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Induction of mucosal immunity using virus-like particle based vaccines

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INDUCTION OF MUCOSAL IMMUNITY USING VIRUS-LIKE PARTICLE BASED VACCINES

BY

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DISSERTATION

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PhD

ABSTRACT

Many viral structural proteins are capable of spontaneously self-assembling into structures that resemble virus particles. These structures, called virus-like particles (VLPs), have multivalent, highly repetitive structures which are capable of inducing robust and enduring immune responses, and, therefore, can serve as the basis for effective vaccines. VLPs can be used as stand-alone vaccines targeting the viruses from which they are derived and also as platforms for presenting heterologous antigens; their ability to evoke strong antibody responses against even poorly immunogenic targets makes them an attractive model for future vaccine and drug delivery vehicle designs. Our lab developed display technologies that allow us to modify VLPs so they can present essentially any potential target antigen on its surface. These technologies have led to the development of several VLP-based vaccines that target molecules derived from microbial and self-antigens. We have previously shown that when given intramuscularly, these

vaccines consistently induce high-titer serum antibodies. Here we present data showing that VLP-based vaccines are compatible with mucosal deliveries to both the genital and respiratory tracts. Specifically, we displayed peptides from the HPV16 L2 coat protein on the bacteriophage VLP platform PP7, and used a second bacteriophage VLP platform, Qβ, to target two domains of the cellular HIV coreceptor CCR5 involved in HIV binding. Vaccines targeting both the viral and self-antigens were successful at inducing mucosal and systemic immune responses, represented by the presence of IgA- and IgG-specific antibodies. The induction of both mucosal and systemic immune responses presents a particular advantage for preventing infection by pathogens transmitted at mucosal surfaces. Indeed, we determined that the L2 mucosal vaccine was successful at preventing genital pseudoviral infection in a mouse model of HPV infection. As a demonstration of the VLP's ability to evoke strong antibody responses against a self - and therefore weakly immunogenic - molecule, we also present data indicating that immunization of macaques with our CCR5 vaccine results in the maintenance of undetectable viral loads in some animals, indicating protection from infection following a high-dose challenge with SIV. Our results provide a general method for the induction of a broad, comprehensive immune response using VLPs as vaccine platforms.

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Chapter 1: Virus-like Particles as Platforms for Antigenic Display

1.1 Introduction to VLPs

Many viral structural proteins have the ability to spontaneously self-assemble into virus-like particles (VLPs). VLPs are morphologically similar to and immunologically indistinguishable from infectious virus, however their lack of a viral genome renders them noninfectious. They are incredibly stable, and can easily be made upon over-expression of coat proteins from both enveloped and non-enveloped viruses. VLPs can be produced from many viruses, including plant, avian and mammalian viruses, as well as bacteriophage. The vast array of viruses available for VLP production and the inherent biocompatibility of the resultant particles are just two of several traits that make VLPs highly flexible materials for a multitude of *in vivo* applications, including targeted drug delivery and vaccine development.

Below, I will describe the vaccine applications of VLPs. I will review how the natural structure of VLPs can elicit strong immune responses and how the capsid can be exploited as an antigen scaffold. Lastly, I will discuss how these features have led to a new era in vaccine design.

1.2 Properties of VLPs that promote immune response

When a viral capsid protein is over-expressed, this protein can often self-assemble into a VLP. VLPs are highly immunogenic, and this immunogenicity is related to structural features of the VLPs. VLPs are particulate structures that contain a dense and repetitive pattern of epitopes on their surfaces. Whereas monovalent, non-particulate

antigens are often ignored by the immune system or induce weak responses, the multivalent particulate nature of the VLP is specifically recognized as harmful by the immune system. This is because particulate antigens are often of microbial origin, and their display on microbial surfaces, such as bacterial pili and viruses, is often highly multivalent. In contrast, these particulate structures rarely occur amongst the self-antigens expressed by vertebrate animals and normally exposed to the immune system. Correspondingly, the immune system has developed mechanisms that allow it to recognize and respond to these structural features.

1.2.1 Interactions with APCs

There are several determinants regulating the uptake of antigen by phagocytic APCs including shape, charge, and hydrophobicity of the antigen complex, as well as the size of the antigen itself. For example, the average size of viruses (and thus VLPs) is between 20 and 100nm in diameter. Dendritic cells (DCs) have been shown to efficiently and preferentially take up particles in this range (Fifis, Gamvrellis et al. 2004) while macrophages, in contrast, take up larger antigens. Thus, it is likely that DCs are the predominant type of APCs first encountering VLPs. Once bound to a DC, VLPs are internalized, processed and presented on MHC class II molecules to activate T helper cells. However, one advantage of the particulate nature of VLPs is that unlike non-particulate exogenous antigens, VLPs can be trafficked to the cytosol for presentation by MHC class I molecules. This is thought to occur in some DC lineages indirectly via cross presentation, or directly by endosomal loading. To briefly review, antigens are processed and presented to T cell pathways by major histocompatibility complex (MHC) class I and class II molecules. In the endogenous processing pathway, peptides derived

from cytosolic proteins are presented by MHC class I for recognition by CD8+ T cells, while in the endosomal (or exogenous) processing pathway, peptides derived from extracellular proteins are presented by MHC class II molecules for recruitment of CD4+ T cells. During cross-presentation, fragments of antigenic extracellular peptides from the phagosome "cross over" and enter the MHC class I pathway following their initial uptake by dendritic cells. The molecules are then transported to the cell surface for presentation to CD8+ T cells (Cutler, Deepe et al. 2007). In this way, VLPs can trigger a CD8+ T cell-mediated cytotoxic T lymphocyte (CTL) response in addition to the CD4+ T helper response.

VLPs can also be designed or selected to enhance APC stimulation. For example, VLPs derived from RNA viruses will have encapsidated single- or double-stranded RNA, which are powerful agonists of toll-like receptors (TLRs) (Lee, Tucker et al. 2009). Alternatively, there are several ways to modify VLPs so as to increase immunostimulation of APCs. These methods will be discussed in more detail below in section 1.4.1. In addition to their interactions with professional APCs, VLPs have a unique effect upon binding to a cognate B cell receptor (BCR).

1.2.2 Interactions with B cells

Just as the magnitude of an immune response following an APCs' uptake of antigen depends on several factors, so does the magnitude of response following the binding of antigen to its BCR. Ultimately, antibody production relies upon the strength of this interaction at the surface of a naïve B cell. The unique spacing of viral epitopes on the VLP surface in a dense, repetitive and multivalent array can enhance the strength of this interaction. When multiple BCRs are engaged, or "cross-linked", it promotes the

formation of stable lipid rafts, which can act as arenas for enhanced signaling to the B cell (Thyagarajan, Arunkumar et al. 2003). Examples of such signaling include upregulation of costimulatory molecules (such as CD80, CD86, CD40L and MHC class II), and stimulation of B cell proliferation, migration and maturation. Downstream effects of these signaling events include engagement of T helper cells and subsequent Ig class switching, affinity maturation, and production of memory B cells. Thus, immunization with multivalent antigens, such as VLPs, results in quantitatively stronger antibody responses than immunization with monovalent or paucivalent antigens.

1.3 VLP Vaccines

Virus-like particles can be used directly as vaccines against the virus from which they were derived. Their morphological similarity to infectious virus and inability to replicate make them naturally potent and safe immunogens, and their particulate nature makes them ideal for fast and easy purifications. As I will describe below, the ability of VLPs ability to display heterologous antigens allows them also to serve as the basis for vaccines against antigens that are typically weakly immunogenic in their native context.

Viral vaccines have traditionally been based on inactivated or attenuated viruses, but there are several advantages to the VLP-based vaccines on the market today. Like VLPs themselves, attenuated viruses are highly immunogenic and can activate both humoral and cell-mediated responses upon immunization. However, attenuated vaccines do not have the inherent safety proffered by VLPs. Viral reversion can occur, leading to the risk of a vaccine-related outbreak (Roberts 2007), and they have also been shown to be dangerous in pregnant and immunocompromised individuals. The manufacturing and

distribution of inactivated viral vaccines also raises safety concerns. Large volumes of virulent pathogens must be handled when manufacturing the vaccine, and there is the risk that the virus may be incompletely inactivated. Perhaps one of the most attractive advantages to developing a VLP-based vaccine is its readiness to market. Unlike the inactivated and attenuated vaccine methods, which require the production of large quantities of virus reliant on replication of a parent strain, VLPs can be produced using recombinant technologies. For example, human-derived VLPs like HPV can be generated in yeast, bacteria, plant, and baculovirus/insect cell expression systems as well as in their natural mammalian cell culture system (Kirnbauer, Booy et al. 1992; Hofmann, Cook et al. 1995; Nardelli-Haefliger, Roden et al. 1997; Warzecha, Mason et al. 2003; Buck, Pastrana et al. 2004). Examples of the wide of variety of VLP-based vaccines in development are shown in **Table 1.**

In addition to inactivated and attenuated viral vaccines, which are based on whole virus preparations, there are also subunit vaccines, which are based on isolated viral proteins. Because they rely on the production of individual viral components, subunit vaccines are also manufactured using recombinant technologies. However, vaccines based on individual proteins are typically less effective than those based on whole virus. The isolated antigen does not always reflect the native structure of the protein, nor are the antibody responses always specific for physiologically relevant epitopes. As a consequence, administration of these vaccines often requires use of strong adjuvants, as well as large and frequent doses of the antigen. In contrast, VLP-based vaccines are strongly immunogenic, often reducing or eliminating the need for exogenous adjuvants.

Table 1.1. Selected virus-like particles used as vaccine platforms

VLP type	Conjugation method(s)	Target epitopes	Refs
Animal Viruses			
Hepadnavirus core Ag (HBV, WHV, DHV)	Genetic and chemical	(B cell or CTL) Malaria, Influenza, FMDV, HCV, Hantavirus and others	(Milich, Peterson et al. 1995; Pumpens and Grens 2001)
HPV	Genetic and chemical	(B cell) HIV, Influenza (CTL) HPV E2 & E7, P1A tumor antigen, HIV	(Slupetzky, Shafti-Keramat et al. 2001; Ionescu, Przysiecki et al. 2006) (Greenstone, Nieland et al. 1998; Peng, Frazer et al. 1998; Nieland, Da Silva et al. 1999; Liu, Liu et al. 2000)
Parvovirus (PPV, B19)	Genetic	(B cell) Dengue Virus, Anthrax (CTL) LCMV	(Amexis and Young 2006; Ogasawara, Amexis et al. 2006) (Sedlik, Saron et al. 1997)
MPyV	Genetic	(CTL) Her2/Neu (Breast Cancer)	(Tegerstedt, Lindencrona et al. 2005)
Plant Viruses			
CPMV	Genetic and chemical	(B cell) HIV, rhinovirus, P. aeruginosa, MEV	(Porta, Spall et al. 1996; Dalsgaard, Uttenthal et al. 1997; Brennan, Gilleland et al. 1999)
PVX	Genetic	HIV	(Marusic, Rizza et al. 2001)
TMV	Genetic and chemical	(B cell) HPV L2 (CTL) Mouse melanoma-associated peptides	(Smith, Lindbo et al. 2006) (McCormick, Corbo et al. 2006)
Bacteriophage			
Qß	Chemical	(B cell) Nicotine, Der p1 allergen (CTL) LCMV	(Maurer, Jennings et al. 2005; Kundig, Senti et al. 2006) (Storni, Ruedl et al. 2004)
MS2	Genetic	(B cell) HIV	(Mastico, Talbot et al. 1993)
Other particles			
Yeast Ty Particles	Genetic	(B cell) HIV, Influenza (CTL) HIV	(Adams, Burns et al. 1994)

1.3.1 Commercial VLP-based vaccines

There are currently clinically approved VLP-based vaccines against two viruses: hepatitis B virus (HBV) and human papillomavirus (HPV). These viruses are known to cause cancer (liver and cervical cancer, respectively) in humans. Vaccines against these viruses protect against infection by generating neutralizing antibodies. The HBV vaccines Recombivax (Merck & Co., Inc.) and Energix (GlaxoSmithKline [GSK]), both released in 1986, were the first commercial vaccines generated using recombinant DNA

technology (Scolnick, McLean et al. 1984). The VLPs, which are approximately 22nm in diameter, are produced in yeast and have the hepatitis B surface antigen (HBsAg) incorporated into yeast lipid membrane particles (McAleer, Buynak et al. 1992).

Two commercial vaccines exist against HPV, Gardasil (Merck) and Cervarix (GSK). Both are composed of VLPs assembled from the HPV major capsid protein, L1 (Lowy and Schiller 2006). Cervarix, released in 2007, is composed of HPV type 16 and 18 VLPs that are generated in L1 recombinant baculovirus-infected insect cells. (HPV types 16 and 18 are found in approximately 70% of cervical cancers.) In contrast to Cervarix, the L1 VLPs for the Gardasil vaccine are produced in yeast. Gardasil, which was released in 2006, targets four HPV types: 16, 18, 6 and 11. Types 6 and 11 cause about 90% of external genital warts.

The HBV and HPV vaccines have excellent safety profiles, and have been shown to induce long-lasting antibodies. In the case of HPV vaccines, which are administered three times over six months, virtually 100% seroconversion has been induced, and titers have remained high throughout the six years since clinical trials first began (Munoz, Kjaer et al. ; Koutsky, Ault et al. 2002; Paavonen, Naud et al. 2009). The antibodies generated following immunization with the HBV vaccines have been detected in vaccinees up to 20 years after vaccination (Poovorawan, Chongsrisawat et al.). Most importantly, rates of acute hepatitis have decreased dramatically where universal vaccination programs have been enforced (Zanetti, Van Damme et al. 2008). In addition to these clinically approved VLP-based vaccines, there are several other vaccines in development and clinical trial that target a variety of heterologous molecules, including

pathogens, chemical agents, and even self antigens. These are discussed in sections 1.5 and 1.6 below.

1.3.2 Adjuvants

One of the major advantages of the VLP platform in vaccine design is the limited need for adjuvants. However, their structure and flexibility also provide a unique opportunity to modify the particle platform to incorporate adjuvants that will specifically stimulate select populations of cells. Structurally, VLPs are composed of viral coat proteins that essentially self-assemble into a hollow particle shell. This shell can then be modified to encapsidate immunostimulatory substances. For example, CpG oligonucleotides can be loaded into VLPs to specifically act as TLR9 agonists, which will then activate CD8+ T cells (Storni, Ruedl et al. 2004). As was discussed briefly above, antigen presenting cells can be specifically activated by incorporating single- or double-stranded RNA into the particle, which will act as an agonist for TLR3 and TLR7/8, respectively. Alternatively, adjuvant molecules can be linked to the particle surface, much like target antigens. This has been previously described using SIV particles chemically linked to cholera toxin B, which is a strong mucosal adjuvant (Kang, Yao et al. 2003). The ability of virtually any VLP to package its own adjuvant is yet another advantage towards its applicability and safety.

1.4 VLPs as scaffolds for antigenic display

As was previously mentioned, VLPs can be derived from any number of viruses and provide strong signals to both the cellular and humoral arms of the immune system. Hence, the VLP provides a flexible platform upon which to display practically any

antigen. Indeed, displaying antigens on the VLP surface has been used to target a diverse number of molecules, particularly those that are poorly immunogenic in their native context. In order to exploit the immunogenic properties of the VLP platform, the displayed molecule must be presented in the same format: a repetitive, multivalent structure.

There are several means by which to achieve arrangement of antigens in this highly dense array on the particle surface. Choosing a method for conjugating a target antigen to the VLP platform can depend on several variables, particularly the surface chemistry of the VLP, the antigen in question, and the type of immune response (CTL, humoral) you want to elicit. Both chemical and genetic approaches, and the advantages and disadvantages of each, are discussed below.

1.4.1 Chemical conjugation

One strategy for presenting target antigens in a highly multivalent fashion on the surface of VLPs is to use bridging molecules to chemically link them to the surface of a preformed VLP. There are several advantages to using a chemical conjugation approach. Notably, target antigens are not limited to proteins; glycans and other small haptens have been attached to VLPs (Raja, Wang et al. 2003). Also, chemical conjugation can be used to attach both peptides and full-length proteins to the viral surface, making for flexibility in vaccine design. While having a linear target peptide as a display epitope can cater to precise targeting of known epitopes critical for vaccine success, a larger protein target can be used to induce a wide range of antibodies capable of recognizing both linear and conformational epitopes on the target molecule. Regardless of the nature of the target antigen, using a chemical conjugation approach requires an understanding of the surface

chemistry of the VLP so that linkage-compatible moieties can be identified on both it and the target.

A number of different strategies have been used to display target antigens on the surface of preformed VLPs. For example, the incredible avidity of the biotin and streptavidin interaction can be exploited by using streptavidin (SA) as a bridging molecule between biotinylated antigens and their VLP platform. Briefly, a preassembled SA tetramer can be reacted with biotinylated target antigens at a molar ratio that will leave one SA binding site unoccupied. The free site on the SA tetramer-antigen complex can then be exposed to and reacted with biotinylated VLPs, with the SA acting as a bridge between the VLP and the display antigens. Our lab has previously used this method with biotinylated papillomavirus VLPs to display a diverse number of biotinylated antigens on its surface (Chackerian, Rangel et al. 2006). The streptavidin bridge also allows for conjugation of larger proteins, such as hen egg lysozyme, to the particle surface (Chackerian, Durfee et al. 2008).

A second strategy for chemical conjugation is to use chemical crosslinkers to display target antigens on the VLP surface. The bifunctional cross-linker, SMPH, has been used extensively in our laboratory to conjugate cysteine-containing antigens to VLP surfaces rich in exposed lysines. This is made possible by the amine- and sulfhydryl-reactive "arms" of SMPH. If a viral coat protein is known to have lysine residues exposed on its surface, the target antigen can be engineered, synthesized, or modified to contain cysteine residues on its terminus (provided the antigen is peptide-based) and the two can be chemically linked via SMPH (**Figure 1.1**).

One consideration when using SMPH is the size of the target peptide. Smaller peptides are more likely to achieve a high-density display on the particle surface, while larger targets will often result in a reduced conjugation efficiency. For example, we have previously shown that the Qβ bacteriophage VLP can support up to 240 molecules of a 12 amino acid peptide on its surface; in contrast, only 18 copies of a 34kD IL-17 homodimer were able to be linked to its surface (Jegerlehner, Storni et al. 2002; Chackerian, Rangel et al. 2006; Rohn, Jennings et al. 2006). While size can affect the density at which a molecule can be displayed, chemical conjugation is the more permissive technique for conjugating targets of diverse size and molecular structure.

Figure 1.1: Chemical Conjugation Method of Antigenic Display

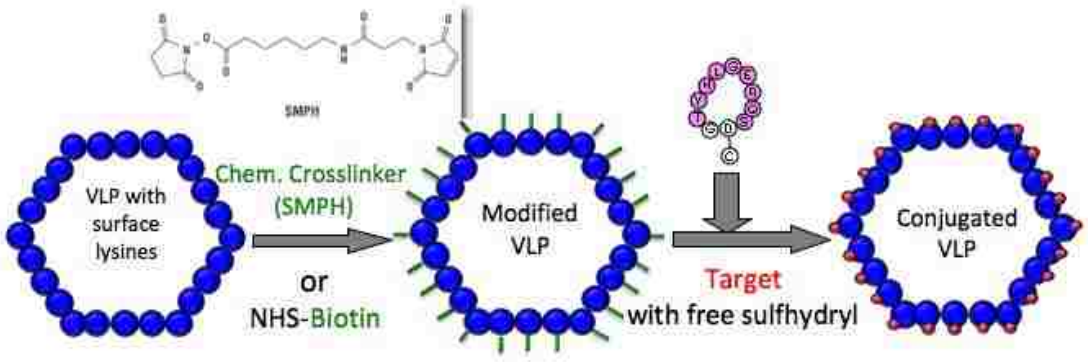


Figure 1.1. An example of the chemical conjugation method. A naked VLP with surface lysines is treated with a chemical crosslinker such as SMPH or biotin, creating a "modified VLP." Target antigens containing a free sulfhydryl, such as the cyclic ECL2 peptide (shown in pink) can then be attached, resulting in a chemically conjugated VLP presenting the target antigen in a repetitive fashion on its surface.

1.4.2 Recombinant VLPs/Genetic Insertion

Another method for displaying heterologous epitopes on the VLP surface is genetic insertion. This method generates a chimeric particle in which a target sequence is successfully incorporated into the viral structural protein. While there are often more technical challenges and a finite range of epitope sizes permitted, the major advantage of this approach is that the target antigen will be displayed in a consistent conformation and density on the particle surface. (**Figure 1.2**) In order for an insertion site to tolerate peptide incorporation, the insertion must not interfere with coat protein folding or VLP assembly. Furthermore, the location of the site must result in the peptide being on displayed on the exposed surface of the particle. Peptide length, hydrophobicity, charge, and structure all need to be taken into consideration when designing a chimeric or recombinant VLP. These barriers have limited the applicability of the genetic approach for peptide display on VLPs.

Numerous technologies have been developed to increase the likelihood of success with the genetic display system. The vast number of viruses amenable to particle formation has greatly spurred these advances, which utilize unique features of insect, plant, bacteriophage, and animal viral platforms to best adapt them for genetic insertion. In our laboratory, we have used a technique in which short peptides can be inserted into the modified coat proteins of two related RNA bacteriophage, MS2 and PP7. The major advantage of this approach is that it allows for near universal display of short peptides on these VLPs.

Figure 1.2: **Genetic Insertion Method of Antigenic Display**

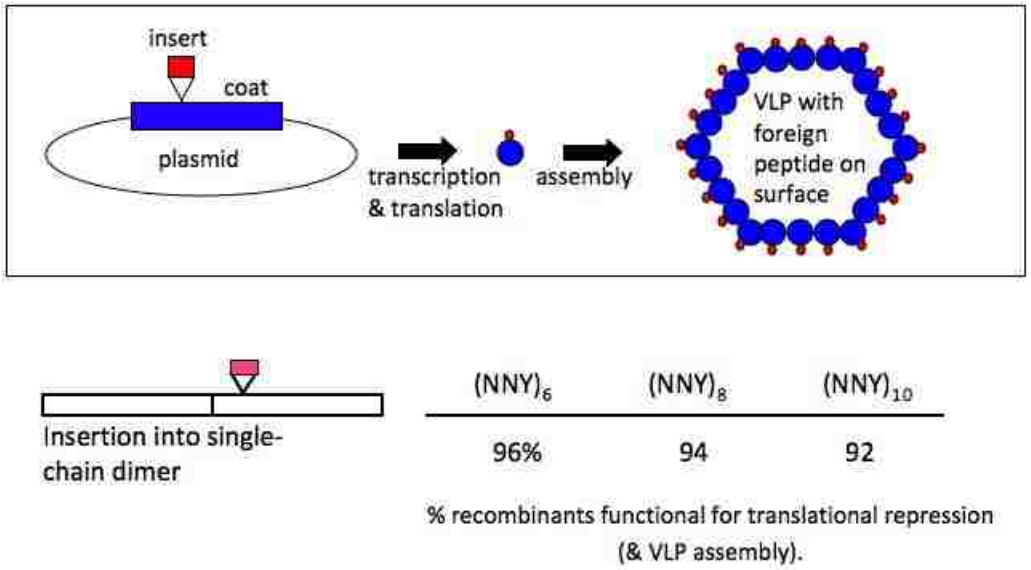


Figure 1.2. Using a genetic insertion model of antigenic display, short peptides can be inserted into modified coat proteins (for example the RNA bacteriophage MS2), and upon translation and assembly, displayed uniformly on the VLP surface (top panel). One disadvantage of this method is as peptide size increases, the VLPs tolerance for insertion (and therefore successful assembly) decreases. An example of this trend is depicted in the bottom panel.

1.5 VLP-based vaccines currently in clinical trial and preclinical development

As was shown in Table 1, there are several VLP-based vaccines that utilize a variety of VLP types and conjugation methods to target a diverse panel of antigens. The hepatitis B core antigen has been used extensively to form VLPs for antigenic display, as its structure has several sites that are well-suited for genetic insertion. One vaccine, ACAM-FLU-A, was generated using recombinant techniques and underwent a phase I clinical trial. ACAM-FLU-A targets the influenza A M2 protein, which is highly conserved amongst influenza A strains. The company Acambis, who developed the vaccine, has not yet reported the results from the human tests, but has indicated that 90%

of volunteers were able to produce measurable antibodies against M2. Previous studies showed protection in animals (Neirynck, Deroo et al. 1999).

A second VLP-based vaccine in clinical trials targets nicotine in an effort to create an anti-smoking vaccine. By generating antibodies against the chemical, the amount and rate of nicotine entering the brain will be reduced, potentially contributing to a permanent cessation of smoking. Nicotine was conjugated to the RNA bacteriophage Qβ using chemical conjugation methods; following immunization it was reported that nicotine-specific IgG responses were generated. The vaccine was tested for efficacy in 227 smokers in a phase II clinical trial. Approximately one-third of the smoking population that received the vaccine was able to continuously abstain from smoking, a proportion nearly twice that of the placebo group (Maurer, Jennings et al. 2005; Cornuz, Zwahlen et al. 2008).

As was previously mentioned, the HBV VLP has been used extensively as a scaffold for other target epitopes. In addition to the influenza vaccine, these include but are not limited to malaria, hantavirus, and hepatitis C virus. Candidate VLP-based vaccines have also been generated for Rotavirus, Norwalk virus, hepatitis E virus, and Parvovirus. These involve formation of a naked, icosahedral VLP; other candidate vaccines involving lipid-envelope containing VLPs are also in development against HIV, hepatitis C virus, and SARS coronavirus. In addition to these infectious pathogens, many VLP-based vaccines currently in development are targeting self-antigens (Roy and Noad 2009)

1.6 Targeting self antigens

While the traditional targets of vaccines are infectious pathogens, the highly immunogenic nature of the VLP, compounded with its ability to display multiple types and sizes of antigen, has led to an increased interest in exploiting the VLP to induce antibody responses against self. Subverting the immune system in order to purposely elicit an anti-self response is desirable in cases where self-molecules are involved in chronic diseases processes, such as cancer and autoimmune disease. Several therapeutics have succeeded in using monoclonal antibodies (mAb) to this end, however a vaccine-based approach overcomes the need for frequent administration, thereby reducing cost, and reduces the likelihood of an inactivating, anti-antibody response.

The prevailing thought is that the mechanisms of B cell tolerance prohibit induction of antibody against self; however, the immune system often fails in this matter, generating up to 50% autoreactive cells in a mature B cell population (Wardemann, Yurasov et al. 2003). In actuality, there are two primary obstacles to producing long-lived, high-titer antibodies against self. First, potentially self-reactive naïve B cells typically downregulate the expression of their B cell receptor. Because of this lower expression, these B cells are only efficiently activated by highly multivalent antigens (Chackerian, Durfee et al. 2008). Secondly, B cells require help from CD4+ T cells in order to undergo class switching, affinity maturation, and generation of memory cells. The mechanisms of both peripheral and central T cell tolerance are more stringent than those in place for B cell tolerance. T cell tolerance results in complete ignorance of the auto-reactive B cell, serving to efficiently prevent its proliferation and subsequent production of potentially harmful IgG auto-antibodies.

Many prior strategies in vaccine design aimed at generating long-lasting anti-self IgG have involved inclusion of foreign T helper epitopes with self-antigens; this is usually accomplished via chemical or genetic approaches, and in the presence of strong adjuvants. While these vaccines have been somewhat successful, a high dose of antigen is often required, yet the IgG titers remain low and tend to diminish rapidly. Data from our laboratory has shown that display of self-antigens on VLPs, which provides antigen multivalency and the presence of foreign T helper epitopes, can efficiently induce antibodies against self (Chackerian, Durfee et al., 2008). VLP display makes self antigens as immunogenic as foreign antigens presented in the same context (Chackerian, Lowy et al. 2001). Several VLP-based vaccines targeting self are currently in clinical trial: these are shown in more detail in **TABLE 1.2**

Table 1.2: Selected VLP-based vaccines targeting self-antigens

Disease	Target Antigen	Status	Refs.
Arthritis, encephalomyelitis, and autoimmune myocarditis	IL-17	Pre-clinical	(Rohn, Jennings et al. 2006; Sonderegger, Rohn et al. 2006)
Hypertension	Angiotensin II	Phase I/II trials (Cytos)	(Ambuhl, Tissot et al. 2007)
Alzheimer's Disease	Aß	Phase I/II trials (Novartis/Cytos)	(Li, Cao et al. 2004; Zamora, Handisurya et al. 2006)
Rheumatoid arthritis, psoriasis, and Crohn's Disease	TNF-α	Pre-clinical	(Chackerian, Lowy et al. 2001)
HIV infection	CCR5	Pre-clinical	(Chackerian, Lowy et al. 1999; Chackerian, Briglio et al. 2004)
Osteoporesis	TRANCE/RANKL	Pre-clinical	(Spohn, Schwarz et al. 2005)
Obesity	Ghrelin	Phase I/II trials (Cytos)	(Dyer, Renner et al. 2006)
Epithelial Cancers	MUC1	Pre-clinical	(Gathuru, Koide et al. 2005)

IL: Interleukin; Aß: Amyloid-beta peptide; HIV: Human Immunodeficiency Virus
CCR5: chemokine (C-C motif) receptor 5; TNF-a: Tumor necrosis factor alpha
TRANCE/RANKL: Tumor necrosis factor-related activation-induced cytokine, also known as receptor activator of nuclear factor-kappa B ligand.

Chapter 2: Delineation of Thesis

2.1 Rationale/Statement of the problem

HIV affects an estimated 33 million people worldwide, with approximately 3 million individuals newly infected with the virus in 2008. The majority of these infections occur in impoverished regions of the world, particularly sub-saharan Africa (UNAIDS/WHO, 2009). Thus, the need for global containment of the HIV pandemic is acute, and the combined goal of prevention and treatment is a focal point in the field of HIV research.

Although the emergence of highly active anti-retroviral therapies (HAART) over the past two decades has vastly improved treatment of HIV infection, there is an ongoing need for prevention. The expense and adverse side effects associated with antiviral drugs argue for further investment in novel vaccine-based therapies, which have the potential for wider spread distribution at lower costs. Yet despite numerous efforts, an AIDS vaccine remains elusive. The promising STEP trial of 2004 tested a "T cell only" vaccine using recombinant adenovirus to express HIV proteins. However, the vaccine failed to provide protection, and was stopped in 2007 at phase IIb after some participants showed enhanced acquisition of HIV-1 infection (Steinbrook 2007). The specific reasons for this occurrence are unknown, though it was shown that pre-existing immunity to the Ad5 vector was an independent risk factor in the increased rate of HIV acquisition among vaccine recipients. Several hypotheses exist to explain the increased susceptibility to HIV-1 infection, including: 1) that receipt of the vaccine boosted adenovirus-specific T cells, which resulted in higher numbers of activated target at cells at mucosal sites following high risk sexual behavior, 2) that prior adenovirus immunity skewed the

immune response to the vector and reduced the innate immune response to HIV-1 infection, and 3) that pre-existing immunity resulted in the production of "enhancing" antibodies that increased susceptibility to infection (Corey, McElrath et al. 2009). Of course, additional hypotheses, or a combination of all three hypotheses is also possible; alternatively, it is still unclear whether the increased acquisition in Ad5 seropositive vaccines actually has an underlying biologic mechanism. Indeed, at least one other group has shown experimentally that seropositive Ad5 status does not predict a significant increase in Ad5-specific CD4+ T cell frequency prior to or following vaccination (Hutnick, Carnathan et al. 2009).

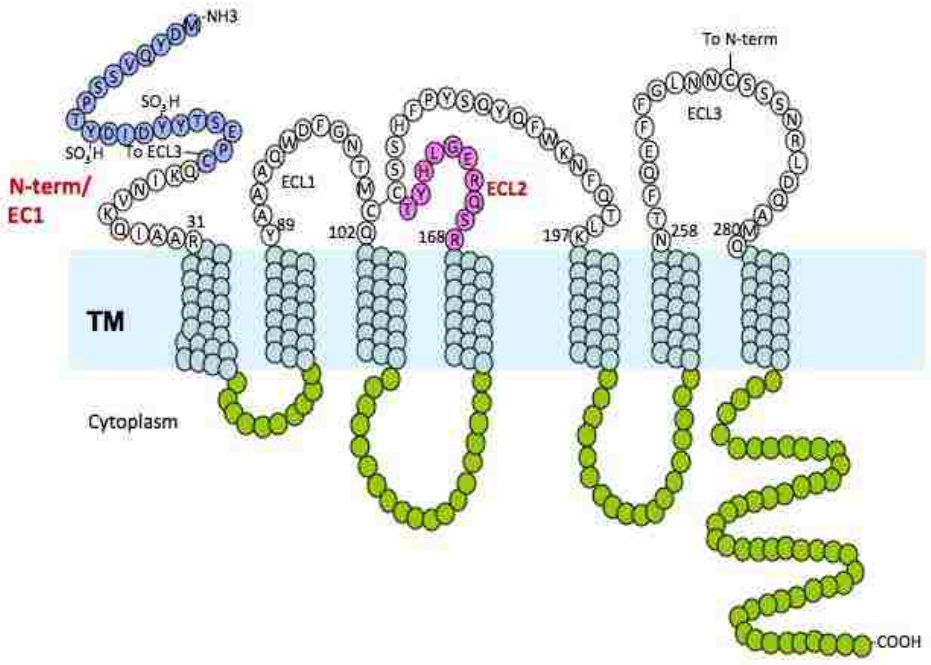
Ultimately, it seems that sufficient concentrations of long-lasting, protective antibodies will have to be present at the time and site of transmission for successful elimination of HIV-infected cells. Inducing and maintaining high levels of antibody against HIV is difficult due to many factors, including but not limited to the vast sequence diversity of the various subtypes, the structure of the virus envelope, and the mechanisms of immune evasion the virus employs.

2.2 CCR5

CCR5 is a cellular protein critically involved in HIV acquisition and replication. It is expressed on a number of immune cells, including but not limited to CD4+ T cells, macrophages and dendritic cells. It belongs to the chemokine receptor family, which are cell surface receptors for chemokines that act to promote cellular migration by chemotaxis. The natural ligands of CCR5 are MIP-1α, MIP-1β, and RANTES; these chemokines can bind, signal through, and promote internalization of CCR5. Structurally,

CCR5 is a G-protein coupled receptor (GPCR), characterized by seven membrane-spanning domains, three extracellular loops and four intracellular loops (**Figure 2.1**).

Figure 2.1: CCR5 Structure



The 3-dimensional structure of CCR5 is thought to be similar to that of another GPCR, rhodopsin, with the TM domains arranged in a cluster. To illustrate this, we have shown on Figure 2.1 where the third extracellular loop (ECL3) connects to the N-terminal extracellular region (EC1, or N-term). The EC1 and ECL2 regions (shown in blue and pink, respectively) have been implicated in HIV entry, and are discussed in further detail in Chapter 3.

CCR5 has a number of proposed functions *in vivo*, including priming of adaptive immune responses, distribution of effector cells to microbial infection sites, and

amplification of inflammation. The ability of one chemokine receptor to bind several ligands is thought to contribute to the overall effectiveness of host response to microbial challenges; it is also one reason that targeted suppression of a chemokine receptor can be tolerated, as many chemokines also bind multiple receptors to carry out overlapping functions. Targeting a stable self-protein such as CCR5, rather than HIV itself, is one strategy to overcome the problem of viral mutation.

2.2.1 CCR5 as therapeutic target

A major barrier to developing an HIV vaccine is the genetic variability of the virus. Instead of targeting the virus itself, one strategy is to develop methods for targeting cellular proteins that are critically important during HIV infection. CCR5, which is utilized by HIV as an entry co-receptor, is a promising candidate for several reasons (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996). Of the coreceptors available to HIV during entry, CCR5 is considered the most physiologically important. Viruses that use CCR5 as a co-receptor are termed CCR5-tropic and predominate throughout the course of HIV infection, thereby making CCR5 an important target for vaccine design. In the early stages of infection, the virus strains isolated are exclusively CCR5-tropic, suggesting a possible selective advantage for these viruses during transmission or early onset of disease (Li, Juarez et al. 1999). In addition to binding its primary receptor CD4, HIV needs to bind to a second chemokine coreceptor, either CXCR4 or CCR5, to gain entry into host cells. Briefly,initial binding of HIV envelope glycoproteins to CD4 results in a conformational change in these proteins, exposing binding sites that permit the coreceptor to bind the virion. Once HIV is bound both to CD4 and the coreceptor, a second conformational change takes place,

allowing for fusion of the HIV virion to the cell surface and subsequent entry of viral contents (Lederman, Penn-Nicholson et al. 2006).

Shortly after the role of CCR5 in viral acquisition was determined, a deletion mutation of 32 base pairs was identified in the coding region of the CCR5 gene. Individuals harboring a homozygous mutation of the CCR5 allele (termed Delta-32) were resistant to HIV infection, while heterozygous individuals progressed slowly from HIV infection to AIDS (Samson, Libert et al. 1996; Winkler, Modi et al. 1998). **Figure 2.2** shows how the Delta-32 mutation prohibits viral entry by truncating the CCR5 receptor. The role of CCR5 in HIV acquisition has prompted numerous investigators to use CCR5 for drug development. These drugs target CCR5 by blocking viral entry either through small interfering RNAs, monoclonal antibodies, or small molecule inhibitors, and one small molecule inhibitor, Maraviroc, is clinically approved (Baba, Nishimura et al. 1999; Trkola, Ketas et al. 2001; Barassi, Soprana et al. 2005).

Figure 2.2: CCR5 structure resulting from the Delta-32 mutation

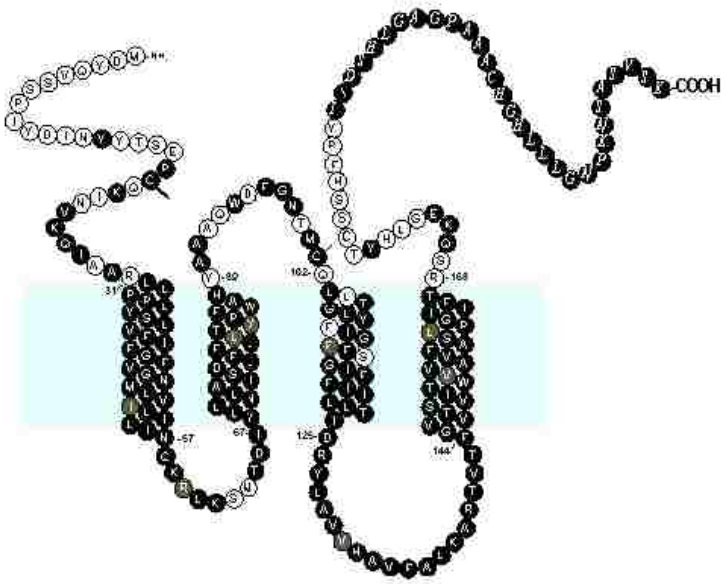


Figure 2.2
The Δ32 mutation results in the loss of three transmembrane domains, two of the three outer loops, and the intracellular signaling domain. The defective CCR5 molecule is not expressed on the cell surface.

While a therapeutic approach is valid, there are also advantages to using CCR5 as a vaccine target. Unlike most viral vaccine targets, which mutate rapidly during the course of infection, CCR5 is a cellular protein and therefore genetically stable. Targeting the receptor itself - either by limiting its expression on the cell surface or by blocking virus-receptor interactions - should have substantial effects on viral pathogenesis. More importantly, a vaccine against CCR5 has the potential for prophylactic use.

2.3 Mucosal immunity may be critical for attenuating viral acquisition

An important consideration in HIV-1 vaccine development is the induction of a mucosal immune response in addition to a systemic response. The conventional parenteral approaches, while successful at inducing a systemic response, do not address the fact that most infectious pathogens (including HIV) initiate infection at a mucosal surface. Thus, induction of mucosal responses could provide better protection at the site of infection. Establishment of HIV-1 infection depends largely on the genital and gastrointestinal mucosas, which respectively play important roles in transmission, as well as viral replication and amplification (Veazey and Lackner 2003). Mucosal antibodies, usually of the IgA subtype, are consequently often found at these sites. Secretory IgA mediates mucosal defense through mechanisms inclusive of, but not limited to, inhibition of inflammatory responses, virus neutralization, and antigen elimination, highlighting the important role of mucosal immunity in combating infection (Dean, Carrington et al. 1996; Holmgren and Czerkinsky 2005).

Inducing a strong mucosal response may be particularly important when targeting CCR5. While HIV can use either CCR5 or CXCR4 as a coreceptor during viral entry, mucosal transmission is almost exclusively restricted to viruses that use CCR5 (Margolis and Shattock 2006). Moreover, large numbers of CD4+ T cells in the gastrointestinal-associated lymphoid tissue (GALT) are rapidly depleted during HIV infection, which is likely due to the high levels of CCR5- expressing cells in the GALT as compared to cells in other locations (Brenchley, Schacker et al. 2004).

The most important determinant for the successful initiation of a broad mucosal response may be the route of vaccine administration. Indeed, the compartmentalization of the mucosal immune system has led to several options for mucosal immunization that are catered to target tissues (i.e. oral, nasal, and vaginal immunizations for gastric, pulmonary, and genital responses, respectively). Furthermore, mucosal immunization can in turn confer systemic immunity, while the reverse is not always attainable. The use of parenterally administered vaccines is often limited in developing countries by high costs and safety considerations (Levine 2003). In contrast, a non-invasive delivery directly to the mucosa would offer a safe and cost-effective approach to establishment of mucosal immunity, with the ultimate goal of global containment of HIV infection through prophylactic and therapeutic means.

2.4 Hypothesis

My hypothesis is that use of a VLP vaccine platform will be compatible with aerosol delivery to mucosal tissues and result in enhanced mucosal immune response against self- and non-self antigens, specifically the HIV coreceptor CCR5 and the HPV16

L2 capsid protein, respectively. (An extensive background and rationale for targeting L2 is provided in Chapter 4, as the primary focus of this work is on vaccines targeting CCR5.) Mucosal delivery of a VLP-based vaccine will protect against viral infection in a relevant animal model.

2.5 Specific Aims

Aim 1. Characterize the immunogenicity of VLP-based CCR5 vaccines. We will construct a bacteriophage VLP platform displaying target epitopes from the macaque CCR5 receptor. The immunogenicity of this vaccine will be assessed by determining 1) its ability to induce CCR5 peptide-specific systemic IgG antibodies upon immunization of mice, 2) whether these antibodies are capable of recognizing and binding the CCR5 receptor in its native conformation *in vitro*, and 3) whether serum-derived antibodies are capable of inhibiting HIV-1 infection *in vitro*.

Aim 2. Induce mucosal immunity via pulmonary delivery of the vaccine. We will nebulize the VLP-CCR5 vaccine developed in Aim 1 and deliver the aerosol to rats via the pulmonary route. The ability of VLP-based vaccines to effectively translate to aerosol and induce local and remote mucosal, as well as systemic, immunity will be assessed.

Aim 3. Investigate alternate routes of mucosal immunization using, if necessary, a variety of VLP conjugates. We will develop and investigate the effectiveness of various immunization strategies using gel- and aerosol- based VLP vaccines applied directly to the genital tract. Because primates are the only relevant animal model for investigating protection from HIV infection, we will conduct preliminary

investigations on the ability of genital vaccines targeting human papillomavirus 16 capsid proteins to elicit systemic and mucosal antibodies that are capable of providing protection against infection in a mouse model.

Aim 4. Select optimal immunization strategy for viral challenge experiments in macaques. Based on the results of the aforementioned aims, we will select an appropriate mucosal VLP-based CCR5 vaccine and administer it to macaques. Following immunization, macaques will be challenged with SIV via the genital tract and the ability of the vaccine to prohibit or reduce infection will be determined. The degree of protection conferred will be quantified by SIV viral loads in immunized animals relative to control (unimmunized) animals.

2.6 Outline

The previous chapter provided extensive background on VLPs and their employment in vaccine development. In Chapter 3, I describe the development of an aerosolized, pulmonary VLP-based vaccine targeting one domain of CCR5 involved in binding HIV, and analyze its efficacy in inducing a robust mucosal immune response. Chapter 4 focuses on the development of a VLP-based vaccine for delivery to the genital tract. In contrast to the pulmonary vaccine, which targeted a self-protein, the genital vaccine targeted the human papillomavirus (HPV) type 16 capsid protein, L2. In targeting L2, we were able to study the ability of the vaccine to inhibit infection in a mouse model. The success of the research described in Chapters 3 and 4 led to the development of an aerosolized vaccine delivered directly to the genital tract of rhesus macaques targeting two domains of CCR5. Whereas the pulmonary vaccine was

delivered in rats and therefore was not amenable to challenge in a relevant animal model, we were able to conduct viral (SIV) challenges in the macaques and determine the vaccine's ability to 1) break B cell tolerance (evidenced by presence of anti-CCR5 antibodies) and 2) inhibit or prevent SIV infection. The progress of this ongoing work is presented in Chapter 5. In Chapter 6, I expand upon the discussion of the previous chapters, summarize the significance of my thesis research, address specific concerns and limitations regarding my research, and propose additional experimental procedures that may function to strengthen my previous findings. Finally, I discuss potential future directions of this project based on my current findings.

Chapter 3:

Induction of mucosal and systemic antibody responses against the HIV coreceptor CCR5 upon intramuscular immunization and aerosol delivery of a virus-like particle based vaccine

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3.1. Abstract

Virus-like particles (VLPs) can be exploited as platforms to increase the immunogenicity of poorly immunogenic antigens, including self-proteins. We have developed VLP-based vaccines that target two domains of the HIV coreceptor CCR5 that are involved in HIV binding. These vaccines induce anti- CCR5 antibodies that bind to native CCR5 and inhibit SIV infection *in vitro*. Given the role of mucosal surfaces in HIV transmission and replication, we also asked whether an aerosolized, VLP-based pulmonary vaccine targeting CCR5 could induce a robust mucosal response in addition to a systemic response. In rats, both intramuscular and pulmonary immunization induced high titer IgG and IgA against the vaccine in the serum, but only aerosol vaccination induced IgA antibodies at local mucosal sites. An intramuscular prime followed by an aerosol boost resulted in strong serum and mucosal antibody responses. These results show that VLP-based vaccines targeting CCR5 induce high-titer systemic antibodies, and can elicit both local and systemic mucosal response when administered via an aerosol. Vaccination against a self-molecule that is critically involved during HIV transmission and pathogenesis is an alternative to targeting the virus itself. More generally, our results provide a general method for inducing broad systemic and mucosal antibody responses using VLP-based immunogens.

3.2. Introduction

Many viral structural proteins can self-assemble into virus-like particles (VLPs) that resemble infectious virus, but lack a viral genome and are therefore non-infectious.

Because of their repetitive, multivalent structures, VLPs are highly immunogenic and make excellent vaccines for the virus from which they were derived; the human papillomavirus (HPV) and hepatitis B virus (HBV) vaccines are two examples of VLP-based vaccines. VLPs can also be adapted as platforms for display of antigens that are either not normally or poorly immunogenic. Heterologous antigens displayed in a highly dense, multivalent format on the surface of VLPs are extremely immunogenic; VLP-displayed antigens can induce high titer antibody responses at low doses and in the absence of exogenous adjuvants (Spohn and Bachmann 2008). VLP display can even be used to induce antibody responses against self-antigens, essentially abrogating the mechanisms of B cell tolerance (Chackerian, Lowy et al. 2001; Chackerian, Lenz et al. 2002; Chackerian, Durfee et al. 2008). This observation has led to the development of a new class of vaccines that target self-molecules involved in a variety of chronic diseases, including Alzheimer's Disease (Chackerian, Rangel et al. 2006; Ambuhl, Tissot et al. 2007), hypertension (Li, Cao et al. 2004; Zamora, Handisurya et al. 2006) and rheumatoid arthritis (Chackerian, Lowy et al. 2001; Spohn, Guler et al. 2007).

As an alternative strategy to conventional HIV vaccines, we have been interested in using VLP display technology to target CCR5, a self-molecule that is critically involved in HIV acquisition. During infection, HIV uses chemokine coreceptors in addition to its primary receptor, CD4, to gain entry into cells (Alkhatib, Combadiere et al. 1996; Doranz, Rucker et al. 1996; Deng, Unutmaz et al. 1997). Although HIV can use several coreceptors, CCR5 is the most physiologically important. In the early stages of infection, the virus strains isolated are exclusively CCR5-tropic, suggesting a possible selective advantage for these viruses during transmission or during the early stages of

disease (Li, Juarez et al. 1999). Furthermore, individuals harboring a homozygous genetic mutation of the CCR5 allele (termed Delta-32) are resistant to HIV infection, and infected heterozygous individuals (who express lower levels of CCR5) progress more slowly to AIDS (Liu, Paxton et al. 1996; Samson, Libert et al. 1996; Winkler, Modi et al. 1998). In 2008 the first small molecule CCR5 inhibitor, Maraviroc (Pfizer), was clinically approved. Maraviroc binds to CCR5 and changes its conformation so that it is not recognized by the coreceptor binding sites present on the HIV envelope glycoprotein, gp120. HIV infected patients receiving Maraviroc monotherapy have dramatically decreased viral loads, often to undetectable levels (Fatkenheuer, Pozniak et al. 2005; Hunt and Romanelli 2009; Yost, Pasquale et al. 2009). These data, in addition to the effects of the Delta-32 mutation on HIV infection, indicate that a reduction in the availability of functional CCR5 on target cells profoundly affects viral pathogenesis.

Unlike most viral vaccine targets, which mutate rapidly during the course of infection, CCR5 is a cellular protein and therefore genetically stable. We hypothesized that a vaccine that targeted CCR5 - either by limiting its expression on the cell surface or by blocking virus-receptor interactions - could block viral replication and affect viral pathogenesis. A number of vaccine strategies targeting CCR5 have been tested, including the use of a recombinant Flock House Virus that contains a CCR5 epitope (Barassi, Soprana et al. 2005), a CCR5-HSP70 fusion protein immunogen (Bogers, Bergmeier et al. 2004; Bogers, Bergmeier et al. 2004), a DNA vaccine consisting of human CCR5 fused to tetanus toxoid (Zuber, Hinkula et al. 2000), and a CCR5 peptide conjugate vaccine (Misumi, Nakayama et al. 2006), among others. Our laboratory has developed several VLP-based vaccines to induce anti-CCR5 antibodies in which CCR5

epitopes are conjugated to the surface of pre-formed VLPs. We have previously shown that a papillomavirus (PV) VLP-based vaccine targeting the N-terminal extracellular domain of macaque CCR5 induced antibodies that bound to native CCR5 and blocked HIV infection *in vitro* (Chackerian, Lowy et al. 1999). Although this initial study was complicated somewhat by the low fitness of the challenge SHIV virus, prophylactic vaccination of macaques with the VLP-CCR5 vaccine reduced viral loads and time to clearance in pig-tailed macaques infected with a CCR5-tropic SHIV (Chackerian, Briglio et al. 2004). These data, and similar data from Misumi and colleagues (Misumi, Nakayama et al. 2006), suggest that prophylactic vaccination against CCR5 may play a role in controlling viral replication in a SHIV/macaque model. In this study, we have developed second generation vaccines based on CCR5-derived peptides conjugated to bacteriophage VLPs. These vaccines target multiple domains of CCR5.

Most current vaccines are administered by intramuscular (IM) or subcutaneous injection. While these routes of immunization are extremely effective for the induction of systemic immunity, they generally result in poor mucosal immune responses. Most infectious pathogens, including HIV, enter the body and infect target cells at mucosal surfaces, so an ideal vaccine against HIV would induce both systemic and mucosal immune responses. Both the genital and gastrointestinal mucosa play crucial roles in the establishment of HIV infection, either as a site of transmission (at the vaginal or rectal mucosa) or as an important and critical site of viral replication and amplification seeding the bloodstream (in the gastrointestinal mucosa) (Veazey and Lackner 2003).

We have been interested in examining the ability of VLP-based immunogens to induce mucosal immune responses. In particular, we have investigated the effectiveness

of pulmonary vaccination using aerosolized VLP-vaccines in inducing broad immune responses. Aerosol delivery to the lung has a number of advantages. First, the lower respiratory tract contains abundant antigen-presenting cells, predominantly pulmonary macrophages and dendritic cells, which play important roles in priming adaptive immune responses. Second, although the mucosal immune system is, by and large, compartmentalized, pulmonary vaccination results not only in local mucosal responses in the lung, but also can give rise to strong mucosal responses in the genital/vaginal mucosa (Holmgren and Czerkinsky 2005). Third, previous studies have shown that mucosal immunization can in turn induce systemic immunity, which could eliminate the need for an intramuscular immunization (Levine 2003).

In this study, we compared the immune responses induced by VLP-based vaccines targeting macaque CCR5 upon intramuscular and pulmonary immunizations. Both routes of immunization resulted in high-titer antibody responses against the vaccine preparation, and anti-CCR5 antibodies were effective at blocking SIV infection. However, only aerosol exposure led to the induction of local mucosal antibody responses.

3.3. Materials & Methods

3.3.1 CCR5-VLP preparation

A 21 amino acid peptide (designated EC1) representing the N-terminal 21 amino acids (MDYQVSSPTYDIDYYTSEPC; sulfated at Y10 and Y14) of pig-tailed macaque CCR5 (ptCCR5) was synthesized by American Peptide (Sunnyvale, CA), and then directly linked to Qß bacteriophage using a bifunctional cross-linker (SMPH, Pierce Endogen, IL), as described previously (Chackerian, Lenz et al. 2002). A second, smaller

peptide representing the second extracellular loop (ECL2) of ptCCR5 was synthesized by Celtek Peptides (Nashville, TN). The ECL2 peptide (DRSQREGLHYTG) is a cyclic peptide spanning amino acids 168 - 177 of ptCCR5 in which the Arg and Thr residues are linked through an Asp-Gly dipeptide spacer. As with the EC1 peptide, the ECL2 peptide was linked to Qß bacteriophage via SMPH. Qß-EC1 VLP preparations, both prior to and after nebulization, were visualized by electron microscopy. VLPs were adsorbed to carbon-coated grids, stained with 1% uranyl acetate, and then were examined with a Philips electron microscope model EM400RT at magnification x36,000.

3.3.2 Animal inoculations

Intramuscular

6-8 week-old female rats (Harlan Sprague Dawley, Indianapolis, IN) were inoculated with 15 µg of Qβ-EC1 VLPs in incomplete Freund's adjuvant (IFA). 6-8 week-old female C57Bl/6 mice were inoculated with either 10 µg of Qβ-EC1 or Qβ-ECL2 VLPs, or 5µg of each VLP preparation, in incomplete Freund's adjuvant (IFA). Inoculations were administered intramuscularly as shown in Table 1. Serum samples (approximately 0.1-0.2 mL) were collected one week following the 1st and 2nd immunization, and every week following the 3rd immunization (in rats) until sacrifice.

Aerosol

Groups of rats were each exposed to 0.1 mg of Qβ or Qβ-EC1 nebulized VLPs (for a total of 0.3 mg in 5mL of phosphate-buffered saline (PBS) in a nose-only exposure chamber (InTox, Albuquerque, NM). The chamber incorporated an aerosol pathway that provides individual supply and exhaust routes in order to ensure uniform delivery of the

test atmosphere. Compressed air was used for both nebulization air and dilution air to ensure adequate air supply. The chamber pressure was maintained just below zero during the dosing. Doses were determined by sampling the nose-only chamber and quantifying the aerosol concentration and using the following equation: Dose = (Aerosol concentration x Respiratory minute volume x Exposure time) / Body weight. Aerosol particle size was determined using a laser diffraction particle size analyzer (Sympatec, Germany). The median diameters of the aerosols were between 0.8 and 1.2 microns, thus ensuring predominant respiratory deposition in rodents(Roy, Hale et al. 2003). Rats were acclimated to aerosol exposure restraint tubes prior to the initial exposure. Shown in **Table 3.1**, two groups (Qß-immunized rats and Qß-EC1 immunized rats) received an IM prime as their first inoculation, with three subsequent aerosol boosts. Two additional groups (Qß-EC1 immunized rats) received only aerosol inoculations, either with or without Cholera Toxin B (CTB) adjuvant. Serum samples were collected as described above unless otherwise indicated (IM prime groups). Animals were housed three per cage in autoclaved, ventilated cages (Tecniplast, Phoenixville, PA) containing autoclaved Tek-Fresh bedding (Harlan). The animals had *ad libitum* access to irradiated chow (Harlan) and autoclaved water. All animal care and experimental protocols were in accordance with the National Institutes of Health and University of New Mexico School of Medicine guidelines.

3.3.3 Quantifying antibody responses

Sera, feces, uterine washes and and bronchial-alveolar lavage fluid (BAL) were tested for antibodies specific for the CCR5-EC1 peptide and Qβ bacteriophage VLPs by

ELISA. Briefly, Immulon II ELISA plates (Dynex Technologies, Chantilly, VA) were coated overnight at 4°C with either 0.5 µg of bovine serum albumin (BSA)-conjugated EC1 peptide or 0.5 µg Qβ VLPs per well. Wells were then blocked with 50 µL of PBS with 0.5% milk (w/v) per well for 2 h at room temperature. An initial 1:40 dilution of serum was serially diluted 4-fold and applied to wells for 2.5 h at room temperature. (All dilutions were done in 0.5% milk (w/v) in PBS unless otherwise noted.) Reactivity to target peptides was determined by using horseradish peroxidase (HRP)-labeled goat anti-rat IgG (Jackson Immunoresearch, Bar Harbor, ME) at a dilution of 1:2000 and incubated for 1 h at room temperature. Upon development, the optical density at 405nm (OD405) was determined using a Thermo Max microplate reader (ThermoLab Systems, Fisher Scientific, Pittsburgh, PA). Absorbancies greater than twice the background were considered positive. ELISAs for IgA in sera were conducted as above, incorporating the following changes: sera was diluted 1:10 in PBS with 0.5% BSA, and presence of antibodies was detected using HRP-labeled goat anti-rat IgA (Open Biosystems, Huntsville, AL). For ELISA analysis of BAL fluids, feces and uterine washes, samples were diluted 1:1 in PBS and the OD405 determined.

3.3.4 ELISPOT

96-well ELISpot plates were activated with 70% ethanol according to manufacturer's instructions (Millipore, Billerica, MA) and coated overnight at 4°C with 0.5 µg Qβ VLPs or BSA-EC1 peptide. Lungs were harvested, perfused and processed for lymphocyte isolation as previously described (Swanson, Zheng et al. 2004). $4x10^5$ cells per well were plated in complete RPMI and incubated overnight at 37°C. Wells were

washed three times with 0.5% fetal calf serum (FCS) in PBS and the appropriate HRP-labeled goat anti-rat secondary (IgG or IgA) antibody was added at a 1:1000 dilution. Following a 2 h incubation at room temperature, wells were washed as described above and developed with TMB substrate (MABtech, Mariemont, OH) until spots appeared. Spots were quantitated using an AID ViruSpot/EliSpot Reader (Cell Technology, Inc., Columbia, MD).

3.3.5 Flow cytometry

Binding to CCR5 was tested by incubating pooled sera from immunized mice with 293T cells that were transiently transfected (or mock-transfected) with a pig-tailed macaque CCR5 expression vector (obtained from the NIH AIDS reference and reagent program). Two days after transfection, CCR5-expressing 293T cells were detached from the plate using 0.5mM EDTA and then washed three times in FACS buffer (0.5% BSA in PBS). Cells were incubated with Protein G-purified IgG isolated from the pooled sera of mice immunized with Qß VLPs, Qß-EC1, or Qß-ECL2. Approximately 10^5 cells were resuspended in 100 μL of staining buffer and then incubated with 10-20 μL of purified mouse IgG followed by a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). As a positive control, cells were incubated with a phycoerythrin (PE)–labeled anti-human CCR5 monoclonal antibody (3A9; BD Biosciences, San Jose, CA). Flow cytometric analysis was performed on a FACS Calibur by using the Cell Quest software package (BD Biosciences, San Jose, CA). Specific IgG binding was measured relative to mock-transfected cells.

3.3.6 SIV inhibition assay

SIVmac251 inhibition was measured using MAGI-ptCCR5 indicator cells (Kimata, Gosink et al. 1999) pretreated with heat-inactivated sera from immunized mice for 30 minutes at 37 degrees and then infected with approximately 100 infectious SIVmac251 particles (obtained from the NIH AIDS Research and Reference Reagent Program). Two days after infection, infected cells were scored by counting the number of blue cells in each well. Inhibition of SIV infection was determined by comparing the number of blue (infected) nuclei in the presence of antibody versus the number of blue nuclei in the absence of sera.

3.4 Results

3.4.1 Vaccine Preparation

Antigens displayed at high density on the surface of VLPs are highly immunogenic. We have used the VLP-display approach to develop vaccines targeting the HIV/SIV coreceptor CCR5. Because of their role in HIV binding to CCR5 (Edinger, Amedee et al. 1997; Wu, LaRosa et al. 1997), we targeted the N-terminal extracellular domain of CCR5, also referred to as extracellular domain 1 (EC1), and a domain from extracellular loop 2 (ECL2). For the EC1 vaccine, a 21 amino acid peptide corresponding to the amino region of EC1 of pig-tailed macaque (pt) CCR5 was synthesized. The tyrosines at positions 10 and 14 of the peptide were sulfated to reflect the fact that sulfation of these residues in native CCR5 is thought to be important in HIV binding (Farzan, Mirzabekov et al. 1999). The ECL2 peptide is a cyclic peptide spanning amino acids 168 - 177 of ptCCR5 in which the Arg and Thr residues are linked through

an Asp-Gly dipeptide spacer. This peptide was originally identified as an immunogen capable of inducing anti-CCR5 antibodies by Misumi and colleagues (Misumi, Nakajima et al. 2001). The EC1 and ECL2 peptides were chemically conjugated to VLPs derived from an RNA bacteriophage, Qß, using a bifunctional crosslinker, SMPH, that allowed us to link the C-terminal cysteine on the peptides to exposed surface lysine residues on the coat protein of Qß **(Figure 3.1 A).** The extent of conjugation was determined by analysis of denatured particles by gel electrophoresis **(Figure 3.1 B).**

Figure 3.1: Generating the Qß-EC1 vaccine

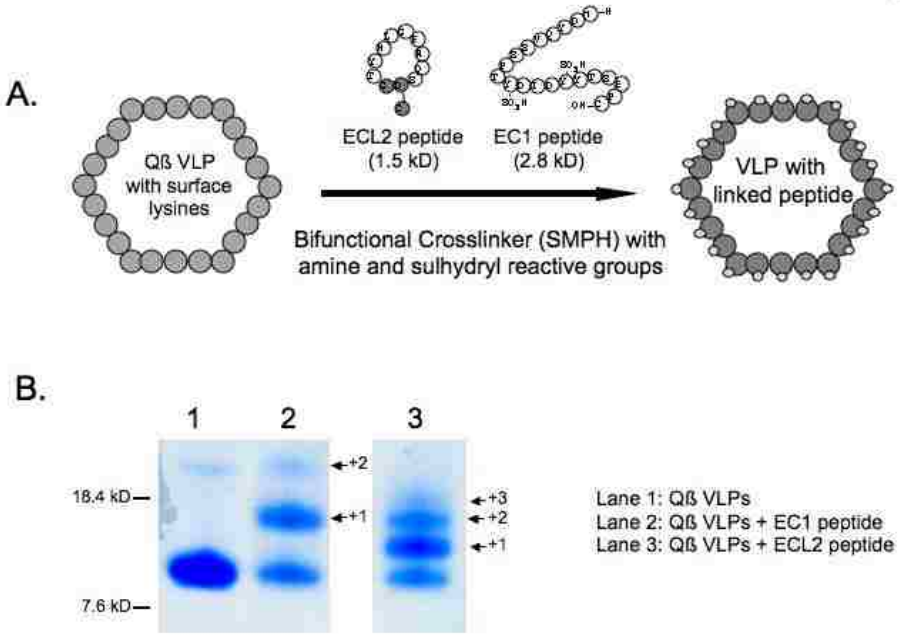

Figure 3.1. Generating the CCR5 vaccines. A) EC1 and ECL2 peptides were linked to Qß VLPs through the use of a bifunctional crosslinker (SMPH). SMPH crosslinks surface lysines on Qß VLPs to a cysteine located at the C-terminus of the EC1 peptide or the base of the cyclized ECL2 peptide. Non-CCR5 derived amino acids are highlighted in grey. Numerous copies of peptide can be attached per coat protein, resulting in peptide presentation in a dense and repetitive array on the VLP surface. B) Polyacrylamide gel analysis of denatured Qß VLPs (lane 1), EC1-conjugated Qß VLPs (lane 2), and ECL2-conjugated Qß VLPs (lane 3). Qß VLPs are comprised of a single protein subunit, coat protein, which migrates with a mobility corresponding to its molecular weight, ~14000 Daltons. Conjugation of the EC1 peptide results in higher molecular weight species, representing individual coat protein subunits modified with 1 (+1), 2 (+2), or 3 (+3) copies of the peptide.

Peptide-modified Qß coat protein displays a mobility shift relative to unmodified Qß coat protein. The degree of the mobility shift reflects the addition of one, two, or three peptides per coat protein molecule. As is shown in **Figure 3.1 B**, the majority of coat protein has been modified with peptide. We estimate that the average Qß-ECL2 vaccine preparation contains 1.5 copies of peptide per coat protein and the Qß-EC1 preparation contains greater than 0.5 copies of peptide per coat protein, resulting in a total of ~270 (ECL2) or >90 (EC1) peptides being presented on the VLP surface. In either case, our earlier studies have indicated that these densities of peptide on the surface of the VLPs should be sufficient to induce a strong antibody response (Chackerian, Lenz et al. 2002; Peabody, Manifold-Wheeler et al. 2008).

Table 3.1: Experimental design for pulmonary immunizations

Species	Inoculum	Delivery & Dose	Vaccination Schedule	Sacrificed @
Mice	Qβ-EC1	IM + IFA (10 μg)	Weeks 0, 2	6 Weeks
Mice	Qβ-ECL2	IM + IFA (10 μg)	Weeks 0, 2	6 Weeks
Rats	Qβ	IM prime (15μg) + aerosol boost (100μg)	Weeks 0 (IM), 2, 6,15 (A)	27 Weeks
Rats	Qβ-EC1	IM prime (15μg) + aerosol boost (100μg)	Weeks 0 (IM), 2, 6,15 (A)	27 Weeks
Rats	Qβ-EC1	IM only (15μg)	Weeks 0, 2	6 Weeks
Rats	Qβ-EC1	Aerosol only (100μg)	Weeks 0, 2	6 Weeks
Rats	Qβ-EC1	Aerosol + CTB adjuvant (100μg)	Weeks 0, 2	6 Weeks

3.4.2 VLP-based CCR5 vaccines are immunogenic

We determined whether Qß-EC1 and Qß–ECL2 could elicit anti-CCR5 antibody responses upon intramuscular (IM) immunization. Groups of three to seven mice were immunized with Qβ-EC1 and/or Qß–ECL2 as shown in **Table 3.1**.

Briefly, animals were given two doses of 10 µg total inoculum (mice immunized concurrently with Qß-EC1 and Qß –ECL2 received 5 µg of each), at a two-week interval and then sera were collected weekly and analyzed for antibodies specific to the EC1 and ECL2 peptides by end-point dilution ELISA **(Figure 3.2).** Immunization with either Qß-EC1 or Qß –ECL2 elicited high-titer (geometric mean titer = ~10^4) IgG antibodies specific to the peptide of interest, but not against the other peptide. Animals immunized with Qß VLPs alone did not produce antibodies that recognized either of the CCR5 peptides. Simultaneous immunization with Qß-EC1 and Qß–ECL2 elicited high-titer IgG antibodies against both CCR5 peptides.

Figure 3.2: Serum antibody responses to intramuscular immunization of rats with Qß-EC1 particles

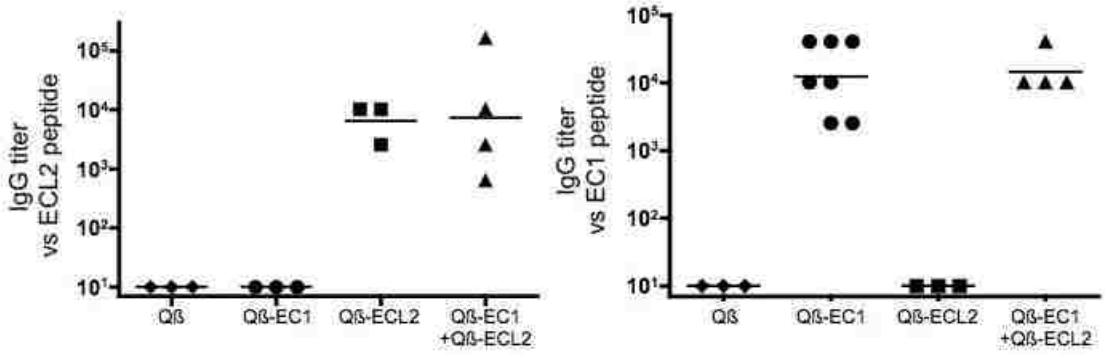

Figure 3.2. IgG antibody responses in C57Bl/6 mice immunized three times at 2 week intervals with 5 µg wild-type Qß VLPs, Qß conjugated to the CCR5 N-term peptide (Qß-EC1), Qß conjugated to the cyclic ECL2 peptide (Qß-ECL2), or a mixture of Qß-EC1 plus Qß-ECL2. This figure shows end-point dilution IgG ELISA titers for sera taken two weeks after the final immunization against peptides representing the CCR5 EC1 (right panel) or ECL2 (left panel). Each data point represents the antibody titer from an individual mouse. Lines represent the geometric mean titer for each group.

3.4.3 Anti-CCR5 antibodies bind native CCR5 in vitro and inhibit SIV infection

Although these data indicated that the two CCR5 peptide-conjugated VLP vaccines elicited antibodies that recognized ptCCR5 peptides, it was possible that these antibodies might not recognize the EC domains in their native conformation on membrane-associated ptCCR5. To examine this question, the ability of anti-EC region antibodies to bind to membrane-associated ptCCR5 was assessed by flow cytometry. IgG was purified from the pooled sera of Qß-EC1 or Qß-ECL2 intramuscularly immunized mice and incubated with ptCCR5- or mock-transfected 293T cells.

Figure 3.3: Qβ-EC1 antibodies bind to native CCR5 receptor *in vitro*

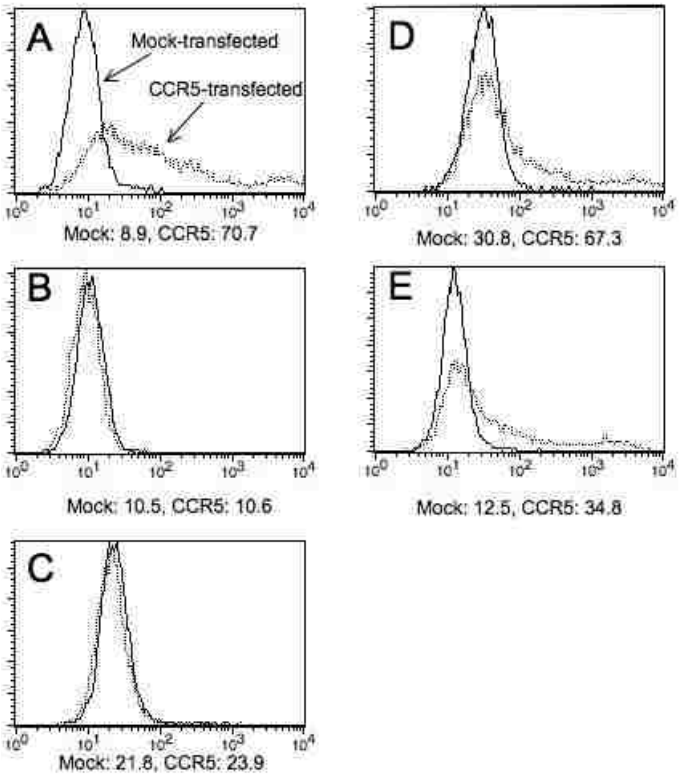

Figure 3.3. Qβ-EC1 antibodies bind to native CCR5 in vitro. 293T cells were mock-transfected (solid line) or transfected with pig-tailed macaque CCR5 (dashed line). Two days after transfection, cells were incubated with (A) a PE-labeled monoclonal antibody (3A9) that binds to the EC1 domain of CCR5, (B) secondary antibody alone, (C) pooled protein G-purified IgG from Qβ-immunized mice, (D) protein G-purified IgG from Qβ-EC1 immunized mice, and (E) protein G-purified IgG from Qβ-ECL2 immunized mice, and then antibody binding was assessed by flow cytometry. Geometric mean fluorescence values are shown below each panel.

As a positive control, some cells were incubated with 3A9, a monoclonal antibody that binds to the EC1 domain of CCR5. As a negative control, transfected cells were incubated with purified IgG from the sera of mice immunized with Qß VLPs alone. As shown in **Figures 3.3 D** and **3.3 E**, IgG from the sera of Qß-EC1 and Qß-ECL2 immunized mice specifically bound to CCR5-expressing cells relative to mock-transfected cells, indicating binding of IgG to CCR5. There was no binding detected in IgG purified from the sera isolated from Qß-immunized mice (**Figure 3.3 C**). Thus, both Qß-EC1 and Qß-ECL2 vaccines elicited antibodies that recognize the conformation of native CCR5 expressed on 293T cells.

To assess the ability of induced sera to inhibit SIV infection, we used a single cycle infectivity assay utilizing the MAGI-ptCCR5 indicator cell line (previously described) to determine whether antibodies elicited from our CCR5 vaccines could inhibit SIV infection *in vitro.* Cells were incubated with increasing dilutions of heat-inactivated sera from mice immunized intramuscularly with Qß-EC1, Qß-ECL2, both Qß-EC1 and Qß-ECL2, or Qß VLPs alone and then infected with the CCR5-tropic strain SIVmac251.

As shown in **Figure 3.4**, sera from animals immunized with either Qß-EC1 or Qß-ECL2 inhibited SIVmac251 infection. Although anti-ECL2 and anti-EC1 antibodies were somewhat weakly inhibitory individually, in combination they displayed 50% inhibition at a 1:40 serum dilution. Although this inhibition titer is not particularly high, it is important to recognize that inhibition of virus infection by blocking the receptor is a much more stringent task than blocking the virus. Every potential target cell expresses multiple copies of CCR5.

Figure 3.4: VLP-CCR5 IgG blocks SIV infectivity *in vitro*

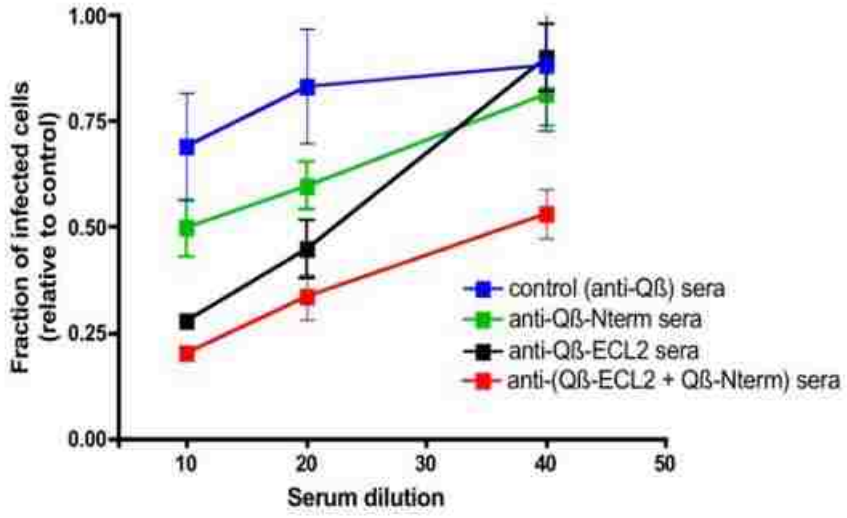

Figure 3.4. Inhibition of SIVmac251 infection. SIVmac251 inhibition was measured using MAGI-ptCCR5 indicator cells pretreated with sera from immunized mice for 30 minutes at 37 degrees and then infected with approximately 100 infectious SIVmac251 particles. Two days after infection, infected cells were scored by counting the number of blue cells in each well. Inhibition of SIV infection was determined by comparing the number of blue (infected) nuclei in the presence of purified IgG versus the number of blue nuclei in the absence of IgG. Data represents the average of two different experiments; error bars show standard error of the mean.

3.4.4 Pulmonary immunization induces systemic antibodies against CCR5

To see whether our VLP-based vaccine could also induce antibody responses upon mucosal immunization, we translated our intramuscular Qß-EC1 vaccine into an aerosolized vaccine for pulmonary administration. We first wanted to determine whether Qß-EC1 VLPs remained intact following the aerosolization process. VLPs were nebulized, recovered, and concentrated, and then visualized by electron microscopy. The nebulized and recovered VLPs had similar morphology to un-nebulized VLPs **(Figure 3.5).**

Pulmonary immunizations were carried out in to 6-8 week-old female rats. We used rats because they have larger tidal volumes than mice, thereby inspired particles are better able to reach the lower respiratory tracts in this species (Parent 1992). Particles that reach the lower respiratory tracts are more difficult to expel, and are in a milieu rich in antigen-presenting cells ideal for initiating an immune response throughout mucosal tissues. Since the induction of anti-CCR5 antibodies in the genital tract was also a primary aim, female rats were chosen so that uterine washes could be collected (along with sera) throughout the study, and the uterus later recovered and analyzed.

Figure 3.5: Qβ-EC1 virus-like particles survive nebulization

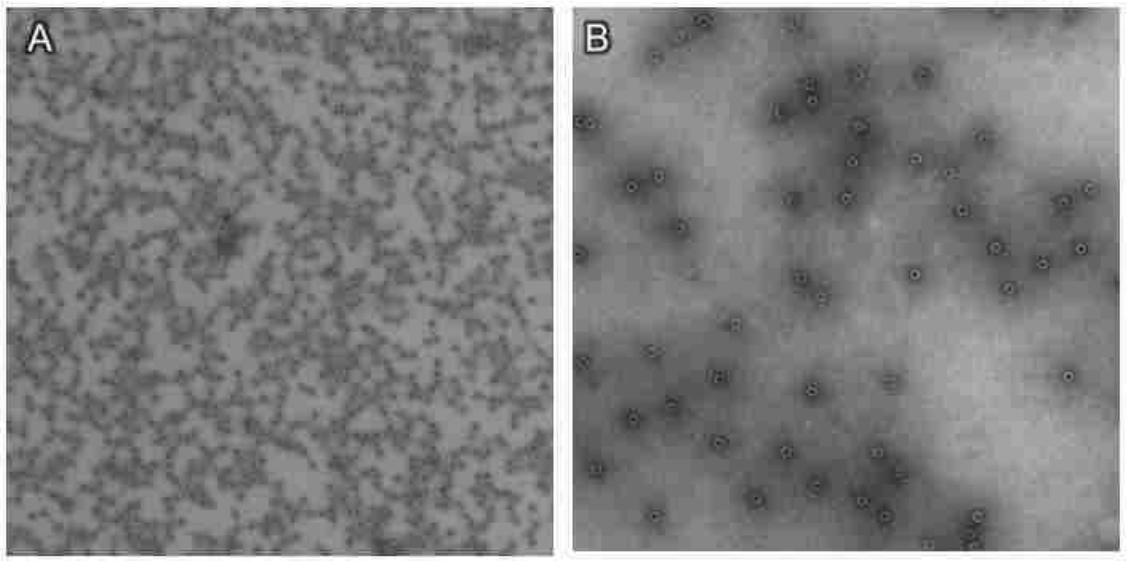

Figure 3.5. Qβ-EC1 virus-like particles survive nebulization. Qß-EC1 was nebulized and then captured and reconcentrated by filter centrifugation. Particles were then adsorbed to carbon-coated grids, were stained with 1% uranyl acetate, and were examined with a Philips electron microscope model EM 400RT at magnification x36,000. Shown is Qß-EC1 prior to (A) or post-nebulization (B).

First, we assessed the antibody responses in rats immunized via the pulmonary route. Rats received aerosol immunizations of 100 µg Qß-EC1 twice at a two-week

interval. One group of rats was immunized with Qß-EC1 formulated with 25 µg of cholera toxin B (CTB). CTB is a non-toxic and recombinantly produced cholera toxin B-subunit that has been reported to have mucosal adjuvant properties. Sera was collected every week until necropsy and IgG and IgA antibodies against Qβ and EC1 were quantitated by end-point dilution ELISA and compared to the antibody responses elicited by intramuscular (IM) immunization. As shown in **Figure 3.6**, aerosol administration of Qß-EC1 elicited anti-EC1 and anti-Qß IgG and IgA antibodies that were comparable to those elicited when the vaccine was delivered intramuscularly. Coadministration of CTB adjuvant had slightly enhanced IgG titers, but elicited no appreciable difference in IgA titers. Rats given IM immunizations had the highest titers against the VLP platform, but had similar anti-EC1 antibody levels as those measured in rats receiving the aerosolized vaccine.

We also wanted to determine whether systemic antibody responses could be increased by "priming" rats with an initial IM immunization followed by an aerosol boost. Groups of rats were immunized with two IM injections, two aerosol exposures, or an IM prime followed by an aerosol boost. One week following the second immunization, sera was collected and IgG and IgA titers against the EC1 peptide were measured **(Figure 3.7 A).** Rats receiving two IM immunizations had the highest serum-associated IgG levels against EC1, but had lower IgA levels. In contrast, rats receiving two aerosol immunizations had lower IgG levels, but higher IgA antibodies. Rats receiving the prime/boost regimen had an intermediate response that achieved a balance between the two other immunization strategies. The IM prime group displayed a 10-fold

increase in IgA titers as compared to rats given only IM immunizations; there was also a moderate increase in IgA titer compared to the group receiving the aerosolized vaccine.

Figure 3.6: Systemic IgG and IgA responses in rats immunized with Qβ-EC1 VLPs

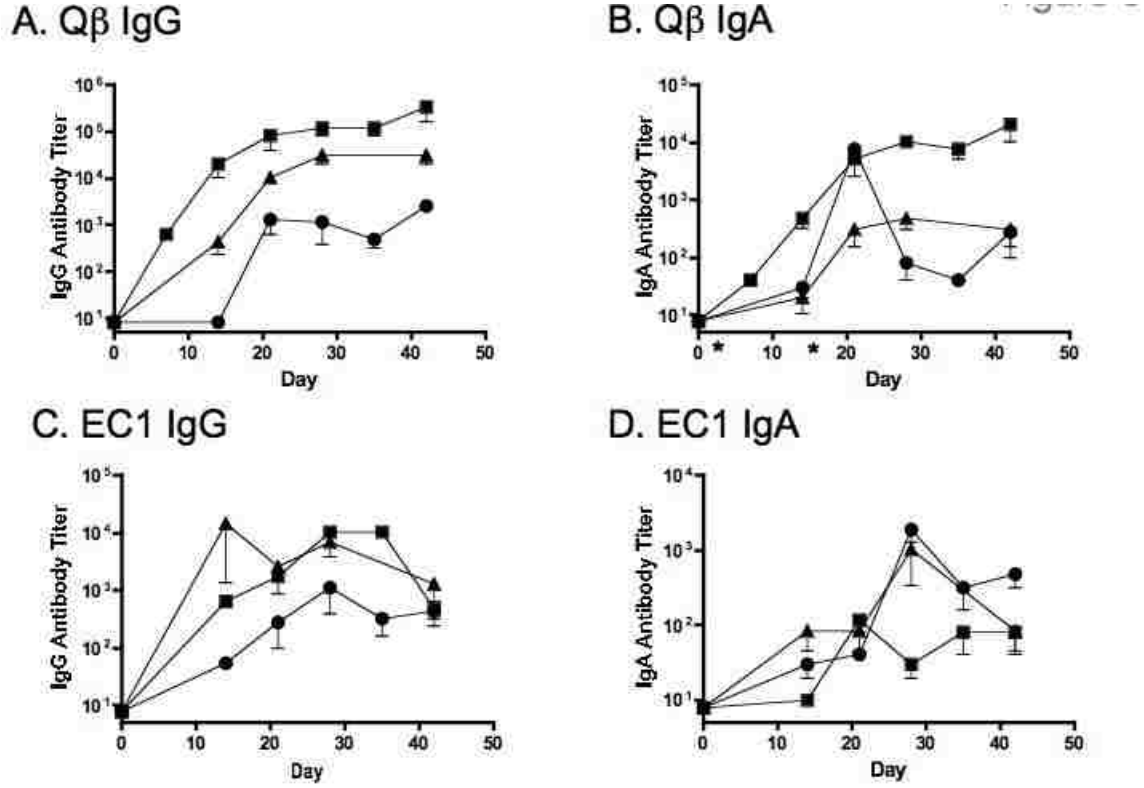

Figure 3.6. Systemic IgG and IgA responses in rats immunized with Qβ-EC1 VLPs. Groups of three rats were immunized intramuscularly (squares) or via the pulmonary route with (triangles) or without (circles) CTB adjuvant. Immunizations were carried out on days 0 and 14, sera were collected at the time points indicated, and antibody levels were determined by end-point dilution ELISA. Shown are (A) anti-Qβ IgG, (B) anti-Qβ IgA, (C) anti-EC1 IgG, and (D) Anti-EC1 IgA antibody titers. The data shows the geometric mean titer of three immunized rats and error bars represent SEM.

Moreover, the level of IgG elicited by the IM group was not lower in the IM prime group, and was slightly higher than that seen in animals receiving only pulmonary immunizations. To investigate the longevity of the immune response and the efficacy of a boost in evoking memory, we continued collecting sera from the prime/boost group

over 27 weeks. Animals in this group were given additional aerosol boosts at weeks 6 and 15, and then sacrificed 27 weeks after the initial immunization. IgG titers against EC1 were sustained, and remained above 10^3 for 12 weeks following the final boost in all three animals **(Figure 3.7 B).** Serum anti-EC1 IgA antibodies diminished more rapidly, perhaps reflecting the shorter half-life of this molecule. However, aerosol boosts increased IgA antibody levels to peak titers (10^4) in 2 out of 3 animals.

Figure 3.7: Antibody titers in rats receiving an IM prime followed by aerosol boosts of Qβ-EC1 VLPs

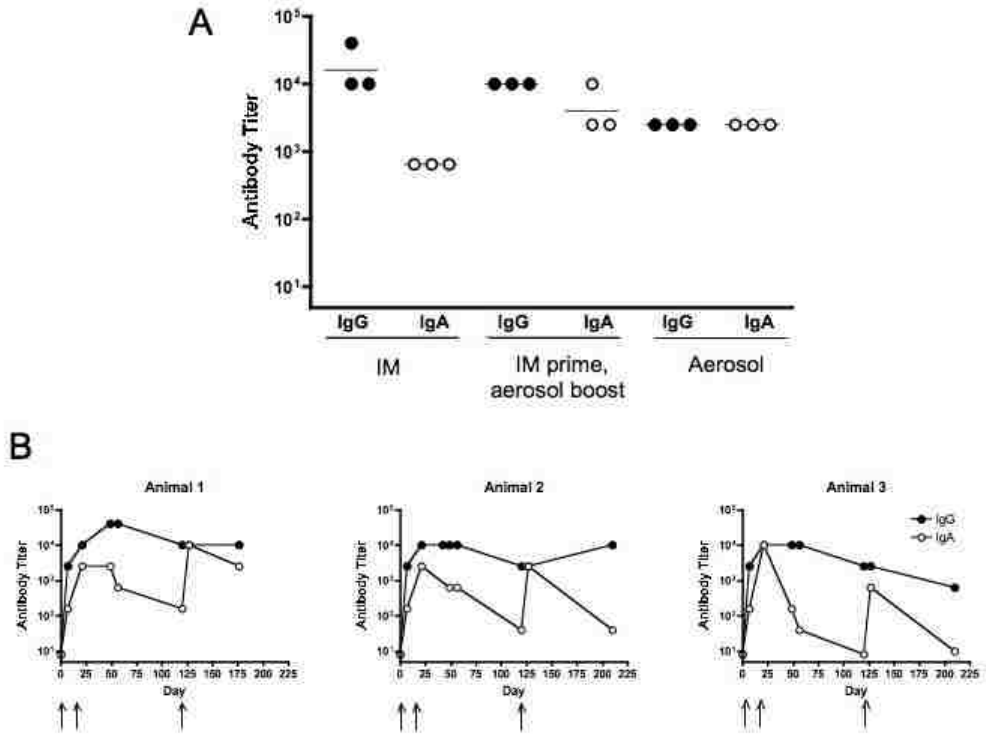

Figure 3.7. Antibody titers in rats receiving an IM prime followed by aerosol boosts of Qβ-EC1 VLPs. (A) Comparison of IgG and IgA serum anti-EC1 titers in rats one week following a 2nd inoculation with Qβ-EC1. Rats received 2 intramuscular inoculations, 2 inoculations via aerosol, or an intramuscular prime followed by an aerosol boost. Bars represent the geometric mean titer of the group. (B) Kinetics of serum anti-EC1 responses in individual rats receiving an IM prime followed by aerosol boosts of Qβ-EC1. Rats were immunized on weeks 0, 2, 6, and 15 (arrows), sera collected at the time points indicated and IgG (closed circles) and IgA (open circles) titers were determined by ELISA.

3.4.5 Pulmonary immunization enhances local antibody secretion in mucosal tissues

Pulmonary administration of our Qβ-EC1 vaccine was successful in inducing both IgG and IgA antibodies in the blood, so we next asked whether antibodies specific to Qβ and EC1 were also present in mucosal secretions of animals immunized by the various strategies described previously. As an additional control, one group of rats was immunized with Qß VLPs alone, via the IM prime/aerosol boosts regimen.

Figure 3.7: Anti-Qβ and -EC1 titers in mucosal washes of immunized rats

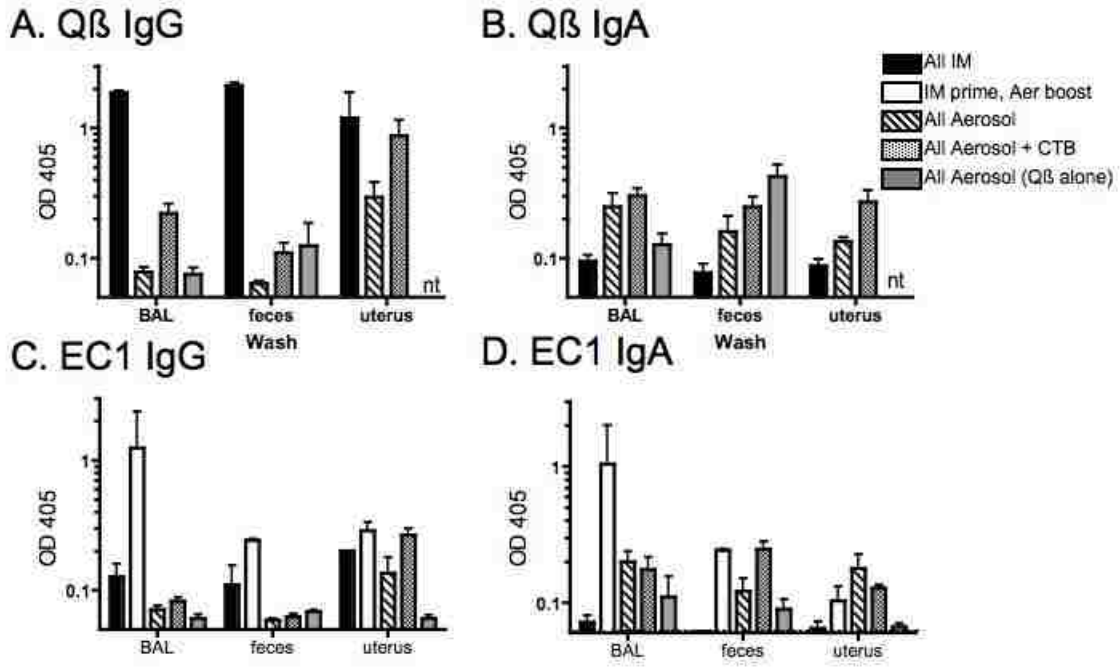

Figure 3.8. Anti-Qβ and -EC1 titers in mucosal washes of immunized rats. Bronchi-alveolar lavage fluid, feces, and uterine lavage samples were collected at necropsy, diluted 1:1 in PBS, and anti -Qβ and -EC1 IgG and IgA antibodies levels were measured by ELISA. Rats were immunized with Qβ-EC1 via the intramuscular route (black), the pulmonary route with or without CTB adjuvant (dotted or lined fill, respectively), or given an IM-prime followed by aerosol boosts (no fill). As a negative control, a fifth group was given an IM prime followed by aerosol boosts of QB VLPs alone (grey). Shown are (A) anti-Qβ IgG, (B) Anti-Qβ IgA, (C) anti-EC1 IgG, and (D) anti-EC1 IgA.

At necropsy, we collected bronchi-alveolar lavage fluid (BAL), and we also collected washes from the uterus and feces of the immunized rats. These samples were diluted 1:1 in PBS with 0.5% BSA, and then anti–Qβ and –EC1 IgG and IgA antibodies were measured by ELISA (note: we did not test the anti-Qß antibody responses of the prime/boost group because of a limited amount of material). As was observed in the sera, rats given the IM vaccine alone and the IM prime/aerosol boost regimen had the highest IgG levels in all three mucosal sites tested **(Figures 3.8 A, C).** Rats immunized via the aerosol route alone had relatively high IgG titers in uterine washes, but surprisingly low IgG levels in BAL fluid. However, only those animals immunized with the aerosolized vaccine secreted IgA at the mucosal sites tested (**Figures 3.8 B, D**)--essentially no IgA response was induced by IM immunization. Co-administration of CTB adjuvant with the aerosolized vaccine somewhat boosted secreted IgG levels relative to aerosol alone, but did not have a pronounced impact on IgA levels in secretions. In general, the IM prime/aerosol boost regimen induced the strongest anti-EC1 antibody response, particularly in the lung.

3.4.6 CCR5-specific B cells in the lung following pulmonary immunization

After determining that pulmonary immunization could confer systemic immunity as well as mucosal immunity in remote tissues, we assessed the local immune response more quantitatively by determining the number of antibody-specific B cells in the lung by ELISPOT. Lungs were collected from animals receiving only aerosol immunizations or only IM immunizations of Qβ-EC1, and processed as previously described for lymphocyte collection (Swanson, Zheng et al. 2004). ELISPOT analysis was performed

on plated B cells and the number of IgG- and IgA- secreting B cells specific for either Qβ or EC1 quantified. As a control, some animals were immunized with Qβ VLPs alone. Consistent with what was observed in mucosal washes, only animals receiving the aerosolized vaccine had detectable Qβ- and EC1-specific B cells. Animals receiving an IM administration of the vaccine had few Qβ-specific IgG secreting B cells in the lung, and no EC1-specific B cells. Coadministration of CTB did not enhance the frequency of EC1-specific B cells, and, if anything, seemed to a have a negative effect **(Figure 3.9),** in contrast to the neutral and/or positive effects observed in the sera and mucosal washes. Rats given the inhaled Qβ-EC1 vaccine without adjuvant had nearly equal amounts of IgA secreting B cells against both Qβ and EC1, and comparable numbers of IgG-secreting B cells as well, indicating an immune response against both platform and target.

3.5 Discussion

Development of a prophylactic HIV vaccine is an important component in battling the worldwide HIV epidemic, but the path towards this goal has been fraught with difficulties. Viral sequence diversity and antigenic variation are major and perhaps insurmountable barriers in the development of vaccines based on the induction of humoral and/or cellular immunity against the virus. As an alternative strategy to conventional HIV vaccines, we have been interested developing a vaccine that targets CCR5, a self-molecule that is critically involved in HIV acquisition, and which is not subject to antigenic variation. However, because CCR5 is a self-protein, the ability to initiate an antibody response against the molecule is seemingly limited by the mechanisms of B cell tolerance, which normally prevent the induction of antibody

responses against self-molecules. In spite of this, we and others have shown that by arraying self-molecules at high density on the surface of virus-like particles (VLPs) we can completely abrogate these tolerance mechanisms and induce high titer IgG antibodies against diverse self-antigens (Chackerian 2007; Jennings and Bachmann 2009).

Figure 3.9: Aerosol exposure, but not intramuscular immunization, results in vaccine specific B cells in the lung

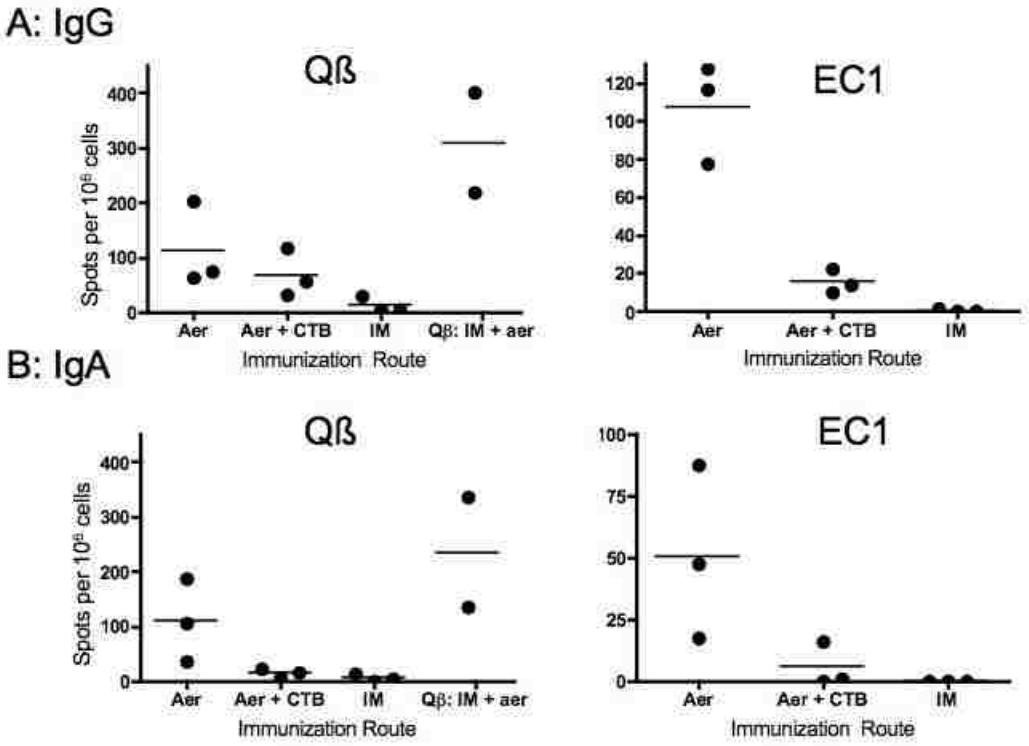

Figure 3.9. Aerosol exposure, but not intramuscular immunization, results in vaccine specific B cells in the lung. Lymphocytes were isolated from lungs of rats receiving intramuscular or pulmonary deliveries of Qβ-EC1 either with or without CTB adjuvant. Cells were analyzed by ELISPOT for IgG (A) and IgA (B) secreting Qβ-specific and EC1-specific B-cells. As a positive control, one group received an IM prime followed by aerosol boosts of Qβ VLPs alone (Far right sample, Qβ only).

Our laboratory has taken advantage of these findings to develop several VLP-based vaccines that elicit anti-CCR5 antibodies. Previously, we developed a

papillomavirus (PV) VLP-based vaccine targeting the N-terminal extracellular domain of macaque CCR5 that induced anti-CCR5 antibodies in macaques that bind to native CCR5 and blocked HIV infection *in vitro*. Moreover, prophylactic vaccination of macaques with this vaccine reduced viral loads and time to clearance in pig-tailed macaques infected intravenously with a CCR5-tropic SHIV (Chackerian, Briglio et al. 2004).

Here, we show that bacteriophage VLP-based vaccines that target two extracellular domains of macaque CCR5 that are involved in SIV/HIV binding induce similarly high titer anti-CCR5 antibodies in rodents. In this study, the question of immune tolerance was not addressed, as we targeted macaque CCR5 in rodent models. However we, and others, have shown that the ability to induce antibody responses in the face of immune tolerance mechanisms is a general characteristic of VLP display (Chackerian 2007; Jennings and Bachmann 2009). Moreover, several laboratories have previously or are currently working on approaches to induce anti-CCR5 antibodies. These include Lucia Lopalco's laboratory, which has developed a recombinant Flock House virus that presents a peptide derived from CCR5 ECL1 (Barassi, Soprana et al. 2005) and Tom Lehner's laboratory, which has developed a CCR5-HSP70 fusion protein immunogen (Bogers, Bergmeier et al. 2004; Bogers, Bergmeier et al. 2004), among others. Three macaque challenge experiments have been reported; our own and the previously mentioned Misumi study (Misumi, Nakayama et al. 2006), in which vaccinated macaques were challenged with a SHIV isolate and which some degree of viral inhibition was reported, and studies by Wahren and colleagues, in which DNA vaccination with a construct containing human CCR5 fused to tetanus toxoid failed to protect macaques from SIVsm challenge (Zuber, Hinkula et al. 2000).

HIV, like many pathogens, most frequently establishes infection at mucosal surfaces. As such, the efficient induction of mucosal immunity by vaccination, and particularly the induction of local immunoglobulin production, including secretory IgA (SIgA), will likely play an important role in future HIV-1 vaccine approaches (Mazzoli, Trabattoni et al. 1997; Devito, Broliden et al. 2000). The genital and gastrointestinal mucosa play crucial roles in the establishment of HIV infection, either as a site of transmission or as an important site of early viral replication and amplification. Although antibodies against CCR5 would presumably not act as classical neutralizing antibodies, it is possible that they could block interactions between virus and the cells that are targeted early in infection, specifically resident activated memory T cells or Langerhans cells (LCs). LCs have been proposed to facilitate HIV infection by capturing virus, migrating to regional lymph nodes, and then transferring virions to susceptible T cells. Although LCs can interact with HIV via a variety of different surface receptors, it has been shown that antibodies that bind CCR5 can partially block the uptake of HIV by LCs (Hladik, Sakchalathorn et al. 2007). Low level CCR5-reactive antibodies (with virus inhibitory activity *in vitro*) have been detected in the seronegative partners of HIV-infected individuals, suggesting that anti-CCR5 antibodies may play a role in protection from natural HIV infection (Lopalco, Barassi et al. 2000).

Numerous mucosal vaccination strategies have been investigated, including administration of vaccines to oral, genital, rectal, and respiratory mucosal surfaces. The mucosal immune system is largely compartmentalized, and the expression of mucosal IgA typically occurs primarily at the site of vaccination (Holmgren and Czerkinsky 2005). Thus, the choice of a mucosal vaccination route needs to be tailored to the

specific target. The respiratory tract is a particularly attractive site for immunization. Not only does immunization of the respiratory tract stimulate a local mucosal antibody response, but it can also elicit a strong genital mucosal immune response [23]. Moreover, administration of vaccines via aerosol to the nasal and bronchial lymphoid tissues is less invasive than other approaches and may facilitate vaccine implementation.

A number of different studies in mice have shown that intranasal vaccination with VLPs or VLP-based immunogens successfully results in local (in the lung) mucosal and systemic antibody responses (Balmelli, Demotz et al. 2002; Barassi, Soprana et al. 2005; Bessa, Schmitz et al. 2008). One advantage of aerosol over intranasal immunization is that aerosol delivery allows for VLP deposition in the lower respiratory tract. The trachea, lungs, and mediastinal lymph nodes act as the major immune inductive sites, allowing for a robust systemic humoral response. The upper respiratory tract, represented by the nasal-associated lymphoid tissue (NALT), although directly stimulated upon intranasal immunization, in some cases appears to play a negligible role in the induction of a mucosal immune response at distal sites (Balmelli, Roden et al. 1998; Balmelli, Demotz et al. 2002; Revaz, Zurbriggen et al. 2007), although at least one report has shown that nasal immunization can result in induction of IgA in the genital tract (Johansson, Wassen et al. 2001). Immunization of the lower respiratory tract is more effective at inducing genital antibody responses in mice (Balmelli, Roden et al. 1998). Moreover, immunization of the lower respiratory tract may be particularly important in inducing mucosal immunity in humans. For example, a comparative study of intranasal versus aerosol immunization of humans with HPV16 VLPs demonstrated that nasal immunization resulted in weak systemic and mucosal anti-VLP antibody responses. In

contrast, bronchial aerosolization of HPV16 VLPs resulted in significantly higher antibody levels and rates of seroconversion (Nardelli-Haefliger, Lurati et al. 2005). Consequently, if the lower respiratory tract is not reached for antigen presentation during intranasal immunization, it could account for reduced levels of mucosal, but not systemic, VLP-specific humoral responses. In this study, we provide the first indication that a VLP-based vaccine targeting a heterologous antigen can be aerosolized to effectively induce IgG and IgA antibodies in both local and distal mucosal tissues. The presence of Qβ–specific IgG antibody in distant mucosal tissues following aerosol immunization suggests that our vaccine was likely able to reach the lower respiratory tract.

We found that Qβ-EC1 VLPs were able to survive nebulization and delivery in a rat model, and that immunization via a pulmonary route was effective at generating both anti-EC1 IgG and IgA antibodies in the sera, the lung, the genital tract, and, to a lesser extent, the gastrointestinal (GI) tract. Presence of IgA in the uterine washes is most likely due to the upregulation of CCR10 on antibody producing cells, which allows for migration from the respiratory tract-draining lymph node to the genital tract (Brandtzaeg and Johansen 2005). Pulmonary immunization does not however typically result in upregulation of CCR9, which directs homing to the intestine (Butcher, Williams et al. 1999; Bowman, Kuklin et al. 2002). While this could explain the decreased amounts of IgG antibody found in the feces, the levels of IgA seen in both the uterine wash and the feces is somewhat surprising. It is possible that some of the inoculum reached the GI tract through animal inspiration during the aerosol exposure.

In accordance with the secreted antibody levels observed in the sera and mucosal tissues following pulmonary immunization, rats receiving the aerosolized vaccine were

also able to maintain high levels of Qβ-EC1 specific B cells in the lung, as well as detectable levels in the spleen and uterus 55 days following final exposure (data not shown). Similar to our study, Bessa et al. recently compared subcutaneous and intranasal deliveries of Qβ VLPs in mice and found that while both routes were effective at generating specific IgG in the serum, only the intranasal route of vaccination yielded Qβ-specific IgA in BAL; levels of mucosal IgG were also generally higher in these mice as compared to the s.c. group (Bessa, Schmitz et al. 2008). Bessa also showed that the numbers of IgG antibody-forming cells in the mesenteric lymph node were higher in mice receiving i.n. immunization of Qβ VLPs, which parallels our finding that aerosol immunization yields higher numbers of Qβ VLP- and EC1-specific B cells in the lung itself.

Unexpectedly, use of the mucosal adjuvant CTB did not appear to enhance immune responses at mucosal sites. Indeed, in some cases, CTB actually dampened antibody levels when compared to the group receiving the aerosolized vaccine without adjuvant. It is possible that the CTB was delivered to different mucosal compartments than the VLPs, or simply that CTB is not compatible with the nebulization process and thus not an appropriate adjuvant for aerosol delivery. Previously, Nardelli et al looked at the ability of mucosal adjuvants to increase both serum-specific IgG and mucosal IgA titers following "aerosol-like" vaccination. In this protocol, mice were anesthetized and immunized with HPV VLPs and either heat labile enterotoxin (HLT) or CpG oligodeoxynucleotides, following priming with influenza peptides. HLT proved a potent adjuvant and was able to increase VLP-specific IgG in the serum by more than 10-fold (Revaz, Zurbriggen et al. 2007). Furthermore, use of HLT was able to restore IgG and

IgA titers in both sera and mucosa that were previously obtained following vaccination with HPV VLPs and CTB adjuvant (without influenza priming). In contrast to our results, these prior experiments with CTB were successful, however titers were higher when HLT was used. The success of this adjuvant could be due to its ability to induce both Th1 and Th2 responses, as well as SIgA and serum IgG and IgA responses (Freytag and Clements 2005). Recent studies have shown that when CTB is used (along with whole cholera toxin [CT]) as a vector, it can give rise to either mucosal immunity or induce peripheral anti-inflammatory tolerance to chemically or genetically linked foreign antigens administered mucosally. More specifically, CTB seems to steer the immune response towards Th2-only immunity or tolerance, while CT favored a broad Th1 + Th2 + CTL immunity (Sanchez and Holmgren 2008). The use of HLT instead of CTB may enhance the IgA titers induced by VLP-based immunogens.

Use of the VLP-based approach for vaccine delivery to the mucosa-associated lymphoid tissue (MALT) has widespread applicability, with relevance to virtually any pathogen infecting at a mucosal surface. A vaccine that elicits both IgA and IgG antibodies at the site of infection prior to exposure could be a powerful deterrent to viral entry and subsequent infection. The innate immunogenic properties of VLPs provide a useful mechanism to generate antibody responses against poorly antigenic molecules both systemically and in the respiratory tract. The simultaneous flexibility and stability of VLPs offer further advantages with regard to mucosal vaccine design. The very type of VLP used for a particular vaccine can be catered to both its destination within the mucosa/environment and to the conjugate antigen, if applicable. For example, VLP size, surface chemistry, and tolerance for antigen insertion or conjugation are all factors in

vaccine design that can be readily manipulated for optimal efficacy. Translating this technology for non-invasive aerosolized delivery, with the result of an enhanced mucosal immune response, significantly broadens its applicability.

Chapter 4:

Aerosol delivery of virus-like particles to the genital tract induces local and systemic antibody responses

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4.1 Abstract

A strong mucosal immune response is desirable for combating pathogens which infect at mucosal surfaces. We had previously shown in rats that immunization via a pulmonary route with an aerosolized VLP-based vaccine elicited a local mucosal antibody response in the lung, as well as a weaker, but measurable, response in the genital tract. In this study, we examined whether direct immunization of the mouse genital tract with a VLP vaccine could induce robust mucosal and systemic antibody responses. We also investigated the importance of the type of VLP platform in inducing an immune response. Specifically, we asked whether the HPV VLP, which typically infects at a mucosal surface, is more immunogenic than bacteriophage VLPs. Lastly, we displayed peptides from the HPV16 L2 coat protein on the bacteriophage VLP platform, PP7. The resultant vaccine, PP7-HPV16L2, was administered intravaginally in mice to determine whether a mucosally delivered vaccine could inhibit infection against a pseudoviral challenge.

4.2 Introduction

Virus-like particles (VLPs) induce strong and long-lasting immune responses. They can be used as stand-alone vaccines that target the viruses from which they are derived and as scaffolds for presenting heterologous antigens. The clinically approved VLP-based vaccines that target hepatitis B virus (HBV) and human papillomavirus (HPV) are safe and effective, and represent the advancement of a new era in vaccine

development. VLPs are also inherently biocompatible and self-assembling, and from a manufacturing standpoint provide a facile and cost-effective solution to the engineering concerns often posed by synthetic materials.

One reason for the VLPs' success as a vaccine platform is its ability to evoke strong antibody responses. These responses are generated by the VLPs' structure: the arrangement of coat proteins provides a dense, repetitive display of immunostimulatory viral epitopes that B cells preferentially respond to. The multivalent nature of the particle allows for cross-linking of the B cell receptors, which in turn sends strong signals precipitating B cell migration, proliferation, and upregulation of surface molecules that promote interaction with T helper cells. This cascade of events initiated by the unique structure of the VLP allows for immunization at much lower concentrations, and without the use of potentially harmful adjuvants, than non-particulate (such as subunit) vaccines.

Our lab has developed VLP-based vaccines that target a number of different molecules derived from microbial and self-antigens. When given intramuscularly, these vaccines consistently induce high-titer serum antibodies. As described elsewhere in this thesis, we have been interested in using VLP-based vaccines to induce mucosal immune responses, particularly for preventing infection by pathogens which are transmitted at mucosal surfaces. Herein we characterize the development of a VLP-based, aerosolized vaccine for the genital tract, and investigate the ability of that vaccine to prevent HPV infection in a mouse model.

The most common sexually transmitted infections are caused by human papillomaviruses. In the United States it is estimated that over 6 million new cases are reported each year, and that over 20 million Americans are currently infected. There are

over 100 different types of HPV, of which a subset of approximately 15-18 "high-risk" types are known to cause cancer. Approximately 500,000 cases of cancer are caused by HPV infection each year, the most common being cervical cancer (Parkin and Bray 2006). Of these cases of cervical cancer, it is estimated that 70% are caused by either HPV16 or HPV18. Not surprisingly, the two HPV vaccines currently on the market target both of these types. The Gardasil vaccine, developed by Merck and approved in 2006, targets 4 HPV subtypes: HPV16 and HPV18 (referenced above), and HPV types 6 and 11, which are associated with genital warts. The Cervarix vaccine, developed by GlaxoSmithKline and approved in 2009, targets only HPV types 16 and 18. Both vaccines are composed of VLPs from the L1 major capsid protein of the HPV types targeted. Consistent with the profile of VLP-based vaccines, Gardasil and Cervarix are highly effective at preventing both infection and disease, induce long-lasting antibody responses, and are incredibly safe (Koutsky, Ault et al. 2002).

The success of the current VLP-based HPV vaccines is largely due to their targeting the major capsid protein L1, which is able to self assemble into a VLP and act as a stand-alone vaccine. However one drawback of the L1-based vaccines is that L1 is type-specific, in that the antibodies produced upon immunization are only able to provide protection against the specific HPV type that was targeted. For example, women vaccinated with HPV16 L1 VLPs were protected against HPV16-related diseases, but not against diseases caused by other HPV types (Koutsky, Ault et al. 2002; Mao, Koutsky et al. 2006). However, HPV virions contain a second structural protein, the minor capsid protein L2, which is highly conserved amongst HPV isolates. It has been hypothesized that a vaccine targeting L2, in contrast to L1, could provide more comprehensive

protection against multiple HPV types. In support of this hypothesis, it has been shown that peptides from the N-terminal domain of L2 contain broadly cross-neutralizing epitopes (Roden, Yutzy et al. 2000).

The specific reasons underlying L2 containing highly conserved, cross-neutralizing epitopes are largely unknown. Although expression of L2 is not required for the formation of VLPs, it is required for the formation of infectious virions. Indeed, there is a positive correlation between viral infectivity and the L2 content of the virion (Buck, Cheng et al. 2008). Yet in spite of L2s role in infectivity, neutralizing antibodies against the protein are absent following both natural infection and immunization with VLPs. Recent studies propose that the neutralizing epitopes are masked on the virion surface until a conformational change exposes them following receptor binding to the virus. Since the epitopes aren't exposed until after HPV has bound its cellular receptor, there would be little evolutionary pressure for L2 to undergo antigenic variation, thus supporting the observation that the minor capsid protein is conserved across several HPV type (Selinka, Giroglou et al. 2003; Richards, Lowy et al. 2006; Day, Gambhira et al. 2008).

Despite its potential to overcome the type-specific limitations imposed by L1-VLP vaccines, an L2-based vaccine poses its own challenges. Immunization with L1/L2-comprised VLPs fails to elicit immune responses against L2, but animal studies have shown that immunization with bacterially-expressed L2 protein or L2-derived peptides is successful at evoking a protective immune response (Lin, Borenstein et al. 1992; Gambhira, Jagu et al. 2007; Alphs, Gambhira et al. 2008). However, unlike L1, L2 is poorly immunogenic. The titers raised after vaccination with L2 are significantly lower

than those elicited by L1 vaccines (Roden, Yutzy et al. 2000). Consequently, our lab has sought to take advantage of our various VLP technologies and target L2 using VLP platform technologies. We anticipate that display of L2 peptides on an appropriate VLP platform will enhance the immunogenicity of L2.

Previously we used the bacteriophage Qβ to display peptides derived from CCR5. In this study, we used a recombinant VLP that had been previously developed by other members of the laboratory (Caldeira Jdo, Medford et al.). In this vaccine, a broadly cross-neutralizing epitope from L2 was inserted into a surface-exposed loop on the coat protein of the single-stranded RNA bacteriophage PP7. Qβ and PP7 are both members of the family *Leviviridae* and their coat proteins have similar structures. Qβ normally infects *E. coli* whereas PP7 infects *Pseudomonas aeruginosa*. We have recently shown that the PP7 coat protein is highly tolerant of peptide insertions, making it a useful platform for genetic display of peptides. For example, intramuscular immunization with PP7 VLPs displaying HPV16 L2 peptides induce high-titer antibodies against HPV16 L2 which can protect from infection in a mouse pseudovirus challenge model (Caldeira Jdo, Medford et al.).

The importance of mucosal immune responses was highlighted earlier in Chapter 3. To summarize, mucosal surfaces are often used by a wide variety of pathogens to gain entry into hosts. One means an invading organism has of securing its survival is to take advantage of the survival means of its host, in particular eating, breathing, and sexual reproduction. The immune system has responded and adapted by having a unique immune environment for each of these receptive mucosal surfaces: the gastrointestinal, respiratory, and genital mucosae, respectively. Because the environment of each of these

mucosal compartments is distinct, it has been suggested that directly immunizing the mucosal compartment targeted by a specific pathogen could result in the induction of protective antibodies at the site of infection (Belyakov and Ahlers 2009). For example, the inhaled FluMist vaccine and oral polio vaccines target the respiratory and gastrointestinal tracts to successfully provide comprehensive mucosal and systemic protection against the influenza and polioviruses (Herremans, Reimerink et al. 1999; Haan, Verweij et al. 2001). However, apart from topical microbicides targeting bacterial pathogens, the genital tract has remained largely under-utilized as a site for possible vaccine delivery (Rusnati, Vicenzi et al. 2009).

The genital mucosa in particular is vulnerable to transmission of a host of bacterial and viral pathogens, many of which can cause serious and incurable diseases. For example, HPV, HIV and herpes simplex virus (HSV) are the most clinically relevant of these sexually-transmitted viruses, and are associated with cervical cancer, AIDS, and genital herpes, respectively. Yet despite numerous efforts, only HPV has been successfully targeted with a vaccine. It is likely that a more comprehensive understanding of the workings of the innate and adaptive immune system at the genital mucosa, and their mechanisms of protection, is necessary for the rational design of other vaccines against STIs.

We previously developed a bacteriophage VLP-based vaccine targeting the HIV coreceptor CCR5 and showed that, following intramuscular administration, it induced high-titer serum antibodies against CCR5 that can inhibit HIV/SIV infection. We have also assessed the ability of this vaccine to induce mucosal immune responses, which is desirable due to the number of pathogens transmitted at mucosal surfaces. In rats, both

intramuscular and pulmonary immunization induced high titer IgG and IgA against the vaccine in the serum, but only the aerosolized vaccine induced CCR5-specific IgA locally in the lung and remotely in the genital tract (Hunter, Smyth et al. 2009).

Here, we present data comparing the magnitude of this "secondary" response in the genital tract to the IgA response invoked following direct immunization of the genital mucosa with different VLP platforms, including VLPs from a virus (human papillomavirus) that normally infects in the genital tract, and VLPs of the bacteriophages Qβ and PP7. Taken together, our results provide a general method for inducing broad systemic and mucosal antibody responses in discrete mucosal compartments using VLP-based immunogens.

4.3 Materials and Methods

4.3.1 Animal Immunizations

Groups of three to five Balb/c mice were immunized with Qß, PP7, or HPV16 L1 VLPs. Mice given an intramuscular vaccine were immunized with 10µg of the appropriate VLP preparation in incomplete Freund's adjuvant, with the exception of mice receiving the conjugated PP7 vaccine, which did not contain any adjuvant. For intravaginal immunizations, either via gel or aerosol (described below), 25 µg of VLPs were applied without exogenous adjuvant. All mice were immunized on days 1 and 15; the group immunized with HPV16 VLPs received a single boost on day 42, the group immunized with PP7 received boosts on days 42 and 200 (**Table 4.1**). Serum samples (approximately 0.1- 0.2 mL) and vaginal lavages (approximately 0.2 mL in PBS) were collected prior to immunization, and every week until sacrifice. To minimize fluctuations

in whole IgA levels, all mice received subcutaneous (sc) injections of Depo-Provera (3.0 mg in 0.1 mL) 4 days prior to immunization, and then every ten days until sacrifice, to synchronize the estrous cycle. Synchronization was verified by microscopic examination of cells obtained by vaginal lavage.

4.3.2 Preparation of immunogen for delivery

For each animal receiving the vaccine in gel form, 25 μg of VLPs were added to a 3% solution of carboxymethyl-cellulose (CMC) in distilled water. VLPs were highly concentrated to ensure that the optimal viscosity was maintained. Using a positive displacement pipette (Gilson, Middleton, WI), 30 μl of the VLP-in-CMC gel was delivered to the vaginal tract. For each animal receiving the aerosolized vaccine, 25 μg of VLPs in PBS (in a volume not to exceed 50 μl) were loaded into a microsprayer high-pressure syringe (Penn-Century, Wyndmoor, PA) and delivered to the vaginal tract.

4.3.3 Disruption of vaginal epithelia

Prior to genital immunization the vaginal epithelia was disrupted either by mechanical or chemical abrasion. Both methods of disruption were performed as previously described (Roberts, Buck et al. 2007; Johnson, Kines et al. 2009). Briefly, mechanical disruption was conducted immediately prior to immunization and performed using a cytobrush (Cooper Surgical, Trumbull, CT). Chemical disruption was conducted 6 hours prior to immunization and performed by introducing 30 μl of a 4% nonoxynol-9 in 3% CMC solution into the genital tract.

4.3.4 VLP preparations

A genetic insertion approach was used to display L2 epitopes on the surface of PP7 VLPs, as described (Caldeira Jdo, Medford et al.). Briefly, the PP7 coat gene sequence was mutated to introduce a KpnI restriction site so that insertion of foreign sequences into the plasmid could be tolerated. PCR was used to insert a 15 amino acid L2 sequence into the PP7 coat. The ability of recombinant proteins to successfully assemble in VLPs was assessed, and the PP7-HPV16L2 VLPs were purified. HPV16 L1-VLPs were generously supplied by Michelle Ozbun (University of New Mexico).

4.3.5 Quantifying antibody responses

Sera and vaginal lavages were collected at the time points indicated, and all samples analyzed by ELISA for antibodies specific to the appropriate VLP. ELISA experiments were performed as previously described (Hunter, Smyth et al. 2008). Specifically, Immulon II ELISA plates (Dynex Technologies, Chantilly, VA) were coated overnight at 4°C with either 0.5 µg of HPV16, Qβ, or PP7 VLPs per well. For analysis of L2-specific antibody in sera from animals immunized with PP7-L2 VLPs, wells were coated in three steps. First, 0.5 µg of streptavidin was applied per well for 2 h at room temperature. After washing in PBS, 0.5 µg of the crosslinker SMPH was added to each well. Lastly, after 2 h at room temperature, wells were washed again in PBS, 0.5 µg of L2 peptide was added to each well, and plates were left overnight at 4°C. Following the appropriate plate-coating procedure, all wells were blocked with 50 µL of PBS with 0.5% milk (w/v) per well for 2 h at room temperature. An initial 1:40 dilution of serum was serially diluted 4-fold and applied to wells for 2.5 h at room temperature. (All dilutions

were done in 0.5% milk [w/v] in PBS unless otherwise noted.) Reactivity to the target VLP was determined by using horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Jackson Immunoresearch, Bar Harbor, ME) at a dilution of 1:4000 and incubated for 1 h at room temperature. Upon development, the optical density at 405nm (OD405) was determined using a Thermo Max microplate reader (ThermoLab Systems, Fisher Scientific, Pittsburgh, PA). Absorbancies greater than twice the background were considered positive. ELISAs for IgA were conducted as above, incorporating the following changes: the blocking was performed with 0.5% BSA in PBS, the lavage samples were applied directly to the wells at a single, 1:1 dilution, and presence of antibodies was detected using HRP-labeled goat anti-mouse IgA (Open Biosystems, Huntsville, AL).

4.3.6 HPV pseudovirus challenge

Following immunization, animals were genitally challenged with HPV16 pseudovirus encapsidating a luciferase reporter plasmid as previously described (Caldeira Jdo, Medford et al. ; Cuburu, Kweon et al. 2009). Briefly, the vaginal epithelium was chemically disrupted as described above. Six hours later, 8.0 μL of 3.7 X 10^8 IU/mL HPV16 pseudovirus in 3% CMC was applied to the genital tract of each animal using a positive displacement pipette.

Infection was detected as a bioluminescent signal using a live animal imager. Forty-eight hours after pseudoviral challenge, anesthetized mice were given 20 μL of the luciferace reporter substrate, XenoLight D-Luciferin Potassium Salt (Caliper Life Sciences), and imaged with a Xenogen IVIS (Caliper Life Sciences). Images were taken

5 minutes after installation of luciferin at medium binning with a 5-minute exposure. The average radiance per mouse was measured in photons/s/cm$^{2/}$sr for a set region of equal dimensions between mice.

Table 4.1: Experimental design for genital vaccines in mice

Inoculum	Disruption Method	Delivery & Dose	Vaccination Schedule	Sacrificed @
Qβ n=3	N/A	Intramuscular + IFA (10 μg)	Weeks 0, 2	6 Weeks
Qβ n=5	Chemical	Gel (intravaginal) (25μg)	Weeks 0, 2	6 Weeks
Qβ n=4	Mechanical	Aerosol (intravaginal) (25μg)	Weeks 0, 2	6 Weeks
Qβ n=4	Chemical	Aerosol (intravaginal) (25μg)	Weeks 0, 2	6 Weeks
HPV16 n=5	Chemical	Aerosol (intravaginal) (25μg)	Weeks 0, 2, 7	12 Weeks
PP7-16L2 n=4	N/A	Intramuscular (10 μg)	Weeks 0, 2, 8, 28	30 Weeks
PP7-16L2 n=5	Chemical	Aerosol (intravaginal) (25μg)	Weeks 0, 2, 8, 28	30 Weeks
HPV16-L1 n=5	Chemical	Aerosol (intravaginal) (25μg)	Week 0	4 Weeks

4.4 Results

4.4.1 Direct immunization of the genital tract induces VLP-specific systemic IgG in recipient mice

We had previously developed a VLP-based vaccine targeting the HIV coreceptor CCR5. This vaccine was delivered via aerosol in rats, and was successful at inducing

both systemic and mucosal immune responses against the target CCR5 peptide. In addition to finding specific antibody in the lung, low-titer antibodies were also found in genital secretions. We asked how this secondary response in the genital tract following delivery of a pulmonary vaccine would compare to the response elicited following direct immunization of the genital tract. To this end, we assessed the responses to genital vaccination with unconjugated VLPs using several different strategies.

Previous data from John Schiller's group at the NIH suggested that vaccination with HPV pseudovirions (for the purpose of inducing T cell responses) is enhanced by disruption of the genital tract prior to immunization (Roberts, Buck et al. 2007; Iwasaki 2010). It is hypothesized that disruption of the vaginal epithelia prior to vaccination allows for maximum uptake of the particles and exposure to resident antigen presenting cells in the genital mucosa. We investigated the efficacy of two separate disruption methods, chemical and mechanical disruption. Mechanical disruption was accomplished by scraping the genital tract with a cytobrush immediately preceding immunization. Chemical disruption was accomplished by applying a solution of 4% nonoxynol-9 in 3% carboxymethyl cellulose (CMC) to the genital tract six hours prior to immunization. Treatment with nonoxynol-9 has been shown to cause small abrasions in the genital tract; these abrasions ultimately create a microenvironment that is more permissive to infection than when left untreated (Roberts, Buck et al. 2007). In addition, we investigated two vaccine application techniques. In the first, the vaccine was delivered to mice in a gel format, with VLPs suspended in 3% CMC. In the second, VLPs were aerosolized and delivered directly to the genital tract through a high-pressure syringe. In each case, mice were immunized twice at a two-week interval with a dose of 25 μg of Qβ VLPs without

adjuvant. As a control, one group of mice received intramuscular immunizations, using 10 µg of Qß VLPs solubilized in incomplete Freund's adjuvant. Sera were collected at the time points indicated and serum antibody titers were determined by ELISA.

Figure 4.1: Aerosol immunization to the genital tract induces systemic antibodies

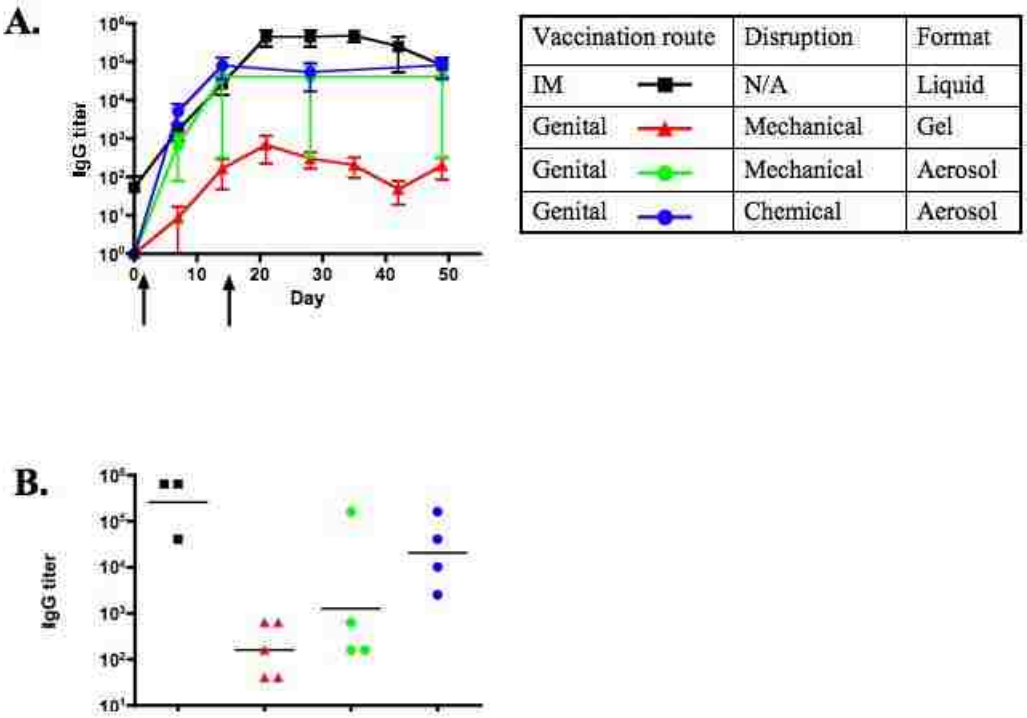

Figure 4.1. Serum IgG antibody titers following various administrations of a Qβ VLP vaccine delivered directly to the mouse genital tract. Mice were immunized on days 1 and 15 with Qβ VLPs in gel (triangles) or aerosol (circles) format. Sera were collected at the time points indicated and antibody titers determined by end-point dilution ELISA. The aerosolized vaccine was administered following mechanical (green circles) or chemical (blue circles) disruption of the vaginal epithelia. As a positive control, one group was immunized intramuscularly (squares). The data shows the geometric mean titer of each group of animals and error bars represent SEM. A) Kinetics of serum anti-Qβ IgG. Arrows indicate days of immunization. B) Comparison of serum anti-Qβ IgG in individual mice two weeks following second immunization. Bars represent the geometric mean titer of each group.

As shown in **Figure 4.1**, the intramuscular route was the most successful at inducing high-titer anti-Qß VLP IgG antibodies in the sera. However, vaginal immunization also resulted in systemic anti-Qß antibody responses. The peak antibody titers in the best-responding vaginal immunization group were about 10-fold lower than in the animals given vaccine via the intramuscular route. Aerosol administration of the vaccine to the genital tract proved more effective than gel administration at inducing antibodies in the sera, with chemical disruption of the vaginal epithelia producing more uniformly higher titers than mechanical disruption. This trend is best illustrated in **Figure 4.1 B**, which shows IgG titers in individual animals at a single time point, one week following the second immunization. Again, with the exception of one highly responsive animal, aerosol immunizations preceded by mechanical disruption elicited titers two-fold lower than those preceded by chemical disruption. Titers were sustained in all groups for five weeks following the final immunization, though the group immunized with a VLP-containing gel consistently had the lowest titers. The ability of the genital vaccines to elicit a systemic response prompted us to determine whether they could also elicit a robust mucosal immune response, and how that response compared to the one elicited by the intramuscular vaccine.

4.4.2 Immunization of the mouse genital tract successfully induces local mucosal antibodies

To assess the ability of the various genital vaccines to elicit a genital mucosal antibody responses, we performed vaginal lavages at the time points indicated and measured Qß-specific IgG or IgA in these secretions.

Figure 4.2: **Anti-Qβ IgG and IgA levels in vaginal washes of immunized mice**

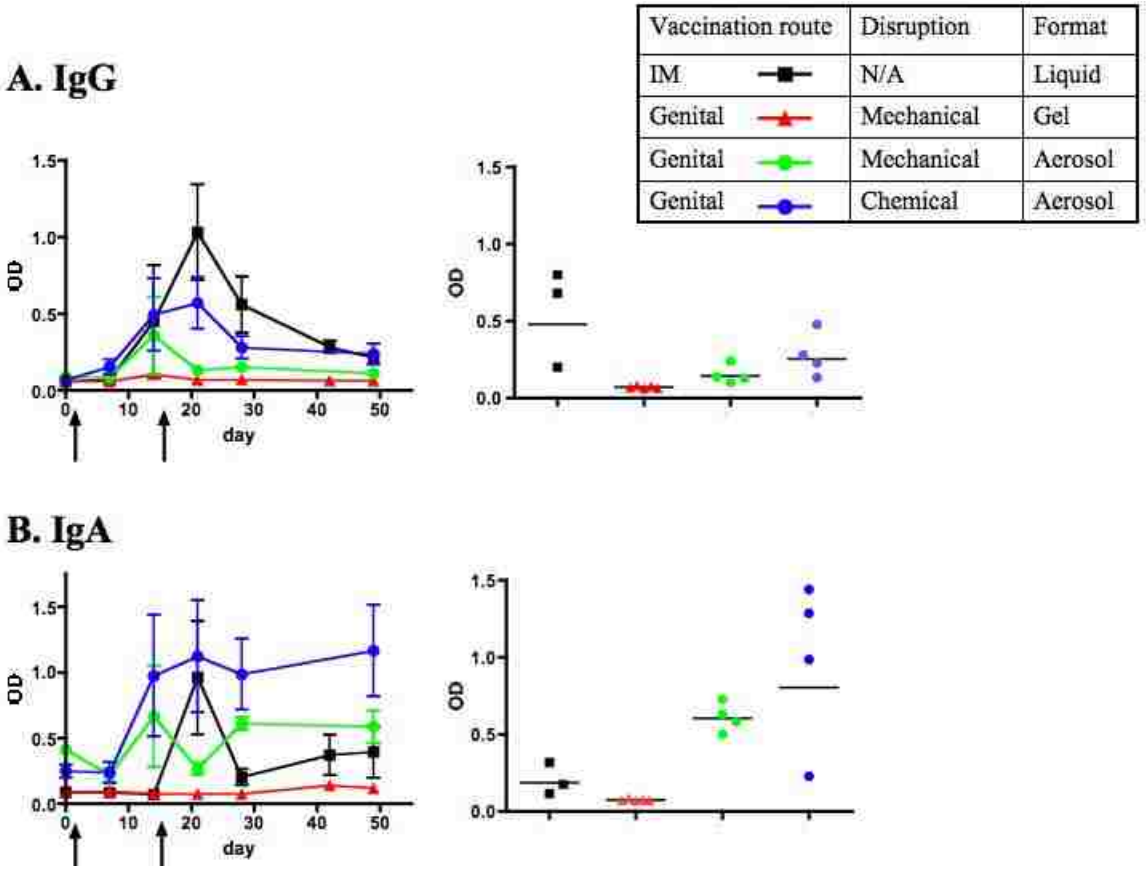

Figure 4.2. Anti-Qβ IgG and IgA levels in vaginal washes of immunized mice. Animals were immunized via different routes as described above, and antibody levels determined by ELISA. The data shows the geometric mean titer of each group of animals and error bars represent SEM. The anti-Qβ IgG (A) and IgA (B) responses are depicted kinetically (left) and at a single time point, two weeks following the second immunization, in individual mice (right).

As shown in **Figure 4.2**, intramuscular administration was able to elicit secreted IgG in the genital tract (panel A), but we only detected IgA at a single timepoint (panel B). Aerosol adminstration of the vaccine was markedly more successful at eliciting both IgG and IgA than the gel, and chemical disruption of the vaginal epithelia prior to immunization greatly improved antibody levels as compared to mechanical disruption. Secreted IgA antibody levels diminished rapidly following the final intramuscular

immunization, while they were sustained for up to 5 weeks in animals receiving the aerosol vaccine. Based on these results, we concluded that the optimal protocol for vaccine delivery to the genital tract was to chemically disrupt the vaginal epithelia prior to immunization via an aerosol spray.

4.4.3 Magnitude of systemic immune response does not depend on type of VLP platform

The next question we sought to address was whether the type of VLP used in the vaccine influenced the magnitude of the immune response. We hypothesized that a VLP derived from a virus that ordinarily infects at the genital mucosa may be preferentially recognized and taken up by resident dendritic cells. As our previous results showed that immunization with the Qβ bacteriophage VLP was successful at inducing both mucosal and systemic antibodies, we repeated these experiments (using only the aerosolized vaccine) with HPV16 VLPs. HPV16 infects at the genital mucosa, is carcinogenic, and is one of the subtypes currently targeted by the commercially available Gardasil vaccine. We compare the mucosal immune response we observed upon immunization of the genital tract with Qβ bacteriophage VLP, which is a bacteriophage and does not infect the mammalian genital tract, with a VLP type that is perhaps more readily recognizable to the resident immune cells in the genital tract. On days 1 and 15, mice were immunized intravaginally via aerosol with either 25 μg of HPV or Qβ VLPs, following chemical disruption of the vaginal epithelia. Sera were collected at the time points indicated and analyzed by end-point dilution ELISA for specific IgG. As shown in **Figure 4.3**, both VLP platforms were successful at eliciting high-titer antibodies in the serum, with mean

titers peaking around 10^5. We followed animals immunized with HPV16 VLPs for two months following the second vaccination. During this period geometric mean antibody titers dropped approximately 10-fold.

Figure 4.3: Comparison of systemic immune responses following immunization with Qβ or HPV VLPs

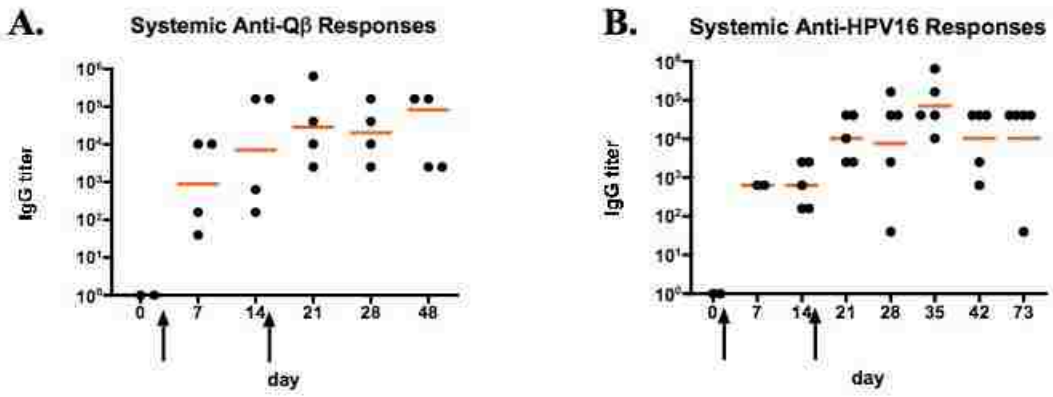

Figure 4.3. Groups of four mice were immunized on days 1 and 15 (black arrows) via aerosol to the genital tract. Mice received 25 μg of either Qβ (A) or HPV16 (B) VLPs six hours following chemical disruption of the vaginal epithelia. Sera were collected at the time points indicated and analyzed for VLP-specific IgG via end-point dilution ELISA. The bars show the geometric mean titer of each group of animals. Mice receiving the HPV vaccine were given a third immunization on day 48 and the efficacy of this final boost tested on day 73.

4.4.4 Mucosal immune responses in mice following aerosolized genital immunization with two separate VLP platforms

To complete our analysis of the mucosal immune response following genital immunization with either Qβ or HPV16 VLPs, vaginal lavages were collected at the time points indicated and analyzed at a 1:1 dilution by ELISA for either IgG or IgA specific

antibodies against the appropriate VLP type. Both Qβ and HPV16 VLP platforms were successful at eliciting secreted IgG and IgA in the genital tract (**Figure 4.4**).

Figure 4.4: Comparison of local mucosal immune responses following immunization with Qβ or HPV VLPs

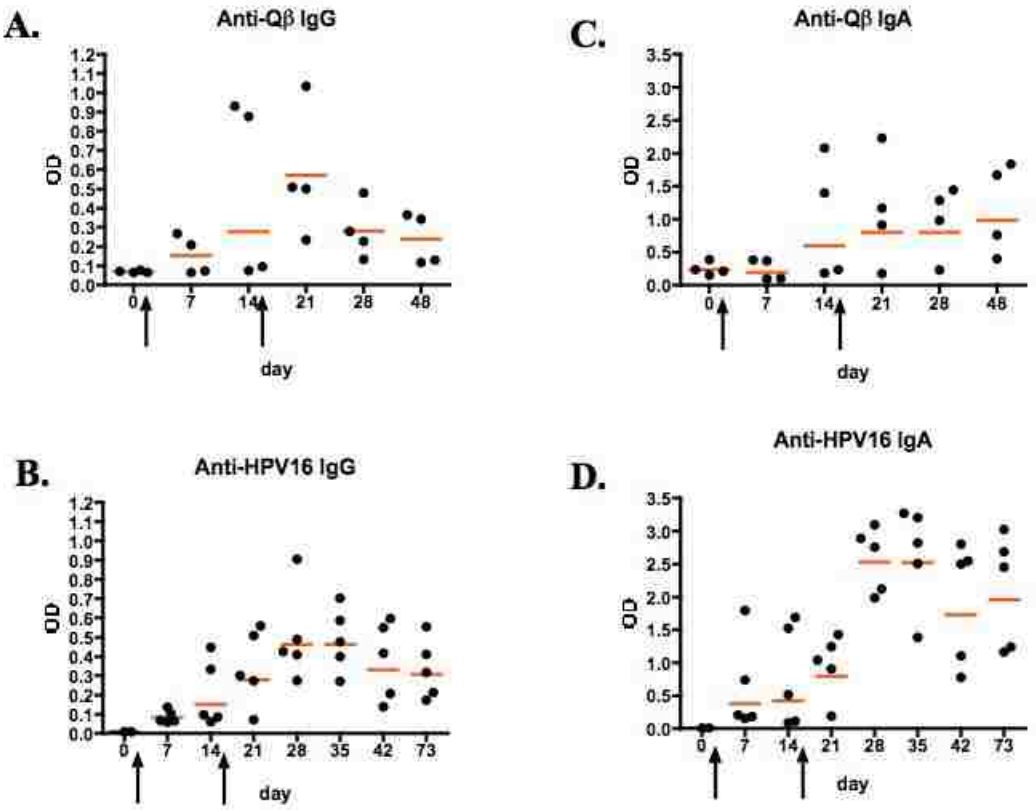

Figure 4.4. Groups of four to five mice were immunized on days 1 and 15 (arrows) via aerosol to the genital tract. Mice received 25 μg of either Qβ (A) or HPV16 (B) VLPs six hours following chemical disruption of the vaginal epithelia. Vaginal lavage samples were collected at the time points indicated and analyzed by ELISA for VLP-specific IgG and IgA, and the OD determined. The bars represent the mean OD of each group of animals. Mice receiving the HPV vaccine were given a third immunization on day 48 and the efficacy of this final boost tested on day 73.

Levels of secreted IgG appeared only slightly higher in animals immunized with HPV16 VLPs, while IgG levels peaked sooner in the animals that received Qβ VLPs. Immunization with HPV16 VLPs elicited higher IgA levels than immunization with Qβ VLPs. In accordance with what was observed in the sera, animals that were

immunized with HPV16 VLPs and observed over two months after the second immunization were still producing and maintaining local IgG and IgA specific antibodies in the genital tract throughout the course of study.

4.4.5 Ability of immunization with HPV16 VLP to confer protection against infection

Next, we assessed whether intravaginal immunization with HPV16 VLPs could protect against HPV16 pseudovirus infection of the genital tract. Because we detected both IgA and IgG responses after a single intravaginal vaccination, we asked whether a single dose was sufficient to provide protection from challenge. A group of 5 mice was given a single vaginal dose of HPV16 VLPs via the chemical abrasion, aerosol immunization protocol. Vaginal lavages and sera were collected prior to application and 20 days following immunization. As shown in **Figure 4.5**, a single immunization was sufficient for the induction of both systemic serum-associated antibodies in all 5 mice, and mucosal antibodies in 4 out of 5 animals. Serum titers were several-fold lower than the peak titers observed in prior groups receiving multiple immunizations.

Mice were challenged with HPV16 pseudovirus on day 24. A second group of unimmunized mice were also challenged as positive controls. Consistent with the immune responses observed prior to challenge, 4 out of 5 animals were completely protected against infection (**Figure 4.6**). Referencing back to Figure 4.5, the mouse that was susceptible to infection was the same animal (#918) that failed to elicit mucosal HPV-specific IgA and IgG antibodies in the genital tract. While this mouse also had lower antibody titers in the sera, a second animal (#916) had equally low titers in the sera,

yet displayed moderate levels of specific antibody in its vaginal washes, and was protected against infection.

Figure 4.5: A single aerosol immunization of HPV VLPs via the genital tract is sufficient for inducing systemic and mucosal antibodies

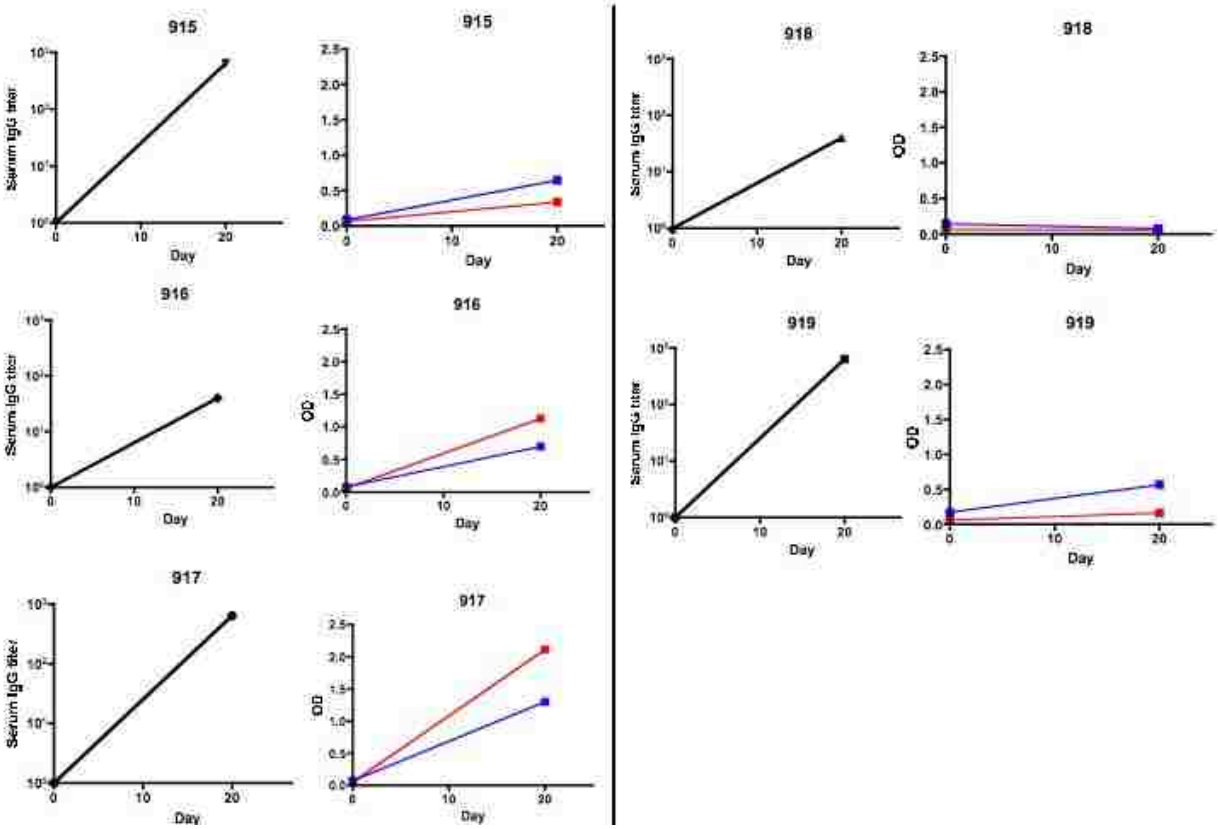

Figure 4.5. Mice (numbered 915-919) were given a single dose of aerosolized HPV16 VLPs via the genital tract. Sera and vaginal lavages were collected on days 0 and 20, and analyzed by ELISA for IgG and IgA specific antibodies. For each animal, serum titers are shown on the left (black); levels of IgG (red) and IgA (blue) from lavages are shown on the right.

These data suggest that specific antibodies at the site of infection could alone be sufficient for resisting infection. In addition, these data indicate that a single immunization with an appropriate VLP platform is able to induce antibody levels in the sera and mucosa that are ample enough to confer protection against viral challenge.

Figure 4.6: **Aerosol immunization of the genital tract with HPV16 VLP confers protection from infection after a single immunization**

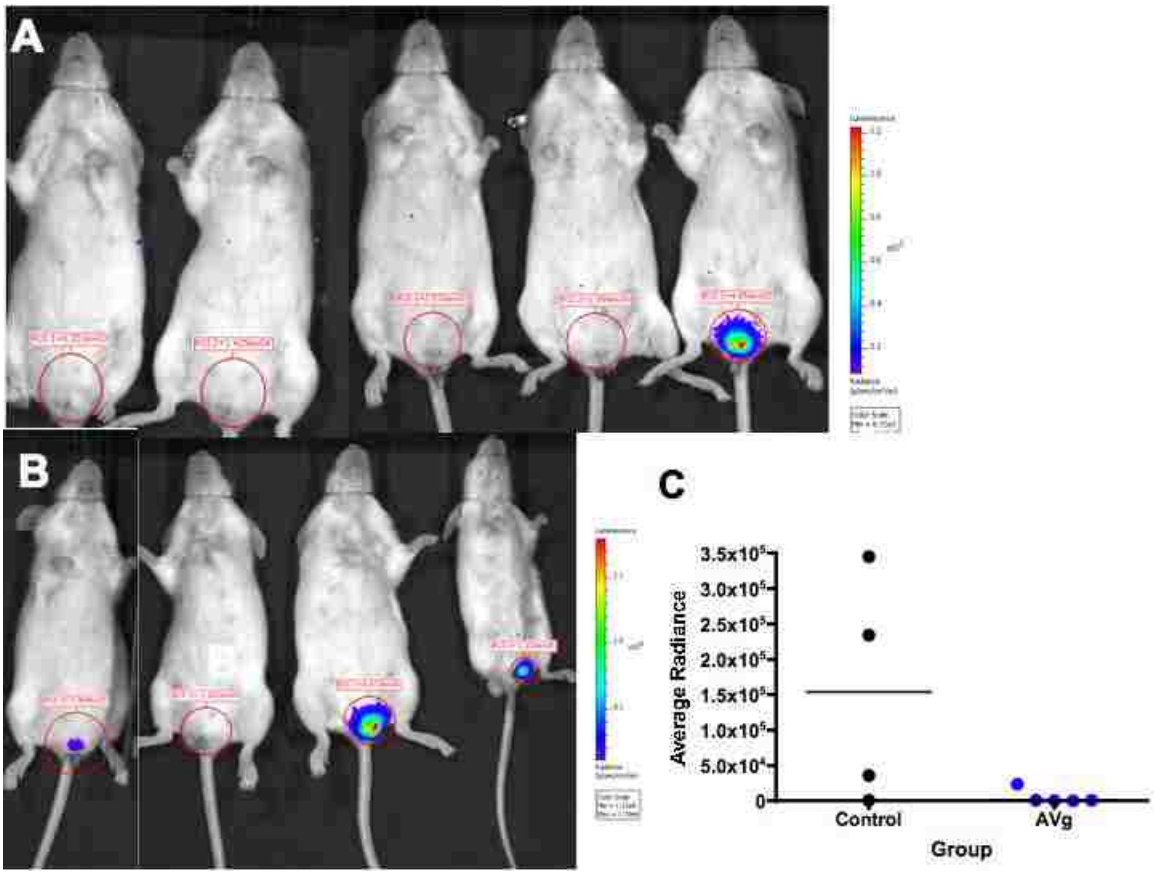

Figure 4.6. Radiance per animal in unimmunized mice (B), and mice immunized via the genital (A) routes. C) Average radiance per group. Data points represent individual animals and data bars represent the mean.

4.4.6 Genital aerosol immunization with a recombinant VLP-based vaccine elicits systemic and mucosal immune responses against both platform and target peptide

As described previously, VLPs can be used as stand-alone vaccines, but they can also be used as platforms for displaying heterologous peptides in a highly immunogenic multivalent format. To this end, we asked whether a recombinant VLP displaying HPV-derived neutralizing epitope could also elicit an antibody response upon intravaginal immunization. We used recombinant VLPs in which a broadly neutralizing peptide

derived from the HPV16 minor capsid protein, L2, was displayed on the surface of VLPs from the RNA bacteriophage, PP7 (Caldeira Jdo, Medford et al.). We characterized the magnitude of the mucosal and systemic immune response following aerosol immunization of the genital tract using these PP7-16L2 VLPs.

Figure 4.7: Systemic immune responses against PP7 VLP and L2 target peptide following aerosol immunization of the mouse genital tract

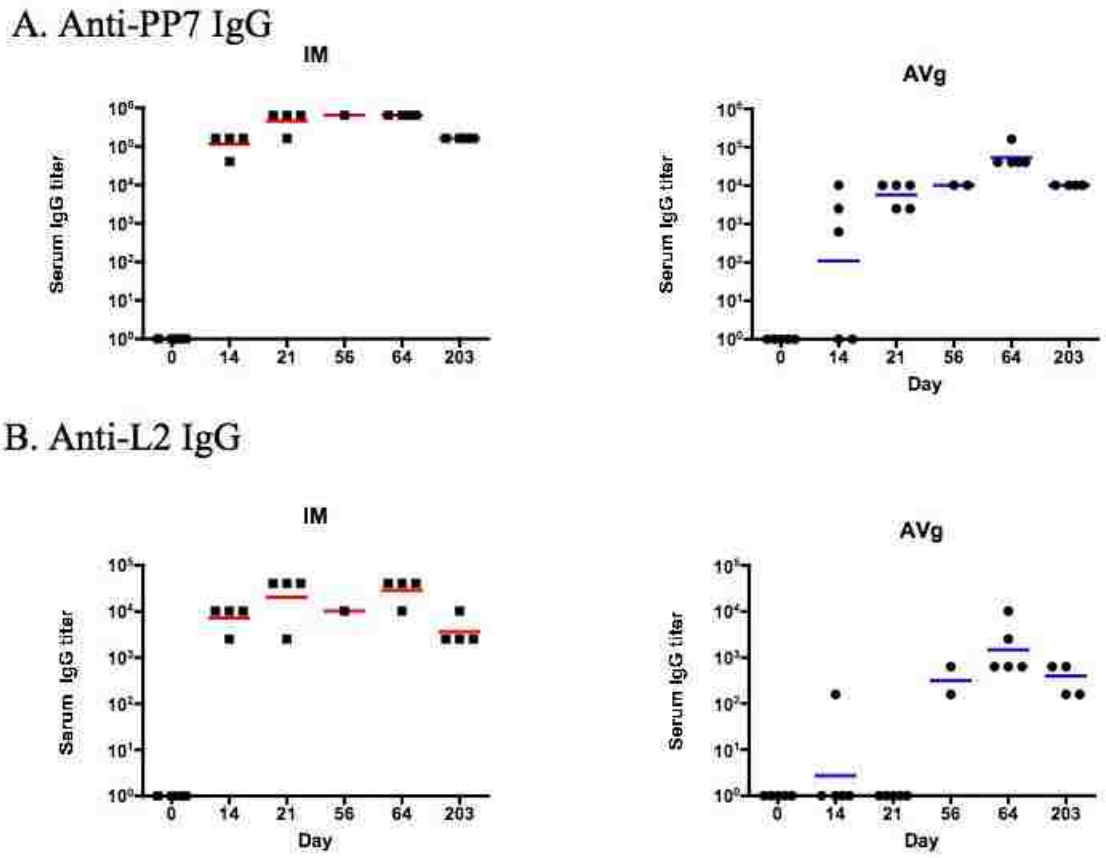

Figure 4.7. Mice were immunized either intramuscularly ("IM", shown in squares) or intravaginally via aerosol ("AVg", shown in circles) with 25 μg of PP7-HPV16L2 VLPs. Immunizations were carried out on weeks 0, 2 and 8; sera were collected and analyzed via end-point dilution ELISA for (A) PP7- and (B) L2- specific IgG on weeks 0, 2, 3, 7 and 9, as indicated. The data shows the geometric mean titer of each group of animals and error bars represent SEM.

On weeks 0, 2, and 8 groups of mice were either immunized intramuscularly (IM) or intravaginally via aerosol (AVg) with 10 or 25 μg of PP7-HPV16L2 VLPs, respectively. Unlike previous IM immunizations, these were carried out in the absence of adjuvant, in order to better compare the two vaccination strategies (as no adjuvant is present in the aerosol preparation). Sera and vaginal lavage fluid were collected prior to immunization and at the time points indicated, and analyzed for IgG and IgA antibodies against both VLP platform and the target peptide. Both the IM and AVg approach elicited high-titer IgG antibodies against PP7 in the sera (**Figure 4.7 A**), though titers were approximately 10-fold higher in the IM immunized group. In IM immunized animals, high-titer IgG against the displayed L2 peptide was achieved after a single immunization, whereas animals immunized via AVg did not have significant anti-L2 IgG until at least 2 weeks after their second immunization (**Figure 4.7 B**).

Antibody responses in the genital tract were consistent with what we observed previously with Qß and HPV VLPs. The amount of vaginally secreted IgG was relatively low in comparison to secreted IgA. The IM vaccine was more effective at eliciting secreted IgG against the PP7 platform, and to a lesser extent the L2 target peptide, than the AVg vaccine (**Figure 4.8 A**), although these responses were short lived. Secreted IgA against PP7 and L2 was seen in the genital tract following both IM and AVg administration of the vaccine, at levels higher than the observed IgG levels (**Figure 4.8 B**). There was no appreciable difference in the amount of IgA against PP7 or L2 at any given time point between animals immunized IM or AVg, although IgA against PP7 was more abundant than IgA against L2.

Figure 4.8: Local mucosal immune responses against VLP platform and target peptide following immunization of mouse genital tract

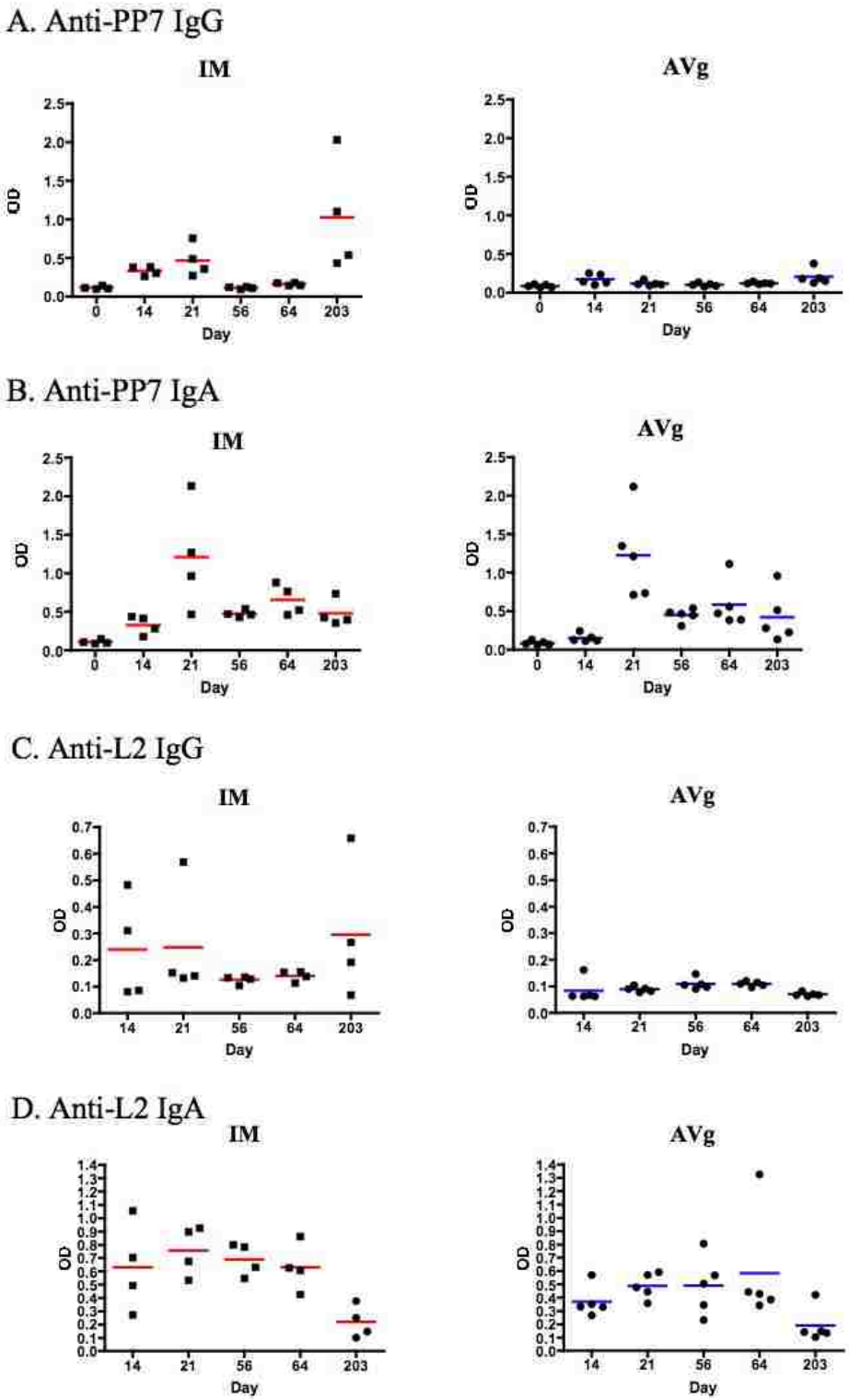

Figure 4.8. Mice were immunized as described above either by IM (squares) or AVg (circles) routes. Vaginal lavages were obtained on weeks 2, 3, 7, 9 and 29, and analyzed by ELISA for IgG (A, C) and IgA (B, D) antibodies against PP7 (A, B) and L2 (C, D). Bars represent the mean OD at 405.

4.4.7 Genital aerosol immunization of the mouse genital tract with recombinant VLPs confers protection from infection

In order to assess the long-term antibody responses in these mice, we followed immunized animals for approximately 6 months after the initial immunization (Figure 4.8). We extended the study in the PP7-L2 group for approximately 5 more months before challenge. Animals were given a final boost of HPV16L2-linked PP7 VLPs, for a total of 4 immunizations, on day 195. On day 203, the IM and AVg groups were challenged with HPV16 pseudovirus carrying a luciferase reporter. As a control, 3 unimmunized mice were also infected. Protection from infection was visualized as a bioluminescent signal 48 hours post-infection using an IVIS live animal imager. The number of infected cells is represented by the amount of radiance detected in a given area per mouse. As shown in **Figure 4.9**, both AVag and IM immunization protected mice from pseudovirus infection, although the protection was more dramatic in the mice immunized through the intramuscular route. Immunization with aerosol via the genital tract conferred only partial protection to the group, with two of the mice showing complete protection (no signal), two showing no protection, and one mouse showing an intermediate degree of protection. This was not surprising based on the pre-challenge antibody titers observed in both groups, and it was anticipated that animals receiving the genital vaccine would not be as protected from infection than those receiving the intramuscular vaccine.

<u>Figure 4.9:</u> Aerosol immunization of the genital tract with conjugated PP7 vaccine confers only partial protection from infection

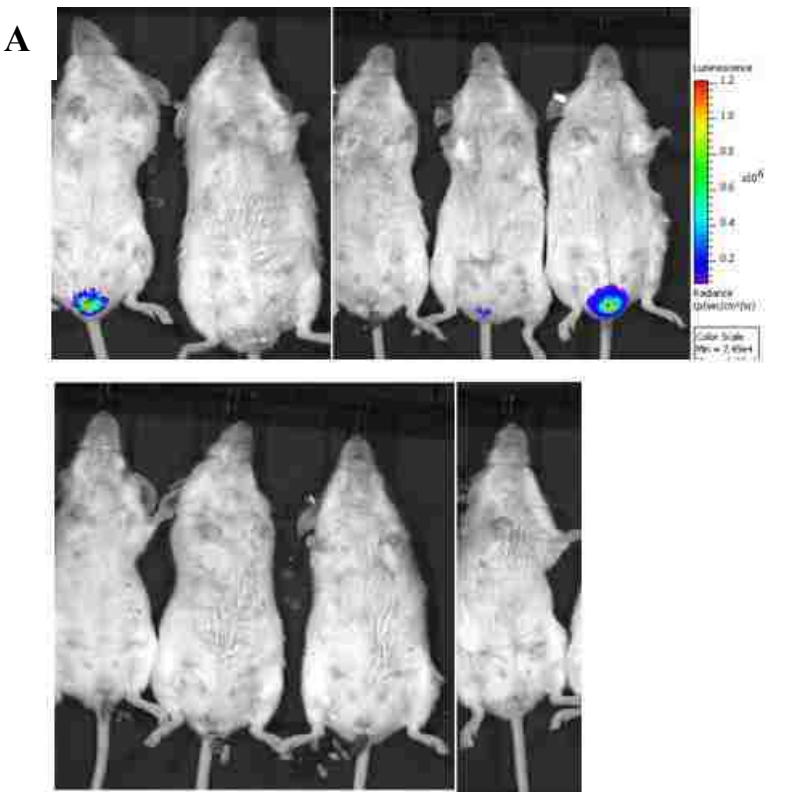

Figure 4.9. A) Radiance per animal in mice immunized via genital (top panel) and intramuscular (bottom panel) routes. B) Average radiance per group. Data points represent individual animals and data bars represent the geometric mean.

4.4 Discussion

The current vaccines targeting HPV are clearly effective, but their high cost and ability to target only a small subset of carcinogenic HPVs argues for further research and development of second generation vaccines that more broadly protect against HPV infection. As the HPVs that cause significant morbidity and mortality establish infections in the genital tract, it would also be greatly beneficial to have

neutralizing antibodies at this site to prevent infection. The local production of antibodies in the genital mucosa is mediated by resident plasma cells, which mostly secrete IgA and can transport efficiently into the mucosal lumen (Holmgren and Czerkinsky 2005). In order to guarantee stimulation of these IgA-producing plasma cells, antigens need access to mucosa-associated lymphoid tissues (MALT). In terms of vaccine design, this will probably best be accomplished via a mucosal administration of the target antigens.

In contrast, existing HPV vaccines are parenterally administered, and likely mediate protection through systemic IgG. Although systemic IgG is able to migrate to the genital tract either through transudation or exudation at sites of microtrauma, IgG titers in the cervicovaginal mucus are often 10-fold lower than in the serum, and continue to diminish during ovulation (Nardelli-Haefliger, Lurati et al. 2005; Stanley, Lowy et al. 2006). Furthermore, traditional L2-based vaccines induce much lower neutralizing (and particularly cross-neutralizing) antibody titers than L1-VLP-based vaccines such as Cevarix and Gardasil, which regularly induce high-titer neutralizing antibodies. Therefore, it is likely that the L2-VLP-based vaccines that we have developed will only be successful if they induce similar high-titer neutralizing antibodies in the serum as L1-VLP vaccines, or if they induce sustained local immune responses in the genital mucosa. To this end, we proposed that a vaccine administered to the genital mucosa would improve upon the IgG antibody levels seen following parenteral vaccination, as well as incorporate IgA antibodies at the site of HPV infection.

The induction of genital immune responses is somewhat complicated however by the compartmentalization of the mucosal immune system. Many studies have

demonstrated to varied success the effectiveness of oral, intranasal and rectal vaccine deliveries at inducing specific antibodies in genital secretions (Bergquist, Johansson et al. 1997; Kozlowski, Cu-Uvin et al. 1997; Johansson, Wassen et al. 2001; Kutteh, Kantele et al. 2001), and our own studies correlate with the observation that presentation of antigen to the lung, either via aerosol or intratracheal deposition, can especially enhance specific antibodies in the genital tract (Balmelli, Demotz et al. 2002; Hunter, Smyth et al. 2009). Yet limited evidence exists supporting intravaginal immunization for the induction of local immunity in the genital tract (Wassen, Schon et al. 1996). This is mostly due to a corresponding lack of reliable vaginal immunization protocols, although some success in this regard has been recently reported using genetically engineered Salmonella (Echchannaoui, Bianchi et al. 2008).

It was of interest, then, that a single mucosal administration of L1 VLPs was sufficient to confer protection from genital challenge with a high-risk HPV. The ability for a single, non-invasive immunization to confer protection may facilitate vaccine implementation on a global scale. The success of the single immunization may in part be due to the VLP platform used, as our studies suggest that HPV VLPs may be more successful at generating high levels of antibody in the genital mucosa than the PP7 and Qβ bacteriophage VLPs platforms that we investigated and employed for antigenic display of L2 peptides.

Here we have shown that a mucosal administration of aerosolized L2-VLPs is effective at inducing IgA and IgG at the genital tract, but did not enhance the protection from pseudoinfection provided by an identical intramuscular vaccine. Since protection from vaginal challenge was not complete in those animals receiving only the genital

vaccine, future studies should be undertaken to determine whether this was a failure of uptake and presentation of the immunogen in the genital environment, or if it is due to the overall weakness of the elicited mucosal immunity. To distinguish between these possibilities, it may also be necessary to determine whether our L2 vaccines can protect against cutaneous challenge with HPV pseudovirions using a cutaneous challenge model described by Roden and colleagues (Alphs, Gambhira et al. 2008).

The specific HPV peptide we chose to display is representative of an N-terminal region on the HPV16 L2 protein that has previously been shown to contain broadly cross-neutralizing epitopes. This epitope is also currently the target of an HPV neutralizing monoclonal antibody, and when linked to a universal T helper epitope can elicit cross-neutralizing antibodies against HPV (Gambhira, Karanam et al. 2007; Alphs, Gambhira et al. 2008). One disadvantage of vaccination with a single L2 peptide is that it induces protection that is greater for a homologous, rather than heterologous, HPV virus. For example, Jagu et al showed that a concatenated multitype L2 fusion protein elicited more broadly neutralizing antibodies than recombinant L2 derived from a single HPV type (Jagu, Karanam et al. 2009). An advantage and future direction of our PP7 genetic display system is that it provides for the rapid development of PP7 VLPs that can display L2 peptides from a variety of HPV types.

A combination vaccine formulated to include additional HPV VLPs types is an attractive possibility as it would probably be more effective at preventing cervical cancer. For example, Merck has developed a nonavalent HPV vaccine that contains L1-VLPs derived from HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58, which is currently in clinical trials. Assuming that it is effective, it is predicted that this vaccine will provide

~90% protection (Munoz, Bosch et al. 2004). Although a nonavalent vaccine would be highly beneficial, it is likely that such a vaccine would be very expensive. Indeed, the current HPV vaccines are already amongst the most expensive vaccines in clinical use. Moreover, because the vaccine is predicted to be ineffective against a small, but still nonetheless significant, fraction of carcinogenic HPV types, costly cytological screening of vaccinees will still be necessary. Together, these factors could present as barriers to worldwide vaccine implementation, especially in the developing world where vaccines are expected to have the most public health impact and where cervical cancer is the most common of women. In support of a mucosally-administered vaccination protocol, which could diminish the need for costly medical personnel and facilities, we can draw from the hindered success of the hepatitis B vaccine in under-developed regions. The vaccine, which requires several intramuscular injections, has been available for over 25 years, but as of 2001 over 80 countries still lacked programs to enforce its successful implementation (Kao and Chen 2002).

Immune responses to the same immunogen can vary dramatically depending on the route of administration, even between different mucosal routes. This was previously observed between the intranasal versus intratracheal routes of immunization, where it was hypothesized that VLPs were presented to the immune system by DCs in the tracheobronchial, but not nasal-associated, lymphoid tissues (Balmelli, Demotz et al. 2002). Likewise, the immune response to differing immunogens in the same mucosal compartment can be variable. Herein we show that our intravaginal L1 VLP vaccine is able to confer protection against intravaginal challenge following a single immunization,

and that intravaginal immunizations with L2-displaying VLPs could also provide some protection from challenge.

Chapter 5: Breaking tolerance in rhesus macaques using a VLP-based CCR5 mucosal vaccine delivered via aerosol to the genital tract

5.1 Introduction

For nearly three decades, the engineering of a successful HIV vaccine has remained an elusive challenge. Sequence diversity and the virus' immense capacity for antigenic variation are two of several hindrances to successful activation of the cellular and humoral arms of the immune system, both of which will likely be needed to effectively offer prophylactic protection against infection. Because the CCR5 coreceptor is a genetically stable self-protein, it is not subject to host immune pressures, unlike viral targets, which can rapidly yield and select for mutated variants. An extensive background on CCR5, its role in virus acquisition, and its emergence as an HIV therapeutic target is given in Chapter 2 of this thesis.

We have previously developed a papillomavirus (PV) VLP-based vaccine targeting the N-terminal extracellular domain (EC1) of macaque CCR5 that induces anti-CCR5 antibodies that bind to native CCR5 and block HIV infection *in vitro* (Chackerian, Lowy et al. 1999). Moreover, prophylactic vaccination of macaques with this vaccine reduced viral loads and time to clearance in pig-tailed macaques infected intravenously with a CCR5-tropic simian-human immunodeficiency virus (SHIV) (Chackerian, Briglio et al. 2004). In this latter study, it was also investigated whether the induction of autoantibodies against CCR5 would contribute to or cause any deleterious effects. We found that the antibodies elicited were not associated with any gross pathology or peripheral blood abnormalities. More importantly, no reduction in T cells (which express CCR5) was detected in the peripheral blood. This data suggests that strong immune

responses against CCR5 may play a role in controlling viral replication. Furthermore, our results correlated to a similar study by Misumi et al (Misumi, Nakayama et al. 2006). In their study, macaques were immunized with a CCR5 ECL2 cyclic peptide (similar to that used and discussed in Chapter 3 of this thesis) and then challenged with the same SHIV isolate that we used. Again, vaccinated macaques had significantly lower viral loads (~100-fold) than controls during the acute phase of SHIV infection.

Despite these successful findings, it is important to note some deficiencies to this study. First, the antibody responses to CCR5 in the vaccinated macaques were variable, underscoring the need to improve the vaccination regime so that high titer anti-CCR5 antibody responses can consistently be induced. Second, the SHIV challenge virus $SHIV_{SF162P3}$ that was selected replicated poorly in macaques and was eventually eliminated by host immunity, making the role of anti-CCR5 autoantibodies in the control of viral replication hard to assess. Third, it is highly possible that intravenous challenge with SIV is an inappropriate model to demonstrate the effectiveness of a prophylactic vaccine targeting CCR5, as transmission more commonly occurs via the anogenital mucosa.

To address these concerns, we have incorporated several changes into our current study. First, we believe that the variability observed in antibody responses could be due in part to the VLP platform and display system. We have since shown that peptide-conjugated Qß VLPs elicit stronger antibody responses than the papillomavirus VLP-based platform that was used previously (Chackerian, Rangel et al. 2006). To this end, we continued developing a Qß-based vaccine, using the Qß-EC1 vaccine that successfully evoked anti-CCR5 systemic and mucosal antibodies in rats as a template.

(This particular vaccine study was presented in Chapter 3 of this thesis, and is summarized briefly below.) Second, we chose to challenge with SIVmac251, a highly pathogenic SIV, rather than SHIV, isolate. Third, we sought to make the challenge more relevant by using an intravaginal immunization strategy, followed by intravaginal challenge.

We believe that intravaginal inoculation with Qß-CCR5 vaccines will result in strong genital mucosal antibody responses. The importance of inducing mucosal immunity has come under increased scrutiny as a vital component of HIV-1 vaccine design (Mazzoli, Trabattoni et al. 1997; Devito, Broliden et al. 2000). Specifically, the genital mucosa plays a crucial role in the transmission of HIV, while the gastrointestinal mucosa acts as an important site for early viral replication and amplification. Denise Nardelli-Haefliger has shown that intravaginal application of human papillomavirus (HPV) VLPs can induce strong anti-HPV antibody responses in mice (Echchannaoui, Bianchi et al. 2008), and our colleagues in John Schiller's laboratory have produced similar results (Graham, Kines et al.). Furthermore, the use of an intravaginal challenge mimics a common route of transmission and will therefore likely be more relevant for assessing the candidate vaccines' ability to prevent infection.

Presumably, antibodies against CCR5 would not be neutralizing, though it is possible that they could block interactions between virus and the cells that are targeted early in infection. For instance, Langerhans cells (LCs) can interact with HIV through a variety of different surface receptors, and it has been shown that uptake of HIV by LCs can be partially blocked by antibodies that bind CCR5 (Hladik, Sakchalathorn et al. 2007). Furthermore, low-level CCR5-reactive antibodies detected in the seronegative

partners of HIV-infected individuals have been shown to possess virus inhibitory activity *in vitro*, suggesting that anti-CCR5 antibodies may play a role in protection from natural HIV infection (Lopalco, Barassi et al. 2000).

As was previously mentioned, we recently tested a bacteriophage VLP-based EC1 vaccine in rats. These results were presented in Chapter 3 of this thesis, but the results can be summarized herein. The vaccine, which was delivered as an aerosol to the lung, resulted in the induction of systemic IgG against CCR5 at similar levels as when rats were immunized intramuscularly. The various pulmonary immunization protocols explored resulted in ~10-fold higher IgA responses in the sera. Rats immunized solely via the IM route had low IgG and undetectable IgA levels in bronchial and uterine lavages and in feces. In contrast, rats immunized with the aerosolized vaccine elicited mucosal IgA responses in the lung, feces, and uterus. IgG was only detected in the uterus (Hunter, Smyth et al. 2009). Based on these results, we have shown that VLP-based vaccines are compatible with mucosal (specifically, pulmonary) delivery and induce high-titer systemic antibodies as well as local and systemic mucosal antibody responses.

Despite the success of the pulmonary vaccine, we felt confident that intravaginal immunization would also elicit CCR5-specific antibodies in the genital tract based on our results with the intravaginal HPV vaccine discussed in Chapter 4. As such, in this chapter we present preliminary data from our study wherein we deliver an aerosolized Qβ-CCR5 vaccine to the genital tract of rhesus macaques, and determine its efficacy at protecting against intravaginal viral challenge.

5.2 Materials and Methods

5.2.1 VLP preparation

EC1 and ECL2 CCR5 peptides (representing extracellular domain 1 and the second extracellular loop, respectively) were conjugated to Qβ VLPs as previously described (Hunter, Smyth et al. 2009). Briefly, the 21 amino acid EC1 peptide, representative of the N-terminal sequence (MDYQVSSPTYDIDYYTSEPC) of pig-tailed macaque CCR5, was synthesized by American Peptide (Sunnyvale, CA). The ECL2 peptide (DRSQREGLHYTG) was synthesized by Celtek Peptides (Nashville, TN). Both peptides have previously been shown in **Figure 3.1**. Each peptide was conjugated to the Qβ surface using the bifunctional chemial crosslinker, SMPH.

5.2.2 Macaque immunizations

Eighteen 4 to 10-year old rhesus macaques (*Macaca mulatta*) were obtained from and housed at the California National Primate Research Center at the University of California, Davis under the supervision of our collaborator, Dr. Koen Van Rompay. Two groups of six macaques each were immunized 3 times at 4 week intervals with 25 μg of Qβ VLP alone (Qβ) or a mixture of Qβ -EC1 and Qβ -ECL2 (Qβ-EC1+ECL2) via the intramuscular route (IM). A third group was given an IM prime (25 μg) of Qβ-EC1+ECL2; the remaining immunizations were given intravaginally via aerosol (AVg) with 100 μg of the mixed VLPs. Serum samples were collected prior to and throughout the course of immunization, as were cervical and vaginal secretions. Cervical and vaginal secretions were collected using a Weck-cell sponge as described (Nardelli-Haefliger, Wirthner et al. 2003). A detailed schedule of animal immunizations and sample collection is shown in **Table 5.1**.

5.2.3 Quantifying antibody responses

Serum specific for EC1- or ECL2- antibody was detected by ELISA. Immulon II ELISA plates (Dynex Technologies, Chantilly, VA.) were coated with 0.5 μg of the appropriate peptide, or Qβ VLP, per well. Serum was serially diluted in 0.5% milk in PBS. Reactivity to target peptides was determined using a horseradish peroxidase-labeled goat anti-monkey IgG (Fitzgerald Industries, Acton, MA) at a dilution of 1:4,000 as a secondary antibody. Upon development, the optical densities at 405 nm (OD_{405}) were read by a Thermo Max microplate reader (Molecular Devices Corp, Sunnyvale, CA). OD_{405} values that were greater than twice the background were considered positive.

5.2.4 Avidity assay

The avidity of serum antibodies was determined by measuring the resistance of antibody target complexes to a 5-minute 8M urea wash by ELISA as described (Chackerian, Briglio et al. 2004) (Mazzoli, Trabattoni et al. 1997). Prior to urea wash, duplicate wells were coated with the appropriate target peptide, blocked, and treated with primary antibody (sera). Serum samples were loaded at the dilution that yielded the antibody titer for each animal at room temperature for 2.5 hours. After washing 3 times in PBS, wells were then treated with either PBS or urea for 5 minutes. Wells were then washed again in PBS; subsequent steps are identical to those described in *5.3.3* above following washing of the primary antibody. The avidity index value was calculated as the ratio of the mean OD value of urea-treated wells to PBS, multiplied by 100.

5.2.5 Macaque challenge with SIV

The eighteen macaques were challenged with 10^5 50% tissue culture infectious doses ($TCID_{50}$) of SIVmac251 and again in the same manner 4 h later, as described

(Stone, Keele et al.). Briefly, virus was introduced nontraumatically to the vaginal canal using a needleless 1-mL tuberculin syringe. At challenge, macaques were transferred to infectious animal housing, and followed for 12 months following infection. During this period, blood samples were collected on weeks 1, 2, 4, 6, 8 and 10 following challenge, and then every 4 weeks until necropsy.

5.2.6 Determination of viral load

Plasma-associated SIV RNA viral loads were determined using a real-time quantitative reverse transcription PCR method as previously described (Chackerian, Briglio et al. 2004) (Lifson, Rossio et al. 2001). Viral loads were transformed to the log base 10 scale since data were approximately normal on that scale.

5.3 Results

5.3.1 Intravaginal immunization with VLP-CCR5 vaccine overcomes tolerance and induces systemic antibodies in Rhesus macaques.

We expanded our previous Qβ-EC1 vaccine to target two regions of CCR5 that are involved in HIV binding: EC1 and ECL2, both of which have been discussed previously. To review, our conjugation protocol resulted in individual Qß VLPs that are coated with multiple copies (>100) of the two CCR5-derived peptides (**Figure 3.1**). Although anti-ECL2 and anti-EC1 antibodies were somewhat weakly neutralizing individually, in combination they displayed 50% inhibition of SIVmac251 infection at a 1:40 serum dilution in a single-cycle replication assay (**Figure 3.4**). While this might not seem like a very high inhibition titer, any potential target cell will express multiple copies

of CCR5, making inhibition of the receptor a more rigorous task than blocking the virus itself.

We delivered this vaccine (designated Qβ-EC1+ECL2) to 2 groups of 6 macaques each. The first immunization was given to both groups via the intramuscular route. The remaining 3 immunizations were given to one group (designated IM) via the intramuscular route; the second group (designated AVg) was given the remaining immunizations intravaginally, and in an aerosolized format. A third group of 6 macaques (designated Qβ) was immunized solely via the intramuscular route with unconjugated Qβ VLPs.

Table 5.1: Experimental Design for Genital Vaccines in Rhesus Macaques

	(-1) wk	0 wk	2 wk	4 wk	6 wk	8 wk	10 wk	14 wk	18 wk	22 wk	24 wk	26 wk
Procedure/Sampling (below)												
Group 1 (IM Qß-CCR5)												
IM immunization [1]		X		X		X				X		
1. Serum for ELISA	X		X		X		X	X	X		X	
2. Blood/PBMCs for flow cytometry (CD4, CCR5)	X		X		X		X	X	X		X	
3. Cervical secretion sampling (Weck Sponge)	X		X		X		X	X	X		X	
4. SIVmac251 intravaginal challenge (2x in 1 day)												X
Group 2 (IVag Qß-CCR5)												
IM immunization		X										
IVag immunization [2]				X		X				X		
1. Serum for ELISA	X		X		X		X	X	X	X	X	
2. Blood/PBMCs for flow cytometry (CD4, CCR5)	X		X		X		X	X	X		X	
3. Cervical secretion sampling (Weck Sponge)	X		X		X		X	X	X		X	
4. SIVmac251 intravaginal challenge (2x in 1 day)												X
Group 3 (IM Qß control)												
IM immunization		X		X		X				X		
1. Serum for ELISA	X		X		X		X	X	X		X	
2. Blood/PBMCs for flow cytometry (CD4, CCR5)	X		X		X		X	X	X		X	
3. Cervical secretion sampling (Weck Sponge)	X		X		X		X	X	X		X	
4. SIVmac251 intravaginal challenge (2x in 1 day)												X

Figure 5.1. Intravaginal immunization with Qβ-CCR5 overcomes tolerance and induces systemic antibodies in Rhesus macaques.

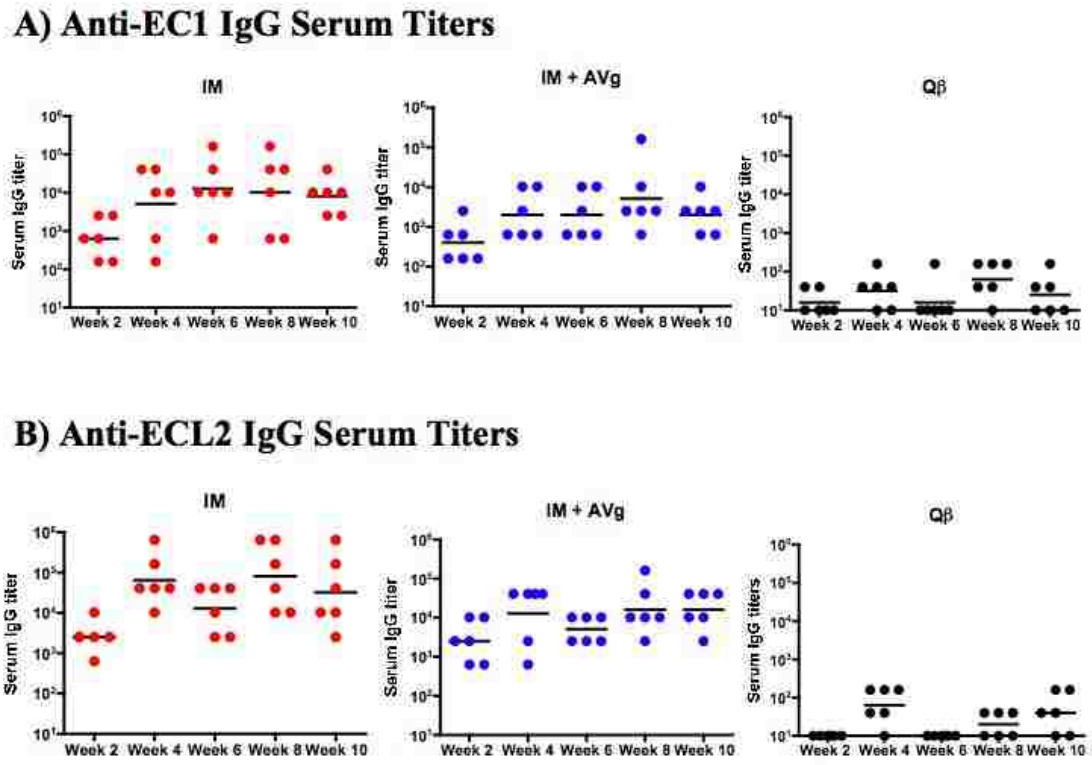

Figure 5.1. Groups of 6 female Rhesus macaques were immunized with Qβ-EC1+ECL2 on weeks 0, 4, and 8, and sera collected at the time points indicated for analysis by ELISA for Qβ-, EC1- and ECL2- specific IgG. Animals were immunized intramuscularly ("IM", red) or received a single IM prime followed by boosts via aerosol to the genital tract ("AVg", blue). As a control, a third group was immunized IM with unconjugated Qβ VLPs (black). Bars represent the geometric mean titer.

To confirm that our Qβ VLP-based vaccines could overcome tolerance and elicit antibodies against macaque CCR5, we immunized Rhesus macaques on weeks 0, 4, 8 and 22 via the IM or AVg protocol with Qβ-EC1+ECL2, or via IM with Qβ alone. Sera were collected at the time points indicated and described in **Figure 5.1** and **Table 5.1**, respectively. Samples were analyzed by end-point dilution ELISA for specific IgG against EC1 and ECL2 CCR5 peptides. Animals immunized both intramuscularly and intravaginally with CCR5 peptides were able to elicit systemic antibodies against both the

EC1 and ECL2 peptides. Titers against ECL2 were slightly higher (10^4-10^5) than titers against EC1 (10^3-10^4) in both groups. There was no appreciable difference in titers between the IM- and AVg- immunized animals **(Figure 5.1)**.

Animals were given a final immunization on week 22 to test the ability of the intravaginal vaccine to elicit a memory response. Serum samples were tested again by ELISA on week 24, and the titer of each animal prior to challenge was determined. These results, along with the approximate avidity of the serum antibodies, are shown in **Table 5.2**. By week 22, titers in the AVg group were lower than those in the IM group. Boosting brought up titers in only a fraction of the animals, with the IM group responding better to the boost than the AVg group. However, all animals had high-avidity antibodies (over 70%) against both EC1 and ECL2 peptides.

Table 5.2: Antibody titer and avidity at time of infection

Animal	EC1 titer wk 22	EC1 titer wk 24	ECL2 titer wk 22	ECL2 titer wk 24	EC1 avidity wk 24	ECL2 avidity wk 24
IM						
787	40,960	163,840	655,360	163,840	High	High
784	2,560	655,360	163,840	655,360	High	High
640	40,960	655,360	163,840	655,360	High	High
469	10,240	163,840	655,360	655,360	High	High
380	640	2,560	10,240	163,840	High	High
393	640	10,240	10,240	163,840	High	High
AVg						
512	10,240	2,560	10,240	163,840	High	High
709	640	2,560	10,240	40,960	High	High
214	640	640	10,240	10,240	High	High
060	2,560	2,560	40,960	10,240	High	High
793	640	160	2,560	2,560	High	High
285	40,960	10,240	163,840	163,840	High	High

High avidity, values $\geq$ 50%; intermediate avidity, values > 30% but < 50%, low avidity, values $\leq$ 30%.

5.4.2 Intravaginal immunization confers moderate protection against SIV challenge in Rhesus macaques

An intravaginal challenge of the highly virulent SIV strain, mac251, was given to all animals on week 26. Serum samples were collected weekly following challenge, and viral load determined. As shown in **Figure 5.2 A**, 3 of the 12 animals immunized with the CCR5 vaccine were substantially protected from infection, with viral loads that were >10,000-fold lower than the mean of the Qß control group. Two of these animals were from the group given the aerosolized intravaginal vaccine. The viral load of these 3 animals continued to decrease for 10 weeks following infection. Upon log-transformation of the viral load values, the AVg group appeared to have better protection against infection than the IM group. Both groups had significantly lower viral loads than control animals immunized with Qβ alone (**Figure 5.2 B**).

5.4 Discussion

Vaccination against CCR5 represents an intriguing alternative strategy to inhibit HIV infection, as conventional vaccines targeting HIV have failed. Here, in a macaque model, we show that upon vaccination with phage VLPs conjugated with CCR5 peptides, we were able to induce high titer antibody responses against those peptides, which represent two regions of CCR5 important in virus entry. We attempted to increase physiological relevance by using a highly-pathogenic SIV challenge virus and an appropriate route of transmission. By delivering the vaccine intravaginally, we sought to produce enhanced levels of local mucosal antibodies present at the time of intravaginal SIV challenge. By comparing between the systemic and mucosal routes of vaccine

administration, we attempt to shed light on the advantages, if any, that both approaches provide.

Figure 5.2: Viral loads by individual and group following viral challenge

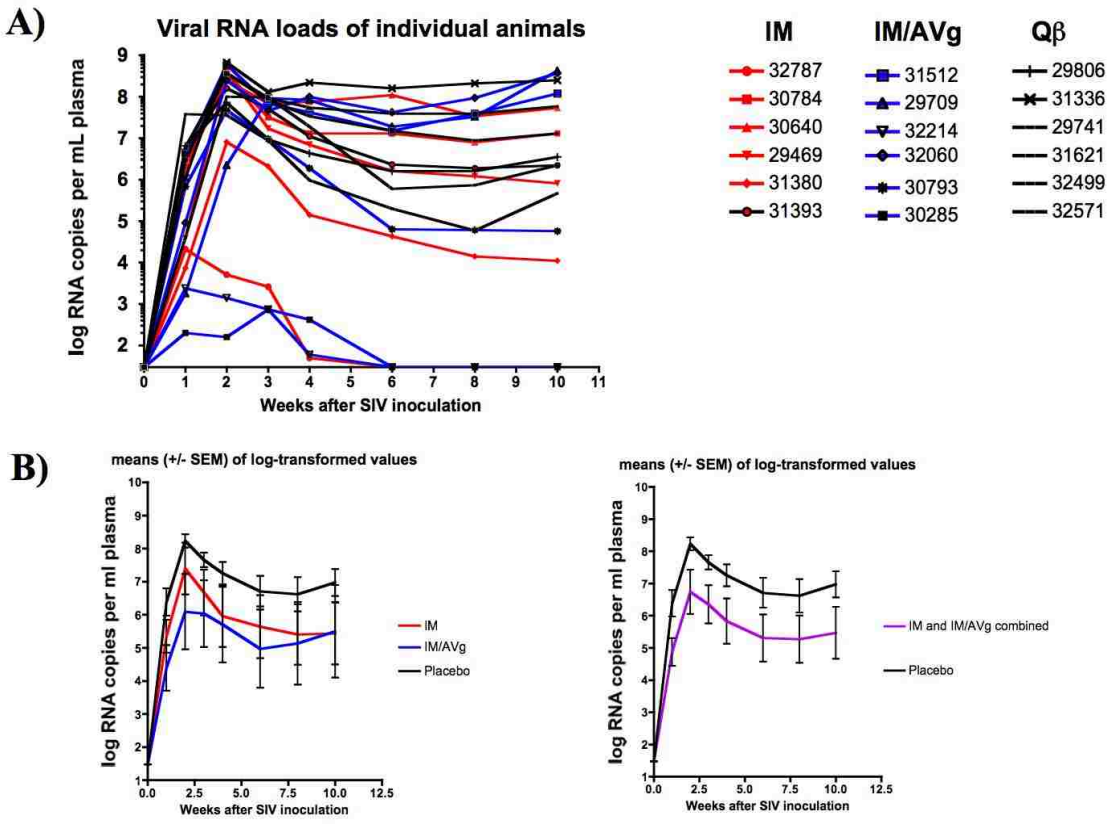

Figure 5.2. Following intravaginal challenge with SIVmac251, plasma was collected at the time points indicated and analyzed by real-time quantitative reverse transcription PCR for plasma-associated SIV RNA viral load. A) Viral loads of individual animals. B) Viral loads of each group (left) and of Qβ-CCR5 immunized versus placebo animals (right). Viral loads were transformed to the log base 10 scale.

In this study, anti-CCR5 antibodies were induced upon intramuscular immunization, as well as after an intramuscular prime followed by vaginal boosts. Macaques were genitally challenged with a high dose (two doses of 10^5 $TCID_{50}$) of the

pathogenic SIV strain mac251. Although vaccination did not prevent infection with SIV, three of the twelve immunized animals had dramatically reduced viral loads. Two of the three protected macaques were in the IM/AVg immunization group. By six weeks after infection, SIV RNA was undetectable in plasma in these three macaques, and remains undetectable as of our analysis ten weeks post infection. The degree of protection did not seem to correlate with anti-CCR5 antibody titers or antibody avidity; two of the three protected animals had quite high anti-CCR5 serum-associated antibodies, but one had lower titers. We are currently evaluating other correlates of protection – these will be discussed in more detail in Chapter 6. Importantly, in the cases where vaccination was able to provide protection, there are significant questions that will need to be addressed regarding the long-term safety of vaccination against the self protein CCR5. These safety concerns are also addressed below in Chapter 6.

In support of targeting CCR5, there are several other laboratories that have either previously or are currently working towards the induction of anti-CCR5 antibodies. For instance, Lucia Lopalco's laboratory has developed a recombinant Flock House virus that presents a peptide derived from CCR5 EC1 (Barassi, Soprana et al. 2005), and Tom Lehner's laboratory has developed a CCR5-HSP70 fusion protein immunogen (Bogers, Bergmeier et al. 2004; Bogers, Bergmeier et al. 2004). Three macaque challenge experiments have also been reported following immunization with a CCR5 vaccine; our own, the previously mentioned Misumi study (Misumi, Nakayama et al. 2006), and studies by Wahren and colleagues, in which DNA vaccination with a construct containing human CCR5 fused to tetanus toxoid was administered. The latter failed entirely to protect macaques from SIVsm challenge (Zuber, Hinkula et al. 2000), while the two

former studies reported only some degree of viral inhibition following challenge with a SHIV isolate. It is important to point out that in these studies antibodies were generated against human CCR5 in macaques. This is not an actual display of autoantibody induction, and it is unclear whether the anti-human CCR5 antibodies cross-reacted with macaque CCR5. In contrast, we have demonstrated previously and herein that our VLP-based system can overcome B cell tolerance and induce genuine autoantibodies against macaque CCR5.

A successful vaccine will have to promote immune responses capable of attenuating virus replication, immune disregulation, depletion of CD4+ T cells, enteropathy and dissemination of virus from mucosal to lymphoid tissues in order to provide sterilizing immunity. In recent years, it has become increasingly clear that in order to accomplish this level of protection, an effective HIV vaccine must be capable of eliciting local mucosal immune responses, both cellular (through CD8+ CTLs) and humoral (specifically IgA antibodies), in addition to a systemic response.

The role of IgA antibodies as a sole defense is largely unclear, however there are several studies supporting its importance in virus neutralization. HIV-1 envelope-specific mucosal IgA antibodies have been found to block transcytosis through epithelia as well as virus entry *in vitro.* Furthermore, highly exposed seronegative women can have secretory IgA in their cervicovaginal secretions, possibly contributing to the rendering of a semi-protective state (Kaul, Plummer et al. 2001). It has been widely proposed that an IgA-mediated mucosal immune response is best obtained through mucosal, rather than parenteral immunization. Still, there appears to be no consensus within the field whether a parenteral immunization route could generate sufficient

mucosal immunity to confer protection, and it is likewise debatable whether immunization via a mucosal route could generate the necessary local immunity necessary for an effective HIV vaccine.

There are studies that have contradicted the need for mucosal immunization entirely, particularly in the induction of T cell responses against HIV. Systemic (IM) immunization alone was able to generate CD8+ CTL responses at multiple mucosal sites in Rhesus macaques, indicating that mucosal immunization could be bypassed and long-lived resident CD8+ T cell memory could be induced both systemically and at mucosal effector sites (Kaufman, Liu et al. 2008). A second macaque study by Pal et al. showed that systemic immunization had decreased the viral set point in blood and mucosal sites, and protected against CD4+ T cell depletion (Pal, Venzon et al. 2006). It should be noted however, that in both of these studies, a comparison was not made between the efficacy of a mucosal immunization strategy versus a systemic one.

Comparisons between mucosal and systemic routes of vaccine administration have however been made. For instance, numerous studies by Belyakov et al. have shown that mucosal administration of a vaccine is more effective at eliciting high avidity mucosal antibodies than when the same vaccine is delivered systemically. More importantly the mucosal vaccine, which was given intrarectally in macaques, was able to better control viral load in gastrointestinal-associated lymphoid tissues (GALT), which comprise a major reservoir for viral replication in early HIV-1 infection. The observed generation of high-avidity CD8+ CTLs in the GALT inversely correlated with viral load in the gut and correlated positively with the maintenance of CD4+ T help following intrarectal challenge with SHIV (Belyakov, Hel et al. 2001; Belyakov, Isakov et al.

2007). Interestingly, these studies also support our observation that a heterologous (prime-boost) immunization strategy is preferential when administering vaccines mucosally; while our observations were restricted to humoral responses in the mucosa, they have shown that a prime-boost regimen is also critical for the induction of high levels of high-avidity CTL responses (Belyakov, Ahlers et al. 2008).

In conclusion, our study to date has shown that immunization with VLP-based vaccines targeting CCR5 - including an intravaginal, aerosolized vaccine - are effective at inducing serum-associated autoantibodies in Rhesus macaques. Some animals were able to maintain undetectable to low viral loads upon challenge with a highly pathogenic SIV strain. It is unlikely that natural transmission of the virus would initially contain such high viral copies; ideally, further studies will determine the maximal dose that can be tolerated and still result in protection. These results indicate that presence of CCR5-specific antibodies may be sufficient for blocking infection, and that protection may be conferred following heterologous (prime-boost) immunization via a mucosal route.

Chapter 6: Discussion

6.1 Summary of Findings

The observation that many viral structural proteins have an intrinsic ability to self-assemble into virus-like particles (VLPs) has led to a new class of vaccines. VLPs have been used as stand-alone vaccines targeting the viruses from which they are derived as well as platforms for presenting heterologous antigens. This dissertation has described the use of virus-like particles (VLPs) as flexible scaffolds for the display of heterologous antigens to the immune system. Clinically approved VLP-based vaccines are safe, effective, and comparatively easy to manufacture. Their inherent biocompatibility provides a facile and cost-effective solution to the engineering concerns often posed by synthetic materials, and their ability to evoke strong antibody responses against even poorly immunogenic targets makes them an attractive model for future vaccines targeting antigens derived from both pathogens and self-molecules.

Our lab has developed a portfolio of display technologies that allow us to deliberately modify VLPs so they can present essentially any epitope as a target antigen; these particles have served as the basis for several vaccines targeting a variety of different molecules derived from microbial and self-antigens. When given intramuscularly, these vaccines consistently induce high-titer serum antibodies. In the previous chapters, we have presented data showing that VLP-based vaccines are compatible with mucosal delivery to both the genital and respiratory tracts. Vaccines targeting both viral and self-antigens were successful at inducing mucosal and systemic immune responses, represented by the presence of IgG and IgA (when relevant) in the sera, and at local and

remote mucosal sites. The induction of both mucosal and systemic immune responses presents a particular advantage for preventing infection by pathogens transmitted at mucosal surfaces. Indeed, we have herein shown that our L2 mucosal vaccines are successful at preventing genital pseudoviral infection in a mouse model of HPV. As a demonstration of the VLPs ability to evoke strong antibody responses against a self and therefore poorly immunogenic molecule, we have also submitted data showing that immunization of macaques with our CCR5 vaccine results in undetectable viral loads and stable CD4 T cell counts in a subset of immunized animals following challenge with SIV.

In the remainder of this chapter, I will address specific concerns and limitations surrounding my research, and identify strategies to address some of these concerns. I also discuss the future directions of my research, and speculate on additional experimental procedures that may strengthen this dissertation.

6.2 Limitations

There are several specific limitations to the various studies and experiments described in this thesis. One common limitation throughout the work however is the use of appropriate mucosal adjuvants. While we have proven that immunization with VLPs is effective at generating an immune response without the use of adjuvants, immunogenicity can still likely be enhanced with their use, which could result in increased protection. Unfortunately, compared with adjuvants for parenteral vaccination, mucosal adjuvants are much less effective. Also, less is known about the compatibility of mucosal adjuvants with the deliveries we are proposing. For instance, our experiments with inactivated cholera toxin B (CTB; Chapter 3) appeared to actually have decreased

antibody responses. With very few exceptions, the group receiving this adjuvant had lower IgG and IgA in the serum and at mucosal sites than the group without adjuvant. Some adjuvants may be incompatible with nebulization, or may become separated from the VLPs and taken up separately. From a technical standpoint, the microsprayers are easily clogged, and any heterogeneity in inoculum could exacerbate this problem. One possible way to correct for this would be to have our VLPs package their own adjuvants. Examples of this were discussed in Chapter 1; to reiterate, CpG oligonucleotides can be encapsidated, and if using an RNA-derived VLP platform, single- and double-stranded RNA will intrinsically be present. While not strictly an example of an internal adjuvant, a more stable option is also available when using VLPs derived from enveloped viruses. For example, it was found that modification of SIV VLPs to incorporate CD40 ligand or GPI-anchored GM-CSF into the lipid bilayer enhanced maturation and functional activation of DCs and increased CD4+ and CD8+ T-cell responses to SIV Env protein compared to VLP alone (Skountzou, Quan et al. 2007).

Generally, and somewhat obviously, this work would likely benefit the most from a more comprehensive understanding of the cellular and molecular events that transpire following vaccination. While it is generally accepted that the repetitive and dense nature of antigenic display on the VLP surface is responsible for the particles' supreme immunogenicity, little is understood of the cellular interactions, trafficking, and downstream events following uptake and presentation of the particles and their cargo, particularly at mucosal sites. This information could impact future vaccine design, especially given the VLP's amenability to manipulation. I interpret this gap in knowledge more as a future direction for this work, rather than a limitation, and therefore propose

additional experiments to pursue in Section 6.3. Below, I acknowledge model-specific caveats and limitations to the CCR5 vaccines investigated in rats and macaques, and the HPV vaccine work in mice.

6.2.1 Limitations Specific to Chapter 3: CCR5 Pulmonary Vaccine in Rats

From a technical standpoint, the potential magnitude of the immune response garnered from the pulmonary vaccine was difficult to assess. The non-invasive chamber allows for steady inspiration and expiration, which is not a realistic mimic of how the vaccine would be administered in human subjects. Rather it is more likely that the inoculum would be received in a single, rapid inhaled burst, similar to the inhaler used for the FluMist vaccine. It can be assumed that there is substantial loss, or at least incomplete lower airway deposition, with the current model. However, it is encouraging that positive results were observed. Presumably, improvements to this model would only enhance the magnitude of response. One way of anticipating how the response would be affected is to use the microsprayer to deliver a single dose through the trachea of anesthetized animals.

There are two possible ways to immunize intratracheally with the microsprayer. The first is to essentially intubate the animal, accessing the lower airway through the mouth. This is ideal, as repeated immunizations can be given. Unfortunately, as no one in our laboratory or the Animal Resource Facility was experienced with small animal intubations, and a lack of small-animal laryngoscopes made visualization of the airway exceedingly difficult, I was not able to consistently ensure successful deposition of the inoculum, which obviously generated erratic results. The second option we discussed was to perform a tracheotomy, wherein a small incision is made directly in the trachea,

and the microsprayer inserted and deployed. The caveat to this approach is that animals do not tolerate multiple tracheotomies, making successive immunizations an impossibility. While it may have been valid to conduct single-immunization experiments similar to those conducted in the HPV studies, as we were hoping to generate a correlation between the immune response generated after multiple immunizations via the exposure chamber, we decided against the invasive tracheotomy approach.

A second caveat to the rat studies is of course the lack of an appropriate challenge model. In many respects, this has been alleviated by our ongoing CCR5 vaccine investigations in Rhesus macaques. Nonetheless, for the macaque studies, we adopted a different vaccination route, making a true assessment of the efficacy of a pulmonary vaccine at generating protective, receptor-blocking antibodies in the genital tract unfeasible. For this reason, I am encouraged by the addition of the mouse model of genital HPV infection to our studies. As is discussed in sections of 6.3 below, there are many future directions we can undertake to fine-tune both the pulmonary and genital VLP-based vaccines, and having a relevant animal model of infection on-site will allow us to assess the success of these vaccine formulations.

6.2.2 Limitations Specific to Chapter 4: HPV Genital Vaccine in Mice

To best discuss the limitations involved in vaccine delivery to the genital tract, further description of the immune environment at this site is required. As was mentioned briefly in Chapter 4, there is a lack of reliable protocols for genital immunization. One reason for this could be the variability in immune response elicited, which can differ greatly depending on the target antigen, carrier, and form of inoculum (gel, aerosol, liquid, etc.). Furthermore, individual mucosal compartments have unique compositions

with regards to their immune environment; for example, the immune cell populations that frequent the lung are different from those in the genital tract.

In general, mucosal surfaces can be divided into either type I or type II tissues. Type I surfaces are composed of simple columnar epithelia characteristic of the gut and lungs, and are likely the surfaces exposed to the inhaled vaccines we investigated. Type II mucosal surfaces are found in the vagina, eyes and mouth, and are covered by a protective stratified epithelial layer. The female genital tract is unique in that it is comprised of both mucosal tissues; type I is found in the endocervix and uterus, while type II is found at the ectocervix and vagina. In addition to histological differences, the tissues are also distinguished by the presence (type I) or absence (type II) of IgA transport mechanisms and mucosa-associated lymphoid tissues. As a result of the former, there is also a major difference in the antibody isotype prevalent at each of these sites. Secretory IgA is found at type I mucosae, while IgG is found at type II. (Iwasaki 2007; Iwasaki 2010).

The immune response evoked following immunization with the gel-based vaccine was somewhat surprising, as antibody titers were negligible, in contrast to the results garnered by our collaborators in John Schiller's laboratory (Graham, Kines et al.). It may have been worthwhile to investigate this disparity to rule out the possibility of human error. Another possible explanation for the observed low IgA response is that the gel application was unable to reach the type I tissues of the upper genital tract, the submucosa of which is constitutively filled with dendritic cells, macrophages and memory lymphocytes, and which favors a secretory IgA response. In contrast, the type II tissues found in the vagina and ectocervix contain a sparse network of DCs and

macrophages, and rare lymphocytes (Wira, Fahey et al. 2005). Presumably, the smaller droplet size and distributive force posited by the aerosolized vaccine would encourage distribution in the upper genital tract. A major limitation with the microsprayer technology is the tendency for small volumes to incompletely nebulize; as volume is reduced, the mist trends towards a fine spray. (Smaller volumes are necessary to avoid loss as the volumetric capacities of the mouse genital tract are finite.) Furthermore, as the immunization is essentially performed blindly, it is difficult to ascertain when/if the inoculum is incompletely nebulized and to make notes of any resulting discrepancies within an immunized group of animals.

From a translational standpoint, there is the problem of the vaccine requiring epithelial disruption prior to administration. It would also certainly raise public concern to continue to use nonoxynol-9, as several reports of its ability to permit sexually transmitted infections have received media attention. Identification of an appropriate adjuvant could sufficiently enhance uptake and negate the need for disruption. Alternatively, it is possible that a prime-boost strategy for vaccine delivery could ameliorate the need for disruption, as a baseline immunity would in theory already be established. Lastly, it is possible that in sexually active subjects sufficient disruption would be in place. Indeed, HIV-1 transmission is considerably increased by the presence of pre-existing genital lesions (caused by other STIs) and microabrasions resulting from sexual intercourse (Shattock and Moore 2003). Ultimately, I believe that the question of epithelial disruption is premature, and that this limitation can be corrected following further research and development.

6.2.3 Limitations Specific to Chapter 5: CCR5 Genital Vaccine in Macaques

To date, the major limitation with the ongoing macaque studies is that the animals, and therefore immunizations and sample collections, are all off-site. While this doesn't necessarily contribute to human error, it is difficult to account for unexpected results. For example, much difficulty was experienced in the extraction of IgA from Weck-Cell sponges, which were used as an alternative to a cervico-vaginal PBS wash. It is doubtful that freezing, shipping, and thawing of the sponges affected the stability of the contained antibodies, however the separate facilities ensure that samples will be limited, prohibiting the opportunity to gather additional ones in the event of any error. Another caveat to consider is the small cohort size, given the genetic diversity between animals. Nonetheless, although statistical significance is difficult to determine, the varying responses displayed are physiologically reflective, and can be helpful when considering any products' readiness for evaluation in clinical trials.

6.3 Overarching Future Directions

As our lab is illustrating with our work towards an L2-targeted HPV vaccine, there are frequently improvements that can be made upon extant vaccines. Both our HIV and HPV vaccine prototypes could undergo several phases of honing before an optimal outcome is reached. These improvements include but are not limited to boosting immunogenicity, decreasing manufacturing cost, increasing the breadth of specificity, making adjustments to particle size and chemistry, and investigating alternate routes of administration. Some more specific experiments geared towards improving vaccine

design of both the HPV and CCR5 VLP-based vaccines are discussed in sections 6.3.1 and 6.3.2 below.

As an overall theme however, I have grown increasingly interested in steering this work towards more mechanistic studies. Specifically, the events that transpire between immunization and antibody production: which cells encounter the VLPs, where VLPs traffic, and possibly how other players participate in the initiation of the immune response. There are several intriguing experiments that can be undertaken to this end. The immense malleability of the VLP platform can allow for alterations to its physical properties such that homing and biodistribution can be monitored, or even controlled. A simple example of this would be delivery of a GFP- or other fluorophore-labelled particle, followed by histology. A more complex approach could involve the incorporation of a highly sensitive high-contrast agent into the particles. Folowing administration, the quantity of particles able to reach relevant tissues, such as draining lymph nodes or spleen, could be assessed by MRI (Song, Kohlmeir et al. 2008). Live animal imaging for particle tracking is also possible using the IVIS (previously described), or a Maestro EX (Cri) multispectral fluorescence imaging device, which is able to obtain images from 550-900 nm in 10-nm-wavelengths. Additionally, surface modifications can also be made to promote internalization and target specific cell populations or receptors. For instance, chemokine ligands could be incorporated on the particle surface and used to bind cognate receptors on cell subsets of interest. The use of fluorophore-coupled particles would make the monitoring of particle uptake by these cell populations possible via flow cytometry. In addition to providing a general method for studying recruitment pathways, a similar tactic has been exploited as an approach for

vaccine development. First, a single immunization via a conventional parenteral route acts as a "prime" to trigger all arms of the adaptive immune response. Following this, selective lymphocyte populations (CD4+ T cells, CD8+ T cells, or B cells) can be reoriented to a desired site using a second signal, or "pull". For example, CXCL-9 has been used to drive CTLs into vaginal tissues following intramuscular immunization (Nakanishi, Lu et al. 2009).

6.3.1 Future directions pertaining to HPV vaccine

The current VLP-based HPV vaccines, in addition to including viral epitopes, stimulate innate immune recognition in dendritic cells (DCs), and it is likely that this stimulation contributes to the success of these vaccines. During natural infection, HPV molecules engage multiple mechanisms to prevent the initiation of a robust immune response. Depletion of LCs by the E6 protein, downregulation of MHC I by E5, and blockade of Type I IFN signaling by E7 are examples of the several viral evasion mechanisms in play that ensure a poor host immune response. Again, I am interested in delving more into the interactions between VLPs and resident antigen presenting cells, and the specific mechanisms by which VLPs are able to initiate an immune response in spite of the several tactics employed by an invading virus. In the case of HPV infection, these cellular interactions will most likely involve DCs.

Cross-presentation of HPV antigens is likely to be carried out by uninfected DCs, as the antigen presentation capacities of these cells are not affected by the aforementioned evasion mechanisms. Presumably DC activation takes place upon recognition of virus-infected cells by an endosomal pattern recognition receptor (PRR). Because HPV occurs only in the type II epithelial layer, LCs in the epithelial layer and

possibly submucosal DCs extending their dendrites towards the epithelial layer are the cells most likely to participate in this process. Similar to what I discussed and proposed in the above section, I think much can be learned of cell to cell interactions through fluorescent labeling of VLPs, and either tissue or live animal imaging. For instance, future studies of the innate recognition system for intact HPV virions, identification of the pattern recognition receptors (PRR) involved in VLP recognition and the nature of pathogen-associated molecular patterns (PAMPs) being recognized would provide further insights into the basic biology of PV detection, and could have an immense impact on vaccine development.

With regards to vaccine design, if the genital immunizations fail to impart more complete protection upon genital challenge, I think it would be worthwhile to return to immunization via the pulmonary route. This would be especially advantageous as our previous work in rats was not able to incorporate relevant viral challenges. The mouse model for HPV infection would allow us to assess how well the inhaled vaccine performs at conferring protection from pseudoviral infection. Also, unlike the viral challenges undertaken in macaques, these animals would be on-site, providing a more fiscally prudent and ethically sound arena with which to fine-tune the pulmonary vaccine. Furthermore, any insights gained from the proposed VLP trafficking and uptake investigations could be translated into vaccine modifications and efficiently assessed for improved efficacy.

Future directions towards the development of an L2-targeted VLP HPV vaccine are also underway on a much larger scale. We are currently collaborating with Drs. Michelle Ozbun and Cosette Wheeler on a project focused on basic and applied HPV

research. In addition to our research, the facility's broader projects include (but are certainly not limited to) the development and evaluation of a macaque model of genital PV infection, and population-based studies to elucidate the effectiveness of current vaccines and the influence of vaccination on the prevalence of HPV genotypes.

6.3.2 Future directions pertaining to CCR5 vaccine

As the study with the CCR5 vaccine in macaques is ongoing, there are several experiments that are yet to be done. Many of these address the physiological effect of inducing autoantibodies against CCR5, how the virus may respond to these antibodies, and the correlation, if any, between antibody titers and clinical outcome. Regarding investigating the effects of autoantibodies against CCR5, we have collected blood samples throughout the course of study to examine whether anti-CCR5 antibodies would lead to the downregulation of CCR5 expression *in vivo*. At monthly intervals, macaque PBMCs will be isolated and CCR5 expression levels determined by flow cytometry. A Quantibrite-based assay can be used to measure absolute numbers of CCR5 on cells. Additional samples of macaque sera were also collected once a month following the final immunization. IgG will be purified from this sera using a Protein A/G column and tested for its ability to block SIV infection using the ptCCR5-MAGI assay described in Chapter 3 of this thesis.

As was previously mentioned, the presence of anti-CCR5 antibodies could block SIV infection in macaques and essentially lead to three different outcomes: 1) protection, 2) sustained reduction of viral loads, or 3) temporary reduction in viral loads. In the cases of outcomes 2 & 3, we will examine the effects of vaccination on the phenotype of the virus that persists in the vaccinated animals. This understanding will be especially

important in the case of outcome #3, with animals whose viral loads rebound, although as of yet the observed trend is that protected animals have been maintaining low viral loads with no indication towards a rebound. To determine phenotype of the persistent virus, PBMCs will be taken from vaccinated and control macaques approximately one year after SIV challenge. DNA will be isolated from PBMCs by standard methods. In order to isolate virus from infected animals, PBMCs will be co-cultured with an equal number of activated macaque PBMCs isolated from naïve rhesus macaques. Virus will be cultured for up to 4 weeks, and virus production assessed weekly by an antigen capture assay measuring p27 gag in the supernatant. In the event we experience difficulty expanding the virus via this method, we could alternatively co-culture infected PBMCs with the CEMx174 cell line, as SIV envelope genotypes have been shown to be stable upon co-culture with this cell line (Rudensey, Papenhausen et al. 1993). After the period of expansion, supernatant will be collected. Virus derived from co-cultures will be tested for coreceptor usage using a panel of coreceptor expressing cell lines (the GHOST cell lines) obtained from the AIDS repository.

Virus will also be tested for sensitivity to anti-CCR5 mouse IgG, using the ptCCR5-MAGI assay previously described. If a shift in coreceptor usage or resistance to anti-CCR5 IgG is detected, we will PCR amplify and sequence envelope clones from infected PBMC DNA, using standard methods (Chackerian, Morton et al. 1994). Because shifts in coreceptor usage typically map to the genetic changes within the V3 region of envelope (Kuhmann, Pugach et al. 2004), we will sequence this region from 4-6 envelope clones from each macaque. Together, these analyses should give us an assessment of the genotypic and phenotypic responses of SIV to CCR5 vaccination.

To assess clinical outcome, all animals will be observed until September of 2011, 12 months following the challenge, unless illness progresses to an unmanageable state and euthanization is required. During this 12-month period, blood will continue to be collected every 4 weeks and analyzed to determine viral load. As a secondary measure of efficacy, we will also examine how the level of anti-CCR5 antibodies affects clinical outcome. This relationship may prove difficult to assess, as it is rarely straightforward. For instance, $CD4^+$ T cells are predicted to be a major sink for cell-associated CCR5 antibodies. A loss of these cells during disease progression might therefore tend to increase serum CCR5 antibodies. This could result in an inverse correlation between CCR5 titers and CD4 counts.

A second confounding factor is that the total amount of CCR5 autoantibodies induced is the sum of free serum and cell-associated antibodies. Antibodies that have low avidity may preferentially reside in the serum compartment, and are least likely to protect against infection. Protection is conferred by cell-associated antibodies, however to date only the serum antibodies have been measured. To correct for this, experiments are currently underway to determine levels of cell-associated CCR5-specific antibodies.

6.4 Regarding the importance of CCR5 antibodies at the genital tract

Several modes of entry have been suggested for HIV-1, including transcytosis through a microfold cell (M cell), uptake by DCs, and entry through microabrasions in the epithelial layer. Early in infection, additional cell types may be targeted, such as resident activated memory T cells or Langerhans cells (LCs), which have been proposed to facilitate HIV infection by capturing virus, migrating to regional lymph nodes, and

then transferring virions to susceptible T cells. It is undetermined how successfully anti-CCR5 antibodies, rather than virus-neutralizing antibodies, in the genital tract at time of virus transmission could inhibit infection. In addition to DCs expressing CCR5, Langerhans cells in the foreskin and CD4+ T cells seem to predominantly express CCR5, providing a selective advantage for R5-tropic virus transmission (Margolis and Shattock 2006). As is suggested by individuals heterologous for the Δ32 mutation, a reduction, rather than complete eradication, of surface CCR5 is sufficient to significantly reduce the incidence of infection. Presumably, anti-CCR5 antibodies would act by blocking the receptor or reducing its surface expression through internalization or sequestration; the most critical factor is whether these antibodies could be long-lasting and present at the time of exposure. It may also be relevant to investigate an oral mucosal route of vaccination using our CCR5 vaccines, given the role of the gastrointestinal tract in the gross depletion of CCR5-expressing T cells during early HIV-1 infection. Lastly, it is also unclear whether the majority of virus during early infection would be cell-associated or free virions. While anti-CCR5 antibodies would only affect cell-associated virus, it is likely that IgA mucosal antibodies would play a more important role in protective immunity against free virus.

6.5 Safety Concerns Regarding Inducing an Immune Response Against CCR5

As our vaccine proposes to induce an antibody response against a self-protein, we have anticipated that safety concerns will likely arise. In response, several points can be raised in defense of a vaccine targeting CCR5. First, we have learned much about the effects of the CCR5-Δ32 allele in human populations, and can apply that knowledge to

anticipate possible effects of anti-CCR5 therapy. To this date the only suggestion that the CCR5-Δ32 mutation may be associated with enhanced susceptibility to disease is in individuals infected with two flaviviruses, West Nile Virus and tickborne encephalitis virus (Glass, McDermott et al. 2006; Kindberg, Mickiene et al. 2008). Obviously, we cannot extend observations made in knockout individuals to predict the consequences of functional inactivation of CCR5 in normal individuals. Therefore a second and better gauge for weighing the potential side effects posed by a CCR5 vaccine may be PRO 140, a humanized CCR5 monoclonal antibody (mAb) for the treatment of HIV infection (Progenics Inc.). PRO 140 is currently in clinical development, and has been reported to exhibit favorable safety and efficacy data (~1.5 log mean reduction in viral loads at the highest dose) in a phase 2 clinical trial (http://www.medicalnewstoday.com/articles/138779.php). If a favorable safety profile emerges from larger phase 3 clinical trials, it would support the possibility that a CCR5 autoantibody inducing vaccine could also be safely delivered. While not related to safety, concerns over manufacturing costs are always raised when approaching vaccine implementation. In this case, cost of delivery favors a CCR5 therapeutic vaccine over CCR5 mAb therapy for use in the developing world, as treatment with currently licensed mAbs costs more than $10,000 per year.

6.6 Conclusions

The recent success of clinically-approved VLP-based vaccines for Hepatitis B Virus and Human Papillomavirus highlight that effective vaccines against sexually-transmitted viruses are possible. The challenges for further success lie in the diversity of these viruses; each STV will likely require a specific type of effector immunity to confer host

protection. For instance, for viruses that undergo rapid mutation, such as HIV-1, antibodies will need to either broadly neutralize highly-conserved epitopes, or be targeted to unconventional or alternative non-viral antigens to elicit the CD4+ and CD8+ T cell and B cell responses necessary to curtail virus propagation. The investigation of strategies to involve the mucosal, in addition to the systemic, immune system in the initiation of an immune response may be critical to the development of future vaccines against a number of pathogens infecting at mucosal sites. Our research contributes to this objective by showing that VLP-based vaccines targeting both self and viral antigens are compatible with a number of mucosal-delivery applications, and are able to generate protective immune responses at mucosal sites in animal models. The relatively non-invasive approaches described herein may facilitate economic vaccine deployment to the developing countries where they are needed most, and minimize risks associated with parenteral vaccines.

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