5-1-2014

Development of HPV next-generation virus-like particle vaccines that are cross-protective

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DEVELOPMENT OF HPV NEXT-GENERATION VIRUS-LIKE PARTICLE VACCINES THAT ARE CROSS-PROTECTIVE

by

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B.A., Biochemistry, University of New Mexico, 2006

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

ACKNOWLEDGMENTS

With thanks and humility, I thank and acknowledge:

My mentor and friend Bryce Chackerian. I will always feel fortunate for the chance to work in your lab and learn from you. Thank you for your patience and understanding, your advice and encouragement.

My wife and family for all your support during the last five years and for the quiet assurance that it would all be worth it and that it would be OK. And to my girls: this is the answer to your oft-repeated question of “When are you going to graduate?”

My committee members, David Peabody, Michelle Ozbun, and Brian Hjelle. for the suggestions, conversations, and encouragement.

My fellow members of the Chackerian lab: Erin, my graduate school comrade-in-arms, Ebenezer, the HPV trailblazer, Julianne, the animal master and my cross-bench conversationalist, and Kathryn, font of wisdom and sage advice. Also, the rest of the Chackerian-Peabody family – Chris, John, Jayne, Paul, and Jerri.

The members of the Ozbun lab: Staci, Mickey, Aga, Pam, Nicole, and Rosa.

Bruce Edwards, for help with the flow cytometry, and giving me great news.

The Infectious Disease and Inflammation training grant staff: Lisa Dillon and Drusilla Soherr-Hadwiger. Thank you for the funding and the travel.

The other groups of the UNM Interdisciplinary HPV Prevention Center: The Wheeler, Ozbun, and Woodall labs.

My Heavenly Father, without whom none of this would have been possible.

DEVELOPMENT OF NEXT-GENERATION VIRUS-LIKE PARTICLES TARGETING HPV

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ABSTRACT

Virus-like particles (VLPs) comprised of viral structural proteins that self-assemble into particles resembling the native virion represent a relatively novel vaccine development strategy. Both safe and immunogenic, VLPs can be used as vaccines against the virus from which they are derived, but can also be used to present heterologous epitopes from other pathogens to the immune system. Both techniques result in high-titer antibody responses against the target epitope. Indeed, vaccines of VLPs are already available, including the two vaccines targeting Human Papillomavirus (HPV). These vaccines are very effective at preventing infection by the HPV types included in their formulation; high-risk HPV

types that are associated with the development of cervical cancer. The elicited antibody response, however, largely does not protect against the other high-risk HPV types. Herein we report the results of studies aimed at developing a next-generation HPV vaccine using bacteriophage VLPs displaying epitopes from the minor capsid protein of HPV L2 that have been found to induce cross-protective antibodies. We first displayed a variety of N-terminal L2 epitopes on PP7 and Qβ VLPs and measured the elicited homologous protection in mice. Finding a type-specific neutralizing epitope, we were able to considerably broaden the observed cross-neutralization by immunizing with a consensus sequence of this epitope drawn from the high-risk HPV types. We also explored displaying a L2 epitope from two different HPV types on one VLP. We were able to construct assembled VLPs that displayed both targets on their surface. We observed that immunization with these hybrid VLPs elicited a more-cross neutralizing response than vaccination with VLPs displaying one target alone. Finally, we investigated the display of two molecular adjuvants on the surface of VLPs. Hypothesizing an increase in the speed and intensity of antibody response, we displayed both the complement receptor 2-minimum binding region, p28, and the monomer component of flagella, flagellin, at low levels on the surface of VLPs. We found, however, that displaying p28 in this way did not increase antibody titers.

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Chapter 1 – Introduction and Background

1.1 Virus-like particles as vaccine platforms

The expression and self-assembly of viral structural proteins produces virus-like particles (VLPs, see Appendix one for list of abbreviations) [1-3]. These VLPs are a relatively novel vaccine platform that boasts several advantages over traditional attenuated or inactivated vaccines[4]. VLPs do not contain viral genomic material and do not self-replicate, which eliminates the danger of reversion of attenuated vaccines [5]. In addition, VLPs are strongly immunogenic and immunologically identical to natural virions, eliciting a robust immune response against the viral proteins from which they are derived. VLPs can be made of non-enveloped or enveloped viruses, can be used as a display platform for heterologous targets and can be produced in a variety of expression systems, increasing their flexibility and decreasing the cost of production [6-8]. Various vaccines comprised of VLPs are both currently available and undergoing clinical trials, representing their utility as an accommodating platform for emerging vaccines.

1.1.1 Immunogenicity of VLPs

The immunogenicity of VLPs stems from several factors. First, the epitopes of the viral proteins are displayed in a dense, repetitive array. This multi-valent display strongly activates B cells, in part through displaying viral epitopes in a spacing that has been shown to be optimal for inducing B cells responses [9-12]. B cells recognize their cognate antigen through binding of the B cell receptor (BCR) to the pathogen. This binding sets off a signaling cascade that results in

proliferation, increased survival, limited production of IgM antibodies, and movement to germinal centers for affinity maturation and class-switching. The efficient activation of B cells is based in part upon the affinity of the interaction between the BCR and its cognate antigen; the stronger the affinity for the target, the stronger the response. It is also based on the cross-linking of BCRs binding to a multi-valent antigen, or the avidity of the interaction [13, 14]. An increase in avidity reduces the affinity threshold of activation for B cells, both in the initial interaction and subsequent interaction with antigen in the germinal center during affinity maturation. In this way, a VLP vaccine that provides repetitive sites for multiple BCRs to bind to increases the avidity of the interaction and promotes a greater immune response.

VLPs are also intrinsically a similar size and shape as natural virions. This encourages the efficient uptake by professional antigen-presenting cells (APCs) [15, 16]. After phagocytosis, the VLP, like any pathogen, is broken down and displayed on MHC Class II molecules to $CD4^+$ helper T cells, which is necessary for the efficient activation and class-switching of B cells. In addition, VLP proteins can be processed for cross-presentation to $CD8^+$ cytotoxic T cells via MHC Class I molecules [17, 18]. These $CD8^+$ T cells are an important component of the body's anti-viral response as they target currently infected cells and kill them, halting the production of more virions.

Another benefit of VLP vaccines is the longevity of the generated immune response. In particular, our own lab has studied the immune response to VLPs in mice and found generated antibody titers to be stable and protective over 18

months [19]. Additionally, studies of the immune response to one of the two Human Papillomavirus (HPV) vaccines, both of which are comprised of HPV VLPs, have shown long-lasting antibody titers after several years (up to 8.4 years) of surveillance [20, 21].

1.1.3 VLPs as an immunogenic scaffold for heterologous epitopes

VLPs can also be used as a display platform for heterologous epitopes [22, 23]. This approach allows for the targeting of pathogens or proteins from which VLPs cannot be normally constructed. Once a suitable epitope is identified, it is possible to display this target on the surface of VLPs. This can be particularly useful in the targeting of epitopes within a pathogen that are poorly immunogenic or masked by non-neutralizing regions in the context of the whole organism. Remarkably, this method of display has also been shown to be able to break B cell tolerance of self-antigens, inducing autoreactive antibodies against the targeted epitope [24]. Heterologous epitope display elicits a high-titer, monoclonal-like response specifically against the displayed target, something that whole virion vaccines can fail to generate.

One method used to present foreign peptides is the genetic insertion of the epitope into exposed loops of a VLP protein [25-28]. This technique requires knowledge of the structure of the proteins making up the VLP to identify regions that are exposed on the surface of the VLP and that might be amenable to insertion. The size and chemical properties of the inserted peptide also determine the success of this technique. Often these genetic inserts result in the misfolding of the protein, leaving them unable to self-assemble into the VLP

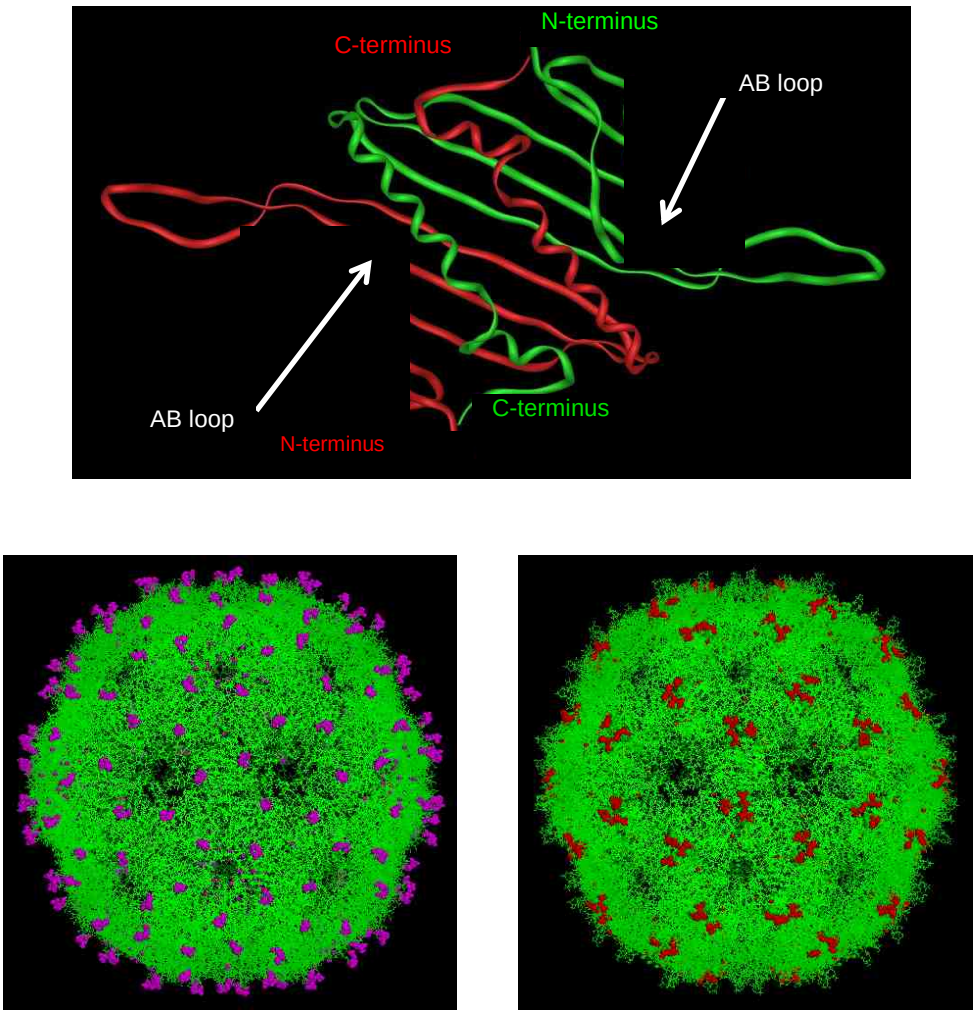

Figure 1.1 – MS2 and PP7 genetic display of heterologous peptides: A) Schematic of two dimerized MS2 coat proteins with relevant genetic insertion sites. The AB loop presents the peptide in a constrained manner, while N- and C-terminal display is less so. B) A representation of the displayed peptides in the AB loop (left) or the N- or C-terminus (right). The displayed peptide is only added to one of the coat proteins of the single-chain dimer (SCD), resulting in the display of 90 peptides per VLP. Images were generated with iMol (http://www.pirx.com/iMol).

structure. Once a VLP is successfully cloned and found to fold correctly, however, these chimeric VLPs are technically simple and inexpensive to produce.

Epitopes can also be linked to the surface of VLPs with chemical linkers [29-33]. This technique allows for the display both longer peptides and non-protein targets as it does not affect the folding of the viral proteins. However, the chemistry of the linkage can preclude certain conjugates. For example, epitopes with internal disulfide bridges are more difficult to conjugate using chemistry that requires the sulhydryl group of cysteine residues. Further, the cost of synthesizing targets to conjugate can be prohibitive. There is no concern about misfolding of the viral proteins, though, since the VLPs are already assembled. In either case, using genetic insertion or conjugation, once the target is successfully displayed, vaccination with these chimeric VLPs leads to antibody responses against the displayed peptide.

In particular, studies in our lab have focused on bacteriophage VLPs as vaccine platforms [34-36]. Bacteriophages are non-enveloped, helical and icosahedral viruses that infect bacteria. The phages used in our lab, MS2, PP7, and Qβ, are icosahedral with a T-3 symmetry, resulting in 180 coat proteins in the capsid. These coat proteins can be recombinantly expressed in a number of different systems, including *E. coli*, and will self-assemble into VLPs which can be purified using size-exclusion chromatography. Previous work done by this lab has identified 3 potential insertion sites for genetic display of peptides in MS2 and PP7: the C- and N-termini and the exposed AB loop [35, 37]. These VLPs,

however, suffer from the same constraints as all VLPs in that they are largely intolerant of the insertion of foreign peptides.

To increase the stability of these coat proteins and allow for the display of diverse peptides, two copies of the coat proteins are genetically fused to form a single-chain dimer (SCD) (Fig 1.1A) [38, 39]. Unique enzyme restriction sites were engineered into the SCD to allow for the genetic insertion of short peptides (12-15 amino acids) into the downstream coat protein's AB loop (Fig 1.1B), as well as the N- and C-termini of the dimer (Fig 1.1C). Display at any of these sites results in 90 peptides being displayed per VLP and leads to a strong immune response against the displayed peptide.

Our lab has aslo made use of the small, bifunctional linker succinimidyl 6-[(beta-aleimidopropionamido)hexanoate] (SMPH) to conjugate peptides to Qβ bacteriophage VLPs (Fig. 1.2) [31, 40]. This linker interacts with exposed lysine groups on the surface of VLPs and free sulfhydryl groups of terminal cysteine residues. Conjugating with SMPH allows us to potentially attach multiple copies of a peptide to each of the 180 coat proteins, often resulting in several hundred peptides being displayed on the surface of each VLP.

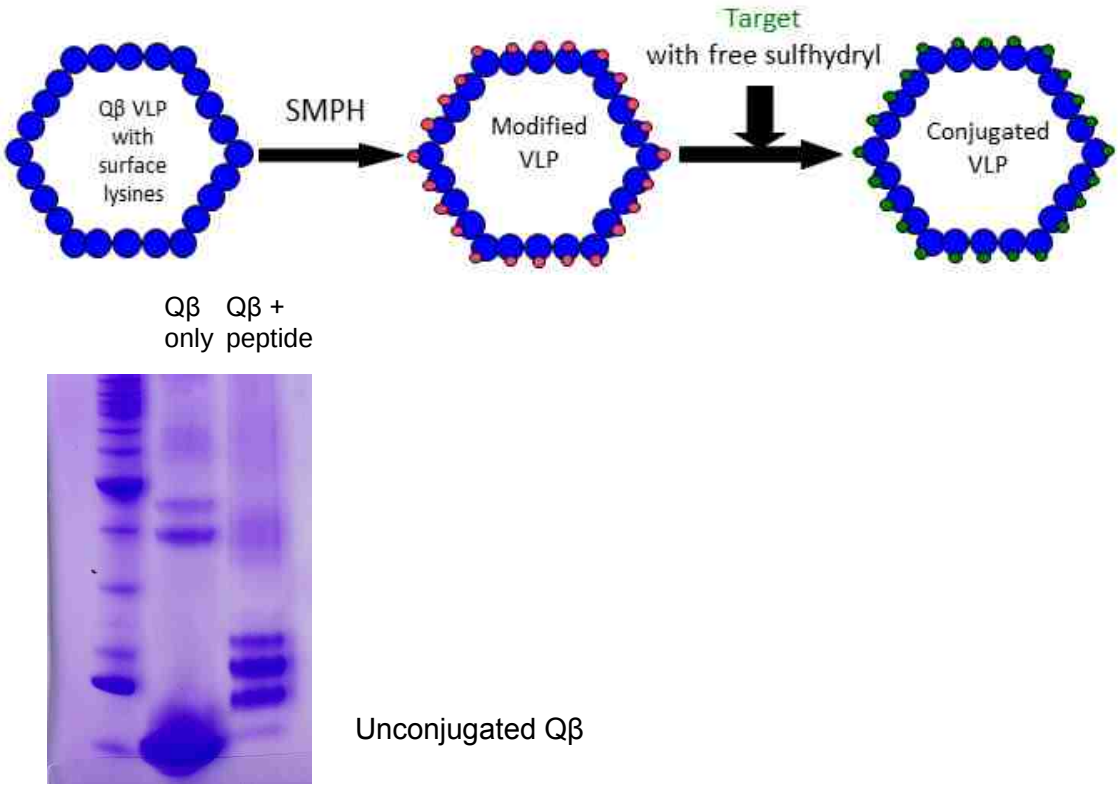

Figure 1.2 – Conjugation of peptides to the surface of Qβ: Qβ VLPs or virions are incubated with SMPH which binds to exposed lysine residues on the surface. Then the Qβ-SMPH particles are mixed with peptides with a free sulfhydryl group. B) SDS-PAGE gel of unconjugated and conjugated Qβ. The ladder of bands in the conjugated lane represent Qβ monomers conjugated to increasing amounts of peptide. Each band represents an additional conjugated peptide. Efficient conjugations results in approximately 300 peptides displayed on the surface.

1.2 Second-Generation Prophylactic HPV Vaccines: Successes and Challenges

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(Published in Expert Reviews Vaccines, 2014 Feb; 13(2):247-55)

1.2.1 Introduction

Human papillomaviruses (HPV) are one of the most common sexually transmitted pathogens in the world, with a reported 11% world-wide prevalence in women with normal cytology [41]. In the United States, over 6 million new HPV infections are reported each year and greater than 20 million people are currently infected. Over 100 different HPV genotypes have been identified, but the most common HPV-associated cancer, cervical cancer, is associated with infection by one of a subset of 14-20 HPVs termed “high-risk” types (reviewed in [42]). Two high-risk HPV types, HPV 16 and HPV 18, are found in approximately 70% of all cervical cancer cases [43]. Cervical cancer is the second most common and fifth deadliest cancer in women worldwide, with over 500,000 new cases and 275,000 deaths each year [44]. Approximately 85% of cervical cancer cases occur in developing countries [45]. High-risk HPV infection is also associated with other anogenital cancers (of the vulva, vagina, penis, and anus) as well as growing percentage of oropharyngeal cancers (reviewed in [46-50]). In

total, HPV infection is estimated to be responsible for about 5% of human cancers worldwide [51].

Cervical cancer is one of the few cancers that can be prevented using a prophylactic vaccine. The current HPV vaccines (Gardasil and Cervarix) are comprised of a mixture of virus-like particles (VLPs) derived from the HPV major capsid protein, L1 of two high-risk HPV types (HPV16 and 18). Gardasil also contains VLPs derived from two low-risk HPV types associated with genital warts (HPV6 and 11). Both Gardasil and Cervarix have excellent safety profiles and strongly protect immunized individuals against infection with the HPV types included in the vaccines [20, 52-55]. However, these vaccines provide modest cross-protection against other high-risk HPV types, leaving vaccinated individuals at a decreased risk, but still vulnerable to the development of cancer. This review will discuss efforts to develop second generation HPV vaccines that will provide broader protection against the HPV types associated with cancer. In particular, we will focus on the considerable progress that has been made in developing vaccines targeting the minor capsid protein of HPV, L2. Vaccines targeting L2 may provide a relatively simple and effective way to generate cross-neutralizing immunity against diverse high-risk HPV types.

1.2.2 HPV Biology

HPV is a non-enveloped double-stranded DNA virus from the family *Papillomaviridae* (HPV biology reviewed in [56]). Its circular, covalently-closed genome is approximately 8kb in length and encodes 8 genes, divided into early (E) and late (L) proteins. Papillomaviruses have a strict tropism for cells of the

squamous epithelium and are peculiar in that their life cycle is dependent upon differentiation of the host cell. In short, upon entering the basal cells, transcription of the viral genome is regulated by E2. Proteins E6 and E7 interact with p53 and retinoblastoma protein, respectively, to deregulate the cell cycle and promote division. As the keratinocytes continue to differentiate and migrate to the skin surface, the late structural proteins, L1 and L2 are produced to encapsidate the viral genome and virions are eventually sloughed off from the dead cells.

HPV virions consist of two viral structural proteins, L1 and L2. L1, the major viral structural protein, assembles into pentamers, 72 of which form an icosahedral capsid with T-7 symmetry. The minor capsid protein, L2, is present in much lower amounts than L1, with a maximum of 72 copies per virion at the vertices [57]. Although both viral capsid proteins are present in virions, natural HPV infection typically results in the induction of low-titer antibody responses directed towards L1 only, demonstrating the immunodominance of L1 epitopes as well as the occlusion of L2. Structural studies have indicated that L2 is poorly displayed on the surface of mature virions, and is only revealed later in the complex infection process, presumably after binding of the virion to the basement membrane, which exposes the amino terminus of L2 [58-60]. Once exposed, 12 or so amino acids at the N-terminus of L2 are cleaved by a furin, a cellular proprotein convertase, leading to surface exposure of one or more domains of L2 on the virion surface [60-62]. Although HPV virus-like particles (VLPs, described below) can be formed by L1 protein alone, L2 is required for productive infection. L2 is required for both HPV endosomal escape and also plays a role in facilitating

trafficking of the viral genome to the nucleus [62-65]. L2 also plays a critical role in the encapsidation of viral DNA prior to virion release [66].

1.2.3 HPV cancer epidemiology

While HPV infection is common, infections rarely progress to cancer. It is thought that most HPV infections are cleared by the immune system. Nevertheless, persistent infection can occur in a subset of individuals, and this persistent infection with high-risk HPV types has been shown to be necessary for the development of cervical cancer (reviewed in [42, 67]). Of the high-risk HPV types, HPV16 and HPV18 stand out. These two HPV types are found in approximately 70% of all cervical cancer cases, and HPV16 infection is associated with 90% of HPV-related oropharyngeal cancers [43, 48], reflecting the enhanced oncogenic potential of these HPV types relative to other high-risk HPVs [68, 69]. Although there are geographic differences in HPV genotype distribution in cancers [68, 70-72], there is strong evidence that about eight HPV types (namely HPV16, HPV18, HPV31, HPV 33, HPV35, HPV45, HPV52, and HPV58) are responsible for at least 90% of the global burden of cervical cancer [73]. Nevertheless, the abundance of high-risk HPV types that cause a small percentage of cancer cases, and regional differences in these types, complicate efforts to protect against all oncogenic types and represent a significant hurdle in efforts to develop a vaccine that provides 100% protection against HPV infection.

1.2.4 Current HPV Vaccines

There are currently two prophylactic HPV vaccines on the market: Gardasil and Cervarix. Both vaccines contain virus-like particles (VLPs)

composed of the HPV L1 protein. The development of these vaccines was made possible by the observation that recombinant L1, when overexpressed, spontaneously self-assembles into VLPs that structurally resemble infectious virus but lack genomic material [2, 74, 75]. Randomized clinical trials of HPV VLP-based vaccines have established that Gardasil and Cervarix are safe and induce high-titer antibody responses. Importantly, vaccination largely protects women from HPV16 and 18 DNA acquisition, and the vaccines are remarkably effective (nearly 100%) at preventing HPV16- and HPV18-associated cervical intraepithelial neoplasia grade III (CIN III), the precursor lesion for cervical cancer [52-55, 76, 77]. Notably, studies in the US and Australia have begun to show a drop in the prevalence of vaccine HPV types both in vaccinated and non-vaccinated populations, indicating that the vaccines may be establishing herd immunity [76, 77].

Although the precise immunological mechanism of protection by the HPV vaccines has not been definitely established, it is likely that the protection provided upon vaccination with HPV VLPs is mediated by neutralizing antibodies. Both Gardasil and Cervarix elicit high titers of neutralizing antibodies in vaccinated individuals after intramuscular immunization [78]. HPV neutralizing antibodies in vaccinated individuals can be measured using sensitive *in vitro* neutralization assays that assess the ability of HPV pseudovirus (PsV; HPV VLPs that encapsidate a reporter plasmid, described in detail in Roberts *et al.* [79]) to infect cell lines [80]. In addition, animal studies have shown that

passively transferred sera from Gardasil-vaccinated mice can protect naïve mice from cervico-vaginal challenge by HPV PsV [81].

Antibody responses to HPV VLPs are also quite durable. Although antibody titers drop about 10-fold in the first year after vaccination, levels are stable thereafter (after 8 years of follow-up, in one study), indicating that the HPV vaccines provide long-lasting protection [20, 21]. These desirable characteristics, the induction of high-titer and long-lasting antibody responses, appear to be general characteristics of VLP-based vaccines. The dense, highly ordered presentation of L1 on VLPs strongly activates B cells through B cell receptor cross-linking. Also, VLPs are readily taken up and presented by professional antigen presenting cells due to their particulate nature (VLPs reviewed in [7, 82]), further enhancing their immunogenicity. It should be noted that Gardasil and Cervarix contain an aluminum salt adjuvant that may contribute to their ability to elicit high titer antibody responses; Cervarix is additionally adjuvanted with monophosphoryl lipid A (MPL), a Toll-like receptor 4 (TLR4) agonist. However, clinical trials have demonstrated that even unadjuvanted HPV VLPs elicit high-titer antibody responses [83], highlighting the innate immunogenicity of VLP-based immunogens.

One major limitation of the current vaccines is that antibodies elicited by L1 VLPs are type restricted, in that they largely protect against infection by the HPV types included in the vaccine and provide suboptimal protection against other high-risk HPV types (reviewed in [84]). Thus, vaccinated individuals are still at risk for cancer. The type-restricted nature of neutralizing antibodies

against L1 has been borne out by epidemiological studies as well as in in vitro studies using the HPV PsV neutralization assay. Although there is evidence that the titer and breadth of cross-reactive antibodies are greater after vaccination with Cervarix than Gardasil [85, 86], in either case the titer of cross-reactive antibodies is quite low compared to those elicited against HPV16 and 18. There are also several aspects of the current vaccines that are barriers to worldwide implementation. Both Gardasil and Cervarix are quite costly, at over US$100 for each of the three immunizations, though recent agreements brokered by the GAVI Alliance have lowered the price to a little less than US$5 in as many as 40 developing countries. Also, the vaccine requires a cold-chain which increases the cost of transportation and storage. Finally, both Cervarix and Gardasil are given as a recommended three-dose series over an extended six-month period (although recent data have shown that two doses of Cervarix are as protective as three doses [87]). Taken together, these factors (price, cold-chain, and requirement for multiple doses) reduce the uptake of the HPV vaccine in developing countries, where it is most needed.

1.2.5 Next Generation HPV Vaccines

The next HPV vaccine will need to address many, if not all, of the issues associated with current vaccines while retaining their effectiveness against HPV 16 and 18. Foremost among them is increasing the number of HPV high-risk types for which immunization confers protection. One way to address this issue using the current vaccine technology is to include additional high-risk HPV VLPs in the vaccine formulation. This strategy has been adopted by Merck, which has

developed a nonavalent HPV vaccine (V503) that is currently in Phase III trials (http://clinicaltrials.gov/ct2/show/NCT00543543). In addition to VLPs of HPV types 16/18/6/11, high-risk types 31, 33, 45, 52, and 58 have been added to the vaccine. Assuming the vaccine is equally effective against all nine HPV types, the immunization with V503 will theoretically prevent over 90% of cervical cancer cases. One study, modeling the increase in protection between current vaccines and the new nonavalent vaccine, estimates that the decrease in cervical cancer cases due to the uptake of the new vaccine could range from 9% to 30%, depending on the region and the amount of cross-neutralization seen after vaccination [88]. While it is somewhat premature to speculate about this vaccine since the results of the trial have yet to be published, our experience in animals is that we can immunize a single animal with a mixture of eight VLPs and still obtain high titer antibody responses to each of the individual components of the vaccine [89]. Thus, inducing high-titer antibody responses against the HPV types included in the formulation is highly possible (although it is also possible that the levels of neutralizing antibodies against the individual components of the vaccine may vary). It is likely, however, that V503 will face many of the same barriers to worldwide implementation as the current vaccines (i.e. high-cost and requirement of a cold-chain). It is also unlikely that the nonavalent vaccine will be universally effective against high-risk HPV infection, so Pap screening will continue, although potentially at reduced frequency.

1.2.6 L2 as the target of Second Generation HPV Vaccines

Another strategy to increase the breadth of protection conferred by vaccination is to target the immune response against epitopes that are more broadly conserved between HPV types. One target that has generated considerable interest is the HPV minor capsid protein, L2. In studies beginning in the early 1990s, several labs showed that vaccination with recombinant L2 protein could provide protection from infection with animal papillomavirus [90-92]. Subsequent studies showed that antibodies targeting L2 could not only mediate homologous neutralization, but could also neutralize diverse papillomavirus types [93]. Mapping studies have shown that broadly neutralizing epitopes within L2 are located in the N-terminal domain of the protein (roughly amino acids 13-120), which is consistent with both the sequence conservation of this region and also the fact that this region of the protein appears to be transiently exposed on the surface of virions. For example, antibodies raised against the N-terminal 88 amino acids of bovine papillomavirus type 1 (BPV-1) are broadly cross-neutralizing against several HPV types, whereas other domains of BPV-1 L2 do not induce cross-neutralizing antibodies [80]. Subsequent studies have identified peptide domains within N-terminus that appear particularly promising targets for vaccines (described in more detail below).

Although vaccines targeting L2 have the potential to cross-neutralization diverse HPV types, they also have challenges to overcome before they can be a viable option as a next-generation HPV vaccine. Most prominently, the antibody titers elicited upon vaccination with recombinant L2 alone are much lower than those elicited by the L1 VLPs. Furthermore, despite the fact that HPV L2 is

relatively conserved amongst types, it still displays sequence heterogeneity. Thus, a broadly effective L2 vaccine will need to protect against as many HPV types as possible. Of course, this must be done while keeping anti-HPV 16 and 18 neutralizing antibody titers high. Finally, the vaccine needs to be cost-effective for uptake in developing countries.

1.2.7 Strategies for Targeting HPV L2.

Most of the efforts to target HPV L2 have focused on vaccines targeting specific epitopes or domains within the N-terminus of the protein and the use of different techniques to increase the immunogenicity of L2-derived peptides (Table 1). Many of these efforts have focused on targeting the L2 domain encompassing amino acids 17-36. This domain is referred to as the RG-1 epitope because it is targeted by a neutralizing monoclonal antibody (RG-1) that binds to this region, strongly neutralizes HPV16 and HPV18, and, upon passive immunotherapy, protects mice from challenge with HPV16 PsV [94]. This region of L2 contains two cysteine residues (which form a disulfide bond in mature virions) that are present in all known papillomavirus types [95]. Although this is a linear epitope, there is evidence that both the oxidation state and structural context of the RG-1 epitope contributes to its immunogenicity [37, 96]. The RG-1 epitope is not the only potential vaccine target; it has been shown that other domains within L2 (for example amino acids 108-120 and 69-81) can also elicit cross-neutralizing antibodies [27, 97, 98].

Because L2 displays some sequence heterogeneity, it is possible that no single L2 epitope will be capable of inducing antibodies that can cross-neutralize

Table 1 – Experimental L2-based vaccines

Vaccine Strategy	L2 displayed as a:	Notable Findings	References
Recombinant L2 Proteins	Concatameric polypeptide	L2 sequences from multiple HPV types may broaden cross-neutralizing potential and also increase immunogenicity by providing a degree of multivalent display	[99-101]
	Lipopeptide fusion	Use of P25, a TLR2 agonist, may enhance immunogenicity. Effective as an intranasal vaccine	[102]
	Thioredoxin fusion	Bacterial fusion protein; potentially low cost.	[96, 103]
Mulivalent display on Virus-Like Particles	Papillomavirus VLPs	Also provokes strong anti-L1 neutralizing antibody titers against the HPV16 platform	[104-107]
	Bacteriophage VLPs	Vaccine effective without requiring exogenous adjuvants Long-lived immunity; mice were protected from HPV pseudovirus challenge 18 months after vaccination Compatible with genetic display and chemical conjugation	[19, 37, 89]
	Adeno-associated VLPs	Particles maintain immunogenicity upon lyophilization	[108]
	Tobacco Mosaic Virus and Potato Virus X	Production in plants	[109, 110]
Recombinant Bacteria	*Lactobacteria casei*	Compatible with oral delivery	[111]

all high-risk HPV types. To account for this possibility, and also to increase the immunogenicity of a recombinant protein-based vaccine, one strategy has been to covalent fuse the N-terminal regions of L2 from diverse HPV types together and expressed this construct as a concatemeric peptide in *E. coli* [99]. Used with a variety of adjuvants, these multimeric recombinant vaccines elicit broadly neutralizing antibodies that were protective against *in vivo* HPV PsV challenge in a mouse genital challenge model of HPV infection. For example, a fusion peptide of HPV L2 amino acids 11-88 from HPV types 1, 5, 6, 16, and 18 (11-88x5), injected five times with Freund's adjuvant, induced high *in vitro* neutralization titers against all of the HPV types included in the polypeptide, as well as types 45, 31, and 58. In another study, the same 11-88x5 peptide was mixed with type HPV 16 L1 capsomeres, a structural component of L1 VLPs comprised of 5 L1 proteins [100]. Capsomeres are less expensive to produce than the full VLP, but elicited type-specific neutralizing antibodies when used in vaccination [112]. Coimmunization elicited antibodies that strongly neutralize HPV16 and also cross-neutralize other HPV types. Finally, in a recent study attempting to optimize their multimeric peptide by determining essential regions needed for neutralization, Jagu *et al.* showed that vaccination with a fusion peptide displaying the 11-88 region from eight different HPV types to be more immunogenic than vaccinating with multimeric peptides representing smaller portions of the same region of L2, implying that the domain contains multiple neutralizing epitopes and suggesting that immunization with a larger portion of the N-terminus may increase antibody titers [101].

Another strategy for presenting L2 epitopes to the immune system is the use of HPV L1 VLPs as a display scaffold [97, 106, 107]. The rationale for these studies is that multivalent display of L2 on HPV16 VLPs will enhance its immunogenicity without sacrificing a strong anti-HPV16 response. The Kirnbauer group, in particular, has had considerable success inserting L2 peptides into exposed loops of BPV and HPV16 L1 VLPs [105]. Schellenbacher *et al.* genetically inserted overlapping sequences derived from the N-terminus representing amino acids 2-200 from L2 into L1 proteins and attempted to generate chimeric VLPs. Although some of the chimeric proteins failed to assemble into intact VLPs, they found that L1/L2 chimeric VLPs displaying L2 aa 17-36 in combination with adjuvants, provoked the greatest amount of cross-neutralizing antibodies in *in vitro* neutralization assays. Specifically, neutralizing antibodies were detected against HPV high-risk types 16, 18, 45, 52, and 58, as well as types 11 and 5. This degree of cross-neutralization was seen when using Freund's adjuvant or the more physiologically relevant aluminum hydroxide-monophosphoryl lipid A (Alum-MPL), though at lower titers with Alum-MPL. Importantly, the insertion of L2 epitopes did not reduce the observed titer of antibodies directed against the VLP vehicle itself, whether BPV or HPV 16. Schellenbacher *et al.* expanded on this study in a recent paper, rigorously investigating the breadth of cross-neutralization induced by vaccination with HPV 16 L1 VLPs displaying the L2 17-35 (RG-1 VLP) epitope with Alum-MPL [104]. Variable *in vitro* cross-neutralization titers were observed against all relevant high-risk types as well as common low-risk and cutaneous types as well. Further,

immune rabbit sera was passively transferred into mice which were then challenged with a comprehensive panel of high- and low-risk HPV PsVs. Protection was seen against all the 21 tested PsVs, even against types for which the *in vitro* neutralization titers were quite low. Crucially, this cross-protection was observed to be long lasting; passively transferred sera drawn 52 weeks after the initial vaccination was still protective against a heterologous PsV challenge.

A similar technique to increase the immunogenicity of L2 epitopes is to display them on non-HPV VLPs. Heterologous vaccine targets can be genetically inserted or conjugated to the surface of VLPs, creating the same dense, ordered display that strongly activates B cells and leads to high-titer antibody production against the displayed epitope. This has been accomplished on a number of different VLPs, ranging from plant viruses to bacteriophages [35, 108-110]. For example, the Palmer group conjugated the streptavidin bound N-terminus of L2 from canine oral papillomavirus (COPV) to biotinylated Tobacco Mosaic Virus (TMV) VLPs, showing an increase in anti-L2 antibodies when compared to immunizing with the L2 peptide alone [110]. Similarly, the Kleinschmidt group made use of Adeno-associated Virus VLPs (AAVLPs), genetically displaying the RG-1 epitope (17-36) from HPV 16 and 31 on the same particle [108]. Immunization with montanide ISA 51 as an adjuvant induced high-titer anti-L2 antibodies that were able to neutralize HPV PsVs 16, 31, 18, 45, 58, and 52. Importantly, Nieto *et al.* demonstrated that lyophilized and re-constituted AAVLPs were also immunogenic, provoking anti-L2 antibody production. This finding

could be advantageous in lowering the cost of storage and distribution of the vaccine for developing countries.

Our lab has focused on the use of bacteriophage VLPs for the display of the RG-1 epitope. Bacteriophage VLPs can be produced in bacterial expression systems, such as *E. coli*, which lowers manufacturing difficulty and cost while generating a high yield of recombinant VLPs. Genetically inserting the L2 epitope into an exposed loop on the surface of PP7 bacteriophage coat protein, we observed induction of high-titer neutralizing anti-L2 antibodies [89]. Notably, when the RG-1 epitope was inserted in a unconstrained format at the N-terminus of MS2 bacteriophage coat protein, we observed a marked increase in the cross-protection against diverse HPV types when compared to display in other regions of the VLP[37]. Using the PsV *in vivo* challenge model, we observed significant protection against HPV types 5, 6, 16, 18, 31, 33, 35, 39, 45, 51, 53, and 58 in mice vaccinated with our L2 displaying VLPs. More recent studies have examined the longevity and potency of immune responses to bacteriophage vaccination ([19] and unpublished data). Mice immunized with PP7 bacteriophage VLPs displaying the RG-1 epitope were found to have high anti-L2 titers for at least 18 months after vaccination. These antibodies were also shown to be protective against PsV challenge after the same time period. We also measured the immunogenicity of PP7 and MS2 bacteriophages with or without adjuvants and found the immune response to be only mildly boosted when mixed with alum, demonstrating the high innate immunogenicity of bacteriophage VLPs. In another study, we have had some success conjugating L2 peptides to the

surface of Qβ VLPs, another bacteriophage. We created a consensus sequence of region aa 65-85 of L2 from the high-risk HPV sequences to increase the cross-neutralization of this region and tested the cross-neutralization in an *in vitro* L2 PsV neutralization assay (assay described in [113]). Sera from mice immunized with VLPs displaying the consensus sequence showed considerably higher titers of neutralizing antibodies against heterologous PsV types than those immunized with non-consensus sequences from the same region.

Finally, L2-derived peptides have been fused to a variety of immune-activating substances. Richard Roden's group fused the RG-1 epitope to p25, a T helper epitope, and P2C, a Toll-like receptor 2 activating lipopeptide [102]. Immunization with this fusion peptide, either sub-cutaneously or intra-nasally, induced a strong anti-L2 response that was cross-neutralizing in both *in vitro* and *in vivo* PsV neutralization assays. Of note is their observation of a high-titer response to the intra-nasal immunization, suggesting that this vaccine could be delivered needle-free, possibly easing its uptake. The Müller group used bacterial thioredoxin as an adjuvant for L2 peptides, displaying one or more L2 peptides into a surface-exposed loop of the protein [103]. Inserting a number of small, overlapping peptides derived from the N-terminus of HPV 16 L2, Rubio *et al.* found that immunizing with these thioredoxin constructs with CFA and IFA adjuvants did induce a strong anti-L2 response. Of the N-terminal regions tested, they found vaccines that displayed aa 20-38, a peptide overlapping the RG-1 epitope, to induce the greatest breadth of cross-neutralization in *in vitro* PsV neutralization assays. Finally, in a rather unique approach, Yoon *et al.*

genetically inserted a large region of the N-terminus of L2, aa 1-240, into a surface protein of *Lactobacillus casei* (*L. casei*) [111]. The lyophilized, recombinant bacteria were given to the mice enterically via intra-gastric gavage, after which anti-L2 antibodies were found in both sera and vaginal washes. Immune sera and vaginal washes also cross-neutralized HPV types 18, 45, 58, and BPV1 in *in vitro* PsV neutralization assays. Immunized mice were modestly protected upon *in vivo* PsV vaginal challenges.

1.2.8 Expert Commentary and Five-year view

Despite the effectiveness of the current HPV vaccines, there remains a need to provide broader protection against rarer high-risk HPV types and to make it more affordable for developing countries. While the nonavalent L1-based vaccine that is in clinical trials may be a potential solution, several groups have aimed to develop cross-protective vaccines based on L2. Although many labs have developed strategies to elicit high-titer broadly cross-neutralizing antibodies against L2, our bias is that multivalent display on VLPs (or some other particulate carrier) is the most promising and potent technique for eliciting the high titer and long-lasting antibody responses that may be required for sustained cross-protection. L2-displaying VLP vaccines confer remarkable *in vivo* cross-protection in HPV PsV challenges against a large variety of HPV types, both high-risk and otherwise. These studies exhibit the versatility and effectiveness of VLP-based vaccines in displaying heterologous targets to the immune system. In all cases, targeting the RG-1 epitope of L2 provokes the broadest cross-neutralizing responses. Nevertheless, there remain obstacles that must be

overcome before any next-generation HPV can be deemed successful, some of which have been examined in the context of non-VLP-based L2 vaccines. For example, several groups have looked for ways to reduce the necessity of a cold-chain in the delivery of the vaccine. The lipopeptide-L2 construct that was shown to be effective when delivered intra-nasally is one example; others have lyophilized their vaccines and shown continued effectiveness. Also, techniques that are needle-free could possibly ease uptake of the virus. It is possible that the VLP-based vaccines that have shown so much promise [37, 104, 108] in cross-protection will also need to incorporate these features in order to be globally successful. Indeed, our lab has begun to investigate formulation and long-term storage issues, including whether bacteriophage VLPs can be stored in a lyophilized format under environmental conditions.

In the long-run, the greatest hurdle may be showing an increase in clinical effectiveness compared to the upcoming nonavalent L1 VLP vaccine. Clinical trials of L2-based vaccines will require the establishment of high-throughput and standardized assays to measure anti-L2 antibody responses and the ability of patient sera to neutralize diverse HPV types. The recent development of an in vitro neutralization assay that is optimized to sensitively detect neutralizing L2 antibodies should prove useful for these evaluations [113]. Although L2-based vaccines have shown effectiveness in preventing infection with cutaneous animal papillomaviruses [114], it remains to be seen whether these successes will translate to protection from genital infection by HPV. Finally, the clinical effectiveness of Gardasil and Cervarix will set a high bar an L2-based vaccine

and an effective nonavalent vaccine could establish even broader protection for a second-generation vaccine that would be difficult to surpass. In clinical trials L2-based vaccines will need to directly compared to the established HPV vaccines and the trials will need to powered sufficiently to demonstrate protection against rare high-risk HPV types. Although these barriers are not insurmountable, they are substantial.

1.3 Displaying adjuvant-like peptides on VLPs

As has been described above, VLPs are very immunogenic. This immune response, however, could be potentiated by the addition of adjuvants [115]. Adjuvants are normally included in vaccines as activators of the innate immune system which further increases the immune response to vaccination. Adjuvants have the potential to decrease the amount of VLPs needed per immunization, increase the antigenic memory of the immune response, or even speed up the production of antibodies. The addition of adjuvant-like moieties to VLPs may further enhance the immune response to vaccination, and may be useful in the design of vaccines targeting HPV L2 or other poorly immunogenic epitopes.

1.3.1 p28 as a molecular adjuvant

The body has a number of pathways that enhance immune responses to pathogens. One is the complement system which leads to the induction of pro-inflammatory signals, increased adaptive immune responses, and in some cases destruction of pathogens [116]. Both the classical and alternative pathways of complement activation involve the cleavage of the complement protein C3 into C3a and C3b. C3b binds to the surface of pathogens to act as an opsonin,

coating the particle and increasing the efficiency of its uptake by APCs. C3b can be further cleaved, leaving a smaller protein, C3d, bound to the surface. C3d is the ligand of complement receptor 2 (CR2), which is expressed by B cells and Follicular Dendritic Cells (FDCs) [117]. CR2 binding to C3d in conjunction with BCR binding of antigen leads to an increase in activation of the B cell, resulting in greater antibody production [118]. CR2 binding on FDCs can lead to more efficient affinity maturation of activated B cells and longer immunological memory [119].

Given its adjuvant-like properties, C3d has been explored as an addition to vaccines [120]. The minimum CR2 binding region, p28, has been shown to activate B cells in much the same way that C3d does when presented in a multi-valent format [121, 122]. p28 has been utilized in a variety of vaccine studies where multiple copies are typically fused directly to the antigen of DNA vaccines and has been shown to increase the antibody titers in response to vaccination [123-125].

1.3.2 Flagellin – the monomer protein of Flagella

Another possible molecular adjuvant is the principle monomer component of flagella, flagellin. Bacterial flagella are a Pathogen Associated Molecular Pattern (PAMP) that is recognized by the immune receptor Toll-like receptor 5 (TLR5) [126, 127]. TLR5 is located at the plasma membrane of epithelial cells and a number of different immune cells [128]. Binding of flagellin leads to the induction of pro-inflammatory cytokines, recruitment of B and T cells to lymphoid tissues, and activation of T cells and dendritic cells [129]. Unlike p28 and other

immunogenic features of VLPs, flagellin does not interact directly with B cells as they do not express TLR5. Instead, antibody production is affected indirectly through activation of other immune cells and general inflammation [130, 131]. Studies have shown that using flagellin as an adjuvant in vaccines, especially when bound to antigen, results in greater production of inflammatory cytokines and higher titers of antibodies [132, 133].

1.4 Overview of Dissertation

As discussed above, there is a need for an HPV vaccine that meets the needs of the countries most affected by cervical cancer. The most important feature of any next-generation HPV vaccine is that it protect against a greater number of HPV types, particularly the high-risk types that are found associated with cervical cancer. Other features include stability of the vaccine at ambient temperatures, negating the need for a cold-chain of transportation and storage, affordability, and a minimum number of boosts required for protection. The inclusion of these characteristics would ease the uptake of the vaccine in developing countries where a majority of cervical cancer deaths occur.

We hypothesize that using bacteriophage VLPs to display L2 epitopes can be the basis of a next-generation HPV vaccine. This platform allows us to target specific epitopes in L2 that are highly conserved or shown to elicit neutralizing antibodies. In addition, the flexibility of bacteriophage VLPs allows us to experiment with their construction, including multiple targets or molecular adjuvants in order to broaden the neutralizing response against heterologous HPV types and lower the dosage or boosts needed.

In chapter 2, various epitopes from HPV16 L2 are displayed on bacteriophage VLPs through genetic insertion or conjugation in order to gauge their immunogenicity and induced protection when displayed in the dense, highly-ordered format of VLP display. We immunized mice with these L2-bacteriophage VLPs and measured the generated antibody response against the L2 peptides as well as the protection conferred when challenged with a homologous HPV PsV. While vaccination with two of these peptides also granted varying degrees of cross-protection against heterologous HPV types, one, aa 65-85, showed no cross-protection. This peptide encompassed a region that is poorly conserved amongst HPV types, and so we hypothesized that immunizing with a consensus sequence derived from the high-risk HPV types may be more effective at eliciting a cross-protective response. After immunizing mice with the consensus peptide-bacteriophage VLPs, we were able to measure the cross-neutralization of the generated antibodies through an *in vitro* neutralization assay developed specifically to measure neutralizing titers of anti-L2 antibodies. We found that sera from mice immunized with bacteriophage VLPs conjugated to the consensus sequence of this region was able to effectively neutralize five different high-risk HPV PsV types, in contrast to sera from mice immunized with VLPs displaying aa 65-85 from either HPV16 or 18.

In chapter 3, we investigated displaying multiple targets on the surface of VLPs. While there are regions of L2 that have great homology among HPV types, it is possible that only immunizing with one peptide might not be able to generate an immune response that protects against all the high-risk types. To

address this, we designed a plasmid that contains two open reading frames of bacteriophage single-chain dimer. Each dimer displays a different target, and these self-assemble to form hybrid particles displaying two targets on the surface. We immunized mice with these hybrid VLPs and compared the immune responses to that of mice immunized with VLPs displaying the traditional one target. We found that immunization with the hybrid VLPs could induce an antibody response against both of the peptides displayed on the surface. In addition, displaying the same regions of L2 from two closely related HPV types resulted in a more broadly cross-neutralizing antibody response as well.

Finally, in chapter 5, we investigate displaying p28, the activating peptide of CR2, and flagellin, the monomer component of flagella, at low levels on the surface of our VLPs in addition to HPV L2 peptides. By displaying p28, we sought to mimic the complement system's own adjuvant-like activities to speed the immune response to vaccination and reduce the amount of VLPs needed. Flagellin is bound by TLR5 which isn't located on B cells but rather on other immune cells like dendritic cells and T cells. Activating this other immune pathway could synergistically lead to greater antibody production in response to our vaccines. We were able to produce VLPs displaying both an HPV L2 epitope and the p28 peptide on the surface through genetic insertion or a combination of genetic insertion and conjugation. Displaying the much longer flagellin fragment proved much more difficult, resulting in misfolding, aberrant translation, or degradation of the coat protein. Immunizing with these p28 displaying VLPs,

however, did not result in a faster or more robust production of antibodies against the included L2 peptide.

Chapter 2 - The use of hybrid Virus-like Particles to enhance the immunogenicity of a broadly protective HPV vaccine

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(Submitted to Vaccine, Feb 2014)

2.1 Abstract

Vaccines targeting conserved epitopes in the HPV minor capsid protein, L2, can elicit antibodies that can protect against a broad spectrum of HPV types that are associated with cervical cancer and other HPV malignancies. Thus, L2 vaccines have been explored as alternatives to the current HPV vaccines, which are largely type-specific. In this study we assessed the immunogenicity of peptides spanning the N-terminal domain of L2 linked to the surface of a highly immunogenic bacteriophage virus-like particle (VLP) platform. Although all of the HPV16 L2 peptide-displaying VLPs elicited high-titer anti-peptide antibody responses, only a subset of the immunogens elicited antibody responses that were strongly protective from HPV16 pseudovirus (PsV) infection in a mouse genital challenge model. One of these peptides, mapping to HPV16 L2 amino acids 65-85, strongly neutralized HPV16 PsV but showed little ability to cross-neutralize other high-risk HPV types. In an attempt to broaden the protection generated through vaccination with this peptide, we immunized mice with VLPs displaying a peptide that represented a consensus sequence from high-risk and other HPV types. Vaccinated mice produced antibodies with broad, high-titer neutralizing activity against all of the HPV types that we tested. Therefore, immunization with virus-like particles displaying a consensus HPV sequence is an effective method to broaden neutralizing antibody responses against a type-specific epitope.

2.2 Introduction

The current HPV vaccines (Gardasil and Cervarix) provide strong protection against two high-risk HPV types, HPV16 and 18, which are associated with roughly 70% of cervical cancer cases [43], but they largely do not protect against the other high-risk HPV types that are associated with cancer [84]. To develop vaccines that provide broader protection against infection by diverse HPV types, many researchers have investigated the minor capsid protein of HPV, L2. Although L2 is component of the viral capsid, natural infection with HPV fails to elicit antibodies against L2. This likely reflects the fact that L2 is only transiently exposed on the surface of the virus particle during the infectious process. Upon viral attachment to the basement membrane and furin cleavage, however, the N-terminal region of L2 is exposed and vulnerable to antibody binding [58, 62]. Moreover, the N-terminus of L2 includes regions that possess a great deal of homology among HPV types, suggesting that vaccination with L2 could potentially elicit broadly cross-neutralizing antibody responses. Early studies using animal papillomavirus models showed that immunization with L2 was protective against homologous and heterologous papillomavirus infection [90-93]. Epitope mapping studies utilizing neutralizing monoclonal antibodies targeting the N-terminus of L2 and recombinant peptide-based vaccines have identified putative neutralizing epitopes within L2 that elicit cross-protective antibodies [94, 105, 134, 135]. In particular, these studies have identified the region encompassing L2 aa17-36 as a broadly neutralizing epitope within this protein. One concern, however, is that vaccination with recombinant L2 typically results in lower neutralizing titers than vaccination with L1-VLPs [136].

Much of the success of the current HPV vaccines is due to the strong immunogenicity of VLP-based antigens. VLPs present viral epitopes in a dense, repetitive pattern, which leads to the efficient cross-linking of B cell receptors and high-titer production of antibodies [82]. VLPs can be used as stand-alone vaccines (as is the case of Gardasil and Cervarix), but they can also be used as scaffolds to display heterologous antigens in a highly immunostimulatory fashion [22, 23]. In general, antigens can be displayed on VLPs by genetic insertion of target sequences into exposed loops on viral structural proteins or by chemically conjugating target peptides to the surface of VLPs with the use of small, flexible linkers that react to exposed residues on the surface of VLPs. Both techniques result in high surface expression of the target antigen and give rise to high titer antibody responses against the targeted peptide when injected into mice or other animals [31, 34].

Vaccines in which L2 epitopes are displayed multivalently can induce potent anti-peptide antibody responses [99, 105, 108]. For example, our laboratory has displayed a the broadly neutralizing L2 epitope (aa17-31) on VLPs and has shown that this vaccine elicits high-titer, long-lasting and broadly neutralizing antibody responses [19, 35, 89]. Here, we hypothesized that this technique could be used to target other potentially cross-reactive epitopes derived from L2. Using two different VLP display methodologies, we assessed the immunogenicity of VLPs displaying a panel of L2 peptides derived from the N-terminal domain of HPV16 L2. We found that this strategy invariably led to a high-titer antibody response against the peptide, but the *in vivo* protection

observed upon vaginal challenge with HPV pseudovirus was quite varied. In particular, we found that vaccination with a VLP displaying HPV16 L2 aa65-85 induced strong homologous protection against PsV16, but little to no cross-protection against heterologous HPV PsV types. We were able to overcome this limitation by immunizing with VLPs displaying a L2 peptide representing the aa65-85 consensus sequence of high-risk HPV types. Sera from mice immunized with VLPs displaying the consensus sequence peptide were able to effectively neutralize heterologous high-risk HPV PsV. We conclude that immunizing with consensus peptides of neutralizing epitopes may be an effective method to generate broadly cross-neutralizing antibodies.

2.3. Materials and Methods

2.3.1. Conjugation of L2 peptides to Qβ

Preparation of Qβ bacteriophage was performed as described previously [31]. Peptides representing 4 regions of the N-terminus of HPV16 L2 (aa34-52, 49-71, 65-85, and 108-120) and a consensus peptide were synthesized by American Peptide Company (Sunnyvale, Ca). Each peptide was synthesized to include a cysteine residue at the C-terminus to allow conjugation to bacteriophage particles. Peptides were conjugated to the surface of Qβ bacteriophage using the crosslinker SMPH (Thermo Scientific) and conjugation efficiency was assessed as described previously [31].

2.3.2. Expression & purification of L2 PP7 VLPs

PCR was used to independently insert four HPV16 L2 peptides (aa17–31, 35-50, 51-65, and 65-79) into the AB-loop of the single-chain dimer version of

PP7 coat protein as previously described [35, 89]. PCR fragments were cloned into pET2P7K32 using KpnI and BamHI restriction sites and constructs were confirmed by sequence analysis. VLPs were made by transforming C41 cells (Lucigen) with L2-PP7 expression vectors. Expression of PP7 VLPs displaying L2 aa(35-50) and (51-65) also required co-expression of the groEL and groES chaperones using the plasmid pGro7 (Takara). Transformed cells were grown at 37°C until they reached an A_{600} of 0.6. L2-PP7 protein expression was induced with 0.5 mM IPTG for 3h. Cell pellets were lysed and VLPs were purified from the soluble fraction as previously described [35].

2.3.3. Immunization of mice and characterization of sera for anti L2-IgG

All animal work was done in accordance with National Institutes of Health and University of New Mexico guidelines. Groups of 3-13 Balb/c mice were immunized three-times at two-week intervals. Immunizations were performed intramuscularly (i.m.) using 5 µg of VLPs plus IFA. Sera from all experimental groups were collected two weeks after the last boost and analyzed by ELISA for anti-L2 IgG. A peptide ELISA was used to assess the titer of anti-L2 IgG in sera. ELISA plates were coated with 1 µg of the appropriate target peptide (representing L2 aa14–40 from HPV16, synthesized by Designer Bioscience, or aa34-52, 49-71, 65-85, and 108-120 from HPV16 and aa65-85 from HPV18, synthesized by American Peptide as described above) conjugated to streptavidin using SMPH. ELISAs were performed as described [89].

2.3.4. Pseudovirus production and purification

HPV6, HPV16, HPV18, HPV31, HPV45, HPV52, and HPV58 PsVs with encapsidated reporter plasmid (pClucf) encoding both luciferase and green fluorescence protein (GFP) genes were produced in 293TT cells as previously described [79, 137] except that matured PsVs were purified by ultracentrifugation on a cesium chloride gradient at 27,000x*g* for 18 hours. Flow cytometry was used to titer the PsV by determining the fraction of GFP-expressing 293TT cells.

2.3.5. Cervicovaginal HPV PsV challenge

Prior to challenge, female Balb/c mice were given 3 i.m. immunizations of 5 µg of control VLPs or VLPs displaying one of the L2 epitopes. Two weeks after the last boost, mice were treated with 3 mg of Depo-Provera subcutaneously (Pharmacia Corp). Five days post-Depo-Provera treatment, mice were vaginally challenged with $1.3 \times 10^5 - 1 \times 10^7$ infectious units (IU) PsV as previously described [79, 137]. Forty-eight hours post-PsV challenge, mice were vaginally instilled with 0.4 mg of luciferin (Caliper Life Sciences) and imaged with a Caliper IVIS Lumina II (Caliper Life Sciences) as described previously [89].

2.3.6. Derivation of an L2 aa65-85 consensus sequence

Twelve high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and the three low-risk types (HPV6, 53, and 66) were used to derive a consensus sequence. L2(65-85) sequences were aligned using ClustalX [138] and a consensus sequence was generated using Jalview 2.7 [139].

2.3.7. In vitro L2 neutralization assay

Neutralization assays were performed as described in [113] except that heparin was not added to PsV solutions prior to infection. Following a two-day

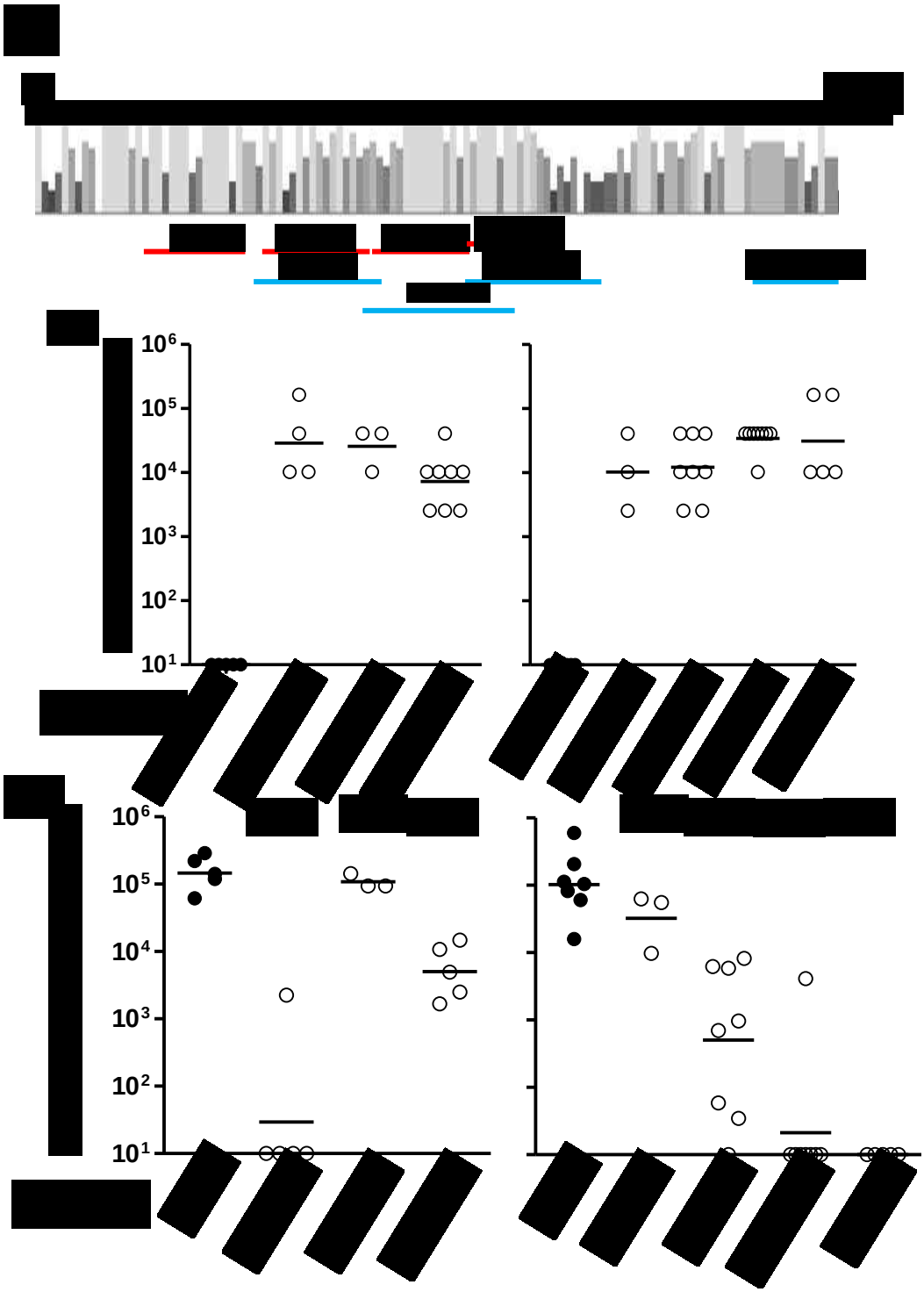

Figure 2.1 - Selection, immunogenicity, and in vivo protection of VLPs displaying L2 peptides. (A) L2 epitopes displayed on VLPs. The reference amino acid sequence listed is that of HPV16 (Genbank #AAA46942.1). The amino acid conservation of L2 was computed by Jalview 2.7 using the sequences of high-risk HPV types 18, 31, 45, 33, 39, 51, 52, 58, and 59. Vertical bars represent relative conservation of residues among included types (taller and lighter indicating greater conservation, shorter and darker indicating less). Red horizontal bars denote

epitopes genetically inserted into PP7, blue bars denote peptides conjugated to Qβ. The numbers above each bar represent the HPV16 L2 aa included in the epitopes. (B) Antibody responses upon immunization with VLPs displaying HPV L2-derived peptides. Mice were immunized with VLPs displaying the indicated HPV16 L2 peptide or, as a control, with the unmodified VLPs (PP7 or Qβ). Sera were collected and anti-L2 peptide IgG titers were determined by end-point dilution ELISA using the synthetic peptide displayed on the VLP as a target. End-point dilution titers indicate the reciprocal of the dilution of serum samples at which reactivity with each of the L2 peptides was at least twice that of background. Data points represent individual mice and lines represent the geometric mean for each group. (C) Vaccination with VLPs displaying L2 peptides protects against homologous HPV16 PsV challenge. Mice were immunized with the VLPs (PP7, left panel; Qß, right panel) indicated on the x-axis and two to three weeks after the last immunization mice were vaginally challenged with 3.0×10^6 IU of HPV16 PsV encapsidating a luciferase reporter plasmid. Two days after PsV challenge, the mice were vaginally instilled with luciferin and imaged for luciferase luminescence. Each data point represents the average radiance for an individual mouse region of interest (ROI) with the line representing the geometric mean of the group. The average radiance ($p/s/cm^2/sr$) was calculated by using Living Image 3.2 software. Data were analyzed by one-way ANOVA with Dunnet's Multiple Comparison comparing each condition to the control. *** - $p \leq 0.001$, ns - not significant.

incubation, the cells were then collected and analyzed by flow cytometry using a Hypercyte autosampler to detect GFP expression as a marker of infection. The dose of PsV used was based on the amount needed to yield 20-40% infection of control pgsa-745 cells. HPV18 PsV stocks were poorly infectious, so infection with HPV18 PsV typically resulted in ~10% of control cells being infected.

2.3.8. Statistical Methods

Statistical analyses of *in vivo* PsV challenges were performed with the Graphpad 5.0 Prism software. Data was log-transformed and then analyzed by unpaired, two-tailed *t*-tests. Multiple comparisons were analyzed by a one-way ANOVA with Dunnett's Multiple Comparison Test.

2.4. Results

We have previously shown that a single-chain dimer version of the PP7 coat protein broadly tolerates the genetic insertion of short peptide epitopes, including aa17-31 of HPV16 L2, allowing the construction of L2-recombinant VLPs [35]. Immunization with HPV16 L2(aa17-31) (either on bacteriophage VLPs or using other display formats) induces cross-neutralizing antibodies against multiple HPV types [94, 102-104, 108]. We asked whether other domains within the N-terminus of L2 were also capable of inducing neutralizing and/or cross-neutralizing antibody responses when displayed on a highly immunogenic VLP platform. In selecting other regions to target through vaccination, we considered both previous L2 mapping studies and the level of L2 homology among HPV types [134, 135]. In addition to aa17-31, we genetically inserted HPV16 L2 aa35-50, 51-65, and 65-79 into the AB loop of the PP7

single-chain dimer (shown schematically in Fig. 2.1A; red lines). In this format, 90 heterologous peptides are displayed on each VLP. Insertion of HPV16 L2 aa35-50 and aa51-65 was compatible with VLP assembly, but insertion of aa65-79 was not. We have also had success linking peptides to the surface of Qβ particles using a chemical crosslinker [31, 140]. While aa17-31 could not be conjugated due to internal cysteine residues and their required disulfide linking, we were able to conjugate peptides representing HPV16 L2 aa34-52, 49-71, 65-85, and 108-120 to the surface of Qβ (Fig. 2.1A; blue lines). Conjugation of each peptide was highly efficient; approximately 360 peptides were displayed per Qβ capsid (not shown).

To assess the immunogenicity of the L2-displaying VLPs, we immunized groups of Balb/c mice with three 5 µg doses of VLPs displaying L2 peptides or, as a control, unmodified VLPs (Qß or PP7). Following the last immunization, sera were collected and analyzed by ELISA for L2 peptide-specific IgG (Fig. 2.1B). Both types of L2-displaying VLPs elicited high-titers of peptide-specific IgG.

To determine whether vaccination conferred protection from genital challenge with HPV16, we utilized the HPV PsV vaginal challenge model developed by Roberts and colleagues [79]. Vaccinated mice were vaginally challenged with a high dose of HPV16 PsV encapsidating a luciferase reporter plasmid. Protection against infection was quantified by comparing the luciferase signal in mice immunized with L2 peptide-displaying VLPs to that of control mice immunized with the vehicle VLP platform alone (Fig. 2.1C). We found that vaccination with PP7-16 L2(17-31), Qß-16 L2(65-85), and Qß-16 L2(108-120)

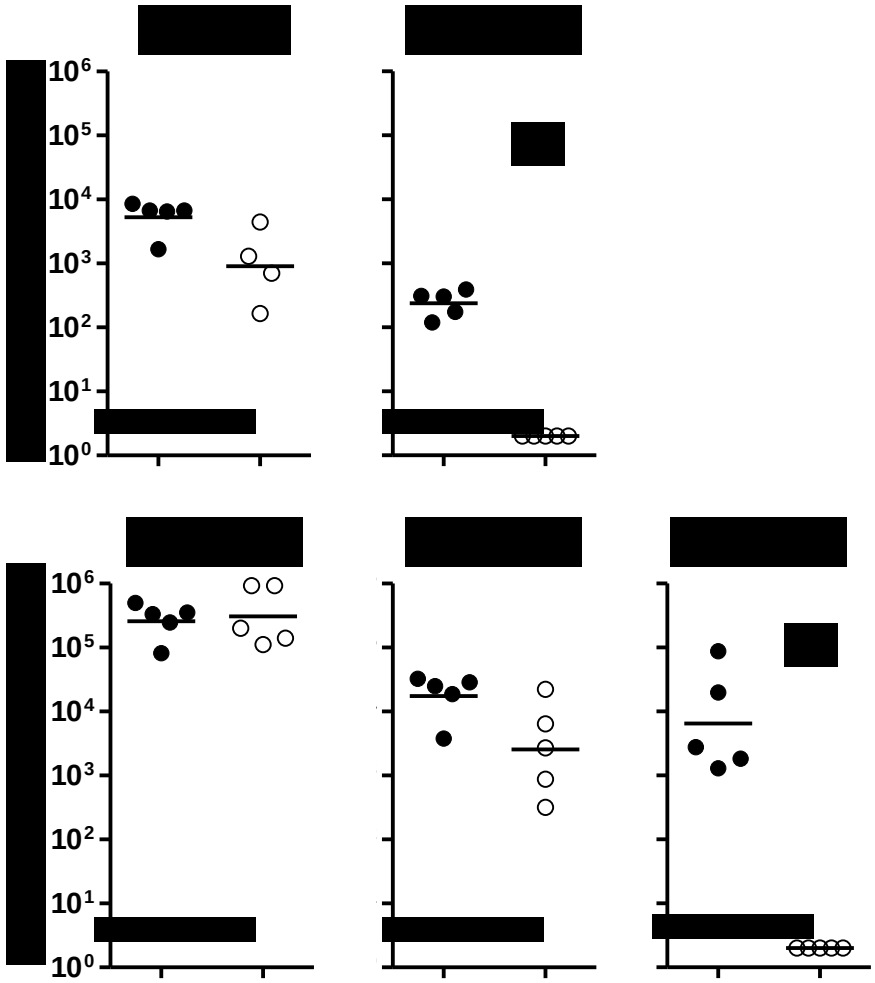

Figure 2.2 - Mice vaccinated with Qβ-16 L2(108-120) show variable levels of cross-protection from heterologous HPV PsV challenges. Two weeks after their last immunization, groups of mice were vaginally challenged with 10^7 IU HPV PsV, as described in Fig. 1C. Solid circles represent mice immunized with wild-type Qβ, empty circles represent mice immunized with Qβ-16 L2(108-120). Data points indicate the average radiance of individual mice and lines represent the geometric mean of each group. Data were analyzed by two-tailed, unpaired t-tests and p-values are listed.

provided strong protection against homologous PsV infection (>99% decrease in mean luciferase signal). Conversely, mice immunized with either Qß-16 L2(34-52) or recombinant PP7-16 L2(35-50) were poorly protected from HPV16 PsV challenge. Immunization with either PP7-16 L2(51-65) or Qß-16 L2(49-71) conferred moderate protection from homologous PsV challenge.

We next investigated whether the vaccines that induced the best homologous protection also conferred protection from challenge with heterologous HPV PsV types. Our studies describing the cross-protection elicited by PP7-16L2(17-31) have been previously published and will not be reiterated in this manuscript [89]. Mice immunized with Qβ-16L2(108-120) showed strong cross-protection against PsV18 and 58, moderate cross-protection (~1 log reduction in average luminescence) against PsV6 and 45, and little protection against PsV31 (Fig. 2.2). In large part, these data agree with previous studies of this region which show variable cross-neutralization elicited by immunization with the 108-120 epitope [27, 141].

Immunization with VLPs displaying a peptide representing HPV16 L2 aa65-85 also strongly protected mice from vaginal challenge with HPV16 PsV. However, mice immunized against HPV16 L2(65-85) were not protected against an *in vivo* HPV18 PsV challenge (Fig. 2.3A), suggesting that this epitope was not cross-neutralizing. To explore this further, we created a vaccine that displayed the same peptide from HPV18. Sera from mice immunized with either Qβ-16L2(65-85) and Qβ-18L2(65-85) were cross-reactive (i.e. recognized both the homologous and heterologous peptide) in an ELISA assay (Fig. 2.3B). Mice

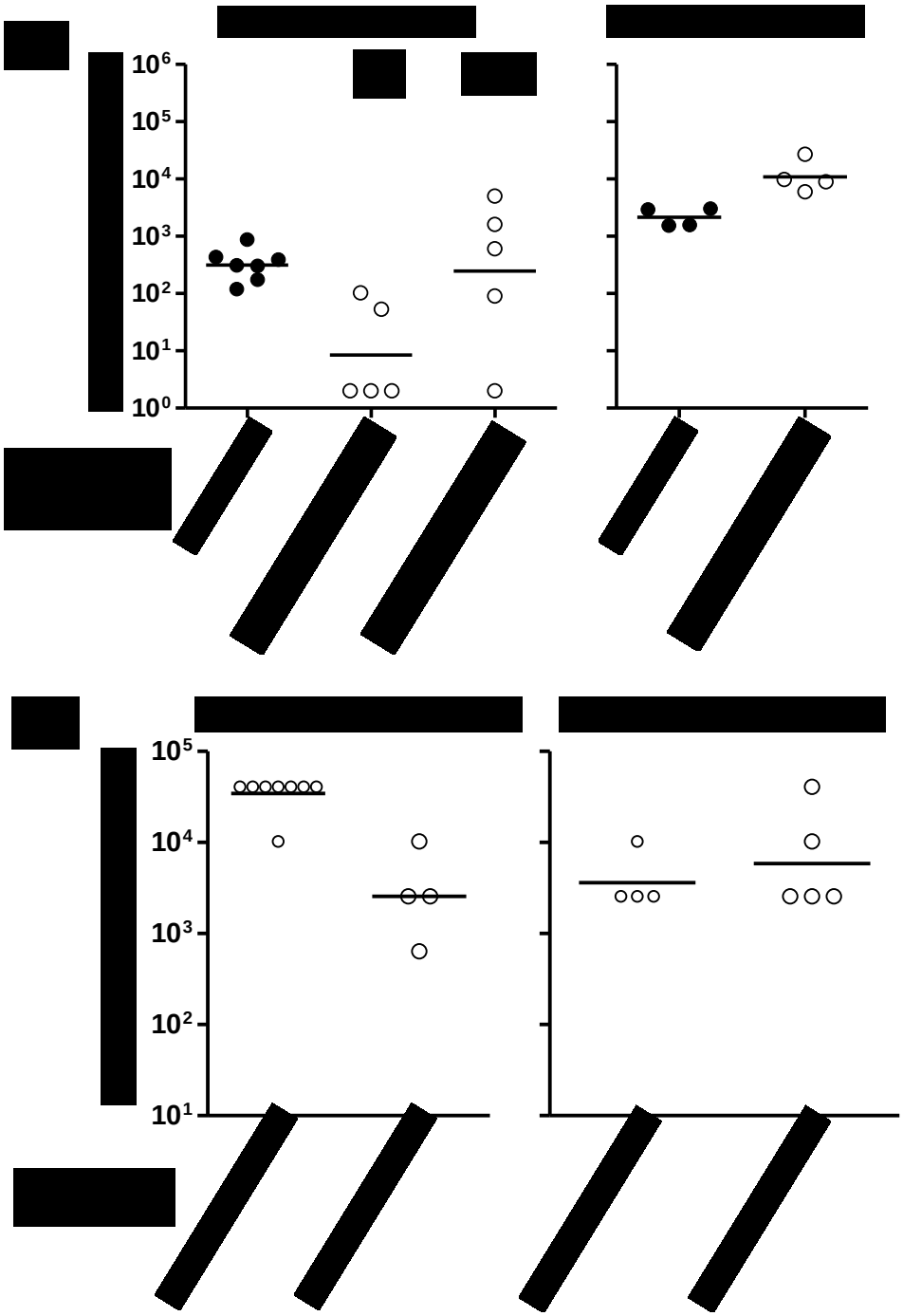

Figure 2.3 - Mice immunized with Qβ-HPV L2(65-85) show type specific in vivo protection despite in vitro cross-reactivity to L2 peptides. Mice were immunized twice with Qβ VLPs displaying either HPV16 or HPV18 L2(65-85), sera was taken for ELISA analysis and then mice were challenged with HPV PsV, as described in Fig. 1C. (A) Immunized mice were challenged with 1.0 x 10^5 IU HPV18 PsV or 1.0 x 10^6 IU HPV16 PsV. Groups of mice were immunized with the VLP indicated on the x-axis. (B) Serum IgG levels against the HPV18 peptide (left) or HPV16

peptide (right) were determined as described in Figure 2. Data points indicate the average radiance of each mouse (A) or the reciprocal endpoint dilution titer of individual mice (B); lines indicate the geometric mean of each group. PsV 18 challenge data were analyzed by an one-way ANOVA with Dunnett's Multiple Comparison. * indicates $p \leq 0.05$, ns – not significant.

immunized with Qβ-18L2(65-85) were strongly protected against 18PsV challenge, but not against 16PsV challenge (Fig. 2.3A). These data are in concordance with previous studies investigating this region of L2 [134]. Given that aa65-74 has the same sequence in HPV16 and 18, our findings suggest that this region of the peptide contains a non-neutralizing, but cross-reactive, epitope. In contrast, the less well-conserved C-terminal region of this peptide is likely a type-specific neutralizing epitope. There are several examples of vaccines that use viral consensus sequences to successfully to induce antibodies with broader specificities [142-145]. To test whether this strategy would work against HPV L2, we synthesized a peptide that represented the consensus sequence of aa65-85 from 12 high-risk HPV types and as 3 low-risk types (Fig. 2.4A). Interestingly, the consensus contains a double proline and a valine-rich region. One of these motifs is present in nearly all of the high-risk HPV types (Fig. 2.4B). As expected, mice immunized with Qβ-consensusL2 (65-85) produced sera that reacted with both the HPV16 and 18 peptides (Fig. 2.4C).

Next, we tested whether the immunization with the consensus peptide elicited more broadly neutralizing antibodies. In order to establish a more high-throughput assay to measure neutralizing antibodies, we made use of an *in vitro* HPV neutralization assay designed specifically to be more sensitive to anti-L2 antibodies [113]. In particular, this assay allows the HPV PsVs to bind to deposited extracellular matrx (ECM), allowing the N-terminus of L2 to be processed by the furin-conditioned media and exposing N-terminal epitopes to anti-L2 antibodies in serum. In addition to quantifying anti-L2 antibodies at a

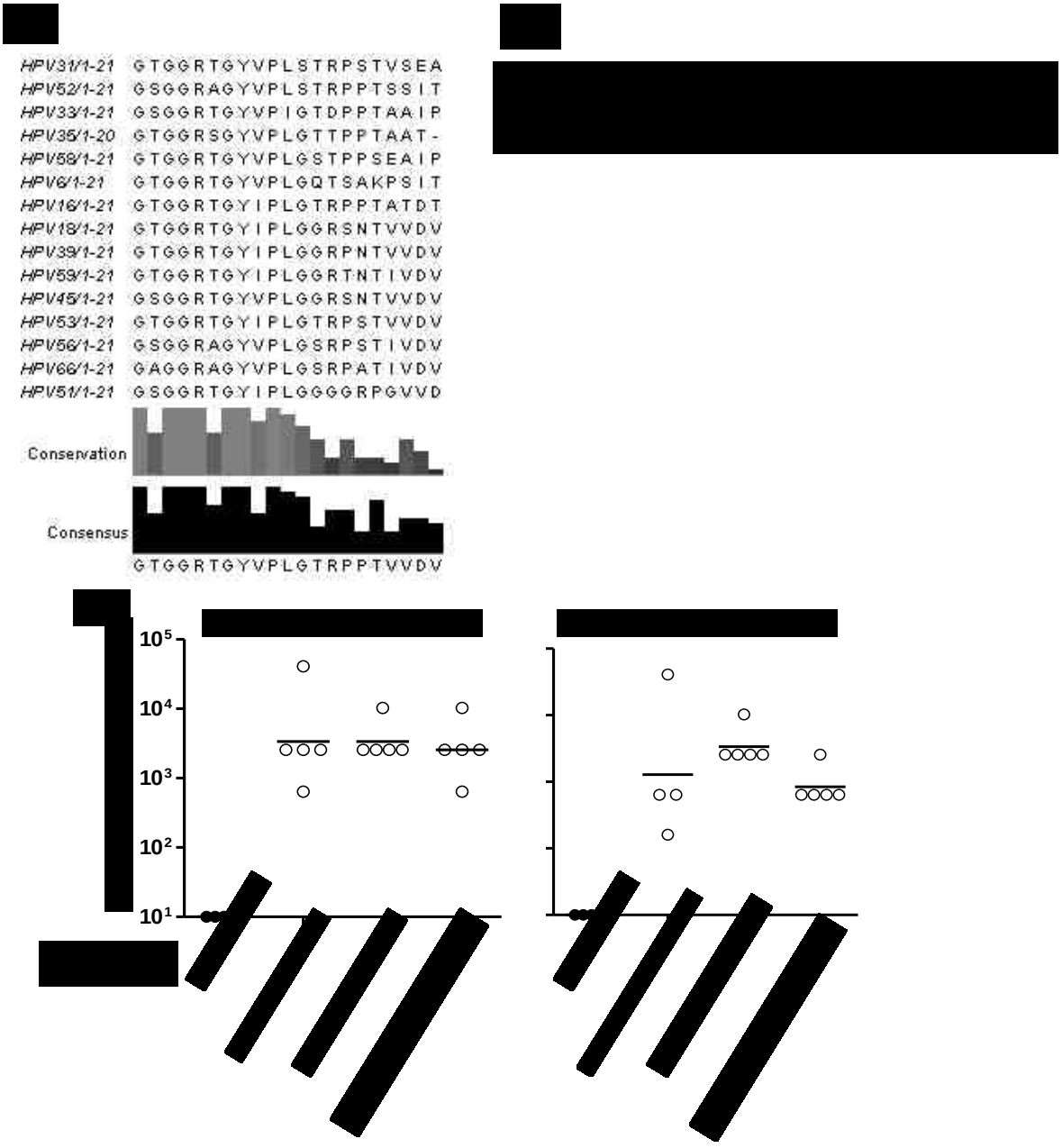

Figure 2.4 - Generation of a L2 (65-85) consensus sequence which elicits anti-serum that exhibits in vitro cross-reactivity to L2 peptides. (A) ClustalX sequence alignment of L2 (65-85) from selected high- and low-risk HPV types. The conservation analysis and consensus sequence was generated using Jalview 2.7, as described in Fig. 3.11A. The numbers after the HPV types are added by Jalview and indicate the number of amino acids included. The vertical bars indicate the relative conservation amongst the selection sequences (upper) or to the consensus sequence (lower). (B) Comparison of the sequence of HPV16 (65-85), HPV18 (65-85) and the consensus sequence. Note that the consensus sequence contains two motifs found in most of high-risk sequences. (C) Immunization with VLPs displaying all three (65-85) peptides (x-axis) elicit HPV16 and HPV18 peptide-binding antibodies. Serum anti-L2 IgG titers were determined by end-point dilution ELISA targeting the 65-85 peptide derived from either HPV16 (left) or HPV18 (right), as described in Fig. 1C. Data points represent the endpoint titer of individual mice and lines represent the geometric mean for each group.

level that correlates with observed *in vivo* protection, this assay also allows the sera from the same mice to be tested for neutralization against a number of different HPV PsV types. First we measured the neutralization of HPV16 PsV by sera pooled from vaccinated mice and compared this to the *in vivo* PsV challenge results previously obtained (Fig. 2.5A and Fig. 2.1C). We found that the *in vitro* results closely mirrored the data from the *in vivo* challenge. Given these data, we concluded that the *in vitro* assay was comparable to the *in vivo* challenge model and could be used going forward as a measure of our vaccines' effectiveness.

To measure the cross-neutralizing activity of serum raised against Qβ-consensus L2(65-85), we tested pooled sera for *in vitro* neutralization against five high-risk PsVs: 16, 18, 31, 45, and 58 Fig. 2.5B & Fig. 2.6). In general, pooled sera from mice immunized with VLPs displaying either the HPV16 or the HPV18L2(65-85) peptide were only poorly cross-neutralizing. We did detect some cross-neutralizing activity, for example sera raised against Qβ-18L2(65-85) against the closely related PsV45. Strikingly, pooled sera from mice immunized with particles displaying the consensus sequence neutralized each of the different HPV types tested at high dilutions, and was the only serum to neutralize HPV31 PsV. Thus, these data indicate that displaying a consensus peptide on Qß bacteriophage can broaden the neutralizing specificity of a region of L2 that normally only elicits type-specific neutralizing antibodies.

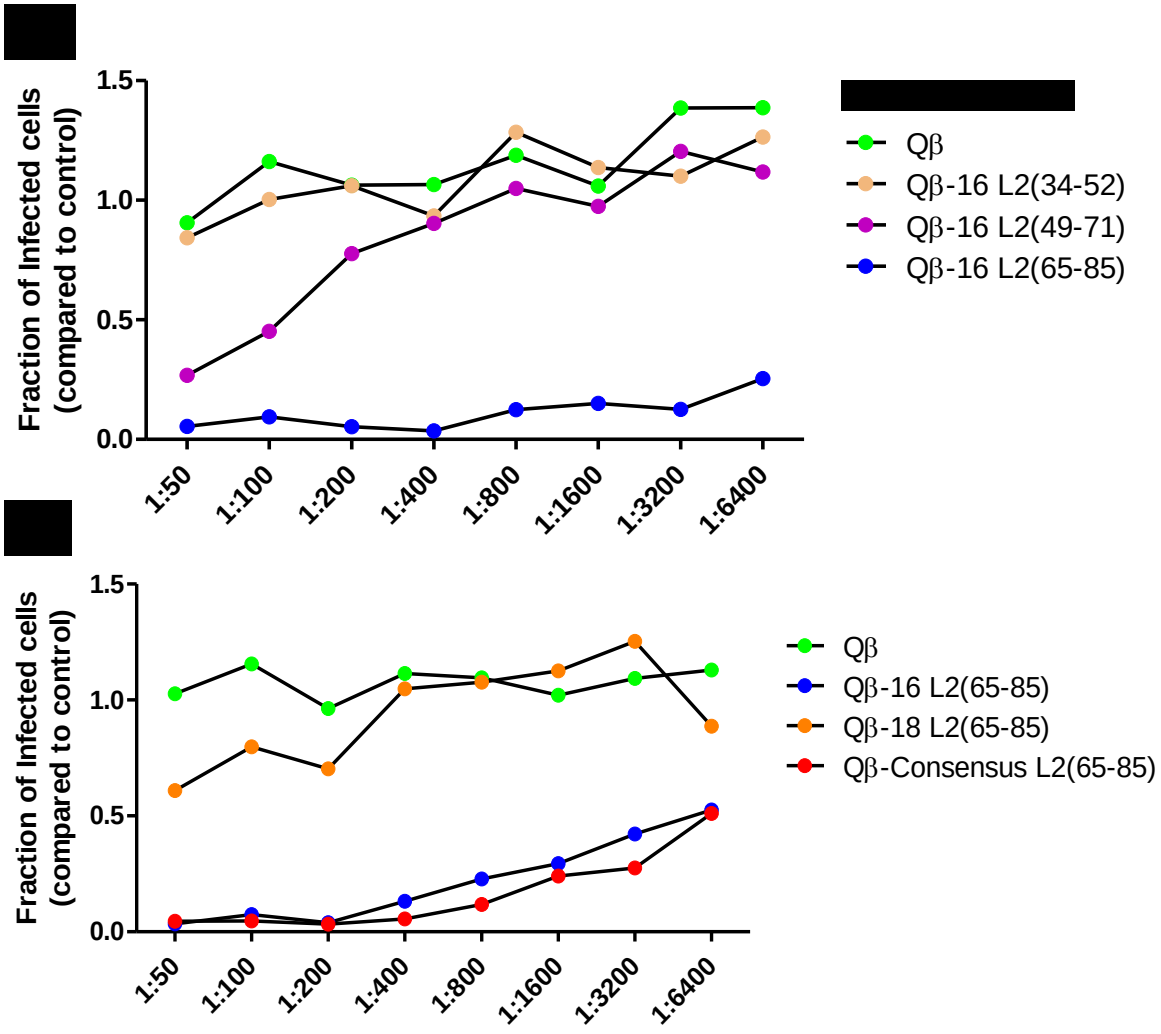

Figure 2.5 - in vitro PsV L2 neutralization assay. (A) *In vitro* data recapitulate previous *in vivo* PsV challenge data. Sera from mice immunized with VLPs indicated were pooled and tested for neutralization against HPV16 PsV at ID_{40} (amount of PsV that infected 40% of cells in wells devoid of sera) at the indicated dilutions. HPV PsV were incubated on deposited ECM in 96-well plates and treated with furin-conditioned media. HPV16 PsV was then incubated with pooled dilutions of sera for 6 hours, after which pgsa-745 cells were added. Infection was measured as GFP production, quantified by a Hypercyt autosampling flow cytometer. Bars indicate the relative amount of infected cells in sera treated wells compared to wells with no sera added. *In vitro* results were compared to previous (Fig. 1C, right panel) *in vivo* HPV16 PsV challenge. (B) Consensus sequence anti-sera neutralizes HPV16 PsV *in vitro.* Sera from mice immunized with the VLPs indicated were pooled (5 mice in each group) and tested for neutralization against HPV16 PsV at ID_{40} at the indicated dilutions as in (A).

2.5 Discussion

The N-terminus of L2 contains cross-protective, neutralizing epitopes [134, 135, 146] including the RG-1 epitope, located within aa17-36 of HPV16 [94]. Nevertheless, other epitopes within the N-terminal third of L2 also have potential to induce neutralizing antibodies. We used two flexible VLP-based approaches to target candidate epitopes within this region. VLP-display elicited high-titer antibodies and allowed us to map potential neutralizing epitopes with heightened sensitivity. We found that targeting aa17-31, 65-85, and 108-120 of HPV16 resulted in near complete protection from homologous HPV PsV challenge. When vaccinated mice were challenged with heterologous HPV PsV types, we found variable amounts of protection from type to type in mice vaccinated with 108-120, but little cross-protection in mice vaccinated with 65-85. However, a consensus sequence peptide derived from this region elicited high-titer cross-neutralizing antibodies.

Developing consensus sequence vaccines to elicit a cross-protective immune response is a technique that has been explored to combat viruses that undergo antigenic variation. For example, consensus vaccines targeting the Env protein of HIV show enhanced ability to generate cross-reactive antibodies [145]. Similarly, consensus norovirus VP1 VLPs elicit broader immune response than VLPs derived from individual norovirus types [142]. Here, we show that this approach can be extended to a short peptide. One concern with the use of consensus antigens, however, is that they may induce low-affinity antibodies.

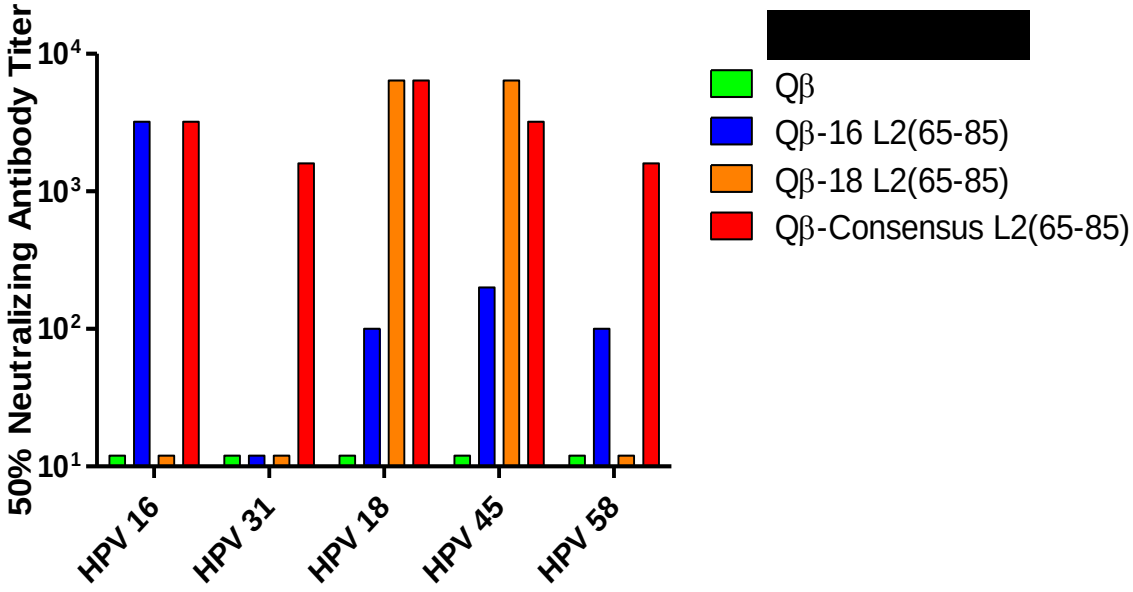

Figure 2.6 - A summary of in vitro HPV PsV neutralization assays showing that Qβ particles displaying a consensus L2(65-85) sequence induce broadly in vitro neutralizing antibodies. Pooled sera from immunized mice (5 mice per group) was measured for cross-neutralizing activity against the indicated heterologous high-risk HPV types as in Figure 7. The PsVs were added at an ID_{20} (amount of PsV that infects 20% of control cells) except for PsV 16 (ID_{40}) and PsV 18 (ID_{10}). Bars indicate the reciprocal of the highest dilution at which the PsV was neutralized greater than 50% compared to wells with no sera added.

Such antibodies may be able to react with a greater number of viral strains, but unable to neutralize any of them effectively. However, the consensus L2 peptide that we engineered efficiently neutralizes five diverse HPV PsV types. The potentiated neutralizing activity of antibodies elicited by consensus peptide VLPs may be due to the inclusion of conserved motifs that are found in most of the high-risk HPV types. The heterogeneous region of the peptide (aa76-85), that likely represents a type-specific neutralizing epitope, contains motifs that are broadly conserved. A majority of HPV high-risk types include either the double proline motif or the valine-rich region. Nevertheless, it is worth nothing that HPV31 does not contain an exact match of either motif, yet was strongly neutralized by sera from mice immunized with the consensus vaccine.

While we have shown a considerable increase in the cross-neutralization of heterologous HPV types when vaccinating with a consensus L2 sequence, there is a high standard of cross-reactivity that will need to be surpassed in order to be a potential candidate as a next-generation HPV vaccine. In addition to the current vaccines, a nonavalent L1-based vaccine currently in clinical trials (http://clinicaltrials.gov/ct2/show/NCT00543543), and other L2-based vaccines have shown great promise in terms of breadth of neutralization [37, 104]. Any next-generation vaccine will be required to show an increase in protection against other high-risk HPV types while still eliciting a strong immune response against HPV 16 and 18 in addition to being cost-effective, safe, and stable. Given that the consensus sequence is a single peptide that can be displayed in a

variety of different vaccine formats and still elicits a cross-neutralizing response, it could be an effective target for next-generation HPV vaccines.

Chapter 3 – Enhancing a next-generation HPV vaccine by using hybrid Virus-like Particles

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Submitted to Biotechnology and Bioengineering, March 2014

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3.1 Abstract

Virus-like particles (VLPs) can serve as a highly-immunogenic vaccine platform for the multivalent display of epitopes from pathogens. We have used bacteriophage VLPs to develop vaccines that target a highly conserved epitope from the Human Papillomavirus (HPV) minor capsid protein, L2. VLPs displaying an L2-peptide from HPV16 elicit antibodies that broadly neutralize infection by HPV types associated with the development of cervical cancer. To broaden the cross-neutralization further, we have developed a strategy to display two different peptides on a single, hybrid VLP in a multivalent, highly immunogenic fashion. In general, hybrid VLPs elicited high-titer antibody responses against both targets, although in one case we observed an immunodominant response against only one of the displayed epitopes. Immunization with hybrid particles elicited antibodies that were able to neutralize heterologous HPV types at higher titers than those elicited by particles displaying one epitope alone, indicating that the hybrid VLP approach may be an effective technique to target epitopes that undergo antigenic variation.

3.2 Introduction

Virus-like Particle (VLP) technology is a promising approach for developing new vaccines. VLPs make attractive vaccines because they are non-infectious and present viral antigens in a dense, ordered manner that leads to efficient activation of B cells, resulting in high-titer and long-lasting antibody responses [82, 147]. VLPs can be used as stand-alone vaccines, but they can also be used as platforms to display practically any antigen in a highly

immunogenic, multivalent format [22, 23]. Linking target antigens, either genetically or chemically, to the surfaces of VLPs causes them to be displayed at high density. This high-density display, in turn, dramatically enhances the ability of linked antigens to induce potent antibody responses.

Chimeric VLPs can be constructed by genetic insertion of a target epitope into a viral structural protein [148]. Unfortunately, generation of recombinant VLPs can be technically challenging because the effects of peptide insertions into viral structural proteins are notoriously difficult to predict and often lead to protein folding failures [6, 82]. As a consequence, the engineering of recombinant VLPs in most systems described to date is a largely empirical process of trial and error. However, we have engineered the structural proteins from two related bacteriophages (MS2 and PP7) so that they are dramatically more tolerant of foreign insertions [34, 35]. These bacteriophages encode a single structural protein, coat protein, which self-assembles into a 27nm-diameter icosahedral particle consisting of 90 coat-protein homodimers. While coat protein monomers of MS2 and PP7 are usually intolerant of genetic insertions, fusing two copies of coat protein into one long reading frame, which is possible because the N-terminus of one monomer lies in close physical proximity to the C-terminus of the other monomer, results in a functional protein that is dramatically more thermodynamically stable, and highly tolerant of short peptide insertions at two display sites (the N-terminus and the so-called AB-loop). Recombinant MS2 and PP7 VLPs created using the single-chain dimer display 90 target peptides on the

surface of each particle and elicit robust epitope-specific antibody responses upon vaccination [89, 149, 150].

Many pathogens have developed strategies to evade immunity by presenting epitopes to the immune system that are antigenically variable, while hiding highly conserved sites that are essential for protein function [151]. One example is Human Papillomavirus (HPV). Over 150 different strains of HPV have been identified and a subset consisting of 14-20 “high-risk” HPV types causes virtually all cases of cervical cancer [42]. VLPs comprised of the HPV major capsid protein, L1, are the basis for the HPV vaccines that are currently available on the market [2, 75]. These vaccines are effective against the two highest risk types, HPV 16 and 18, which account for approximately 70% of cervical cancer cases worldwide [53, 54]. However, antibodies raised against L1 VLPs are largely type-specific, thus the vaccines do not provide protection against other high-risk HPV types. Therefore, there is an impetus to develop more cross-protective HPV vaccines that will provoke immune responses that will protect against more of the high-risk HPV types.

In order to develop a more broadly protective HPV vaccine, we have used a VLP platform approach to target a highly conserved epitope in the HPV minor capsid protein, L2. L2 is essential for the virus life cycle but is normally shielded from immune recognition [93]. Previous studies have shown that vaccination with recombinant L2 elicits immune responses that protect from papillomavirus infection [90-92] and immunization with epitopes derived from the N-terminal region of L2 can elicit antibodies that broadly inhibit infection by diverse HPV

types [104, 146, 152]. In general, the titers of neutralizing antibodies elicited by recombinant L2 vaccination are, unfortunately, lower than those elicited by vaccination with HPV L1 VLPs [136]. Further, while anti-L2 antibodies are more cross-protective than anti-L1 antibodies, the breadth of cross-protection has to be sufficient to protect against most, if not all, of the high-risk HPV types [89, 94]. As one solution, we have developed vaccines in which we immunize with a cocktail of VLPs displaying L2 epitopes from different HPV types [89]. However, there are obvious manufacturing and cost advantages to using a single antigen that can provoke broadly protective responses.

We hypothesized that one method for broadening protection would be to display multiple L2 epitopes on the surface of a single VLP. We designed a plasmid that encodes two open reading frames of bacteriophage coat protein, each displaying a different epitope. This enabled the production of hybrid VLPs that display two different epitopes on the same particle in the same highly immunogenic display context. We hypothesized that these hybrid particles could elicit antibodies that could bind to both the displayed peptides and other similar targets as well. We found that immunization with VLPs displaying L2 epitopes derived from two different high-risk HPV types induced a broader cross-neutralizing response than immunizing with VLPs targeting one epitope. These hybrid particles may be an effective way to broaden the utility of rationally designed, epitope-based vaccines.

3.3 Methods

3.3.1 Construction of expression plasmids

PCR was used to independently insert the peptides representing the L2 amino acids 17-31 from HPV 1, 18, and 16, as well as the FLAG epitope into the AB-loop of the single-chain dimer version of PP7 coat protein (using the expression vector pET2P7K32) as previously described [35, 89]. Similarly, the L2 sequence representing HPV16 and HPV 31 L2 amino acids 17-31 were cloned onto the amino-terminus of a single-chain dimer version of the MS2 coat protein (using the expression vector pDSP62) by PCR as previously described [37]. All constructs were confirmed by sequence analysis.

3.3.2 Construction of the dual expression plasmid

PCR was used to engineer complementary, unique restriction sites bracketing the single-chain dimer expression cassette. As a template for the PCR, we used MS2 or PP7 single-chain dimer expression plasmids containing insertions in the AB loop (PP7) or at the N-terminus (MS2). The plasmid was constructed as shown in Figure 1A. Briefly, the upstream expression cassette was amplified using a forward primer that contained a BglII restriction site and reverse primer that contained a PstI restriction site. The downstream single-chain dimer was amplified similarly, except that the forward primer included a PstI restriction site and the reverse primer contained an EcoRI site. After amplification, the original plasmid was digested with the BclI (leaving an end compatible with a Bgl II cut end) and EcoRI. The dual expression plasmid was then constructed by three-piece ligation and then confirmed by restriction digest analysis.

3.3.3 Expression, purification, and characterization of VLPs

Recombinant VLPs were made by transforming C41 cells (Lucigen) with the PP7 or MS2 expression vectors and VLPs were purified from the soluble fraction as previously described [35]. Intact VLPs were visualized on a 1% agarose gel with ethidium bromide (Invitrogen) and quantified by polyacrylamide gel electrophoresis.

3.3.4 Capture ELISA

ELISA wells were coated with 500 ng/well of RG-1, an anti-L2 antibody that binds the L2 17-31 epitope (provided by Richard Roden) overnight at 4°C [94]. The wells were then blocked with 0.5% milk in PBS for one hour. Purified recombinant PP7 VLPs were added at 5 µg, 1 µg, or 0.5 µg/well for 2 hours at room temperature. The wells were then probed with a biotinylated anti-FLAG M2 monoclonal antibody (Sigma) diluted 1:2000 for 1 hour at room temperature. Finally, a HRP conjugated streptavidin (Life Technologies) was added, which was diluted to 1:4000 for one hour. ABTS was added as the developer and reactivity was determined by measuring the mean optical density (OD) values at 405 nm.

3.3.5 Immunizations and characterization of antibody responses

All animal work was done in accordance with National Institutes of Health and University of New Mexico guidelines. Groups of Balb/c mice were immunized twice intramuscularly (i.m.) at a two-week interval with 5 µg of PP7-L2 (displaying L2 amino acids 17-31 from HPV18, 16, 1, 18/1 or 18/16), or MS2-L2 VLPs 16L2 (displaying L2 amino acids 17-31 from HPV16, 31, or 16/31), or, as negative controls, unmodified MS2 and PP7 VLPs. Vaccine was prepared with incomplete

Freund's adjuvant (IFA). Two weeks after the second immunization, sera were collected and anti-L2 IgG titers were determined by peptide-based ELISA using disulfide-constrained L2 peptides representing amino acids 14-40 from HPV1, 5, 6, 16, and 18 (American Peptide company, as described [89])

3.3.6 HPV pseudovirus (PsV) production and purification

HPV6, 16, 18, 31, 45, and 58 PsVs with encapsidated reporter plasmid (pClucf) encoding both luciferase and green fluorescence protein (GFP) genes were produced in 293TT cells as previously described [89, 153, 154]. PsV-infectivity titer was characterized using flow cytometry by determining the fraction of 293TT cells expressing the GFP protein.

3.3.7 Cervicovaginal HPV PsV challenge

Prior to challenge, female Balb/c mice were given 3 i.m. immunizations of 5 µg of control VLPs or VLPs displaying one of the L2 epitopes. Two weeks after the last boost, mice were treated with 3 mg of Depo-Provera subcutaneously (Pharmacia Corp). Five days post-Depo-Provera treatment, mice were vaginally challenged with 1.0×10^5 infectious units (IU) of the PsV stock as previously described [79, 137]. Forty-eight hours post-PsV challenge, mice were vaginally instilled with 0.4 mg of luciferin (Caliper Life Sciences) and imaged with a Caliper IVIS Lumina II (Caliper Life Sciences) as described previously [89].

3.3.8 In vitro L2 neutralization assay

These assays were performed as described in [113] except that heparin was not added to PsV solutions prior to infection. Following a two-day incubation, the cells were then collected and analyzed by flow cytometry using a

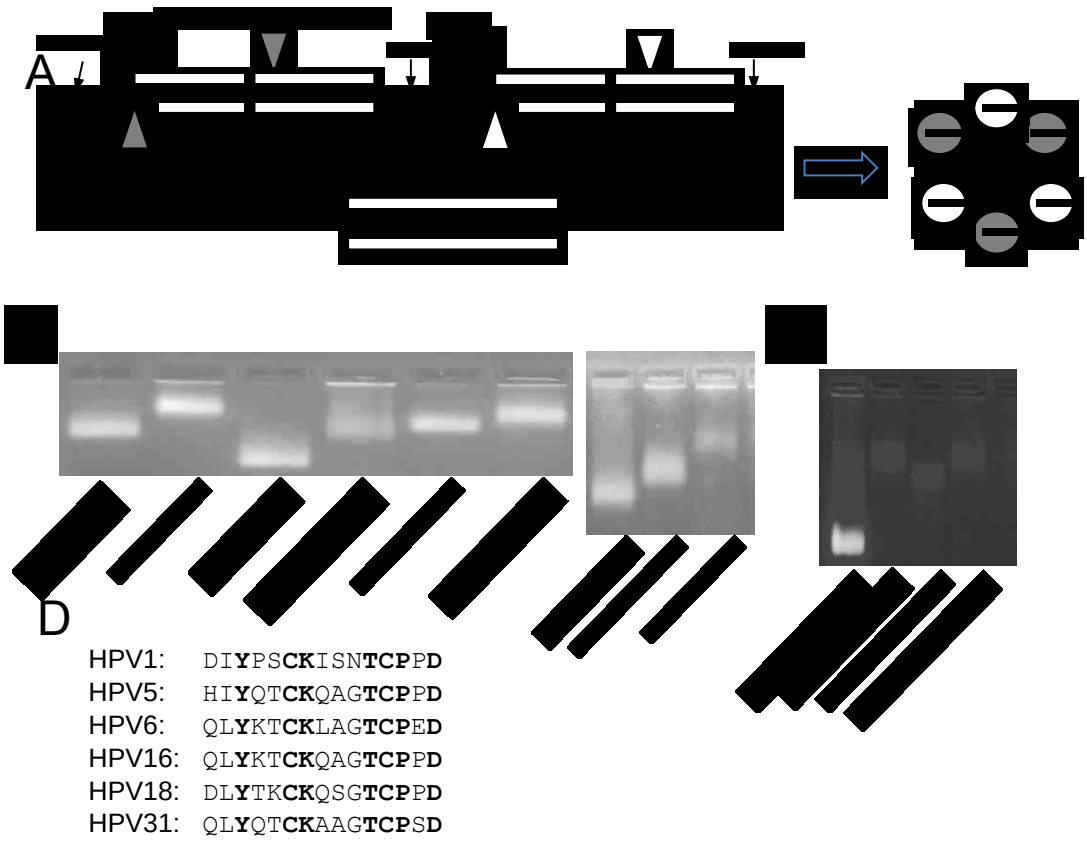


Figure 3.1 - Design and characterization of hybrid bacteriophage VLPs. (a) Design of the hybrid VLP expression plasmid. Peptide targets can be displayed at either the N-terminus or the AB-loop of the coat protein single-chain dimer. Each expression cassette is engineered separately, amplified by PCR, and then plasmids are assembled by three-piece ligation using the restriction sites listed. PP7 (b) or MS2 (c) VLPs were analyzed using a 1% agarose, non-denaturing gel stained with ethidium bromide (which binds to the genomic material encapsidated by the VLPs). The mobility of the bands were compared to VLPs of unmodified PP7 or MS2 coat protein. (d) An alignment of selected HPV sequences representing L2 aa 17-31 (or the equivalent). Conserved amino acids are indicated in bold text.

Hypercyte autosampler to detect GFP expression as a marker of infection. The dose of PsV used was based on the amount needed to yield 20-40% infection of control pgsa-745 cells. HPV18 PsV stocks were generally poorly infectious, so cells were infected with amounts of HPV18 PsV resulting in ~10% of control cells being infected.

3.4 Results

3.4.1 Production and characterization of hybrid VLPs

In order to produce hybrid VLPs, we designed a plasmid that contains two identical expression cassettes, each containing a T7 promoter, an open reading frame encoding the single-chain dimer version of either PP7 or MS2, and a transcriptional terminator (Figure 1A). As a preliminary test of the ability of the PP7 version of this plasmid to produce hybrid VLPs, we engineered one of the coat proteins to display a sequence derived from HPV18 L2 (aa17-31) and the other to display the FLAG epitope. Upon expression, VLPs were purified and then characterized by agarose gel electrophoresis. Because VLPs migrate through the gel due to their overall electrophoretic charge and can be visualized using ethidium bromide by virtue of the RNA that is encapsidated by the particles, this assay can be used to measure charge differences that are conferred by the epitopes that are displayed on the surface of the VLPs. As predicted, the L2/FLAG hybrid particles show a mobility that falls between VLPs that display either the L2 or FLAG peptide alone, suggesting that both peptides are displayed on the surface of the VLPs (Figure 1B). To confirm this, we performed a sandwich ELISA, in which an anti-L2 monoclonal antibody was used

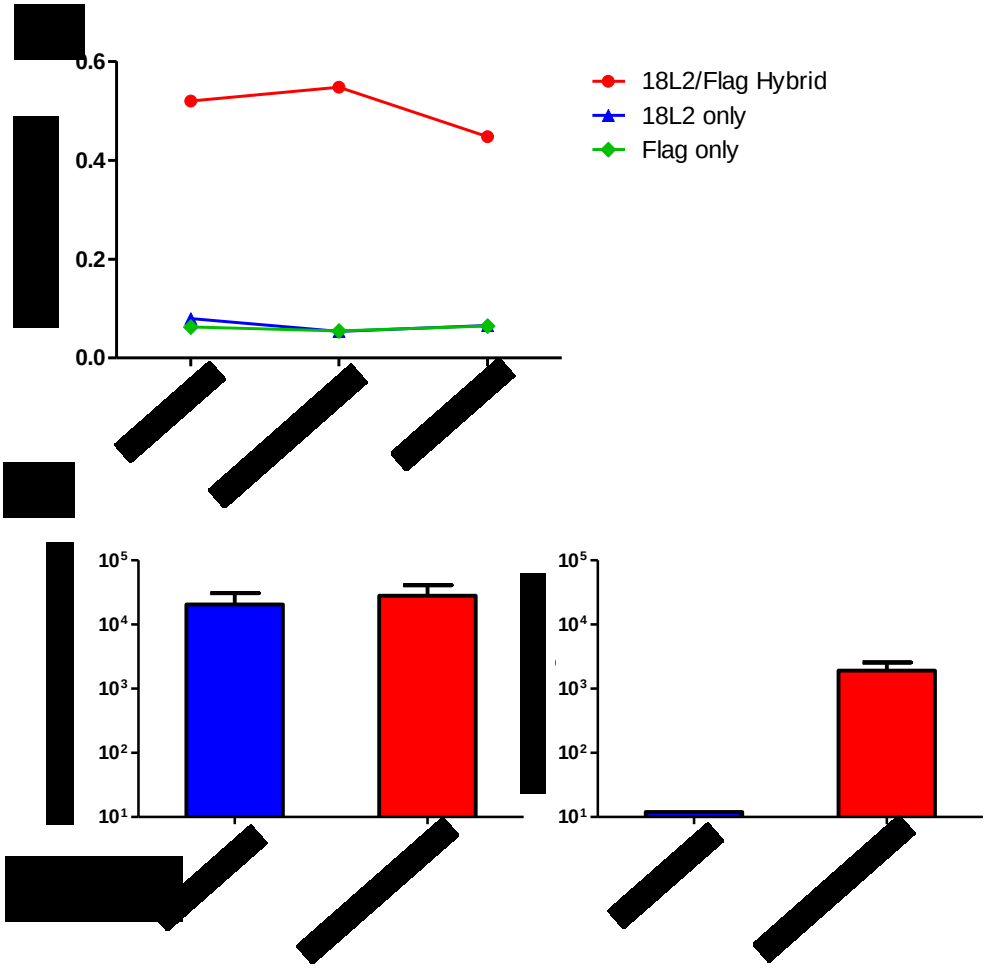


Figure 3.2 - Characterization of PP7-18L2/FLAG hybrid VLPs. (a) Hybrid VLPs were characterized via capture ELISA. Different amounts of VLPs were added to wells coated with RG-1, a monoclonal antibody that binds to the aa 17-31 region of L2, and then probed with a biotinylated anti-FLAG antibody followed by HRP-labeled streptavidin. Data points indicate the mean absorption of each well at 405 nm. (b) Mice (three per group) were immunized with 5 µg of indicated VLPs twice with two week intervals and sera were collected 2 weeks after the last immunization. Serum anti-L2 peptide or anti-FLAG IgG titers were determined by end-point dilution ELISA using synthetic peptides. Titers indicate the reciprocal of the lowest dilution of serum samples at which reactivity with the immobilized peptide was at least twice that of background. Bars indicated the geometric mean of the group, with error bars indicating standard deviation.

to capture the VLPs, and an anti-FLAG antibody was used as a probe, allowing us to only detect particles that display both peptides on their surfaces. Hybrid HPV/FLAG particles were detected readily using this assay, whereas VLPs displaying either the FLAG epitope or the L2 peptide alone were not, indicating that hybrid particles display both epitopes on their surfaces (Figure 2A).

To assess the immunogenicity of the FLAG/L2 hybrid particles, we immunized mice and then measured the IgG antibody responses against the L2 and FLAG peptides by ELISA (Figure 2B). Mice immunized with the hybrid particles made high-titer antibodies that bound to both target peptides. In contrast, sera from mice immunized with VLPs displaying only HPV18 L2 peptide only reacted with the 18L2 peptide. Taken together, these data indicate that our dual expression plasmid produces hybrid VLPs displaying both peptides on the surface and can elicit an antibody response against both targets.

3.4.2 Hybrid VLPs can elicit more broadly reactive antibodies against HPV L2

Previous studies in our lab showed that immunization with PP7 VLPs displaying an HPV16 L2 sequence (aa17-31) elicited antibodies that were only modestly cross-reactive with L2 sequences from other HPV types [89]. To determine if hybrid particles could induce more broadly cross-reactive antibody responses, we produced two hybrid PP7 VLPs that displayed L2 epitopes from two HPV types (HPV18/1 and HPV18/16 VLPs, respectively). The HPV16 and 18 L2 sequences are closely related (13 of the 15 amino acids are identical), whereas the HPV1 and 18 L2 peptides are less so (10 of the 15 amino acids are

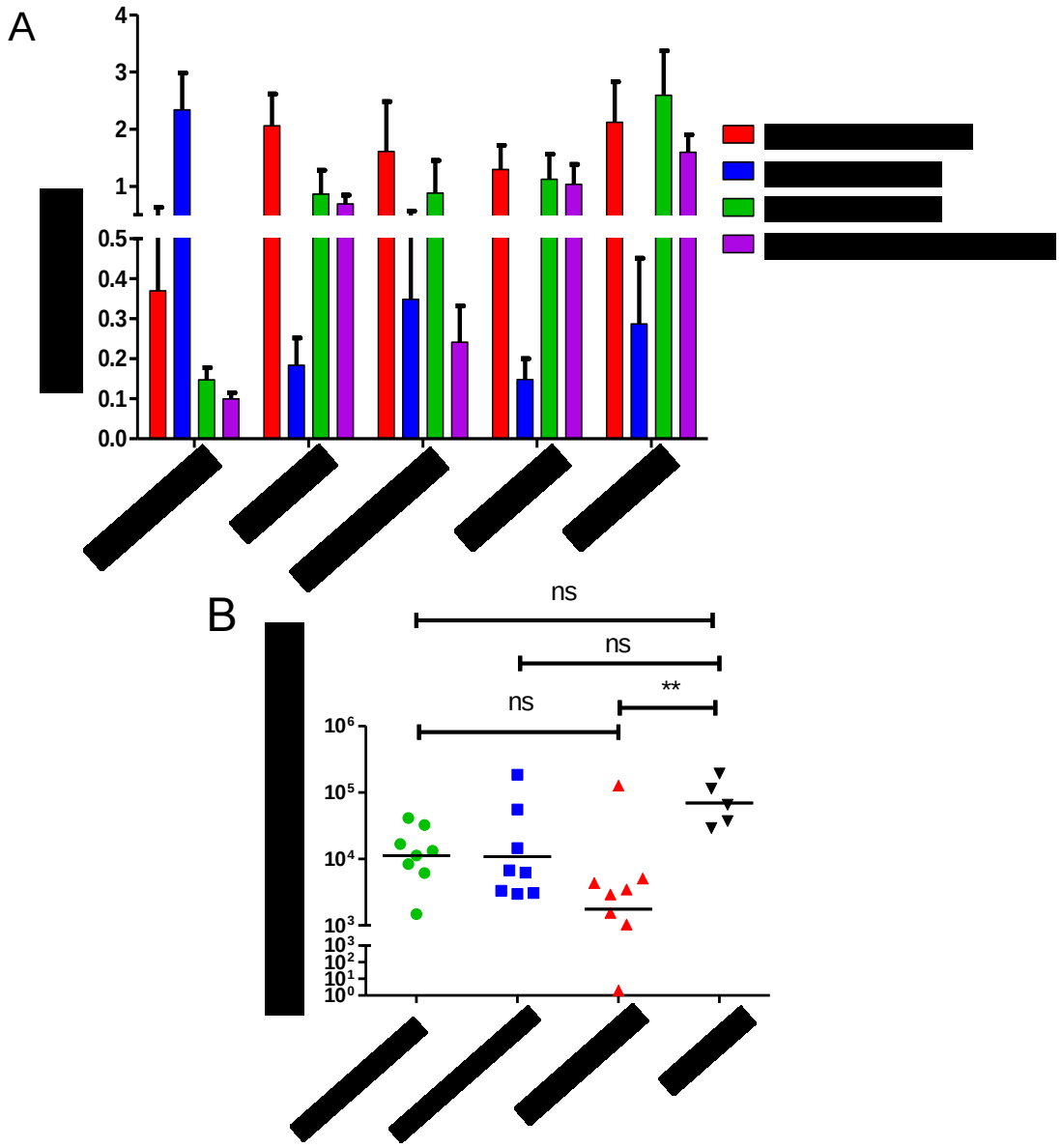


Figure 3.3 - Immunogenicity and in vivo neutralizing activity of hybrid VLPs. (a) Groups of eight mice were immunized three times with 5 µg of PP7 VLPs displaying the L2 aa 17-31 peptide from HPV18 alone, HPV18/1 or HPV18/16. Sera collected two weeks after the last immunization was tested for IgG binding to a selection of peptides representing amino acid 14-40 from five HPV types. Sera was diluted at 1:160. Bars indicate group means with standard deviations. (b) Mice immunized with the indicated VLPs were vaginally challenged with 10^5 IU of HPV6 PsV encapsidating a luciferase reporter plasmid. Two days after PsV challenge, the mice were vaginally instilled with luciferin and imaged for luciferase luminescence. Each data point

represents the average radiance for the region of interest (ROI; genital tract) of individual mice; lines representing the geometric mean of each group. The average radiance (p/s/cm^2/sr) was calculated by using Living Image 3.2 software. Data were analyzed by a Kruskal-Wallis test with Dunn's multiple Comparisons Test; ** indicates $p \leq 0.01$, ns, not significant.

the same; Figure 1D). Analysis of the electrophoretic mobility of the L2 hybrid VLPs on an agarose gel suggested that the hybrid VLPs incorporated both L2 peptides (Figure 1B). Mice were immunized with either PP7-18L2 VLPs or the hybrid VLPs and then antibody binding to peptides representing the L2 sequence from five diverse HPV types was measured by ELISA (Figure 3A). In agreement with our previous results, the PP7-18L2 anti-serum had strong reactivity to HPV18 L2, moderate reactivity with L2 peptides from HPV5, 6, and 16, and including little to no reactivity with the peptide derived from HPV1, which is the most evolutionarily distant type that we tested [89]. Unexpectedly, immunization with the PP7-18/1L2 hybrid VLP elicited antisera that only reacted with the HPV1 peptide. There was little reactivity with the other four peptides, including the HPV18 peptide that was included in the VLP, suggesting that the HPV1 L2 peptide was immunodominant. In stark contrast, the PP7-18/16L2 hybrid VLPs elicited antibodies that bound strongly to both the 16L2 and 18L2 peptides, as well as the other three heterologous L2 peptides that we tested, including HPV1. As a comparison, we also immunized mice with a mixture of VLPs displaying the HPV16 L2 sequence and the 18L2 sequence alone. The hybrid 16/18L2 VLPs elicited higher levels of cross-reactive antibodies than the mixture of VLPs, and only the hybrid VLPs were able to elicit antibody responses that reacted with the HPV1 peptide. These data indicate that more broadly cross-reactive antibodies were elicited by hybrid VLPs than by simply mixing two L2-VLPs together.

To assess whether hybrid VLPs were capable of providing protection *in vivo* from challenge with a heterologous HPV type, vaccinated mice were

vaginally challenged with a heterologous HPV pseudovirus (HPV6 PsV) encapsidating a luciferase reporter (Figure 3B). The mice immunized with the PP7-18/16L2 hybrid VLPs were the only group that showed a significant reduction (97.5%) in the geometric mean luciferase signal compared to control mice vaccinated with wild-type PP7 VLPs.

3.4.3 Hybrid VLPs can enhance the cross-neutralizing potential of an already potent L2 immunogen

Our lab previously has shown that VLPs that display HPV16 L2 aa17-31 in an unconstrained fashion at the N-terminus of coat protein can elicit broadly cross-reactive antibodies that can provide significant in vivo cross-protection from a panel of eleven diverse HPV PsV types [37]. The one outlier was HPV31, which was not as strongly cross-neutralized as other HPV types. The sequence of the HPV16 and 31 L2 epitope only differs by two amino acids, so this result was somewhat surprising. We hypothesized co-display of HPV16 and HPV31 L2 peptides on a hybrid VLP would enhance the already robust cross-protection. To test this, we produced hybrid 16/31 MS2 VLPs (Figure 1C) and compared the antibody responses in vaccinated mice with mice immunized with either MS2-16L2 or MS2-31L2 VLPs alone, or mice immunized with a mixture of MS2-16L2 and MS2-31L2 VLPs. As shown in Figure 4, immunization with MS2-16L2 VLPs alone elicited antibody responses with considerable cross-reactivity to a panel of L2 peptides, whereas MS2-31L2 VLPs elicited antibodies with weaker cross-reactivity. Sera from mice immunized with MS2-16/31L2 hybrid VLPs reacted strongly to all the peptides tested, including HPV1 L2. Thus, co-display of the

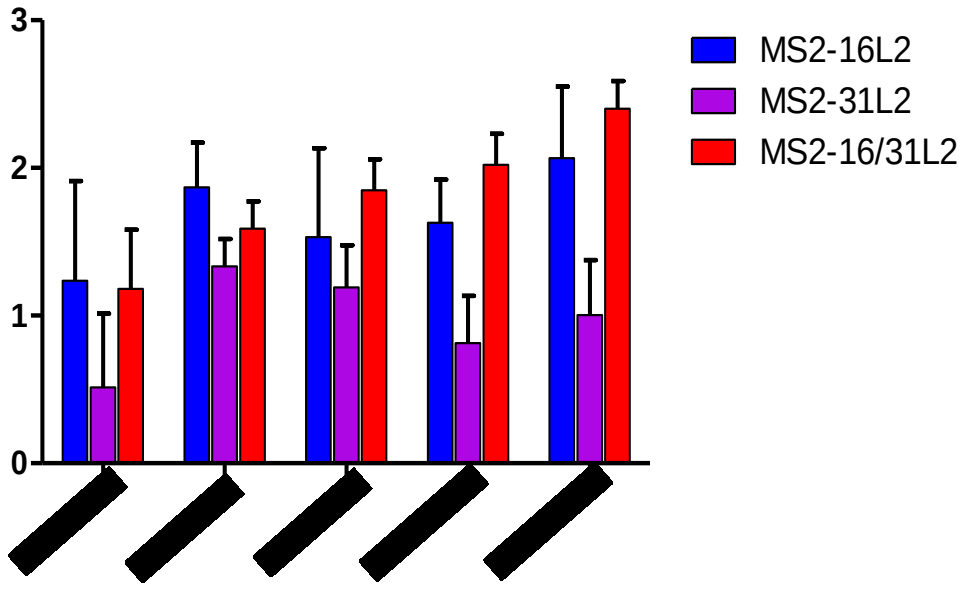


Figure 3.4 - Sera from mice immunized with MS2 displaying N-terminal L2 peptides bind to heterologous HPV peptides. Mice (five per group) were immunized three times and the resulting sera were diluted 1:160 and analyzed by ELISA as in Figure 3.3A. Bars indicate group means and error bars indicate standard deviations.

HPV31 peptide on the hybrid VLPs does not detract from the broad cross-reactivity that we had previously observed.

To measure the neutralizing activity of antisera, we utilized a new L2 neutralization assay developed by Day *et al* that is tailored to measure the neutralization titers developed in response to anti-L2 vaccines (Figure 5) [113]. Sera from mice immunized with MS2-16L2 VLPs neutralized all five of the high-risk HPV PsV types that were tested, but the lowest neutralization titer was against HPV31 [in agreement with the in vivo data that we had previously generated [37]]. Serum from mice immunized with MS2-31L2 showed a distinct pattern of neutralization; this serum strongly neutralized the homologous HPV31 PsV, but neutralized the other HPV PsV types at lower titers. Importantly, serum from mice immunized with the either the hybrid VLPs or mixed VLPs neutralized all of the HPV PsV types at high titers. Thus, immunization with hybrid L2-VLPs can enhance the breadth of HPV neutralization without sacrificing the ability to neutralize individual HPV types.

3.5 Discussion

The strong antibody responses elicited by peptide epitopes displayed on VLPs results from the dense, repetitive manner in which the antigen is displayed to B cells [9]. This multivalent display allows for increased cross-linking of B cell receptors, strong B cell activation, and enhanced antibody production. By displaying two related HPV-derived peptides in highly immunogenic context on the surface of a single hybrid VLP, we hoped to take advantage of these avidity effects to activate B cells that could produce antibodies that reacted with a broad

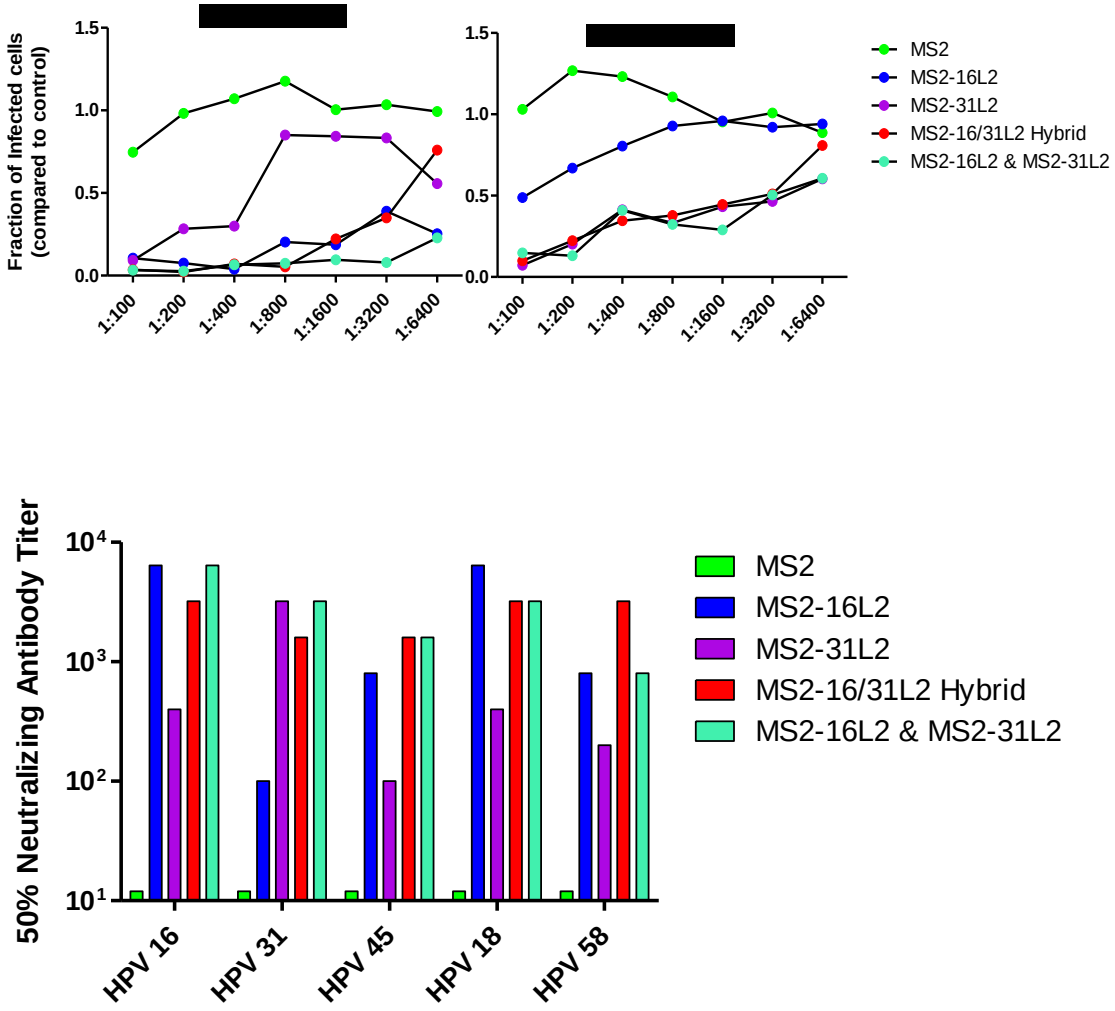


Figure 3.5 - In vitro neutralization of HPV PsV by hybrid MS2 VLP immune sera. (a) Sera from groups of 5 mice immunized with the indicated VLPs were pooled and tested for neutralization against (Left) HPV16 PsV at ID_{40} (Infectious Dose of PsV that results in 40% of control cells being infected) and (Right) HPV31 PsV at ID_{20} at the indicated dilutions. HPV PsV were incubated on deposited ECM in 96-well plates and treated with furin-conditioned media. HPV PsV was then incubated with pooled dilutions of sera for 6 hours, after which pgsa-745 cells were added. Infected cells were measured by GFP expression, quantified using a Hypercyt autosampling flow cytometer. Data points indicate the relative amount of infected cells in sera treated wells compared to wells with no sera added. (b) Pooled sera from mice immunized with the indicated VLPs was tested for neutralization against five high-risk HPV PsV types. HPV45 and 58 PsV were tested at ID_{20}. HPV18 PsV was added at ID_{10} due to low infectivity. The bars represent the highest titer at which the PsV was neutralized greater than 50% compared to control (no sera) wells.

spectrum of HPV L2-derived peptides and had enhanced neutralizing activity against diverse HPV types [13]. We anticipated three possible outcomes at the onset of this study: (1) hybrid VLPs would elicit an immunodominant response against only one of the two epitopes, (2) the VLPs would elicit antibodies that against both epitopes, but the response would not be any different from immunizing with a mixture VLPs displaying each peptide separately, or (3) the VLPs would elicit a broadly-reactive response that would recognize other similar epitopes as well. Our results indicated that all three of these outcomes are possible depending on which peptides are being displayed on the hybrid VLPs.

Immunization with hybrid PP7 VLPs displaying 18/1 L2 elicited high titer antibody responses against HPV1 L2, but largely failed to elicit antibodies that bound to four other L2 peptides, including the HPV18 peptide which was displayed on the VLPs. There are several possible explanations for this result. First, it is possible that these hybrid VLPs preferentially incorporated the HPV1 L2-displaying coat protein. Although we cannot absolutely rule out this possibility, analysis of the mobility of the 18/1 hybrid VLP on an agarose gel showed that the hybrid VLPs displayed an electrophoretic mobility that was midway in between VLPs displaying only the HPV1 or HPV18 L2 peptides, suggesting that both peptides are displayed on the VLPs (Fig 1B right). Second, it is possible that the HPV1 L2 peptide is much more immunogenic than the HPV18 peptide. Previous studies of the immune response to this region of HPV L2 being displayed on PP7 VLPs did not show that PP7-1L2 VLPs elicit higher antibody titers than PP7 VLPs displaying other L2 sequences [89].

Moreover, co-immunization with a mixture of eight L2-displaying PP7 VLPs elicited balanced responses against our panel of L2 peptides [89]. Third, it is possible that Balb/c mice preferentially responded to the HPV1 peptide due to an increased frequency of precursor B cells specific for the unique elements in the HPV1 L2 peptide (i.e. immunodominance). Interestingly, when we immunized a different strain of mice (C57BL/*6)* with hybrid 18/1L2 VLPs we also observed immunodominance of the HPV1 L2 peptide (data not shown). Thus, these data indicate that epitope immunodominance is a potential consequence when immunizing with hybrid particles. This potential consequence will need to be carefully evaluated when considering the use of hybrid antigens.

When two highly related L2 peptides were displayed on the same hybrid VLPs, more broadly reactive antibody responses were generated. Both the PP7-18/16L2 and MS2-16/31L2 hybrid particles elicited more cross-reactive IgG responses when compared to that elicited by VLPs displaying only one of the targets. Hybrid VLPs also elicited more broadly neutralizing antibodies than when we simply immunized with mixtures of VLPs, indicating that there are distinct B cell responses to the hybrid particles. In a recent study Nieto and colleagues displayed two L2 peptides (from HPV16 and 31) at two separate display sites on adeno-associated VLPs (AAVLPs) [108]. These VLPs elicited strongly neutralizing antibodies, but antibody responses against one of the peptides (HPV31) was somewhat weaker, suggesting that one of the display sites on the AAVLP was less exposed to the immune system. One of the advantages to the approach that we describe is that both targeted peptides are displayed in the

same highly immunogenic structural context and spatial arrangement that we think is critical for induction of high-titer strongly neutralizing antibodies against HPV [37].

In this study we targeted a single vulnerable neutralizing epitope from HPV that shows a limited degree of sequence heterogeneity. Although our study focused on HPV, there are many pathogens that frustrate vaccination efforts due to antigenic variation and could potentially be targeted using the hybrid VLP approach. Similarly, we have shown that hybrid VLPs can also elicit strong antibody responses against two unrelated epitopes (i.e 18L2 and FLAG). This feature may be useful for targeting pathogens where more polyclonal antibody responses are required. In the context of displaying unrelated peptide epitopes, we think that it is unlikely that hybrid particles will elicit qualitatively distinct antibody responses than co-immunization with two VLPs. However, there are certain manufacturing advantages to using a single hybrid VLP as opposed to a mixture of individual VLPs. Taken together, the use of hybrid VLPs expands the capabilities of an already useful platform for vaccine design.

Chapter 4: Display of molecular adjuvants on the surface of VLPs

4.1 Introduction

Virus-like particles are a promising platform for novel vaccines in large part due to their high immunogenicity. As discussed above, VLPs have a variety of characteristics that synergistically work to induce high antibody titers. Foremost among these is the dense repetitive array in which they present their epitopes; either components of the capsid proteins that make up the VLP or the heterologous epitopes displayed on the surface. The combination of potent immunogenicity and safety, as VLPs do not contain viral genomic material, makes them doubly attractive as vaccines.

However effective VLPs are, there is always room for improvement. In particular, uptake of VLP based vaccines, such as the current HPV vaccines, may be improved in developing countries if there was no cold chain requirement or if the dosage or number of boosts could be lowered. While there are studies examining the efficacy of the HPV vaccines after only one dose, three total shots are still the recommended dosage. Reducing the number of return trips to the doctor by increasing the immune response to a single vaccination could aid efforts in inoculating populations.

In order to increase the effectiveness of vaccination and address these issues, vaccines often include adjuvants [115]. Adjuvants are substances added to vaccines to potentiate the immune response. Often these substances act to pool the antigen and slow its release after injection, thereby increasing the time

of antigen stimulation. Adjuvants can also facilitate the uptake and presentation of antigen by Antigen Presenting Cells (APCs). Other vaccines include microbe components that are recognized by the immune system as Pathogen Associated Molecular Patterns (PAMPs). The binding of the receptors to PAMPs leads to increased inflammation and stimulation of the adaptive immune system. Studies have also shown that adjuvants can modulate T cell responses, speed the reaction to pathogens, and broaden the cross-neutralization of elicited antibodies [155, 156]. Adjuvants often are essential components of an effective vaccine.

4.1.1 p28: The activating region of complement protein C3d

Similarly, the body has endogenous mechanisms to speed up and bolster the immune response. One of these mechanisms is the complement system [116]. Complement plays an important role in the clearance of microbes in part through opsonization, direct lysing of pathogens, and increased inflammation. A small aspect of the complement response to pathogens involves C3d, a downstream product of the cleavage of other complement proteins. All three of the complement activation pathways result in the cleavage of the protein C3, either through the activity of the assembled C3 convertase in the classical and lectin pathways or by spontaneous cleavage in the alternative pathway. This leaves the C3b fragment that can bind hydroxyl and amine groups on the surfaces of pathogens through an exposed thioester group [157]. While C3b can opsonize pathogens, making them easier to be taken up by macrophages and other APCs, it can also be further acted upon by complement Factor I, cleaving C3b and leaving the smaller portion, C3d, still bound to the surface of the

pathogen. C3d is the ligand for the receptor Complement Receptor 2 (CR2), which is found on B cells and Follicular Dendritic Cells (FDCs) [119]. When a B cells binds to its cognate antigen on a pathogen CR2 can also bind to embedded C3d and cross-link with the BCR. This interaction lowers the B cells activation threshold, reducing the required amount of BCR binding and increasing antibody production [118, 121, 158].

Germinal center FDCs are a key participant in the process of B cell affinity maturation. FDCs express Fc receptors and CR2 and are able to capture and present antibody or C3d-bound antigen to B cells [117, 119]. CR2 binding can retain the antigen within the germinal center as well as increase the stimulatory signals that the binding B cells receive in order to survive and become memory cells. Further, C3d coated antigens will stay in the germinal centers longer, prolonging the affinity maturation process.

As C3d is a natural adjuvant, there is interest in incorporating it into vaccines [159]. Previous studies have found that the use of tandem repeats of p28, the 28 amino acid minimum CR2-binding region of C3d, in DNA vaccines expressing recombinant antigen can lead to the increased production of antibodies specific for the fused epitope [120, 123-125]. Thus, we hypothesized that p28 could potential enhance the immunogenicity of VLP-based vaccines as well.

4.1.2 Flagellin, the ligand of Toll-like Receptor 5

Another family of immune receptors is called Toll-like receptors (TLRs) [126, 160]. These receptors are found on a variety of cells including cells of the

innate and adaptive immune systems. Some are found at the plasma membrane and bind extracellular ligands while others are located within endosomes and interact with phagocytosed microbes or antigen. TLRs bind PAMPs; structures that are common to invading microbes. PAMPs include single-stranded and double-stranded RNA, lipopolysaccharides, unmethylated single-stranded DNA, yeast cell wall components and others. Activation of any of the TLRs typically leads to the induction of pro-inflammatory cytokines, and the recruitment and activation of various immune cells.

TLR5 is found on epithelial cells and a number of different immune cells, particularly $CD11c^+$ cells (such as dendritic cells, macrophages, and monocytes) and T cells but not on B cells [129]. It is located at the plasma membrane and recognizes flagellin, the monomer component of flagella. TLR5 binding sets off an immune-activating signaling cascade through the IL-1R pathway resulting in NF-κB activation. In epithelial cells, flagellin binding results in the production of cytokines that recruit immune cells to the area [128]. The response of dendritic cells (DCs) is even more potent [161]. In general, TLR binding activates DCs, increasing the expression of co-stimulatory molecules their surface and stimulating the production of cytokines such as IFN-γ. Similar effects have been shown in T cells [130, 162]. While flagellin can indirectly lead to increased stimulation of T cells through the activation of APCs like dendritic cells, it can also act directly on T cells, leading to increased IFN-γ production and cell proliferation. Flagellin and TLR5 binding has also been shown to promote the immunosuppressive T_{reg} cells, possibly as a self-regulating mechanism of the

immune system [163]. Although flagellin does not directly act on B cells, it does stimulate various cells involved in the efficient activation of B cells. Thus a relatively small dose of flagellin leads to a large increase in antibody production [164].

Flagellin has been explored as an adjuvant in various vaccine systems, either fused to antigen or having antigen genetically inserted into flagellin itself [132, 165]. For example, linkage of a membrane-bound form of flagellin to influenza VLPs found that membrane bound flagellin increased the antibodies responses [133]. Indeed, clinical trials of vaccines that include flagellin as an adjuvant are ongoing. Bacteriophage VLPs, that activate B cells so strongly, may benefit from the activation of dendritic cells and T cells that flagellin can provide.

We hypothesized that display of flagellin or p28 on VLPs could enhance immunogenicity and could perhaps lead to vaccine dose-sparing effects. Using a genetic approach, we displayed p28 and flagellin at the C-terminus of MS2 coat protein. This was accomplished by using an amber stop codon, which allows for limited read-through of the stop codon in the presence of special tRNAs that are co-transfected into *E. coli* with the expression plasmid. Using a low-efficiency tRNA that allows 1-2% read-through of the amber stop codon, we were able to purify MS2 VLPs displaying one or two copies of p28 or flagellin. Further, we were able to display a target from HPV16 on the p28-displaying VLPs through either genetic insertion or conjugation. We found that co-display of p28 did not enhance the antibody response against this epitope.

4.2 Methods and Materials

4.2.1 Construction of p28 peptide by assembly PCR

The mouse analog of the identified CR2 binding site, p28, was taken from the GenBank file accession number ABD66220. A single copy of p28 or a two copies in tandem connected with three glycines were constructed by assembly PCR. In short, two overlapping forward and reverse primers (four forward and reverse for p28-double) spanning the entire peptide and including a Pst1 site at the 5' end and a stop codon and BamH1 site at the 3' end were constructed by Eurofins MWG Operon (Huntsville, AL). The first round of PCR contained equal amounts of all primers and progressed for 8 cycles. The PCR product was then used as the template for the second round of PCR with only the 5' and 3' primers were used for 25 cycles. This PCR product was then run through a 1% agarose gel and extracted.

4.2.2 Construction of C-terminal display plasmids

The plasmid pDSP62(am) contains an open reading frame with two copies of MS2 coat protein genetically fused as a single-chain dimer with an amber stop codon (TAG), Pst1 site, and BamH1 site sequentially downstream. A plasmid (pUC57-Kan) encoding *Salmonella fliC* (one of three flagellin genes, GenBank accession number NP_460912) flanked by the Pst1 site and four glycines at the 5' end and BamH1 at the 3' end was synthesized by GenScript (Piscataway, NJ). pDSP62am, pUC57-Kan, pDSP62am and the p28 constructs were digested with Pst1 and BamH1 and gel purified. The cut *fliC* and p28 constructs (single or double) were then ligated into the cut pDSP62am

individually. Correct ligation was confirmed by sequencing. HPV16 aa17-31 was then ligated into the N-terminus as previously described [37]. Correct ligation was again confirmed by restriction digest analysis and sequencing.

4.2.3 Expression and purification of VLPs

Recombinant VLPs were made by transforming C41 cells (Lucigen) that are stably transfected with pNMsupA with the MS2 expression vectors. pNMsupA uses the replication origin and chloramphenicol resistance of pACYC18422, and the lac promoter of pUC19 to express an alanine-inserting amber suppressing tRNA. VLPs were purified from the soluble fraction as previously described [35] except that chloramphenicol was added (25 mg/ml) during the selection process. Intact VLPs were visualized on a 1% agarose gel with ethidium bromide (Invitrogen) and quantified by polyacrylamide gel electrophoresis. LPS was removed by incubation with Triton X-114 (Sigma-Aldrich) [19]. Triton X-114 was added at 1% of the total volume of VLP solution, then incubated at 4°C for five minutes. The solution was then incubated for 5 minutes and centrifuged at 16,000x*g* at 37°. The supernatant was then transferred to an endotoxin-free collection tube (MO BIO Laboratories, Inc., Carlsbad, CA). These steps were then repeated.

4.2.4 Conjugation of HPV16 L2 65-85 peptide to p28 displaying MS2 VLPs

A peptide representing HPV16 L2 amino acids 65-85 was synthesized by American Peptide (Sunnyvale, CA) to include a C-terminal cysteine residue, to allow for chemical crosslinking to VLPs. Purified MS2 VLPs with or without C-terminal p28 inserts were conjugated with this peptide as previously described

[31]. In short, MS2 VLPs were incubated with SMPH (Thermo Scientific) at a single-chain dimer to SMPH molar ration of 10:1 for two hours at room temperature. Excess linker was removed using a centrifugal filter unit (100 KDa cut-off, Millipore) and VLPs resuspended in PBS. The VLPs were then incubated with the HPV peptide at a 10:1 molar ratio overnight at 4°C. Excess peptide was removed by centrifugal filtration. Conjugation efficiency was visualized by polyacrylamide gel electrophoresis.

4.2.5 Characterization of p28 VLPs by ELISA

ELISA wells were coated with 500 ng of MS2 VLPs displaying C-terminal insertion of p28_single, p28_double (MS2-p28_single or MS2-p28_double) or wild-type MS2 VLPs overnight at 4°C. 5 µl of mouse sera, containing mouse complement proteins, in 45 µl of PBS was used as a positive control. Wells were blocked with 0.5% milk in PBS for one hour. Next, the wells were probed for p28 expression with a goat anti-mouse C3d antibody (R&D Systems, Inc., Minneapolis, MN) at dilutions of 1:250, 1:1000, and 1:4000 for 1.5 hours. MS2 VLPs that also displayed HPV16L2 17-31 at the N-terminus were probed similarly with an anti-L2 antibody, RG-1, provided by Richard Roden [94]. A HRP-conjugated donkey anti-goat IgG antibody (R&D Systems, Inc., Minneapolis, MN) was added to the C3d wells at 1:1000 dilution, and a HRP-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA) was added to the L2 wells at 1:2000 dilution. After addition of the substrate ABTS the mean optical density (OD) values at 405 nm were determined.

4.2.6 Capture ELISA of MS2-fliC-L2 VLPs

ELISA wells were coated with 500 ng of the anti-L2 antibody, RG-1 (described above) overnight at 4°. Wells were then blocked with 0.5% milk in PBS for one hour. 5 µg, 1 µg, or 500 ng of LPS-removed VLPs were added to the wells for 2 hours. An anti-flagellin antibody (Abcam Inc, Cambridge, MA) was added to each well at a dilution of 1:4000 for 2 hours. Binding of the anti-flagellin antibody was probed with a HRP-conjugated, goat anti-rabbit antibody (Jackson Immunoresearch, West Grove) at 1:2000 dilution. ABTS was added as the developer and reactivity was determined by measuring the mean optical density (OD) values at 405 nm.

For the TLR5 capture ELISA, wells were coated overnight with 250 ng of soluble, chimeric TLR5 (RD Systems), then blocked with 0.5% milk. A 1:50 dilution of MS2-fliC VLPs or wt MS2 VLPs was added. Wells were then incubated with a rabbit anti-MS2 antibody (1:2000, 1 hour) followed by HRP goat anti-rabbit secondary antibody (1:10,000, 1 hour, Jackson Laboratory). Bound VLP/antibody complexes were detected using ABTS solution (Calbiochem) and the absorbance was read at 405 nm 1 hour later.

4.2.7 Western Blot of MS2-fliC VLPs

VLP solutions were first separated by gel electrophoresis. Proteins were transferred from the electrophoresis gel for one hour at 25 V. The membranes were then blocked in 5% milk. The anti-L2, RG-1, and anti-flagellin (Abcam) antibodies were added at 1:15000 dilutions, with secondary goat anti-mouse and goat anti-rabbit antibodies added at 1:20000 (Jackson Immunoresearch). The

membrane was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and detected with Blue Basic autoradiography film (BioExpress, Kaysville, UT).

4.2.8 Immunizations and characterization of antibody responses

All animal work was done in accordance with National Institutes of Health and University of New Mexico guidelines. Groups of Balb/c mice were immunized with MS2 VLPs. Mice were immunized with MS2 displaying HPV16 L2 17-31 at the N-terminus alone or with either p28_single or p28_double at the C-terminus. The next set of mice was immunized with MS2 conjugated with HPV16 65-85 alone or with either p28_single or p28_double at the C-terminus. Both sets of mice were immunized with 2 week intervals with sera collected one week after each immunization. The mice immunized with the N-terminus MS2 were vaccinated with 250 ng twice and 500 ng once. The mice immunized with conjugated MS2 were vaccinated with 500 ng three times and 5 μg once. Sera were analyzed by endpoint dilution ELISA for binding of the target peptide, either HPV16 17-31 or 65-85, both from American Peptide (Sunnyvale, CA), as previously described [89].

4.3 Results

4.3.1 Displaying p28 at the C-terminus of MS2 single-chain dimers.

Previous studies utilizing p28 as an adjuvant have shown that a single copy of p28 can act to inhibit antibody production, but that multiple copies, or polyvalent p28 ligands enhance B cell activation [120, 121, 124].

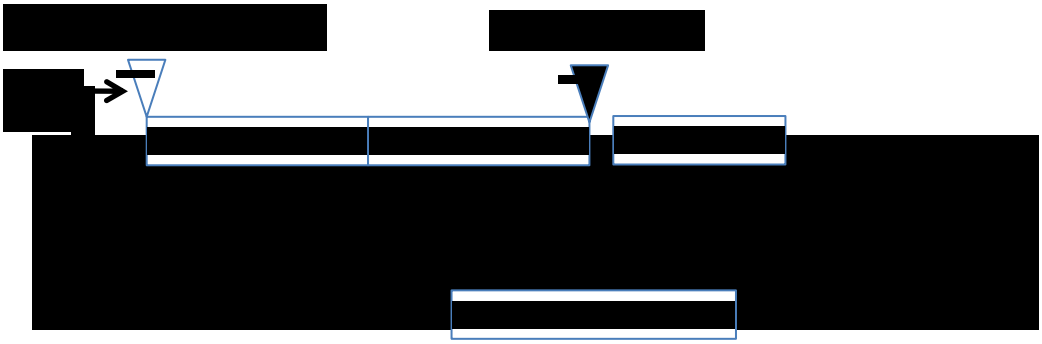
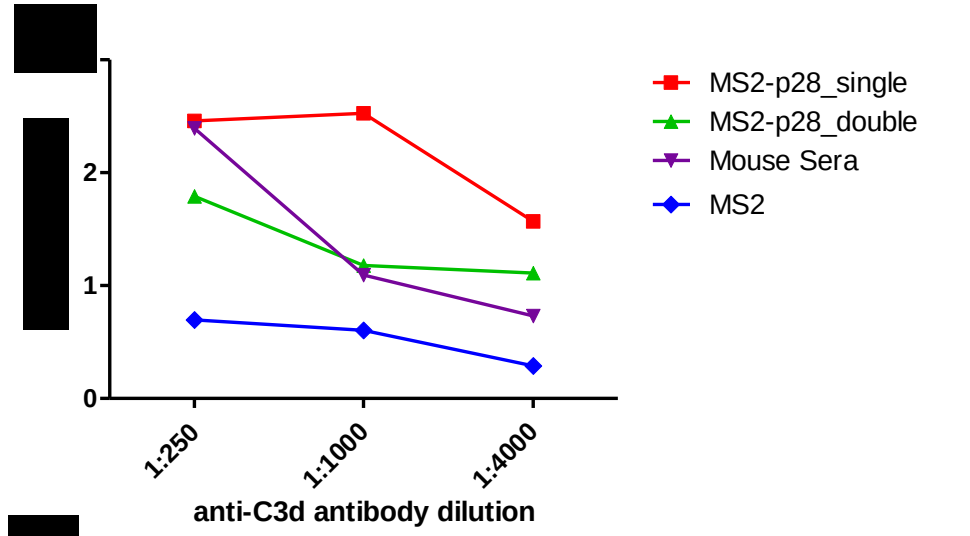


Figure 4.1 – Schematic of pDSP62am: The adjuvant peptide was ligated downstream of an amber stop codon to allow for limited read-through. *E. coli* C41 cells were co-transfected with this plasmid and the pNMSupA plasmid encoding the Sup A tRNA suppressor, allowing low levels of adjuvant peptide expression.

Accordingly, we constructed DNA encoding either a single copy or a double copy of murine p28 with a four glycine linker and ligated it into the pDSP62am plasmid, downstream

of a single-chain dimer and an amber stop codon (Figure 4.1). Co-expression of an amber suppressor tRNA can promote translational read-through. The efficiency of read-through depends on the specific tRNA suppressor co-transfected into the *E. coli* with pDSP62am. We anticipated that expression of p28 on every single-chain dimer could result in the inability to assemble into VLPs or could even lead to an immune response against the p28 peptide [24]. Indeed, we were only able to produce assembled VLPs when we expressed coat protein in the presence of SupA, which results in a low level of translational suppression (~1-2%). The use of other suppressors that result in higher efficiency read-through failed to produce VLPs. These MS2-p28 VLPs were probed for p28 display by ELISA using a polyclonal anti-mouse C3d antibody (Figure 4.2A). Although wells containing unmodified MS2 VLPs showed some background, the p28 displaying VLPs showed as much, if not more, reactivity as the mouse sera control. This indicated that these VLPs were displaying p28 on their surface.

We next constructed a plasmid that contained the C-terminal p28 as well as the HPV16 L2 17-31 epitope. To verify expression of both of these genetic inserts, we probed the MS2 VLPs with both an anti-L2 antibody, RG-1, that binds to this region of L2 [94], and the anti-C3d antibody. We found that the VLPs displaying p28_double and the L2 epitope bound both antibodies, indicating that


[redacted]
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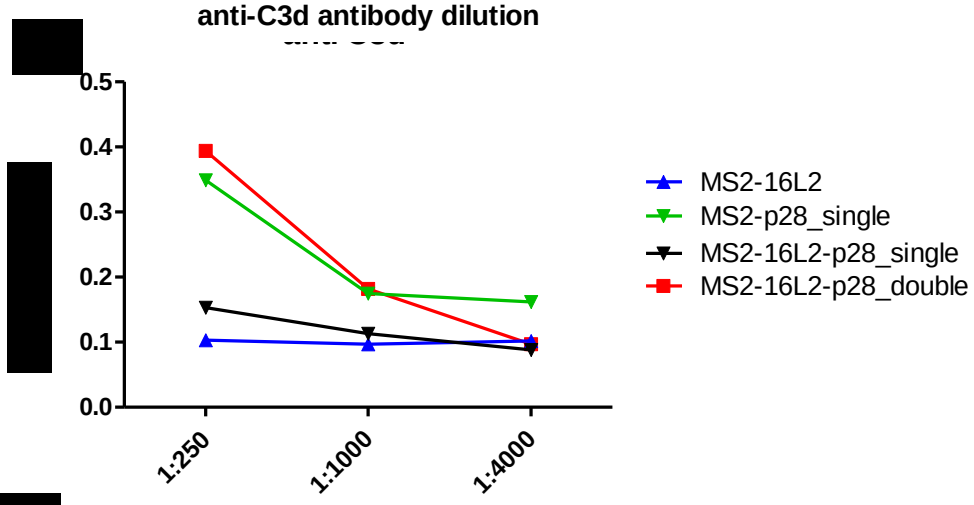
1:1000
1:4000
anti-C3d antibody dilution
MS2-p28_single
MS2-p28_double
Mouse Sera
MS2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anti-C3d antibody dilution
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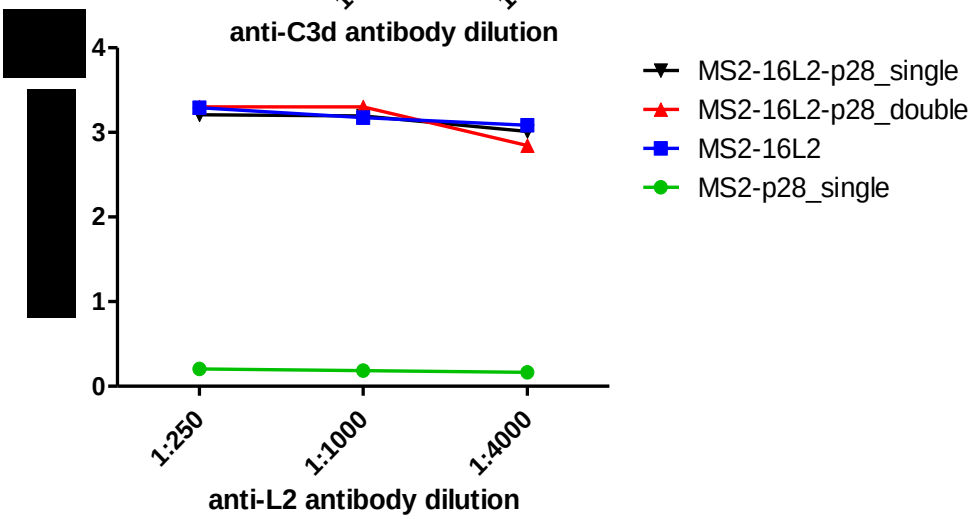
MS2-16L2-p28_single
MS2-16L2-p28_double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MS2-p28_single

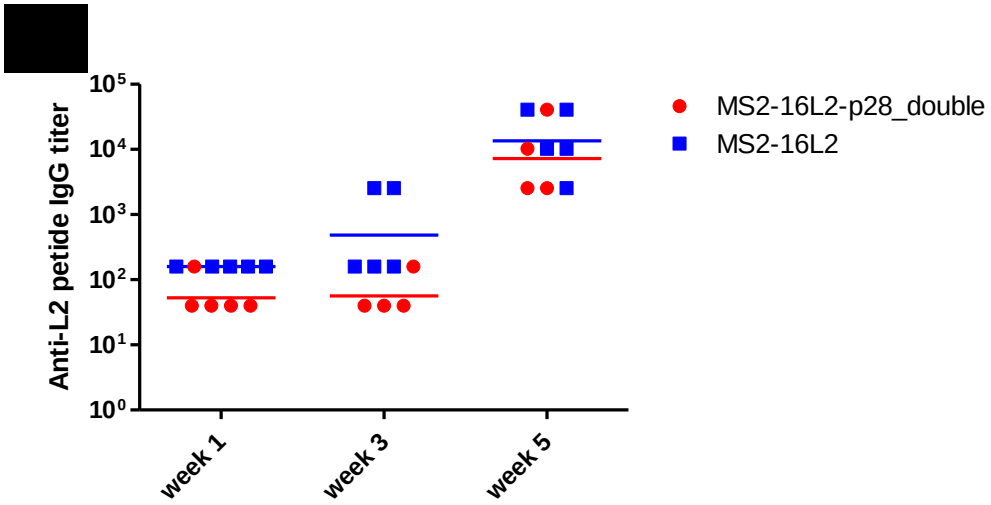

Figure 4.2 – Characterization of p28-displaying and p28/16L2 VLPs: 500 ng of VLPs were plated and probed with anti-C3d or anti-L2 antibodies to verify expression of the peptide on the surface of the VLPs. A&B: 500 ng of VLPs were probed by the indicated amount of anti-C3d antibody. A secondary HRP-conjugated donkey anti-goat antibody was then added and developed using ABTS. Absorbance was read at 405 nm. Fig 4.2A was allowed to develop overnight. C: VLPs analyzed as above except an anti-L2 antibody, RG-1, was used. A HRP-conjugated goat anti-mouse was used to detect binding. D: Groups of 5 mice were initially immunized with 250 ng of the indicated VLPs and boosted at week 4 with 500 ng. Data points indicate the titers of individual mice. Bars indicated the group geometric mean.

both were present on the surface of the VLPs, while the MS2-16L2-p28_single VLPs was not bound by the anti-C3d antibody (Figure 4.2B&C). This result seemed to indicate that the p28_single epitope would similarly not be bound by CR2.

4.3.2 Immunization with MS2 displaying p28

In order to gauge the effect of p28 display on our VLPs, we immunized groups of mice with a low dose (250 ng) of MS2-16L2 alone or MS2-16L2-p28_double (Figure 4.2D). Sera were taken one and three weeks after immunization to detect any changes in the kinetics of the antibody response. Mice were then boosted after four weeks with 500 ng of VLPs, with sera taken one week after the final immunization. Sera were tested for IgG antibody titers by peptide ELISA targeting the HPV16 L2 17-31 epitope. Both VLPs elicited high-titer responses against L2. Co-expression of p28_double on the surface of the VLPs, however, did not enhance anti-L2 antibody titers.

4.3.3 Conjugating an L2 peptide to MS2 VLPs displaying p28

Display of peptides at both the N- and C-termini of MS2 coat protein is somewhat problematic. These two sites are in close proximity in the assembled VLPS, so it is possible that there may be steric issues that prevent binding of the p28 peptide to CR2. Indeed, we observed an anti-C3d antibody failed to bind to the MS2-16L2-p28_single VLPs. As an alternative method, we displayed a different HPV16 L2 peptide on the surface of the MS2-p28_single and double VLPs by chemical conjugation (Figure 4.3A).

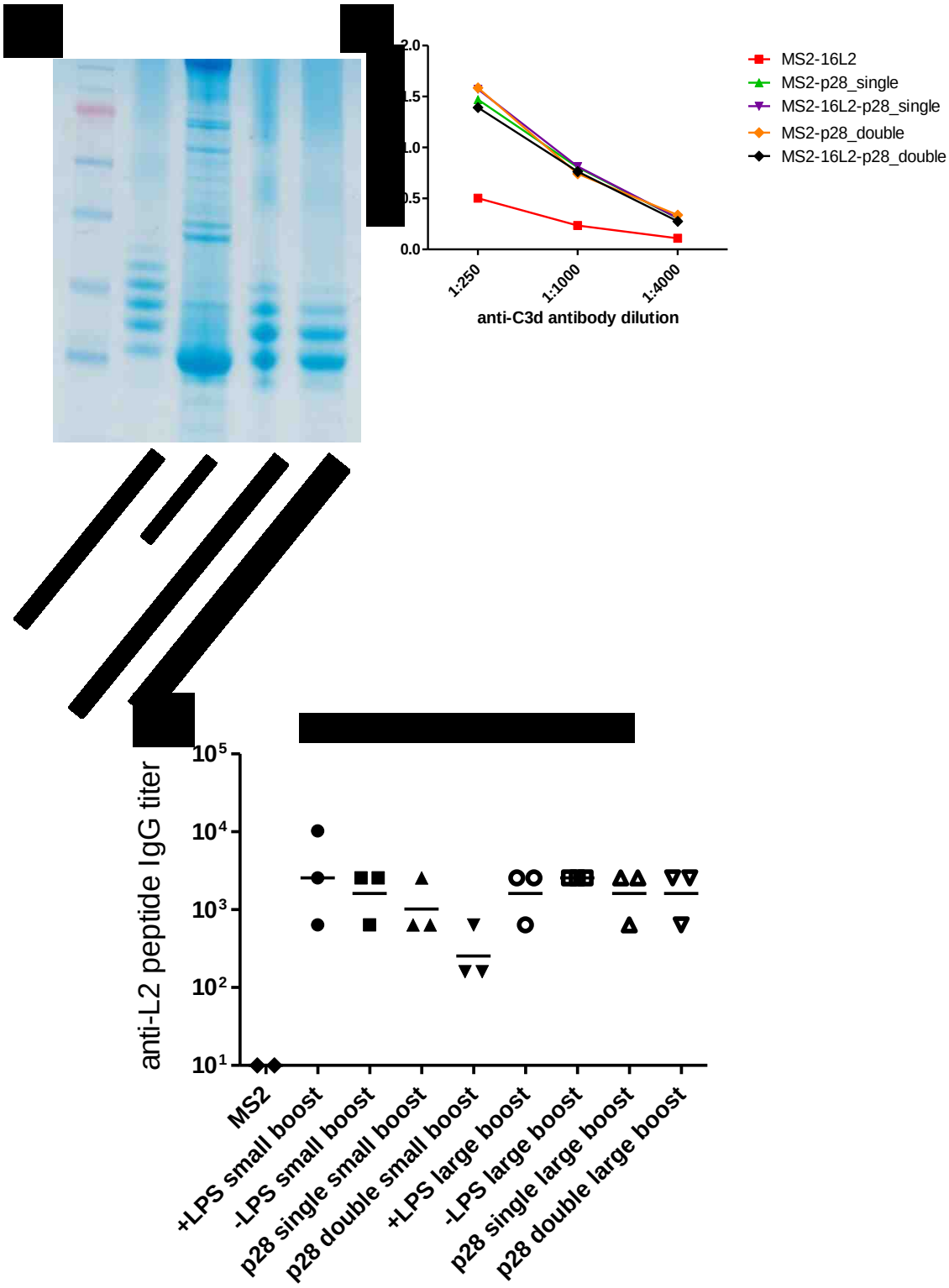

Figure 4.3 – Conjugating a L2 peptide to MS2 VLPs displaying p28 peptides. A) Electrophoresis gel visualizing the efficiency of the conjugation of HPV16 L2 65-85. MS2 VLPs with or without p28 peptides were incubated with SMPH and then with the 65-85 peptide with a terminal cysteine. Each successive band represents a single-chain dimer with another peptide

conjugated to it. B) Measuring the binding of anti-C3d to conjugated VLPs by ELISA as in Fig 4.2 A. C) Mice were immunized with 500 ng of conjugated VLPs. LPS was removed from all of the groups except the +LPS control. The low boost sera was taken one week after a 500 ng boost. A week after, the mice were immunized with 5 μg (high boost) and sera was taken one week after. Anti-L2 peptide titers were obtained as in Fig 4.2D. Data points represent the titers of individual mice and bars represent group geometric means.

To verify that p28 was accessible to the anti-C3d antibody, we again performed an ELISA to measure its binding (Figure 4.3B). Both MS2-p28_single and double as well as the conjugated MS2-16L2-p28_single and double bound to the anti-C3d antibody, demonstrating that both p28 peptides were, at this point, unblocked by the conjugated peptides.

4.3.4 Immunization with conjugated/p28 VLPs

We vaccinated groups of five mice with low doses of MS2 16L2 65-85 with and without the p28 peptide. Since our VLP solutions were contaminated with bacterial endotoxin, we also compared the immunogenicity of VLPs that had endotoxin removed and those that had not. We then boosted with a larger dose (5 μg). Sera were collected one week after the second and third immunizations (Figure 4.3C). Similar to previous results seen in our lab, the removal of LPS did not have a significant effect on the endpoint titers elicited by vaccination [19]. Further, the display of p28_single also did not impact the kinetics of antibody production nor the titers of antibodies produced. Interestingly, the p28_double VLP consistently showed lower titers than the other sera after the first two immunizations.

4.3.5 Displaying flagellin at the C-terminus of MS2 single-chain dimers

To create the flagellin displaying VLPs, we followed a similar procedure as described above. The open reading frame of the *Salmonella* flagellin gene, *fliC*, was inserted at the 3' end of MS2 single-chain dimer of expression plasmids for wild-type MS2 and MS2-HPV16 L2 17-31. Due to the large size of the f*liC* insert (~1500 base pairs), we co-transfected *E. coli* with only the lowest tRNA

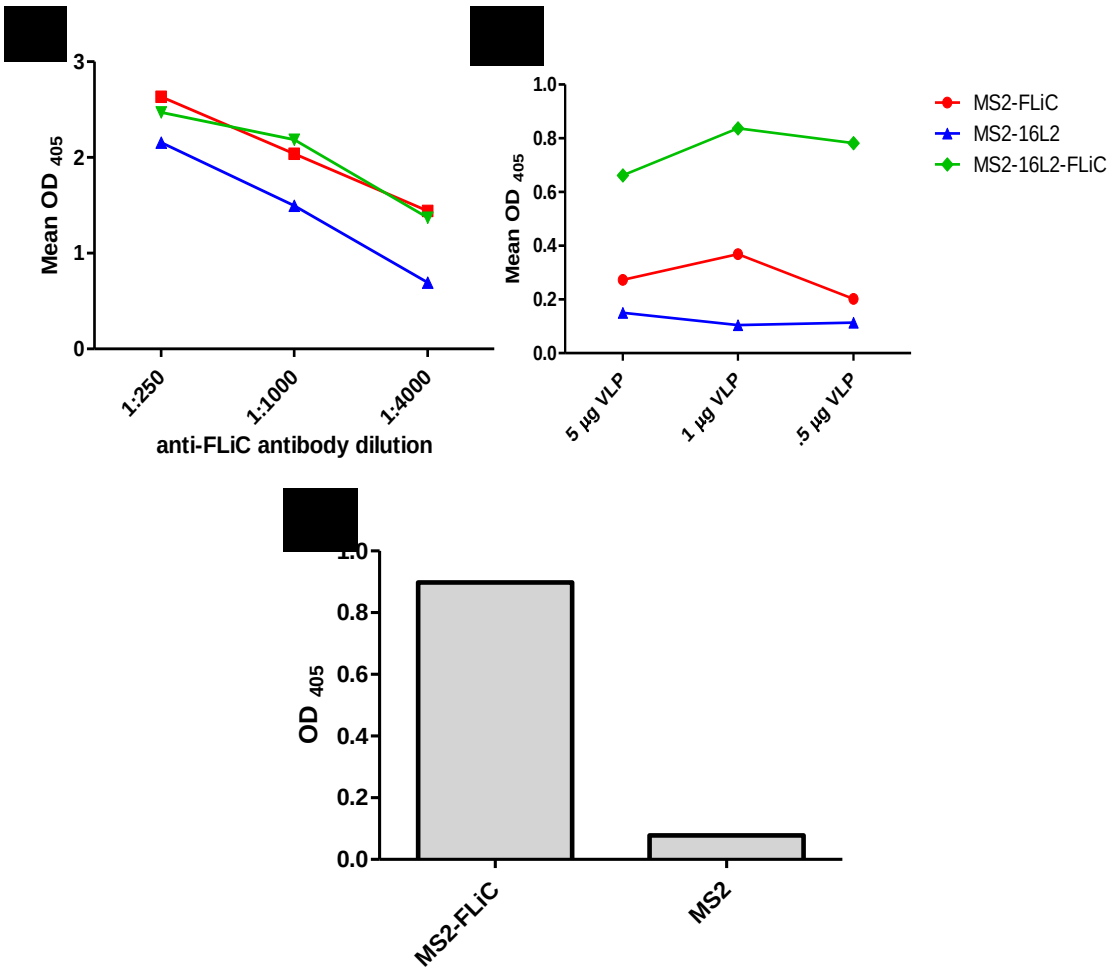

Figure 4.4 – Characterization of MS2-FLiC VLPs. A) 500 ng of the VLPs were probed with the indicated dilution of an anti-FLiC antibody as in Fig 4.2A with a goat anti-mouse 2° antibody. B) A capture ELISA was performed on LPS removed VLP solutions. The anti-L2 antibody, RG-1, was used to capture the VLPs, which were then probed with the anti-FLiC antibody. C) Capture ELISA with 250 ng of a soluble, chimeric TLR5. Wells were blocked with .5% milk then incubated with the indicated VLPs. An anti-MS2 antibody was added at a 1:2000 dilution then probed with a HRP-conjugated goat anti-rabbit 2° at 1:10000 dilution and developed with ABTS.

suppressor plasmid, pNMSupA. We sought to confirm expression of flagellin on the MS2 VLPs by ELISA with an anti-flagellin antibody (Figure 4.4A). We found, however, a considerable amount of background in control MS2 solutions. This seemed to indicate that flagellin from the *E. coli* used to produce the recombinant VLPs remains in solution with our purified VLP solutions. We removed LPS by incubation with Triton X-1114 and assayed the VLPs using two capture ELISAs. First we used a soluble, chimeric TLR5 to capture the MS2-fliC particles and probed with an anti-MS2 antibody (Figure 4.4B). Our MS2-fliC was bound by the TLR5 whereas MS2 was not. Second, we tested the MS2-16L2-fliC VLPs by capturing them with the anti-L2 antibody RG-1 and probing with the anti-flagellin antibody (Figure 4.4C). While there was still some background in the control solutions, the MS2 displaying both L2 and flagellin showed the greatest reactivity.

To confirm that MS2 was displaying flagellin, we performed Western Blots using the same anti-L2 and anti-flagellin antibodies (Figure 4.5). While the anti-L2 blot seemed to show a high-weight band, possibly due to MS2 single-chain dimer displaying L2 and flagellin, the anti-flagellin blot did not show a corresponding high-molecular weight band. Indeed, the anti-flagellin blot had a number of non-specific bands, seeming to indicate that flagellin degradation products were present in our VLP solutions. Further, the high-molecular weight band in the anti-L2 had an estimated weight of over 98 kDa, whereas the predicted weight of the MS2 single-chain dimer, 16L2 peptide, and flagellin is 82 kDa.

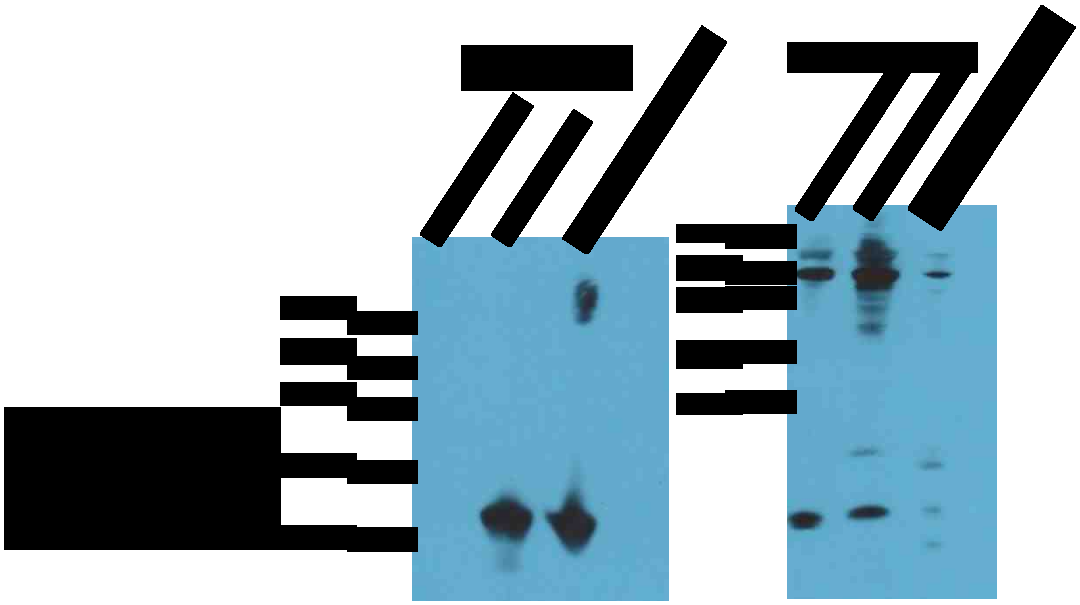

Figure 4.5 - Western Blot with anti-L2 and anti-FLiC antibodies. Endotoxin was first removed through incubation with Triton X-114. Solutions were then separated on an electorophoresis gel. Proteins were transferred from the electrophoresis gel for one hour at 25 V. The membranes were then blocked in 5% milk. Both the anti-L2 and anti-FLiC antibodies were added at 1:15000 dilution, with secondary antibodies added at 1:20000. The membrane was developed with SuperSignal West Pico Chemiluminescent Substrate and detected with autoradiography flim.

The high-molecular weight band that reacted with the anti-L2 antibody is difficult to explain; however, it seems clear that our VLP solutions have a great deal of flagellin in them, likely masking any effect of our displayed fagellin.

4.4 Discussion

The design of vaccines requires considerations of immunogenicity, safety, and effectiveness. Virus-like particles have been shown to be potent vaccines that induce long-lasting, protective immunity. Further, their flexibility as display platforms creates the opportunity to display adjuvant peptides on their surface. In addition, VLP vaccines have been shown to be very safe. To enhance the effectiveness of our VLPs, we investigated displaying two potential adjuvants on MS2 VLPs: the minimum binding region of C3d, p28 and the monomer protein component of flagella, flagellin. While we were able to assemble VLPs displaying these epitopes, various obstacles presented themselves in their effective implementation.

Primarily, the immunogenicity and adjuvant properties of the VLPs themselves may have overwhelmed any effect that our displayed adjuvants may have had. VLPs have been shown to activate B cells to such an extent that they are able to break B cell tolerance to self-antigens, producing autoantibodies. Moreover, these VLPs were expressed in and purified from *E. coli* cells and so have considerable endotoxin contaminant, a PAMP that is recognized by TLR4 [126]. VLPs additionally encapsidate RNA, which is another PAMP that is recognized inside the endosomes of phagocytic cells by TLR7. Previous studies in our laboratory have shown the importance that the ssRNA in our VLPs plays in

their immunogenicity [19]. Interestingly, LPS removal had little effect. However, both TLR4 and TLR7 activation lead to similar downstream effects as TLR5 when binding flagellin [160]. It may be that these endogenous adjuvants, even with LPS removed, were already having the desired effect of including flagellin on our VLPs.

The studies of VLPS expressing the flagellin peptide were primarily confounded by the presence of flagella in our solutions. Despite our purification efforts, all of our VLP solutions showed reactivity with anti-flagellin antibodies by both ELISA and Western blot analysis. The capture ELISA, using anti-flagellin and anti-L2 antibodies to confirm that both targets were being displayed on the MS2 particles, reacted most with the MS2-16L2-fliC constructs, but also reacted with preps displaying only 16L2, implying that flagellin was associated with the VLPs. Production in a different expression system, in plant, mammalian or insect cells, which would not produce any extra flagella, may be an area where this adjuvant peptide may be of more use.

C3d is one of the body's own types of adjuvants, binding to CR2 on B cells to enhance the elicited antibody response. Other studies have investigated the use of p28, the 28 amino acid binding region of C3d as an adjuvant and have seen dramatic increases in the generated titers against the target. Display on our VLPs, however, seemed to have no effect on either the speed or intensity of the immune response to vaccination, despite changes in where the target peptide was being displayed. One possible reason is that the dense, ordered display of epitopes by VLP already activates B cells to such an extent that CR2 binding

becomes irrelevant. Surprisingly, vaccination with conjugated MS2-16L2-p28_double seemed to result in lower L2 specific antibodies. Previous studies of p28 have shown that monovalent p28 could inhibit immune response, so it is puzzling why the double peptide would have that effect. The lower titers, however, were not noticeable after the larger, 5 μg boost. Other vaccines studies in which p28 was successfully used were primarily based on recombinant protein or DNA vaccines [120, 123, 166]. Our VLPs are fully formed capsid particles which have many more sites that could be bound by endogenous C3d in the body itself after vaccination. Also, we were only able to display p28 at very low valency without affecting the correct folding and assembly of the VLPs. At an estimated one to two copies per VLP, there simply may not have been enough displayed to achieve the adjuvant effect. Any or all of these factors could have contributed to the lack of effect.

There are other adjuvants that have been explored for use with VLPs such as loading empty particles with other TLR activating PAMPs or simply injecting them with VLPs [167-169]. Adjuvants have the potential to ease the uptake of new vaccines by reducing the amount of VLPs needed or by lowering the number of boosts. Further study into the endogenous immunogenicity of VLPs will assist in targeting new immune-activating pathways that aren't already being triggered by the VLPs themselves.

Chapter 5 – Conclusions

5.1 Summary of research

These studies have focused on optimizing and enhancing the use of bacteriophage virus-like particles targeting the minor capsid protein of HPV, L2. We identified a type-specific, neutralizing epitope of L2, amino acids 65-85, that strongly protected mice from the homologous HPV PsV type, but that did not protect against heterologous types. We were able to broaden the elicited neutralizing response through the use of a consensus sequence peptide of that region derived from the high-risk types of HPV. We were also able to broaden the cross-reactivity of elicited antibodies by immunizing with VLPs displaying two peptides. Vaccination with these hybrid particles induced an immune response against both targets displayed as well as a "hybrid response" that was able to better neutralize similar heterologous PsVs than antibodies elicited by VLPs with only one target. Finally, we were able to display two molecular adjuvants on the surface of VLPs at low levels: the CR2-minimum binding region of C3d, p28, and the monomer component of flagella, flagellin. Despite using two different methods to display the L2 epitope, amino acids 17-31, on MS2, the p28 VLPs did not show any effects on antibody production. Further, the flagellin VLPs were confounded by the ubiquitous presence of flagellin contaminant in our VLP solutions.

These projects, though concentrated on L2-displaying vaccines, demonstrate techniques that could be used in vaccines targeting a variety of pathogens. Vaccines with consensus sequence epitopes would seem to be of most use targeting highly mutagenic pathogens with a large number of endemic

types or strains. Consensus vaccines have already been investigated in studies of norovirus and HIV as a method of increasing the breadth of cross-protection generated by immunization. Additionally, incorporating consensus sequences of type-specific neutralizing epitopes in L1-based HPV VLPs may be a way to increase the breadth of protection afforded by the current HPV vaccines.

The hybrid vaccines present an alternative method to achieve the same end of increased cross-protection. By presenting two targets on the VLP, we take advantage of the high avidity of VLP-B cell interactions to induce an immune response that, presumably, can recognize both the displayed targets and similar targets as well. Perhaps the greatest utility of the hybrid display may be the ability to target two separate epitopes from the same pathogen or two epitopes from two different pathogens. It is possible that two vaccinations could be combined into one by displaying both neutralizing epitopes on the same VLP.

The molecular adjuvants that we displayed may be attempting to stimulate the immune system through pathways already activated by the VLPs themselves. It is very possible that VLPs are opsonized and tagged with C3d naturally during immunization like a normal virus would. If this is the case, then our low expression of p28 would be redundant. Further, we discovered that our VLP solutions already have flagellin contaminant in them from the *E. coli* cells used to recombinantly express them. However, given the variety of expression systems that can be used to produce VLPs, it is possible that displaying flagellin, at least, may still be a viable option.

5.2 Future Directions

One of the strengths of these studies is that the aims synergistically build upon one another and this is one future direction this research can take. The neutralizing epitopes identified in Chapter 2, consensus or otherwise, could be displayed on a hybrid particle. There is some evidence that immunizing against two HPV16 L2 epitopes increases the heterologous cross-neutralization of HPV types [135]. We have observed the ability of these hybrid particles to elicit immune responses against both of the displayed targets. This may be an ideal way to target two epitopes of L2. Further, L2 epitopes that are fairly well conserved, such as amino acids 108-120, may benefit from the same type of hybrid display as performed with the 17-31 epitope. By displaying the 108-120 epitope from two different HPV types, the cross protection we observed in Chapter 2 could be increased. Finally, while not feasible in this study, both the consensus and hybrid VLPs may benefit from the display of flagellin as an adjuvant, if produced in a flagellin-free system and with VLPs that do not encapsidate unmethylated RNA, another TLR ligand.

In the short term, the immune response elicited by both the consensus and hybrid VLPs would need to be further tested for neutralization against other high and low risk HPV PsVs. To be a viable option as a next-generation HPV vaccine, either would have to demonstrate protection against most or all of the other high-risk HPV types while equaling the strong protection against HPV16 and 18 generated by the current vaccines.

It would also be worthwhile to investigate genetically inserting the 65-85 consensus sequence into our bacteriophage VLPs. Previous work in our lab has

shown that inserting this region into PP7 VLPs resulted in misfolded coat protein, despite the use of chaperone proteins. The cost of synthesizing peptides for conjugations, however, is such that it could hinder its development for clinical trials. Possible strategies could include displaying the peptide on MS2 instead of PP7 or inserting only the consensus sequence of the poorly conserved region of this peptide, 76-85, that seems to be the neutralizing epitope. It may also be useful to determine whether displaying a shorter peptide in a more constrained fashion would affect the cross-neutralization properties of the elicited antibodies, similar to previous studies in our lab of the L2 17-31 epitope [37].

Among the hybrid vaccines, the immune response against the PP7-18/1L2 stands out as an anomaly. The strong *in vitro* reactivity with the HPV1 peptide was expected, but the lack of any reactivity with the other peptides, especially the HPV18 peptide that was also displayed on the surface of the VLP, was not. The possible explanation that this skewing of the immune response is caused by an immunodominant response against the HPV1 peptide requires further study. Certainly it cautions against the use of hybrid particles in every situation as a similarly skewed response is a possibility.

The adjuvant studies would benefit from a better understanding of the body's response to our VLP vaccines. The inclusion of p28 seemed to be a way to assimilate an adjuvant-like aspect of the body's own immune response into our VLPs. If it were to be confirmed that the VLPs are already bound by C3d during immunization and activating CR2, then perhaps p28 VLPs administered at a different site would have more effect. It is possible that VLPs displaying p28 that

are administered in an alternative route, either through mucosal sites or orally, would elicit a greater antibody response than those without p28 as they are more likely to enter local lymphatic tissues before being exposed to complement proteins in the blood. Thus, the chances of be bound by B cells before serum complement proteins would be higher.

Also, the finding that p28_double expression seemed to slow the antibody response runs contrary to previous studies showing that tandem repeats or polyvalent p28 were immunoactivating. Using *in vitro* cell-based assays using B cells incubated with VLPs displaying p28_double, it may be possible to identify any activated pathways within the B cells which would inhibit antibody production.

The apparent contamination of our VLP solutions by flagellin needs to be addressed. While previous studies in our lab have investigated the role of endogenous and exogenous adjuvants administered with our VLPs, flagellin was not one that was examined [19]. While it is possible that soluble flagellin not associated with the VLP would not have a strong adjuvant effect, it is difficult to say for certain what effect it has on antibody production. Though we were unable to find a bacterial expression system that was flagellin free, other non-bacterial VLP expression systems would allow us to measure the effect of displaying flagellin. Also, a more stringent purification protocol may be able to remove any soluble flagellin left in our solutions.

5.3 Conclusion

These studies represent several techniques that could be used in the rational design of VLP-based vaccines targeting any number of pathogens. Our bacteriophage VLPs are a highly versatile vaccine platform, as the single-chain dimer bacteriophage VLPs can tolerate a wide range of genetic insertions. With the increasing number of neutralizing monoclonal antibodies being discovered and used as potential therapies, epitope based vaccines like our VLPs may be able to elicit a robust immune response that mimics the monoclonal therapies but at a lower cost. Importantly, VLPs that can be rapidly produced and targeted to neutralizing epitopes may be extremely useful in combating emerging diseases. As our understanding of how VLPs are processed and elicit such strong immune responses grows, we will be able to better fine-tune these strategies to the greatest effect.

Appendices

Appendix 1 – Abbreviations used

AAVLP – Adeno-associated Virus-like Particles

Alum-MPL - Aluminum Hydroxide-monophosphoryl Lipid A

APC – Antigen Presenting Cell

BCR – B Cell Receptor

BPV – Bovine Papillomavirus

CIN III – Cervical Intraepithelial Neoplasia Grade III

COPV – Canine Oral Papillomavirus

CR2 – Complement Receptor 2

DC – Dendritic Cell

ECM – Extracellular Matrix

FDC – Follicular Dendritic Cell

GFP – Green Fluorescent Protein

HPV – Human Papillomavirus

i.m. – Intra-Muscular

ID – Infectious Dose

IFA – Incomplete Freund's Adjuvant

IU – Infectious Units

MPL - Monophosphoryl Lipid A

OD – Optical Density

PAMP – Pathogen Associated Molecular Pattern

PsV – Pseudovirus

ROI – Region of Interest

SCD – Single-chain Dimer

SMPH - Succinimidyl 6-[(beta-aleimidopropionamido)hexanoate]

TLR – Toll-like Receptor

TMV – Tobacco Mosaic Virus

VLP – Virus-like Particles

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