Published in final edited form as:

Vaccine. 2014 July 23; 32(34): 4267–4274. doi:10.1016/j.vaccine.2014.06.054.

Immunization with a consensus epitope from Human Papillomavirus L2 induces antibodies that are broadly neutralizing

Mitchell Tyler, Ebenezer Tumban, Agnieszka Dziduszko, Michelle A. Ozbun, David S. Peabody, and Bryce Chackerian*

Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, NM 87131, United States

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.

Keywords

HPV vaccine; Virus-like Particles; L2; neutralizing epitope

1. 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

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel: +1 505 272 0269; fax: +1 505 272 6029. bchackerian@salud.unm.edu (B. Chackerian).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cancer cases [1], but they largely do not protect against the other high-risk HPV types that are associated with cancer [2]. To provide broader protection against infection by diverse HPV types, many researchers have investigated vaccines based on 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 [3, 4]. Moreover, the N-terminus of L2 includes regions that possess a great deal of identity 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 [5–8]. Epitope mapping studies utilizing neutralizing monoclonal antibodies targeting the N-terminus of L2 and recombinant peptide-based vaccines have identified neutralizing epitopes within L2 that elicit cross-protective antibodies [9–12]. 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 [13].¹

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, strong activation of B cells, and production of high-titer antibody responses [14]. 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 [15, 16]. 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 [17, 18].

Vaccines in which L2 epitopes are displayed multivalently can induce potent anti-peptide antibody responses [11, 19, 20]. Given the potent immunogenicity of VLPs, one technique has been to display L2 epitopes in a repetitive format on the surface of heterologous VLPs. Groups have successfully used a number of different VLP platforms to display L2, including HPV [11, 21], adeno-associated virus type 2 [20], and tobacco mosaic virus [22]. Our laboratory has displayed a broadly neutralizing L2 epitope (aa17–31) on bacteriophage VLPs and has shown that this vaccine elicits high-titer, long-lasting and broadly neutralizing antibody responses against HPV [23–25]. 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

¹Abbreviations used: HPV – Human Papillomavirus, aa – amino acids, VLP – virus-like particles, PsV –pseudo-virus, SMPH - succinimidyl 6-[(beta-aleimidopropionamido)hexanoate], IFA, Incomplete Freund's Adjuvant, DMSO - dimethyl sulfoxide, ABTS - 2, 29-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, ECM – extracellular matrix

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 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. Materials and Methods

2.1. Conjugation of L2 peptides to Q β

Preparation of Q β bacteriophage was performed as described previously [17]. 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 Q β bacteriophage using the crosslinker SMPH (Thermo Scientific) and conjugation efficiency was assessed as described previously [17].

2.2. 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 [23, 24]. 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 *E. coli* cells (Lucigen) with L2-PP7 expression vectors. Expression of bacteriophage 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 [23].

2.3. 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 μ g 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 μ g 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 [24].

2.4. 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 [26, 27] except that matured PsVs were purified by ultracentrifugation on a cesium chloride gradient at 27,000xg for 18 hours. Flow cytometry was used to titer the PsV by determining the fraction of GFP-expressing 293TT cells.

2.5. 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 [26, 27]. 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 [24].

2.6. 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 [28] and a consensus sequence was generated using Jalview 2.7 [29].

2.7. In vitro L2 neutralization assay

Neutralization assays were performed as described in [30] 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 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.

2.8. 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.

3. Results

Bacteriophage VLPs can be used as flexible platforms for immunogenic display of target antigens. Although a number of different techniques can be used to display targets in a

multivalent fashion on the surface of VLPs, we have primarily relied on two complementary approaches, genetic insertion and chemical conjugation, to engineer VLP-based vaccines. Because these two display methodologies have certain capabilities and limitations (for example, not all peptides can be displayed using both approaches [14]), here we used both approaches to map neutralizing epitopes in HPV L2.

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 [23]. Immunization with HPV16 L2(aa17–31) (either on bacteriophage VLPs or using other display formats) induces cross-neutralizing antibodies against multiple HPV types [10, 20, 31–33]. 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 amino acid identity among HPV types [9, 12]. 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. 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 Q β particles using a chemical crosslinker [17, 34]. 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 Q β (Fig. 1A; blue lines). Conjugation of each peptide was highly efficient; approximately 360 peptides were displayed per Q β capsid (not shown).

To assess the immunogenicity of the L2-displaying VLPs, we immunized groups of Balb/c mice with three 5 μ g doses of VLPs displaying L2 peptides or, as a control, unmodified VLPs (Q β or PP7). Following the last immunization, sera were collected and analyzed by ELISA for L2 peptide-specific IgG (Fig. 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 [27]. 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. 1C). We found that vaccination with PP7-16L2(17–31), Q β -16L2(65–85), and Q β -16L2(108–120) provided strong protection against homologous PsV infection (>99% decrease in mean luciferase signal). Conversely, mice immunized with either Q β -16L2(34–52) or recombinant PP7-16L2(35–50) were poorly protected from HPV16 PsV challenge. Immunization with either PP7-16L2(51–65) or Q β -16L2(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 [24]. Mice immunized with Q β -16L2(108–120) showed strong cross-protection against PsV18 and 58, moderate cross-protection ($\sim 1 \log_{10}$ reduction in average luminescence) against PsV6 and 45, and little protection against PsV31 (Fig. 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 [35–37].

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 HPV16L2(65–85) were not protected against an *in vivo* HPV18 PsV challenge (Fig. 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 Q β -16L2(65–85) and Q β -18L2(65–85) were cross-reactive (i.e. recognized both the homologous and heterologous peptide) in an ELISA assay (Fig. 3B). Mice immunized with Q β -18L2(65–85) were strongly protected against 18PsV challenge, but not against 16PsV challenge (Fig. 3A). These data are in concordance with previous studies investigating this region of L2 [9]. 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 [38–41]. To test whether this strategy would work against a region of HPV L2 that is a type-specific neutralizing epitope, 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. 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. 4B). As expected, mice immunized with Q β -consensus L2(65–85) produced sera that reacted with both the HPV16 and 18 peptides (Fig. 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 a *in vitro* HPV neutralization assay designed specifically to be more sensitive to anti-L2 antibodies [30]. In particular, this assay allows the HPV PsVs to bind to deposited EMC, 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 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 Q β -L2 vaccinated mice and compared this to the *in vivo* PsV challenge results previously obtained (Fig. 5A and Fig. 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 Q β -consensus L2(65–85), we tested pooled sera for *in vitro* neutralization against five high-risk PsVs: 16, 18, 31, 45, and 58 (Fig. 5B & Fig. 6). Pooled sera from mice immunized with VLPs displaying either the HPV16 or the HPV18L2(65–85) peptide was only poorly cross-neutralizing, although we did detect some cross-neutralizing activity (for example sera raised against Q β -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 Q β bacteriophage can broaden the neutralizing specificity of a region of L2 that normally only elicits type-specific neutralizing antibodies.

4. Discussion

The N-terminus of L2 contains cross-protective, neutralizing epitopes [9, 12, 42] including the RG-1 epitope, located within aa17–36 of HPV16 [10]. 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. In order to determine if we could convert a type-specific neutralizing epitope into a broadly neutralizing epitope we designed a consensus peptide representing this (aa65–85) sequence. Strikingly, VLPs displaying the consensus sequence peptide 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 [41]. Similarly, consensus norovirus VP1 VLPs elicit broader immune response than VLPs derived from individual norovirus types [38]. 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. Such antibodies may be able to react with a greater number of viral strains, but unable to neutralize any of them effectively. Nevertheless, the consensus L2 peptide that we engineered elicits antibodies that efficiently neutralize 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 heterogenous region of the peptide (aa76–85), that likely represents a type-specific neutralizing epitope, contains motifs that are broadly conserved. While a majority of HPV high-risk types include either the double proline motif or the valine-rich region, it is worth noting that HPV31 does not contain an exact match of either motif, yet HPV31 PsV 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 [31, 43]. 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.

Acknowledgments

We thank Julianne Peabody for assistance with animal studies and Bruce Edwards for assistance with the Hypercyte autosampler. L1/L2 expression and reporter plasmids were generously provided by Chris Buck, Susana Pang, John Schiller, Martin Müller, and Tadahito Kanda. We would also like to thank members of the University of New Mexico Interdisciplinary HPV Prevention Center for helpful discussions. This study was supported by a grant from the US National Institutes of Health (U19 AI084081) to the University of New Mexico Sexually Transmitted Infections Cooperative Research Center (STI-CRC). M Tyler was funded by a T32 training grant award by the US National Institutes of Health (T32 AI007538) to the University of New Mexico Infectious Disease and Immunity Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer*. 2007; 121:621–32. [PubMed: 17405118]
2. Schiller JT, Castellsague X, Garland SM. A review of clinical trials of human papillomavirus prophylactic vaccines. *Vaccine*. 2012; 30 (Suppl 5):F123–38. [PubMed: 23199956]
3. Day PM, Kines RC, Thompson CD, Jagu S, Roden RB, Lowy DR, et al. In vivo mechanisms of vaccine-induced protection against HPV infection. *Cell Host Microbe*. 2010; 8:260–70. [PubMed: 20833377]
4. Richards RM, Lowy DR, Schiller JT, Day PM. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. *Proc Natl Acad Sci U S A*. 2006; 103:1522–7. [PubMed: 16432208]
5. Christensen ND, Kreider JW, Kan NC, DiAngelo SL. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. *Virology*. 1991; 181:572–9. [PubMed: 1707567]
6. Campo MS, Grindlay GJ, O'Neil BW, Chandrachud LM, McGarvie GM, Jarrett WF. Prophylactic and therapeutic vaccination against a mucosal papillomavirus. *J Gen Virol*. 1993; 74 (Pt 6):945–53. [PubMed: 8389809]
7. Lin YL, Borenstein LA, Selvakumar R, Ahmed R, Wettstein FO. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. *Virology*. 1992; 187:612–9. [PubMed: 1312271]
8. Roden RB, Yutzy WH, Fallon R, Inglis S, Lowy DR, Schiller JT. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. *Virology*. 2000; 270:254–7. [PubMed: 10792983]
9. Rubio I, Seitz H, Canali E, Sehr P, Bolchi A, Tommasino M, et al. The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies. *Virology*. 2011; 409:348–59. [PubMed: 21074234]

10. Gambhira R, Karanam B, Jagu S, Roberts JN, Buck CB, Bossis I, et al. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. *J Virol.* 2007; 81:13927–31. [PubMed: 17928339]
11. Schellenbacher C, Roden R, Kirnbauer R. Chimeric L1-L2 virus-like particles as potential broad-spectrum human papillomavirus vaccines. *J Virol.* 2009; 83:10085–95. [PubMed: 19640991]
12. Kondo K, Ishii Y, Ochi H, Matsumoto T, Yoshikawa H, Kanda T. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology.* 2007; 358:266–72. [PubMed: 17010405]
13. Karanam B, Jagu S, Huh WK, Roden RB. Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. *Immunol Cell Biol.* 2009; 87:287–99. [PubMed: 19421199]
14. Chackerian B. Virus-like particles: flexible platforms for vaccine development. *Expert Rev Vaccines.* 2007; 6:381–90. [PubMed: 17542753]
15. Schodel F, Wirtz R, Peterson D, Hughes J, Warren R, Sadoff J, et al. Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. *Journal of Experimental Medicine.* 1994; 180:1037–46. [PubMed: 7520465]
16. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, Kundig T, et al. A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine.* 2002; 20:3104–12. [PubMed: 12163261]
17. Chackerian B, Rangel M, Hunter Z, Peabody DS. Virus and virus-like particle-based immunogens for Alzheimer's disease induce antibody responses against amyloid-beta without concomitant T cell responses. *Vaccine.* 2006; 24:6321–31. [PubMed: 16806604]
18. Peabody DS, Manifold-Wheeler B, Medford A, Jordan SK, do Carmo Caldeira J, Chackerian B. Immunogenic display of diverse peptides on virus-like particles of RNA phage MS2. *J Mol Biol.* 2008; 380:252–63. [PubMed: 18508079]
19. Jagu S, Karanam B, Gambhira R, Chivukula SV, Chaganti RJ, Lowy DR, et al. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. *J Natl Cancer Inst.* 2009; 101:782–92. [PubMed: 19470949]
20. Nieto K, Weghofer M, Sehr P, Ritter M, Sedlmeier S, Karanam B, et al. Development of AAVLP(HPV16/31L2) particles as broadly protective HPV vaccine candidate. *PLoS One.* 2012; 7:e39741. [PubMed: 22761884]
21. Varsani A, Williamson AL, de Villiers D, Becker I, Christensen ND, Rybicki EP. Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J Virol.* 2003; 77:8386–93. [PubMed: 12857908]
22. Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, et al. Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. *Virology.* 2006; 348:475–88. [PubMed: 16466765]
23. do Caldeira JC, Medford A, Kines RC, Lino CA, Schiller JT, Chackerian B, et al. Immunogenic display of diverse peptides, including a broadly cross-type neutralizing human papillomavirus L2 epitope, on virus-like particles of the RNA bacteriophage PP7. *Vaccine.* 2010; 28:4384–93. [PubMed: 20434554]
24. Tumban E, Peabody J, Peabody DS, Chackerian B. A pan-HPV vaccine based on bacteriophage PP7 VLPs displaying broadly cross-neutralizing epitopes from the HPV minor capsid protein, L2. *PLoS One.* 2011; 6:e23310. [PubMed: 21858066]
25. Tumban E, Peabody J, Peabody DS, Chackerian B. A universal virus-like particle-based vaccine for human papillomavirus: Longevity of protection and role of endogenous and exogenous adjuvants. *Vaccine.* 2013; 31:4647–54. [PubMed: 23933337]
26. Buck CB, Pastrana DV, Lowy DR, Schiller JT. Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods Mol Med.* 2005; 119:445–62. [PubMed: 16350417]
27. Roberts JN, Buck CB, Thompson CD, Kines R, Bernardo M, Choyke PL, et al. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat Med.* 2007; 13:857–61. [PubMed: 17603495]

28. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007; 23:2947–8. [PubMed: 17846036]
29. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*. 2009; 25:1189–91. [PubMed: 19151095]
30. Day PM, Pang YY, Kines RC, Thompson CD, Lowy DR, Schiller JT. A human papillomavirus (HPV) in vitro neutralization assay that recapitulates the in vitro process of infection provides a sensitive measure of HPV L2 infection-inhibiting antibodies. *Clin Vaccine Immunol*. 2012; 19:1075–82. [PubMed: 22593236]
31. Schellenbacher C, Kwak K, Fink D, Shafit-Keramat S, Huber B, Jindra C, et al. Efficacy of RG1-VLP Vaccination against Infections with Genital and Cutaneous Human Papillomaviruses. *J Invest Dermatol*. 2013
32. Alphs HH, Gambhira R, Karanam B, Roberts JN, Jagu S, Schiller JT, et al. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. *Proc Natl Acad Sci U S A*. 2008; 105:5850–5. [PubMed: 18413606]
33. Rubio I, Bolchi A, Moretto N, Canali E, Gissmann L, Tommasino M, et al. Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20 – 38) peptide displayed on bacterial thioredoxin. *Vaccine*. 2009; 27:1949–56. [PubMed: 19368776]
34. Hunter Z, Smyth HD, Durfee P, Chackerian B. Induction of mucosal and systemic antibody responses against the HIV coreceptor CCR5 upon intramuscular immunization and aerosol delivery of a virus-like particle based vaccine. *Vaccine*. 2009; 28:403–14. [PubMed: 19849995]
35. Kawana K, Yoshikawa H, Taketani Y, Yoshiike K, Kanda T. Common neutralization epitope in minor capsid protein L2 of human papillomavirus types 16 and 6. *J Virol*. 1999; 73:6188–90. [PubMed: 10364381]
36. Slupetzky K, Gambhira R, Culp TD, Shafit-Keramat S, Schellenbacher C, Christensen ND, et al. A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine*. 2007; 25:2001–10. [PubMed: 17239496]
37. Pineo CB, Hitzeroth, Rybicki EP. Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimeras. *Plant Biotechnol J*. 2013; 11:964–75. [PubMed: 23924054]
38. Parra GI, Bok K, Taylor R, Haynes JR, Sosnovtsev SV, Richardson C, et al. Immunogenicity and specificity of norovirus Consensus GII.4 virus-like particles in monovalent and bivalent vaccine formulations. *Vaccine*. 2012; 30:3580–6. [PubMed: 22469864]
39. Gao F, Weaver EA, Lu Z, Li Y, Liao HX, Ma B, et al. Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group m consensus envelope glycoprotein. *J Virol*. 2005; 79:1154–63. [PubMed: 15613343]
40. Zhang M, Zhang L, Zhang C, Hong K, Shao Y, Huang Z, et al. DNA prime-protein boost using subtype consensus Env was effective in eliciting neutralizing antibody responses against subtype BC HIV-1 viruses circulating in China. *Hum Vaccin Immunother*. 2012; 8:1630–7. [PubMed: 23111170]
41. McBurney SP, Ross TM. Human immunodeficiency virus-like particles with consensus envelopes elicited broader cell-mediated peripheral and mucosal immune responses than polyvalent and monovalent Env vaccines. *Vaccine*. 2009; 27:4337–49. [PubMed: 19389453]
42. Pastrana DV, Gambhira R, Buck CB, Pang YY, Thompson CD, Culp TD, et al. Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2. *Virology*. 2005; 337:365–72. [PubMed: 15885736]
43. Tumban E, Peabody J, Tyler M, Peabody DS, Chackerian B. VLPs displaying a single L2 epitope induce broadly cross-neutralizing antibodies against human papillomavirus. *PLoS One*. 2012; 7:e49751. [PubMed: 23185426]

Highlights

- Using a VLP platform, we sensitively mapped neutralizing epitopes in HPV16 L2
- Vaccines targeting L2 aa 65–85 and 108–120 protected against HPV16 PsV challenge
- The 108–120 vaccine provided modest cross-protection against other HPV types
- HPV16 and 18 (65–85) vaccines elicited type-specific neutralizing Abs
- An L2 65–85 consensus sequence vaccine elicited broadly cross-neutralizing Abs

Fig. 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; Qβ, 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/cm²/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. *** - $p < 0.001$, ns - not significant.

Fig. 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 10⁷ 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.

Fig. 3.

Mice immunized with Q β -HPV L2(65–85) show type specific *in vivo* protection despite *in vitro* cross-reactivity to L2 peptides. Mice were immunized twice with Q β 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 1.0×10^5 IU HPV18 PsV or 1.0×10^6 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 $p < 0.05$, ns – not significant.

Fig. 4.

Generation of a L2 (65–85) consensus sequence that elicits sera with *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. 1A. 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). The Genbank Protein accession numbers for the HPV sequences used are: 6 (AAK01851.1), 16 (AA4692.1), 18 (ADC35722.1), 31 (AAA46955.1), 33 (AAA4693.1), 35 (AAA4971.1), 39 (AAA47055.1), 45 (ABP99854.1), 51 (ACV88632.1), 59 (CAA54855.1), and 66 (AAA79504). The UniProtKB Protein Knowledgebase database numbers for the remaining HPV types are: 52 – P36763.1, 53 – P36764.1, 56 – P36765.1, and 58 – P26538.1. (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.

Fig. 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 ID₄₀ (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. Data points 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, shown again here) *in vivo* HPV16 PsV challenge. (B) Consensus sequence anti-sera neutralize 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 ID₄₀ at the indicated dilutions as in (A).

Fig. 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) were measured for cross-neutralizing activity against the indicated heterologous high-risk HPV types as in Figure 7. The PsVs were added at an ID₂₀ (amount of PsV that infects 20% of control cells) except for PsV 16 (ID₄₀) and PsV 18 (ID₁₀). Bars indicate the reciprocal of the highest dilution at which the PsV was neutralized greater than 50% compared to wells with no sera added.