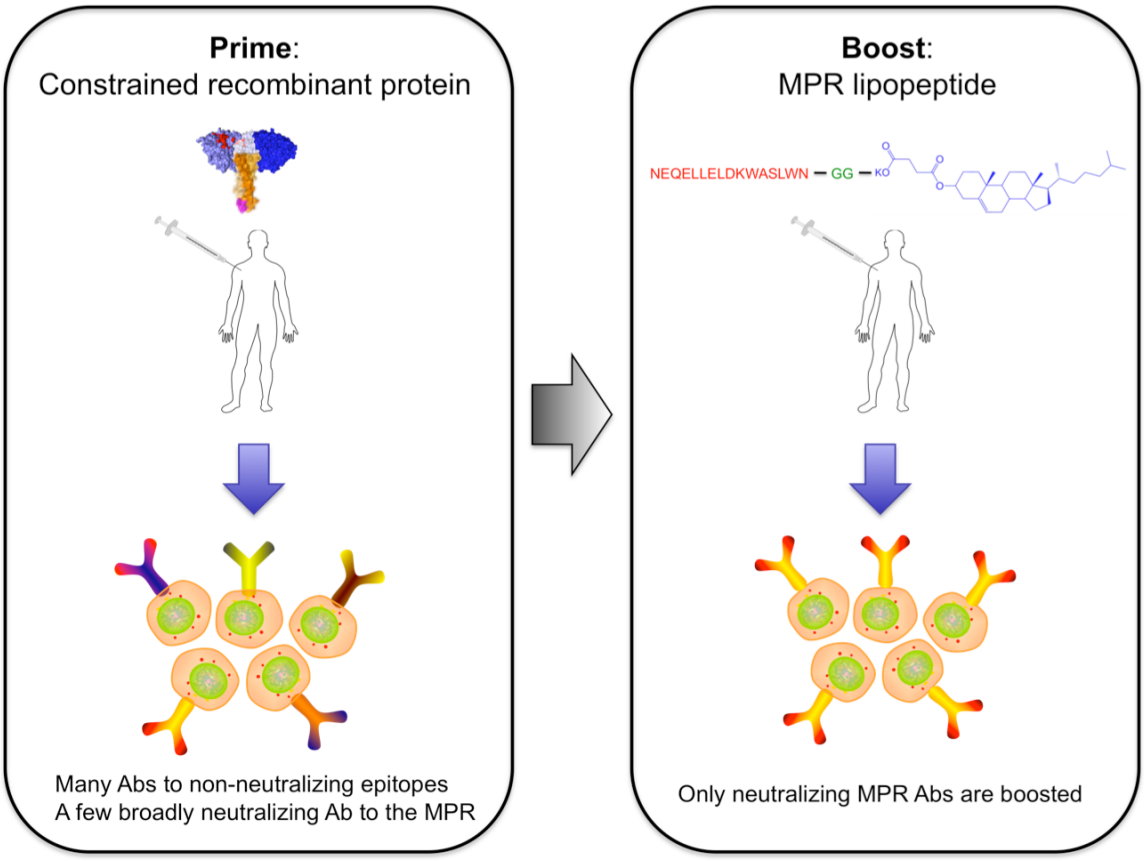

Figure 6-4. Proposed prime-boost approach to elicit neutralizing antibodies to the MPR.

in the priming antigen that are inherently more immunostimulatory. This approach remains dependent on a structurally accurate priming immunogen, however.

6.4.3 Outlook for antibody-mediated neutralization in prevention of HIV-1

Despite recent focus on T cell-mediate vaccination against HIV, the observation that neutralizing antibody cocktails prevent infection in primates justifiably continues to motivate investigation of antibody-mediated vaccines [23-25]. Indeed, it is widely believed that an effective HIV vaccine will contain both humoral and cellular components

[26-29]. Given the rapid mutation rate of HIV, any vaccine that targets a single epitope or a few epitopes will inevitably generate escape mutants. Thus, any antibody-mediated HIV vaccine should be comprised of as many critical neutralizing epitopes as possible. One could envision a minimal chimeric recombinant protein in which key epitopes are grafted together but immunodominant non-neutralizing sequences are omitted. Efforts by Shaw, Kwong and others to graft the antibody-interacting surfaces of neutralizing epitopes onto xenogeneic protein scaffolds represent a promising step in this direction [30].

Progress continues to be made in the design of immungens that target neutralizing determinants other than the MPR, such as the CD4 binding site [31] and oligomannosyl self glycans [32]. New targets are also being identified as increased throughput techniques are deployed to analyze the neutralization specificity of patient sera [33] and to clone human antibody responses [34]. Also, genomic approaches have produced envelope glycoprotein immunogens with consensus sequences that could dramatically enhance the coverage of antibody-mediated vaccines [35]. These ongoing advances are fostering creative new approaches that will accelerate progress in HIV vaccine development.

The first HIV vaccine may not completely prevent infection. It may not prevent infection at all; instead it may simply mitigate the devastating effects of the disease. A great philosopher once claimed that “the difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather a lack of will.” Three decades into the global HIV/AIDS epidemic, the failed Merck trial and other blows to the gut have not shaken the will of the HIV research community. As fundamental understanding of

HIV immunobiology improves, the development of an effective vaccine in my lifetime becomes an increasing probability.

6.5 Acknowledgements

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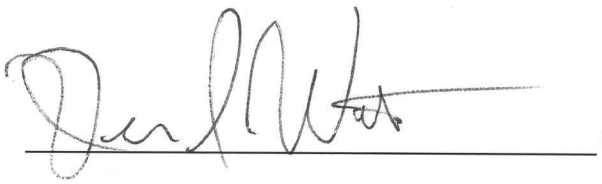

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