DEVELOPMENT OF PROPHYLACTIC

HUMAN PAPILLOMAVIRUS VACCINES

by
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Abstract

The demonstration of the etiologic role of oncogenic type HPV infection in cervical cancer has led to the development of the prophylactic vaccines Cervarix and Gardasil. Despite their licensure, current vaccines do not elicit complete protection against all 15 oncogenic HPV genotypes, and the high cost of these vaccines is a major hurdle in access to vaccination. During my thesis, I have compared several promising technologies as candidate preventive vaccines with the potential to target a broad swath of medically significant HPV types using approaches that are simple and inexpensive to manufacture, and thus represent potential alternatives to highly multivalent L1 VLP vaccines. An initial critical step was the development of pseudovirions derived from medically significant HPV types, and examining their assembly and infectious pathway for commonalities using inhibitors and antibodies. The HPV pseudovirions were utilized for measuring the neutralizing antibody responses to candidate vaccines and for vaginal challenge studies in mice. In passive transfer studies neutralizing antibodies by all vaccines tested were sufficient to mediate protection against experimental vaginal challenge, implying their central role in effecting immunity and their relevance as a correlate of protection. We compared four L2-based HPV vaccine candidates: 1. in vivo electroporation of a DNA vector expressing a codon-optimized L1 gene, 2. HPV16 L1 capsomers linked with amino acids 13-47 of HPV18, HPV31, and HPV45 L2 in tandem (GST-HPV16L1Δ-L2x3), 3. A chimeric VLP presenting HPV16 L2 amino acids 17-36 in an immunodominant loop of HPV16 L1 (RG1-VLP), and 4. L2 multimer fusions comprising L2 protective domains (amino acids 11-88) derived from 8 or 5 different

HPV subtypes (11-88×8 and 11-88×5). Electroporation of L1 DNA vector elicited type-restricted immunity but when different HPV type vectors were co-administered heterotypic L1 interactions produced interference. GST-HPV16L1Δ-L2x3 and RG1-VLP induced high titers of HPV16 L1-specific neutralizing antibody but moderate titers of L2-specific antibody, although sufficient for cross-protection against other HPV types. L2 multimers induced substantially broader protection against vaginal challenge with medically significant HPV types than either Gardasil or Cervarix in the mouse model. Our data suggest the potential of L2 multimer and RG1-VLP as next generation prophylactic HPV vaccines.

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A. **Introduction**

a) Human papillomavirus associated cancers

Like the hepatitis B vaccine, the development of the two licensed HPV vaccines is a medical triumph in both infectious disease and cancer prevention because the viruses targeted are each responsible for approximately 5% of all cancer cases worldwide. The two licensed HPV vaccines, Cervarix® (GSK) and Gardasil® (Merck), are composed of virus-like particles (VLPs) derived from the major capsid protein L1 of the most prevalent types in cancer, HPV16 and HPV18, and Gardasil also includes L1 VLP of the two benign types HPV6 and HPV11 that cause ~90% of genital warts. While L1 VLPs are highly immunogenic alone, both vaccines utilize an adjuvant; Gardasil contains amorphous aluminum hydroxyphosphate sulfate (AAHS), whereas Cervarix utilizes aluminum hydroxide and a toll-like receptor (TLR)-4 agonist, 3-O-desacyl-4'-monophosphoryl lipid (MPL) A.

The primary etiologic role of persistent HPV infection in causing ~500,000 cervical cancer cases worldwide each year is firmly established, and thus with the advent of HPV vaccines and continued efforts in cytologic and viral DNA-based screening for precursor lesions (Figure 1) and ablative therapy of high grade squamous intraepithelial neoplasia (SIL), cervical cancer is a preventable disease [1,2]. Further, 40% of penile cancers, 40% of vaginal and vulval cancers, 90% of anal cancers, 3% of mouth cancers, and 12% of oro-pharyngeal cancers are also triggered by HPV and potentially preventable through vaccination [3]. HPV is a small, non-enveloped DNA tumor virus

and over 120 HPV genotypes have been described [2]. With increasingly sensitive new technologies investigators continue to find new types, but only approximately a dozen sexually transmitted ‘oncogenic’ HPV types are responsible for more than 95% of cervical cancer cases [1].

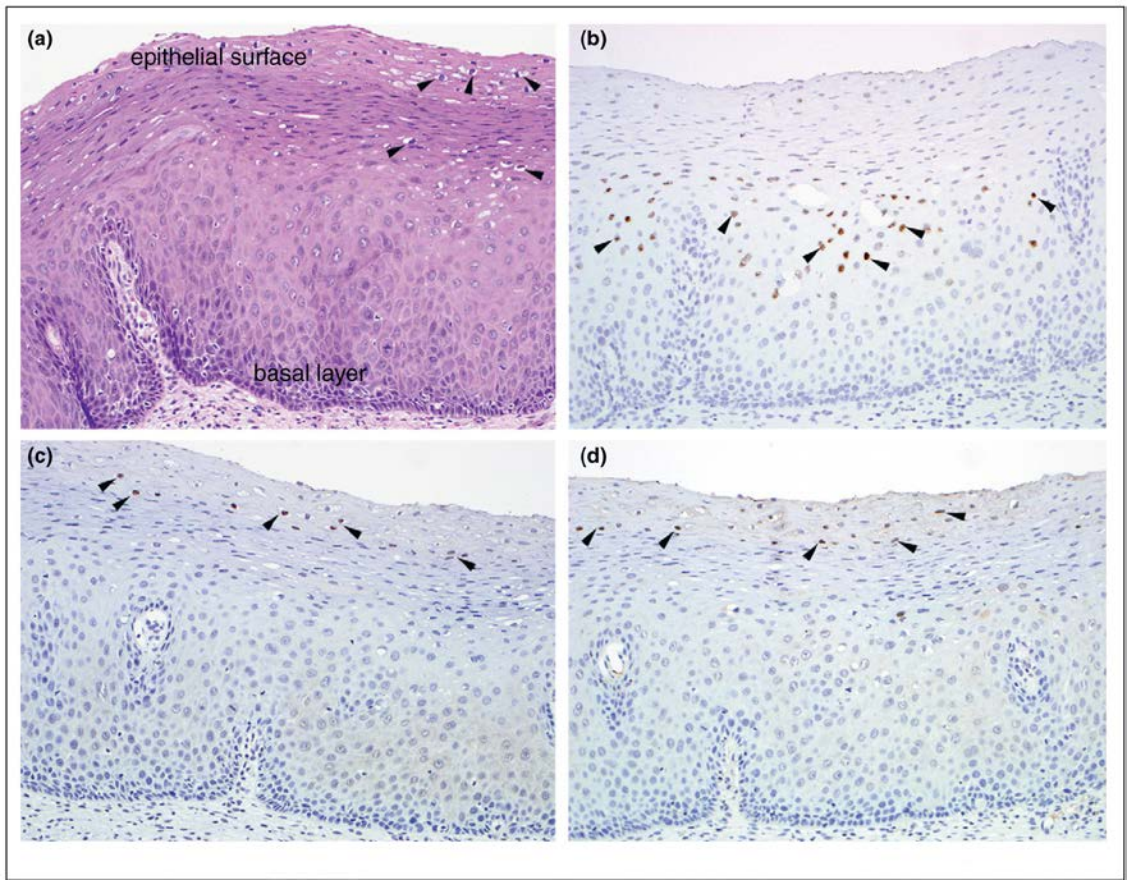


Figure 1. Localization of capsid protein expression and viral genomes in a productive HPV16 lesion (low grade squamous intraepithelial lesion (LSIL/CIN1)).

(a) Hematoxylin and eosin-stained section of cervical squamous epithelium; keratinocytes with markedly enlarged nuclei surrounded by cytoplasmic halo (arrowheads) in the upper epithelial layers characteristic of LSIL. (b) In situ hybridization for HPV16 genome; discreet nuclear signals (arrowheads) predominantly in the middle zone of the epithelium where the proliferating cells are just beginning to differentiate. (c) Immunohistochemistry of L1 using monoclonal antibody 1H8. (d) Immunohistochemistry for L2 using monoclonal antibody RG-1. Positive immunolabeling in the occasional keratinocyte nuclei limited to the upper epithelial

layers (arrowheads). The exquisite temporal/spacial regulation of viral genome copy number and late gene expression is apparent in this productive HPV16 lesion. In the basal epithelial cells the viral genome copy number is low (~100 episomes/nucleus), but as the cells divide and move up through the epithelium their differentiation triggers vegetative viral replication (producing a high copy number of ~10^4 episomes/cell), and in the uppermost regions of the lesion expression of the major L1 and minor L2 capsid proteins.

b) Immunodominant and conformationally dependent neutralizing epitopes of L1 VLP

Since HPV utilizes the host DNA polymerase for replication of its genome at a low error rate, HPV sequences have remained very stable, although it is likely that the different HPV genotypes have emerged over eons based on distinct tissue tropism (e.g. cutaneous versus mucosal epithelia) and to escape immune responses [4]. Indeed, while the sequences of the internal regions of L1 are highly conserved, the surface loops are hypervariable between HPV types and correspond to type-restricted and immunedominant neutralizing epitopes [5]. This suggests that the preferential accumulation of these changes in the hypervariable loops may reflect both selection to escape protective antibody responses and a lack of structural constraints in these loops. Thus L1 VLP-reactive neutralizing antibodies predominantly recognize only the type against which they were raised and consequently most genotypes are different serotypes [6]. Another notable feature is that the neutralizing antibodies overwhelmingly recognize conformational/non-linear epitopes, and this may also contribute to the specificity of binding [7]. Thus the licensed vaccines target the two most common HPV types found in cervical cancer, HPV16, and HPV18 (and one also targets the two most prevalent types in benign genital warts, HPV6, and HPV11) and this produces robust protection against persistent infection and intraepithelial lesions caused by these HPV types, but variable efficacy of protection of potentially reduced duration for other related HPV types [8,9]. For example vaccination with HPV16 L1 VLPs can also provide strong protection against HPV31 [10,11]. This is presumably because the sequence of HPV31 L1 is evolutionarily most closely related to HPV16 [12]. However the cross-neutralizing antibody titer is orders of magnitude lower than to the HPV16-specific response (T Kemp et al., abstract

BS2, 26th International Papillomavirus Conference, Montreal, Canada, July 2010). While this suggests that low titers of neutralizing antibody are sufficient for protection, it raises the question of the longevity of the cross-protective response. Similarly, HPV18 L1 VLPs can trigger an HPV45 cross-neutralizing and cross-protective response, but protection against other more divergent types is weak [10,11,13]. This contrasts the T cell responses to HPV L1 VLP which show some type restriction but in general are very broad, likely reflecting recognition of epitopes within the conserved internal portions of the L1 capsid [14]. As a consequence of the type-restricted nature of L1 VLP vaccines, there are ongoing clinical trials of a highly multivalent vaccine, comprising L1 VLPs of the two types found in ~90% of genital warts (HPV6 and HPV11) and the seven most prevalent HPV types detected in ~90% of cervical cancer cases (HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58). While both of the licensed vaccines utilize an adjuvant, L1 VLPs are remarkably immunogenic and produce robust neutralizing antibody responses even when patients are vaccinated without an adjuvant [15]. Indeed, a single dose of the licensed vaccine provides at least short-term protection, although the standard regimen is three immunizations (AR Kreimer et al., abstract LBA, 26th International Papillomavirus Conference, Montreal, Canada, July 2010). It is likely that several features of L1 VLP contribute to this immunogenicity. Firstly, their highly regular and close-packed display of neutralizing epitopes on the surface of the VLPs provides increased avidity for and crosslinking of reactive B cell receptors lowering the threshold for their activation compared to a monovalent antigen [16]. Secondly, L1 VLP can inherently activate both immature human myeloid and plasmacytoid dendritic cells (but not Langerhans cells) [17-19]. Thirdly, in contrast to typical protein antigens, L1 VLP are

rapidly taken up by immune cells facilitating presentation of MHCI and II epitopes, and producing robust cellular immune responses to vaccination [20].

c) Mechanism of protection and immune correlates

An important unanswered question is how vaccination with L1 VLP mediates protection. This question has been addressed in animal models, notably cottontail rabbit papillomavirus (CRPV) challenge of rabbits and canine oral papillomavirus (COPV) challenge of dogs. Specifically, passive transfer of sera from L1 VLP immunized animals protected na¨ive animals from experimental viral challenge [21,22]. This implies that neutralizing antibodies are sufficient to mediate protection, but does not rule out a contribution of cell-mediated immunity in protection. However, parenteral vaccination with L1 VLP did not impact wart growth in animal models, suggesting minimal impact of L1-specific T cell responses on established lesions. It should also be noted that vaccination with L1 VLP induced type-specific neutralizing antibodies and protection in animals, and that vaccination with denatured L1 failed to induce significant titers of neutralizing antibodies and was not protective.

Clinical studies of potential immune correlates of protection have centered upon L1-specific serum antibody responses, as measured by Competitive Luminex-based Immuno-Assay (CLIA), L1 VLP ELISA, and *in vitro* neutralization studies [23]. Each of these assays indicates that almost all vaccinated patients generate a robust type-restricted serum antibody response to L1 VLP, corresponding with the high efficacy of type-restricted protection. The serum antibody titers wane to a plateau a few months after the final immunization and appear stable thereafter. In one study of the licensed quadrivalent HPV vaccine, HPV18 L1 VLP-specific antibody titers determined by CLIA waned below the threshold of detection in 40% of patients by 48 months although the titers to the three other types in the vaccine remained detectable [24]. Despite the absence of CLIA-

detectable HPV18 L1 VLP-specific antibodies, no new HPV18 infections were observed, suggesting that immunity remained [24]. This suggests that either immunity to HPV18 in these patients is cell-mediated, possibly by the reactivation of memory B cells, or the titer of neutralizing antibody required for protection is very low and its measurement by CLIA lacks adequate sensitivity. Although L1 VLP vaccination induces robust T cell responses in patients, the absence of a profound therapeutic effect of the licensed vaccines upon established HPV infection suggest that it is unlikely that the protection against HPV18 in the absence of a CLIA-detectable antibody response is cytotoxic T cell-mediated. Alternatively, it is possible that exposure to the viral inoculums triggers a reactivation of the B cell response and local production of neutralizing antibody in time to prevent an initial infection. However, several factors point to the limited sensitivity of the CLIA for detection of HPV18 L1-specific antibody as an explanation for this observation, and that low, but protective levels of neutralizing antibodies are maintained. Firstly, the threshold of this assay is set using antibody levels in natural infection, and it is possible that these titers are protective. Secondly, CLIA detects responses via competition of patient antibodies with a high avidity neutralizing monoclonal antibody to L1 VLP, and thus primarily detects high avidity responses to a single epitope. It is known that multiple neutralizing epitopes are displayed by L1 VLP, and thus these patients may have protective antibodies to other epitopes, and/or protective antibodies of lower avidity that are poorly detected by CLIA.

While the L1 VLP ELISA assay is more sensitive than CLIA, it also detects non-neutralizing and presumably nonprotective antibodies and may provide false positives. *In vitro* neutralization assays measure functional antibodies, but native HPV virions are hard

to generate for all types and in sufficient quantities. Furthermore, the readout of infection is early spliced viral mRNA assayed by quantitative RT-PCR, and this is relatively cumbersome. A technology based on infection of 293TT cells with HPV pseudovirions that carry marker genes has greatly simplified the *in vitro* neutralization assay [25]. Nevertheless, recent passive transfer studies in mice suggest that the *in vitro* neutralization assay may lack the sensitivity to detect the minimal protective level of antibody, and that the passive transfer approach may provide a better approach, albeit low throughput [26]. This may reflect differing mechanisms of infection and thus neutralization *in vivo* within the genital mucosa [26] as compared to *in vitro* with 293TT target cells [27] (Figures 2 and 3, respectively). *In vitro* neutralization assays also depend upon extensive dilution to measure antibody levels, and it is possible that low avidity antibodies are protective and poorly detected by this *in vitro* assay [28]. Nevertheless, the range of HPV types cross-neutralized by the sera of L1 VLP-vaccinated patients appears to correspond to the breadth of protection.

That serum L1 VLP-specific antibody titer better correlates with protection than the robust L1-specific cell-mediated response raises several questions. Firstly, why does the L1-specific cellular immune response not clear established HPV infections? This may be explained in part by the unique biology of HPV [29]. HPV does not produce viremia, but rather is confined to epithelial lesions above the basement membrane and systemic T cell responses may not reach these lesions or may be suppressed therein. Most importantly, while all HPV infected cells express early genes E6 and E7, the capsid proteins are only expressed in the upper differentiating and dying layers (Figure 1). Consequently, L1-specific cellular immune responses do not target the basal epithelial

cells that harbor the infection, unless some type of bystander response can be triggered [29]. The second surprise is that serum neutralizing IgG titers are the relevant correlate for a purely mucosal infection and begs the question of how these antibodies reach the viral inoculum since systemic vaccination typically fails to induce a local IgA response (Figure 2). L1 VLP-specific IgG is detected in the vaginal fluid of vaccination patients and its level correlates with serum titer, although it varies with the menstrual cycle [30]. This indicates the occurrence of either active transport or passive transudation of the IgG into the vaginal fluid wherein it neutralizes the viral inoculum. However, it does not explain protection at cutaneous sites against HPV6 and HPV11 infections induced by vaccination. A second, not mutually exclusive, possibility is that the microtrauma that is associated with infection during intercourse and facilitating access for HPV to the basal epithelia may trigger a local exudation directly from plasma to the site of infection (Figure 2). It is unclear whether a minimal level of neutralizing antibody must be maintained sufficient to provide sterilizing immunity, or whether the viral inoculum can trigger a rapid anamnestic activation of memory B cells to produce neutralizing antibody locally [31]. The slow course of HPV infection and the ability to neutralize the virus many hours after binding to cell surfaces suggest that the latter is a possibility [32]. Indeed re-vaccination of individuals clearly triggers a robust anamnestic antibody response, but it is unclear if it happens within the window for neutralization [33].

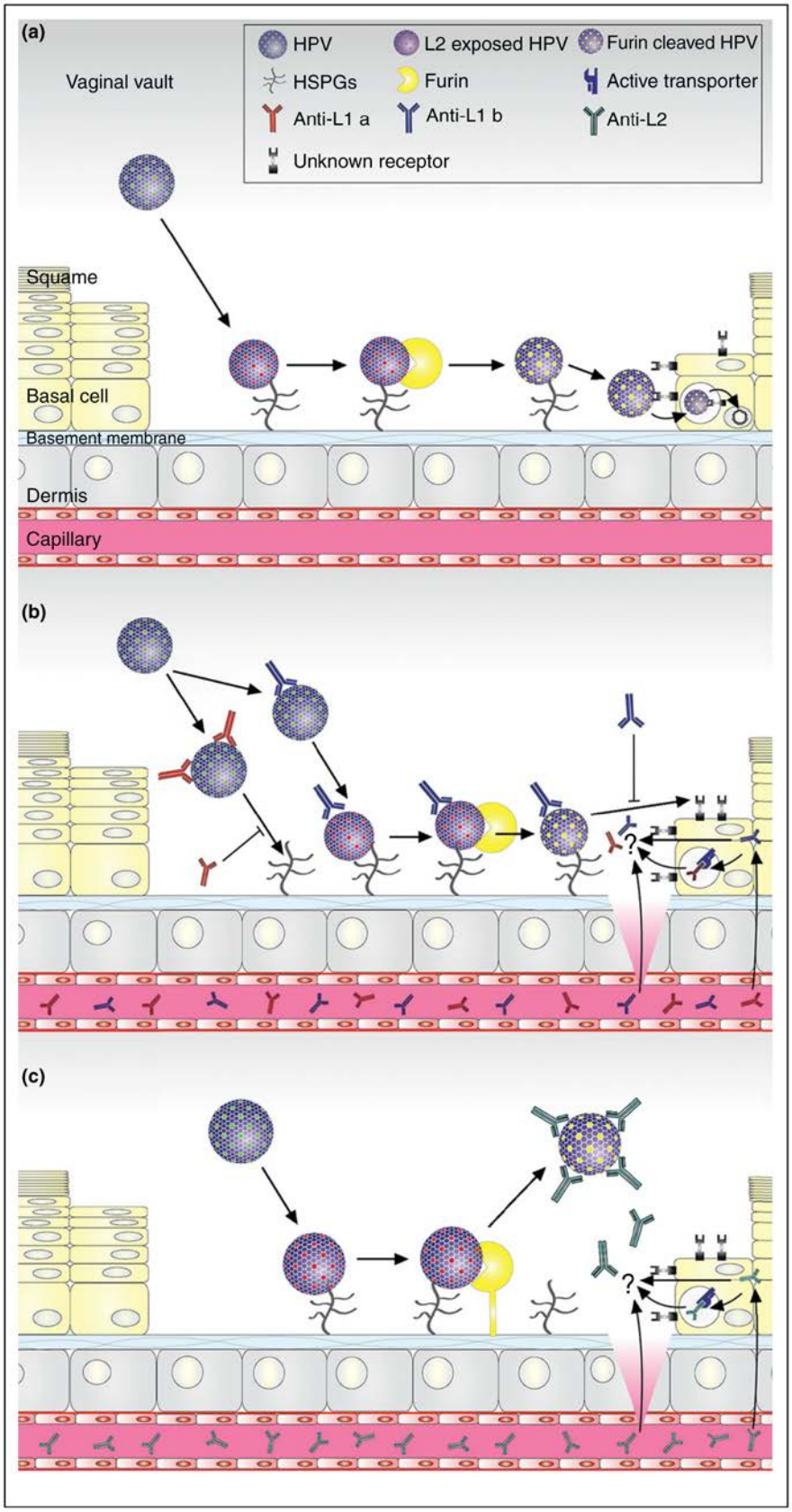

(a)
Vaginal vault
HPV
L2 exposed HPV
Furin cleaved HPV
HSPGs
Furin
Active transporter
Anti-L1 a
Anti-L1 b
Anti-L2
Unknown receptor
Squame
Basal cell
Basement membrane
Dermis
Capillary
(b)
?
(c)
?

Figure 2. Models for the mechanisms of HPV infection *in vivo* and antibody-mediated protection.

Day et al. have proposed models of infection and immunity based upon microscopic studies of HPV pseudovirions during vaginal challenge of naı̈ve mice and those immunized with L1 or L2 [26]. (a) Infection of vaginal epithelium. Microtrauma to the squamous epithelium of the vagina/and or cervix associated with intercourse provides HPV access to the basement membrane. HPV binds to the basement membrane via heparan sulfate glycosaminoglycans (HSPGs) and this triggers a conformational change in the capsid exposing L2 for N-terminal clipping by secreted furin. The furin-cleaved HPV binds to a viral receptor on the surface of basal epithelial cells during wound healing to initiate infection. (b) L1 VLP-specific antibody-mediated protection against vaginal infection. In hosts vaccinated with L1 VLPs, neutralizing IgG passively transudates from the capillaries into the cervical/vaginal fluid and/or is actively exchanged. If the trauma is sufficient, direct exudation from plasma is possible at the site of wounding. High concentrations of L1 VLP-specific antibodies can prevent virions binding to the basement membrane. In the presence of lower antibody levels the binding of HPV to the basement membrane, and the cleavage of L2 by furin still occurs, but the virions are unable to transfer to the viral receptor on the basal epithelial cells and the virion-antibody complexes are released. (c) L2-specific antibody-mediated protection against vaginal infection. In the presence of L2-specific antibody, the binding of HPV to the basement membrane and the exposure of L2 occur. However, the antibodies bind to epitopes in the N-terminus of L2 after its cleavage by furin and the virions are unable to transfer to the viral receptor on the basal epithelial cells, leading to release of the virion-

antibody complexes.

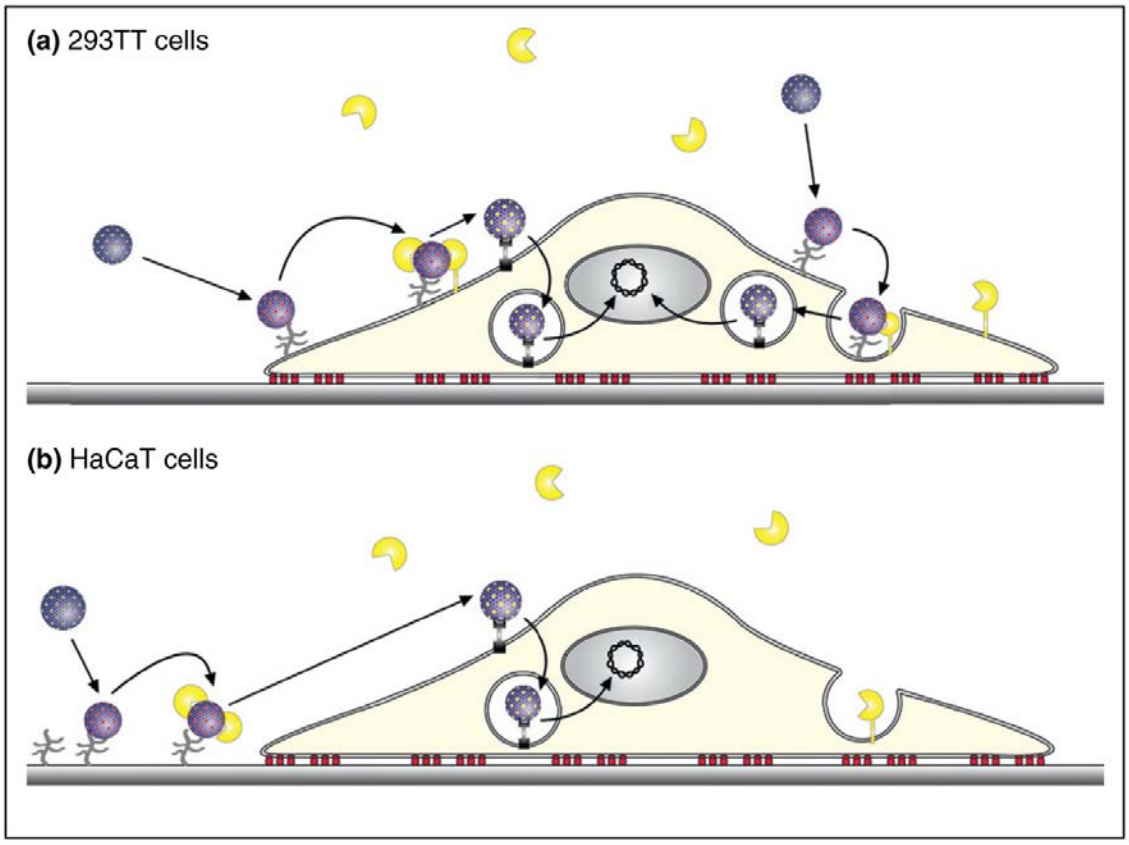


Figure 3. Model of *In vitro* infection of 293TT and HaCaT cells by HPV (based upon [26,27]).

(a) *In vitro* infection of 293TT cells. Early studies of *in vitro* infection by HPV utilized 293TT or other transformed cell lines as target cells. HPV binds directly to the 293TT cell surface via HSPGs which triggers a conformational change in the capsid such that the amino terminus minor capsid protein L2 becomes exposed. Exposure of L2 renders it accessible to cleavage by furin [50]. Furin-cleavage of L2 is essential to infection and is associated with escape of L2 and the viral genome from the endosome. g secretase activity is also required for infection, but it is unclear what it cleaves [51]. The viral genome–L2 complex is too large to cross the nuclear envelope, but gains access to the nucleus as during mitosis while its membrane has dissolved [52]. (b) *In vitro* infection of

HaCaT cells or primary keratinocytes. In contrast to 293TT cells, HPV binds first to the laminin-5 associated extracellular matrix via HSPGs secreted upon the culture surface before infection of the immortalized keratinocyte line HaCaT or primary keratinocytes. Upon attachment to the extracellular matrix, the HPV virions undergo a conformational shift that exposes L2 for cleavage by secreted furin. Only then can the virions bind to a secondary receptor on the keratinocyte surface for subsequent uptake. Antibodies reacting with particular loops on the capsid surface (e.g. H16.V5 and H16.E70 monoclonal antibodies) can prevent virions binding to the extracellular matrix, but these antibody-bound virions can still bind the cell surface, although infection does not occur. In the presence of antibodies reacting with other surface L1 loops (e.g. H16.U4 monoclonal antibody), the binding of HPV to the basement membrane, and the cleavage of L2 by furin still occur, but the virions are unable to transfer to the viral receptor on the basal epithelial cells [27]. Likewise, L2-specific antibodies (e.g. RG-1 monoclonal antibody) allow the binding of HPV to the basement membrane, and the exposure of L2. The antibody binds to the exposed L2 after its cleavage by furin and the virions are unable to transfer to the viral receptor on the basal epithelial cells [27].

d) Candidates for second generation HPV vaccines

The identification of a correlate of protection is important to identify whether an individual immunization is successful, to validate batches or sources of L1 VLP vaccine produced by different manufacturers and/or in different systems (e.g. bacteria or plants [34,35]) and also in the development of second generation preventive HPV vaccines. One such potential second generation vaccine comprises L1 capsomers (the pentameric subunit of the VLP) that can be readily produced in bacteria [36]. The L1 capsomer-based vaccine offers potential advantages of reduced cost, stability at ambient temperature that could facilitate introduction into low resource settings where HPV vaccines are most needed. Although L1 capsomers induce lower neutralizing antibody titers than VLP, protection is robust even without adjuvant and the use of an adjuvant can potentially close this gap [36,37]. Another approach of HPV vaccination is the use of naked DNA vectors, which are simple to manufacture, heat stable and delivered by gene gun, tattoo or electroporation to express codon-modified L1 *in vivo* [38]. The use of needles for immunization also provides a barrier to widespread use, and there are efforts to use live recombinant vectors, such as an L1 recombinant version of the orally administered typhoid vaccine *Salmonella typhi* [39]. The VLP vaccine could also be combined with other vaccines, either by mixing them together or by introducing the L1 gene, for example into measles vaccine, or the tuberculosis vaccine BCG to deliver L1 [40,41].

In selecting a correlate of protection, it should be both simple to use, readily standardized into predefined international units and ideally not specific to L1. The latter point is relevant because of several efforts to second generation HPV vaccines based

upon the minor capsid antigen L2 [42]. Vaccination with L2 protects animals from experimental viral challenge, but this immunity lacks the type restriction associated with L1 VLP vaccines [43,44]. The protection is mediated by broadly neutralizing antibodies that recognize conserved linear epitopes in the N-terminus of L2 [43,45]. Residues within the protective region of L2 play a critical role in viral infection and thus their sequence is conserved even in diverse HPV types [46,47] (Figure 3). The L2 cross-neutralizing epitope appears to be displayed on the capsid surface only during infection, and thus vaccination with virions or L1/L2 VLPs induces limited, if any, L2-specific antibody [27,44]. Unfortunately L2 does not form a particulate structure alone and therefore is significantly less immunogenic than L1 VLP, suggesting the need for an adjuvant for vaccination or for multimeric display of L2 in an immune-dominant epitope of a recombinant VLP [48,49].

B. Chapters

I) Impact of inhibitors and L2 antibodies upon the infectivity of diverse alpha and beta human papillomavirus types

a) Introduction

Human papillomaviruses (HPV) comprise a family of at least 120 non-enveloped epitheliotropic viruses which contain a double-stranded circular DNA genome and are phylogenetically classified into five genera; alpha, beta, gamma, mu and nu [12]. Papillomavirus infections generally produce benign papillomas or warts of either skin or mucosa, such as condylomata accuminata (anogenital warts) associated with 'low-risk' types HPV6 and 11. However, the sexually transmitted 'high-risk' members of the alpha genus mucosal HPVs are essential etiological agents in cervical cancer, and also in a significant fraction of anal, penile, vaginal, vulval and oropharyngeal cancers [2,53]. HPV16 and HPV18 are the most impactful high-risk HPV types, together causing 70% of cervical cancer, with a dozen or so other alpha HPVs associated with the remaining cases [3,54], although it is important to recognize that the majority of infections are cleared by patients. The beta HPVs infect skin beginning early in childhood and are associated with non-melanoma skin cancer in sun-exposed areas of immunocompromised patients or those with the rare hereditary disease epidermodysplasia verruciformis (EDV), notably HPV5 and HPV8 [55]. Conversely, beta HPV infections are generally clinically inapparent in immune competent patients, but may cooperate with UV-induced DNA

damage in the development of cutaneous squamous cell cancers [55]. Infections with HPV of the gamma, mu and nu genera typically produce benign and self-limiting skin warts [12].

Papillomavirus virions have a non-enveloped 60nm diameter capsid with T=7*d* iscosahedral symmetry [56]. The capsid is formed from 360 molecules of the major capsid protein L1 via assembly of 72 star-shaped capsomers or pentamers, each comprising five L1 molecules. The capsid also contains as many as 72 molecules of the minor capsid protein L2 and, while its location is not totally clear, at least a portion of L2 is buried at the base of central cavity at the center of each capsomer [57]. Five surface loops of L1 with high variation in amino acid sequence among different types contain the immunodominant neutralization epitopes and act as domain linkers for the conserved internal jelly roll structure [5].

Recombinant expression of L1 is sufficient to form virus-like particles (VLP) that mimic native virus morphologically and immunologically. Similar to infectious virions, VLP can bind to heparan sulfate proteoglycans (HSPG) on the cell surface, and HSPG mimetics such as soluble heparan sulphate or carrageenan compete this interaction [58-64]. Indeed, consistent use of the carrageenan-based vaginal microbicide, Carraguard, with condoms was negatively associated with the acquisition of high-risk HPV infections in a randomized, double-blind and placebo controlled trial [65]. L2 facilitates viral genome encapsidation during virion assembly and is critical for infection [6,66]. At the initiation of infection, virions undergo a conformational change that reveals the amino terminus of L2 on the capsid surface such that it is cleaved by the cellular protease furin [50,67]. Furin cuts L2 at residue 11 in HPV16 and this cleavage is required for infection

[50]. L2 interacts with several cellular components, including cyclophilin, annexin, syntaxin-18 and sorting nexin 17, believed to contribute to infectious cell entry, although the mechanisms are not fully elucidated [68-71]. L2 also enables virion egress from the endosome and translocates with the viral genome to the nucleus to establish infection [72]. The regulated intra-membrane protease γ-secretase is required for endosomal escape, but its cellular target remains to be defined [51,73].

The licensed HPV vaccines, Cervarix and Gardasil, were both developed to prevent HPV16 and HPV18 associated persistent anogenital infection and disease, in particular epithelial dysplasias and cancers, and Gardasil to also prevent benign genital warts associated with HPV6 and HPV11 [74]. Vaccination elicits type-restricted neutralizing antibodies and protection due to immunodominant epitopes formed by hyper-variable surface loops of the L1 VLP antigens [5]. Both vaccines provide limited cross-protection, particularly toward HPV31 or HPV45, close phylogenetic relatives ofHPV16 and HPV18, respectively [11,75,76]. However the duration of cross-protection is unclear, and is weak to ineffective for more distantly related types [77]. A nonavalent L1 VLP vaccine is currently in development to broaden protection among the high-risk mucosal alpha HPV types. Another approach is vaccination with the amino terminus of L2 including residues 11-88 [48,78,79]. This region of L2 is well conserved and can elicit broadly neutralizing antibodies, although at lower titer than L1 VLP, because L2 does not form an immunogenic VLP structure. However, because L2 protective epioptes are linear, those derived from multiple HPV types can be concatenated into a single antigen, e.g. α11-88×8 that compromises a fusion of the 11-88 region of the eight medically significant alpha HPV types 6, 16, 18, 31, 39, 51, 56 and 73. Vaccination with L2 α11-

88×8, or passive transfer of its antiserum, provides broad immunity against vaginal challenge with alpha HPV, but its activity against other genera has not been examined [78].

Our mechanistic understanding of papillomavirus infectious process(es) is based on studies generally performed with only a few HPV types. Here we examine if medically significant HPV types of different genera, carcinogenic potential and tropism share a common infection mechanism. As the known inhibitors and vaccines have also been tested predominantly against a limited number of HPV types, their breadth of activity cannot be fully appreciated. Further, several studies indicate differences in receptor usage and uptake pathways by different HPV types [62,80,81]. Because of the technical difficulties of culturing and measuring the infectivity of papillomaviruses *in vitro*, PsV are used as surrogates for biologic studies of native virions, particularly given their capacity to deliver a reporter construct upon its encapsidation by over-expression of L1 and L2 [82]. In light of this, we generated PsV for 34 HPV types from the alpha and beta genera to better understand the commonalities and differences in the infectious pathway of diverse HPV types with distinct biology.

b) Materials and Methods

1) Pseudovirion (PsV) production

L1/L2 expression plasmids were provided by Martin Müller and Lutz Gissmann (DKFZ, Heidelberg, Germany), Christopher Buck and John Schiller (NCI, Bethesda, MD), and Helena Faust (Lund University, Malmö, Sweden) and Joakim Dillner (Karolinska Institutet, Stockholm, Sweden). Additional constructs were synthesized by Biobasic (Supplementary Table 1). Briefly, the L1 and L2 genes for individual isolates of HPV genotypes documented in Genbank were codon-optimized for expression in human cells and cloned with a 5' Kozak sequence (GCCACC) and two 3' stop codons (TAATAG) into the mammalian double expression vector pVITRO1-neo-mcs (InvivoGen) as listed in Supplementary Table 1. The L1 gene flanked by BamHI-XbaI was cloned into the BglII-NheI region of MCS2, followed by the L2 gene flanked by BamHI-XbaI which was cloned into the BamHI-AvrII region of MCS1. All HPV PsV were produced as described in http://home.ccr.cancer.gov/Lco/pseudovirusproduction.htm. Briefly, plasmids containing L1 and L2 late genes of different HPV types were transfected with plasmid expressing luciferase reporter gene into 293TT cells utilizing TransIT-2020 transfection reagent. Cells were incubated for 48 h at 37°C, harvested, washed with DPBS-Mg (DPBS supplemented with 9.5 mM $MgCl_2$), and lysed with equal volume of lysis buffer (DPBS supplemented with 0.5% w/v Brij58 and 0.2% v/v benzonase). For PsV maturation, cell lysates were incubated for 24 h at 37°C and adjusted to 850mM NaCl. For extraction lysates were centrifuged at 10,000 x *g* for 10 min, and clarified supernatants loaded onto

a 12mL step gradient of Optiprep (39, 33, 27% *w/v*), and spun at 40,000 rpm in a SW40 rotor for 15 h at 16°C. After centrifugation, 1mL fractions were collected from the top layer and the fraction demonstrating the highest infectivity was selected for further experiments.

2) HPV Infectivity assays

1.5×10^4 293TT, HeLaT or HaCaT cells in 100 μL DMEM-10 or KH-SV cells in 100 μL KSFM were infected with 250, 500, 1000, or 2000 million particles of each HPV types in 100 μL medium in triplicate and incubated for 72 h at 37°C. Luciferase activity was measured as described in inhibition assays. All experiments were performed in triplicate.

3) HPV Inhibition assays

293TT cells were seeded at 1.5×10^4 cells in 100 μL 10% FBS DMEM (DMEM-10) per well in 96-well plates and allowed to attach overnight. For heparin and carrageenan inhibition studies, heparin (Sigma H-4784) and carrageenan (Sigma C1138) were serially diluted in DMEM-10 and incubated with 250 million PsV particles of different HPV types in 100 μL DMEM-10 for 1 h at 37°C. Mixtures were added to 293TT cells and incubated at concentrations of 64, 128, 256, 512 μg/mL of heparin or carrageenan for 72 h at 37°C. For furin inhibition studies, furin inhibitor 1 (Decanoyl-RVKR-CMK) was mixed with 250 million particles of each HPV type in 100 μL DMEM-10 and the mixtures were immediately transferred to 293TT cells and incubated at 20 μM concentration of furin inhibitor 1 for 72 h at 37°C. For γ-secretase inhibition

studies, XXI was mixed with 250 million particles of each HPV type in 100 μL DMEM-10 and the mixtures were immediately transferred to 293TT cells and incubated at a final concentration of 500nM XXI for 72 h at 37°C. As a positive control, 4 μL of L1 antiserum from mice vaccinated i.m. with L1 DNA utilizing electroporation in 50 μL of culture medium was mixed with 250 million particles of each HPV type in 50 μL. Mixtures were transferred to 293TT cells and incubated for 72 h at 37°C. Following infection cells were washed with 1X PBS, and lysed with 30 μL of Cell Culture Lysis Reagent on rocking shaker for 15 min at room temperature. Lysates were transferred to 96-well black plate and luciferase activity was measured by GloMax®-Multi Detection System after adding 50 μL of luciferin substrate to each well. All experiments were performed in triplicate.

4) Neutralization assay

293TT cells were pre-plated at 1.5×10^4 cells per well in 96-well plate and incubated 24 h at 37°C. 4 μL of rabbit L2 α11-88×8 antiserum [78] was serially diluted two-fold in 50 μL of culture medium. As a negative control, pre-immune serum was applied. 250 million particles of each HPV type in 50 μL of culture medium were mixed with serially diluted serum in triplicate and then transferred to pre-plated 293TT cells and cultured for 72 h at 37°C. Luciferase activity was measured as described above.

5) SDS-PAGE and Western blotting

Each HPV PsV preparation was normalized by the amount of L1 protein. Samples were boiled for 5 min with reducing gel sample buffer, subjected to SDS-PAGE analysis

using 4-20% Tris-HCl gels, and proteins were transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk in PBST (PBS containing 0.1% Tween-20) at room temperature for 1 hour, incubated with a rabbit L2 α11-88×8 antiserum at room temperature for 1 hour and binding detected with HRP-conjugated goat anti-rabbit IgG secondary antiserum.

6) Quantitative real time PCR measurement of luciferase reporter plasmid

Reporter plasmid was extracted from 20 μL of each HPV PsV preparation using PureLink Viral RNA/DNA extraction kits. The real time PCR was performed with 25 μL of universal PCR Master Mix, 900 nM of forward and reverse primers (TTG ACC GCC TGA AGT CTC TGA and ACA CCT GCG TCG AAG ATG TTG), 250 nM of TaqMan probe (6FAM-CCG CTG AAT TGG AAT CCA TCT TGC TC-TAMRA), and 5 μL of extracted reporter plasmid in 50 μL final volume using an I-Cycler IQ (Bio-Rad). Each sample was conducted in duplicate.

7) Data analysis and Statistics

To calculate the neutralization titer value (the reciprocal of the dilution that causes 50% reduction in luciferase activity), the non-linear model Y=Bottom + (Top-Bottom) /(1+10^((LogEC50-X)*HillSlope)) was fitted to the $\log_{10}$ transformed neutralization data using Graphpad Prism 6 and the estimated EC50 [with 95% confidence intervals] is reported as the titer. Comparisons between the infectivity of members of papillomavirus species was determined by the Mann Whitney test, and means ± standard error are presented graphically using Graphpad Prism 6.

c) RESULTS

1) Generation and characterization of 34 HPV PsV types

PsV are useful surrogates for biologic studies of native virions, particularly given their capacity to recapitulate early events of papillomavirus infection and neutralization, deliver a reporter gene of choice and their relative ease of production [82]. Here we encapsidated a vector expressing firefly luciferase (pY-luc) into HPV PsV because of this reporter's low background, high sensitivity and utility in the mouse challenge model [78]. Recombinant mammalian expression of papillomavirus capsid genes is dramatically enhanced by their codon modification [83,84], presumably reflecting the disruption of negative regulatory sequences [85]. Several PsV vectors have been previously described [25,82,86-88] and we generated constructs for additional HPV types that were not previously available (Sequences in Supplementary Table 1 and constructs may be obtained from Addgene (www.addgene.com)). HPV PsV were prepared by co-transfection of 293TT cells with pY-luc and the L1/L2 expression vectors per standard methodology [82].

We first tested infectivity of each HPV PsV type normalized to 250 million particles (as estimated based on L1 concentration) by measuring luciferase activity (relative light units, RLU) 72 h post-infection in 293TT cells. The HPV PsV infected cells showed dramatic differences in reporter expression (Figure 4A). Overall, the cutaneous HPV type PsV showed lower infectivity compared to mucosal tropic types ($p<0.0001$), and the PsVs derived from alpha species HPV showed greater infectivity that those derived from beta HPV types ($p=0.01$). However, even for the weakest PsV types,

the signal observed was still more than 100-fold higher than background signal. Furthermore, pre-incubation of the PsV with rabbit antiserum raised against the cognate L1 VLP prevented reporter delivery, consistent with antibody-mediated neutralization of an infection rather than a non-specific transfection. Rabbit antiserum to L1 VLP was not available for a number of HPV types. Instead, sera were harvested from mice two weeks after electroporation i.m. three times at two week intervals with the an HPV L1 expression vector [89]. Pre-incubation of the HPV PsV with these mouse antisera similarly inhibited reporter transfer (Figure 5)

It is possible that the weak infectivity of the PsV derived from cutaneous HPV types reflects use of 293TT cells a target cell line, rather than one derived from skin cells. Therefore we also tested infectivity of diverse alpha and beta HPV PsV in HeLaT clone 4 cells, a human cervical cancer line transduced with SV40 large T antigen [90], as well as in the spontaneous immortalized human keratinocyte cell line HaCaT [91], and KH-SV, a human keratinocyte line immortalized by transduction with SV40 large T antigen [92]. In both human skin keratinocyte-derived lines (HaCaT and KH-SV), the cutaneous HPV PsV demonstrated minimal or undetectable signals, whereas mucosal HPV PsV showed more robust infectivity. Similar findings were seen for infection of HeLaT cells, implying that the weaker infectivity of the cutaneous HPV PsV does not reflect the use of 293TT as target cells or the need to infect a line derived from skin keratinocytes (Figure 6).

L2 is known to play several important functions during virion assembly and infection, and therefore differences in the incorporation of L2 may account for the wide range of PsV infectivity by HPV type. Thus, the level of L2 incorporation into the

gradient-purified preparations of HPV PsV was analyzed by Western blot (Figure 4B) using a broadly reactive rabbit antiserum to the L2 antigen α11-88×8 [78]. This Western blot analysis suggested that the PsV preparations of several cutaneous HPV types that exhibited low infectivity (Table 1) also contained low levels of L2, notably HPV23, 27, 38 and 57 (Figure 4B). This does reflect the failure of the antiserum to recognize the L2 of these HPV types because this antiserum, but not the pre-immune serum, can neutralize their infection. Furthermore, the L2 band was not apparent on Coomassie-stained SDS-PAGE analysis of these PsV. Rather, this reflects poor expression of L2 and thus low incorporation into the PsV, as Western blot analysis of 293TT cells transfected with these constructs shows low levels of L2 (data not shown).

Differences in the infectivity of each type of HPV PsV might also reflect variable efficiency in reporter plasmid encapsidation. Therefore the number of copies of benzonase-resistant, and therefore presumably encapsidated, reporter plasmid DNA present within 315ng of each gradient purified HPV PsV preparation was measured by quantitative real time PCR. The lowest levels of reporter DNA encapsidation were observed for PsV of HPV27, 57, and 34 (Figure 4C). Since L2 has been shown to facilitate genomic DNA encapsidation, the low DNA encapsidation by HPV types 27 and 57 may reflect the low levels of L2 incorporated in these PsV. However the amounts of encapsidated plasmid and/or L2 were not always proportional to the relative infectivity (Figure 4A,C), as seen for HPV18 and HPV35 PsV.

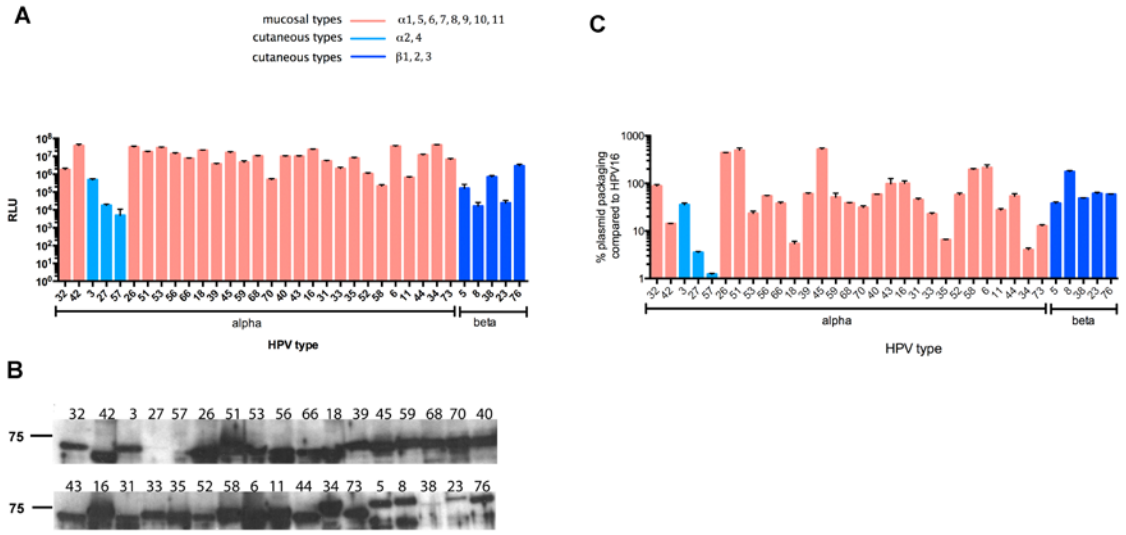


Figure 4. Assessment of infectivity, L2 incorporation, and reporter DNA encapsidation by PsV preparations of 34 HPV types.

(A) 250 million particles (as estimated based on L1 concentration) of HPV PsV were transferred to 293TT cells, incubated at 37°C for 72 hours before cell lysis. Luciferase activity was measured in cell lysate (n=4). (B) Western blot analysis detecting L2 in 100ng of each HPV PsV preparation using rabbit antiserum to L2 α11-88×8. (C) Reporter plasmid copy number of each PsV preparations for each HPV type sample was measured by quantitative real time PCR. Black and blue bars represent alpha and beta type, respectively, and mean ± standard error plotted.

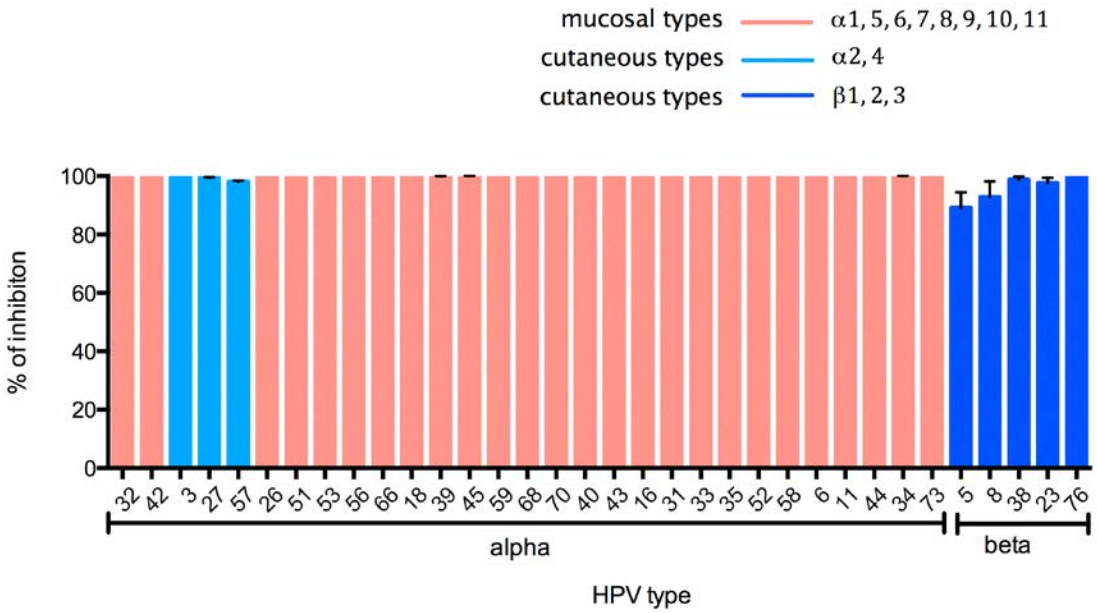


Figure 5. Neutralization of PsV by L1 VLP antiserum

PsVs of each indicated HPV type, each carrying a luciferase reporter gene, were mixed with mouse or rabbit L1 VLP antiserum or their respective pre-immune serum (each at 1:50 dilution) for two hours at 37°C, then the mixtures were transferred to 293TT cells and cultured for 72 hours (n=3). Cells were then lysed and luciferase activity was measured. Percent neutralization by L1 VLP antiserum was plotted. Pre-immune serum was non-neutralizing in all cases. Red and blue bars represent mucosal and cutaneous HPV types, respectively.

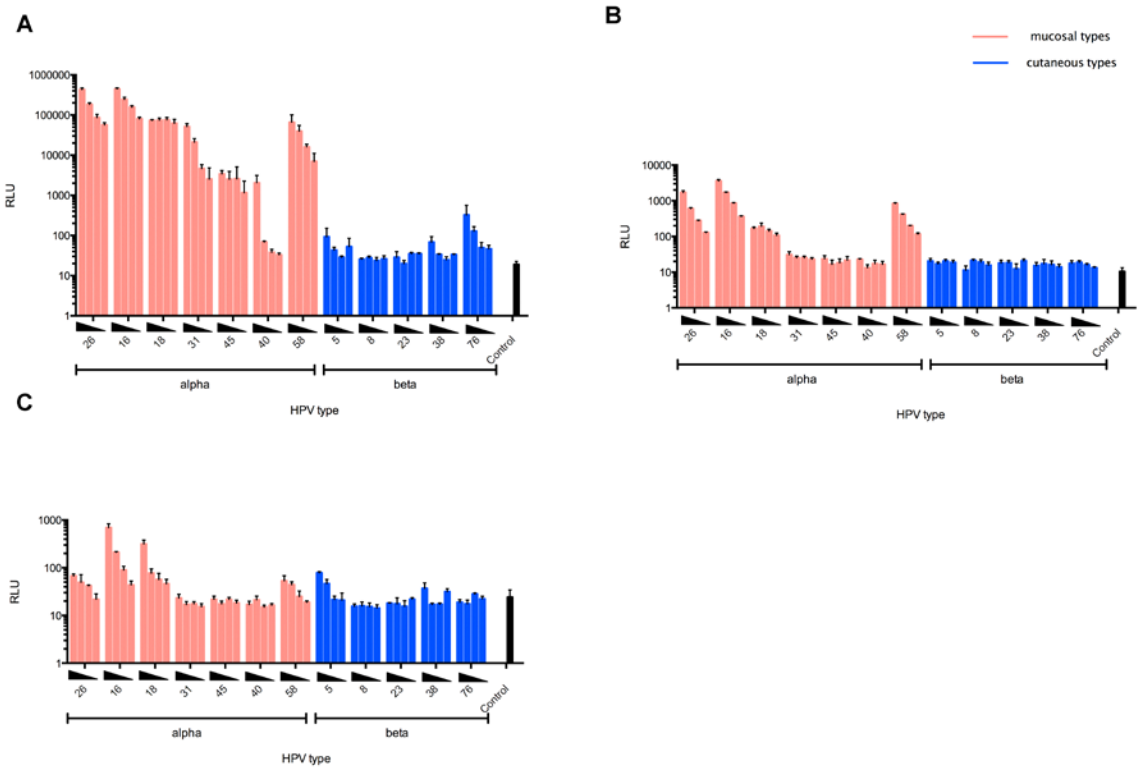


Figure 6. Infectivity of diverse HPV on mucosal and skin cell lines.

HeLaT (A), HaCaT (B), KH-SV (C) cells were treated with titrations of diverse HPV PsV from alpha and beta subfamilies and incubated at 37°C for 72 hours. Cells were lysed after incubation and luciferase activity was measured. Red and blue bars represent mucosal and cutaneous HPV types, respectively.

Subfamily	HPV type	Mucosal / Cutaneous	Predicted net charge at pH 7.4	Neutralization titer for L2x8 antiserum	95% confidence interval in neutralization titer
α1	32	M	5.2	3,360	2,225 to 5,074
	42	M	3.4	11,450	9,456 to 13,865
α2	3	C	2	11,140	7,314 to 16,967
α4	27	C	-4.2	3,942	350 to 44,402
	57	C	1	3,440	858 to 13,789
α5	26	M	5.2	11,223	7,786 to 16,177
	51	M	5.2	4,420	3,755 to 5,202
α6	53	M	6.3	352	208 to 595
	56	M	5.2	1,536	1,243 to 1,898
	66	M	7.2	870	517 to 1,465
α7	18	M	5.9	12,422	10,780 to 14,312
	39	M	5.1	6,216	3,798 to 10,174
	45	M	5.9	4,913	3,021 to 7,991
	59	M	7.1	8,214	7,255 to 9,300
	68	M	3.1	1,403	980 to 2,008
	70	M	4.1	2,359	1,382 to 4,027
α8	40	M/C	4.2	834	380 to 1,830
	43	M/C	6.2	1,580	1,063 to 2,349
α9	16	M	2	31,266	28,513 to 34,284
	31	M	8	2,212	940 to 5,207
	33	M	4.8	16,207	9,942 to 26,420
	35	M	7	7,458	5,151 to 10,800

	52	M	5.9	927	651 to 1321
	58	M	4.5	14,807	8,904 to 24,622
α10	6	M	7.1	5,553	4,315 to 7,146
	11	M	7.2	27,760	23,455 to 32,855
	44	M	7	5,041	2,716 to 9,358
α11	34	M	5.7	13,140	11,689 to 14,770
	73	M	4.9	8,810	6,853 to 11,325
β1	5	C	-4.7	3,561	492 to 25,770
	8	C	-2.8	17,679	7,256 to 43,073
β2	38	C	-4.8	1709	489 to 5976
	23	C	-3.9	2475	834 to 7347
β3	76	C	-4.9	2110	1154 to 3861

Table 1. Summary of the phylogeny, tropism, charge and *in vitro* neutralization by L2 11-88×8 antiserum of the 34 HPV genotypes tested.

Phylogeny and tropism were taken from de Villiers et al, [99] predicted net charge of L1 at pH7.4 was calculated as described in [80]. PsVs of each indicated HPV type carrying a luciferase reporter gene were mixed with titrated rabbit L2 α11-88×8 antiserum for two hours at 37°C, then the mixtures were transferred to 293TT cells and cultured for 72 hours. Cells were then lysed and luciferase activity was measured. Neutralization titer and 95% confidence interval are shown.

2) Inhibition of HPV infection by Heparin and ι-carrageenan

CHO cells deficient in heparan sulfate proteoglycan (HSPG) production are no longer infected by HPV PsV [58], suggesting that HSPG is an important entry factor. Further soluble heparan sulfate can bind to capsids and is an inhibitor of infection for at least some HPV genotypes, but not all [62]. To explore the generalizability of these observations, we tested if heparin universally inhibited infection by PsV of all 34 HPV types within the alpha and beta HPV subfamilies. Ten-fold serial dilutions of heparin (Sigma H-4784), ranging from 10 to 1000 μg/mL, were incubated with the PsV of each HPV type for 1 hour at 37°C prior to transfer to 293TT cells to assess infectivity. Near complete inhibition was observed for PsV of all 29 alpha HPV types at 1000 μg/ml heparin, including HPV 31 (Figure 7A) for which the role of HSPG in infection is controversial [93]. With the exceptions of HPV8 and HPV23, infection by PsV of the beta HPV types was not impeded even at the highest concentration of heparin tested (1000 μg/mL), and paradoxically several demonstrated increased levels of infectivity [94]. Notably, the addition of 10 or 100 μg/mL heparin profoundly increased infectivity of HPV5, HPV57 and HPV76 PsV, slightly increased the infectivity of HPV38 PsV, but inhibited HPV8 and HPV23 PsV (Figures 7A and C). In contrast, even at 10 μg/mL heparin, most alpha HPV type PsVs exhibited significant inhibition.

Carrageenan is also a potent inhibitor of HPV infection via a mechanism resembling that of heparan sulfate [60-63], although the breadth of its activity has been questioned recently [81]. It is currently being tested as a vaginal microbicide, and may be broadly protective against genital HPV transmission [65]. Therefore, we also tested the impact of carrageenan on *in vitro* infectivity of PsVs of diverse HPV types. The findings

resembled the inhibitory profile of heparin [62,80,95]. PsV of most alpha types were inhibited except HPV27 and HPV57, but carrageenan had limited impact on beta types (Figure 7B, D). Carrageenan was more potent in inhibiting alpha HPV PsV infection than heparin at 10 μg/mL (Figure 7), consistent with earlier findings [60]. Carrageenan also showed some differences in inhibitory efficacy against PsV of beta HPV types compared to heparin. For example, carrageenan did not inhibit HPV27 infection whereas heparin did, suggesting subtly distinct mechanisms of inhibition by carrageenan and heparin. Taken together, the results imply that different HPV subfamilies bind HSPG differently during infection, which may contribute to their differing tropism [62]. Further, heparin and carrageenan are broadly inhibitory against PsV of most alpha species of HPV (including all of the high-risk mucosal HPV types tested), but not against PsV infection by beta or certain benign alpha types that can also infect cutaneous sites.

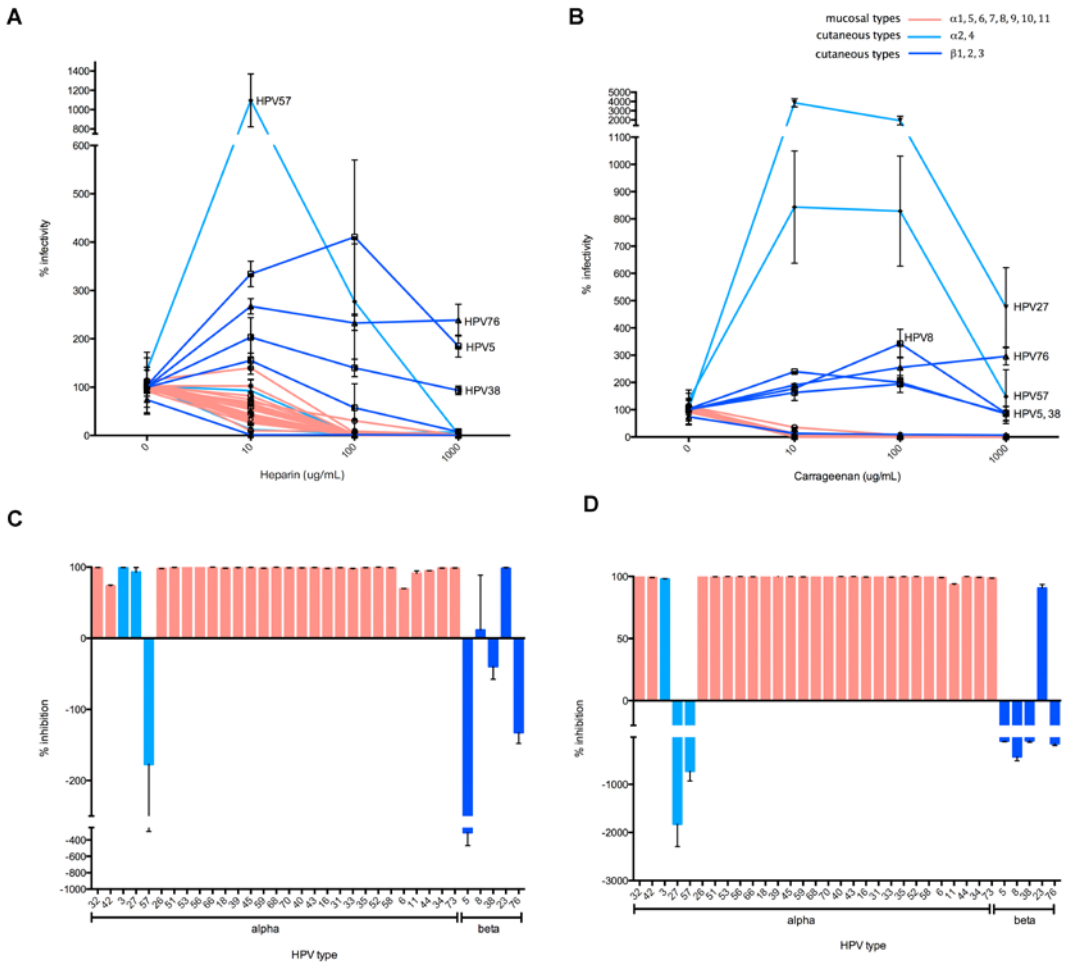


Figure 7. Inhibition of HPV PsV infection by titrations of heparin and carrageenan. PsVs of each HPV type carrying a luciferase reporter gene were incubated with 1000 µg/mL, 100 µg/mL, 10 µg/ml or 0 µg/ml heparin (A) or carrageenan (B) at 37°C for 1 hour. The mixtures were transferred to 293TT cells for 72h. After incubation cells were lysed and luciferase activity was measured, and percent infection compared to control was calculated (n=3). The percent inhibition of infection by each HPV PsV in the presence of 100 µg/mL of heparin (C) or carrageenan (D) was also plotted separately. Red and blue bars and lines represent mucosal and cutaneous HPV types, respectively.

3) Conservation of furin cleavage as an essential step for infection

After binding to HSPG on extracellular matrix, HPV16 undergoes a conformational change and exposes the previously buried amino terminus of L2 on the capsid surface prior to cell entry [50,67]. The exposed amino-terminus of L2 is sensitive to furin cleavage as it contains a conserved consensus cleavage site, Arg-X-(Arg/Lys)-Arg, and mutation of this site prevents HPV16 PsV infection. The furin inhibitor decanoyl-RVKR-cmk also can prevent HPV16 infection, suggesting cleavage of L2 by furin is a critical step for infection [50]. However, only a few HPV types have been tested and overall significance of furin cleavage among diverse HPV types has not been thoroughly addressed so far. Sequence alignment indicated that the furin target sequence, RX(R/K)R, is present in the L2 of all the HPV types for which we developed PsV, suggesting that the function provided by furin cleavage is conserved (data not shown). Therefore we tested whether application of the furin inhibitor at 20 μM (a concentration at which no cell toxicity was observed by XTT assay, data not shown) inhibited infection of 293TT cells by PsV of all 34 HPV types. Infection of 293TT cells by PsV of every alpha HPV was strongly inhibited, although it was less effective against some beta types such as HPV5 (Figure 8). Nevertheless, it is clear that the requirement for furin cleavage during PsV infection is ubiquitous among the 34 diverse HPV types tested.

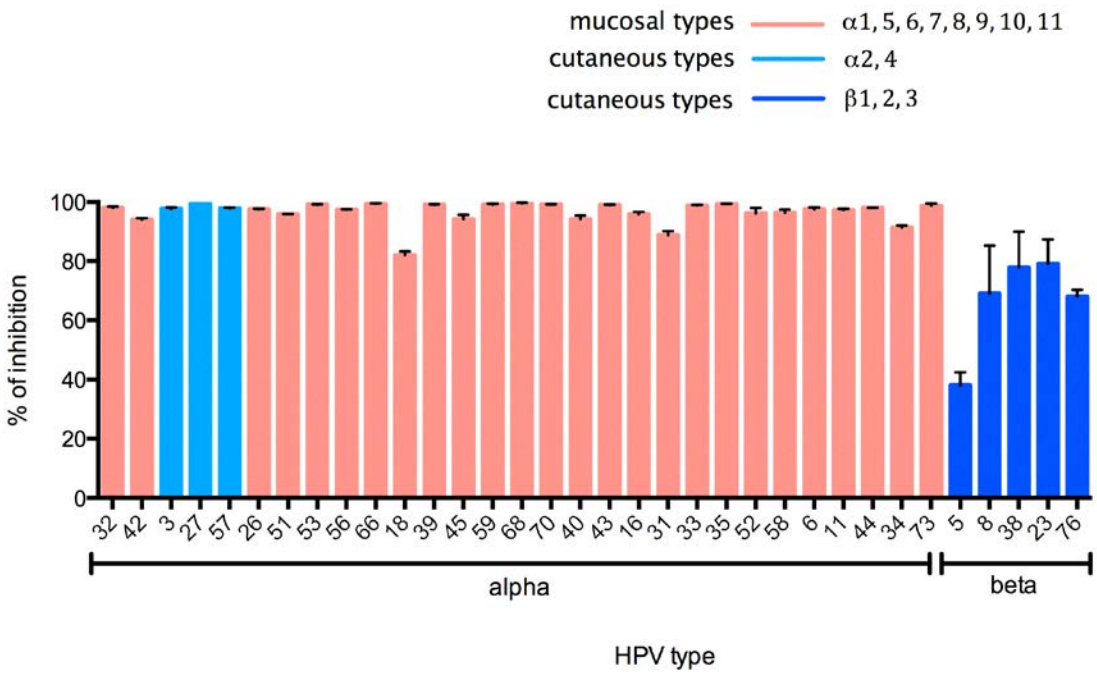


Figure 8. Impact of furin inhibitor on infection by PsV of diverse HPV.

PsVs of each indicated HPV type carrying a luciferase reporter gene were transferred to 293TT cells for 72 hours in the presence or absence of 20 μM furin inhibitor. After incubation, cells were lysed, luciferase activity was measured and percent inhibition of infectivity compared to control calculated. Red and blue bars represent mucosal and cutaneous HPV types, respectively.

4) Conservation of need for cleavage by γ-secretase during infection

The γ-secretase inhibitor XXI potently blocks HPV16 infection, and homozygous deletion of γ-secretase component subunits nicastrin or presenilin-1 also prevents HPV16 infection [51]. To determine whether the need for γ-secretase function during infection is conserved across diverse HPV types, we examined the impact of 500nM XXI upon infection of 293TT cells by each PsV types. The γ-secretase inhibitor XXI dramatically reduced the infectivity of the PsV of all 34 HPV types tested (Figure 9), implying that γ-secretase is a key cellular factor that diverse genotypes utilize during infection and its inhibition can broadly inhibit HPV infection [51,73].

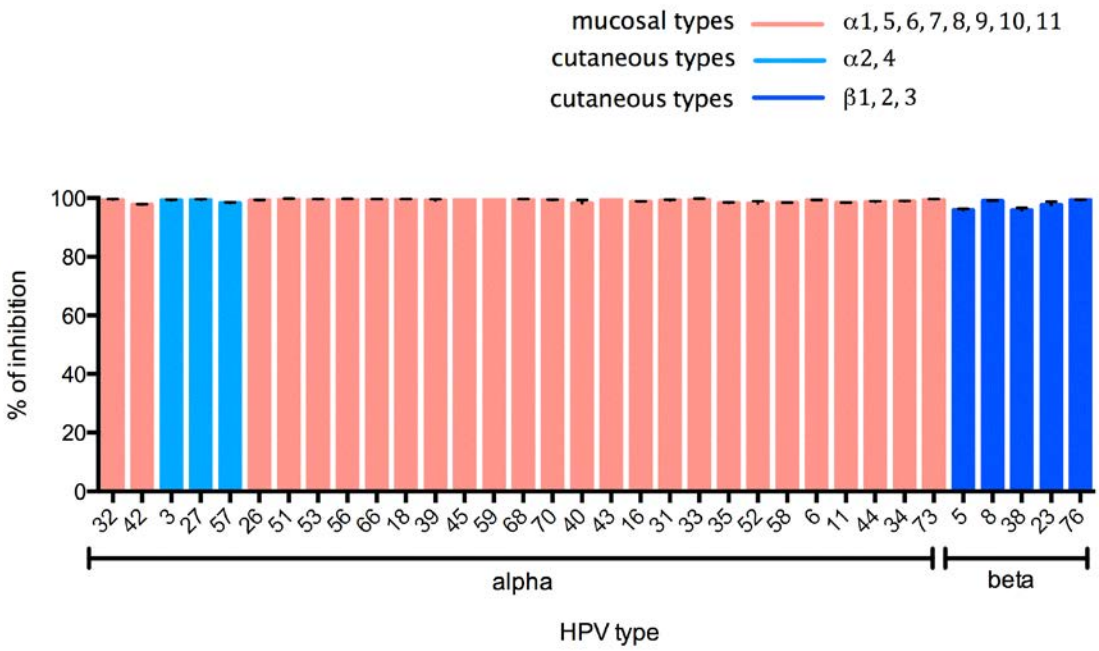


Figure 9. Impact of γ-secretase inhibitor on infection by PsV of diverse HPV.

PsVs of each indicated HPV type carrying a luciferase reporter gene were transferred to 293TT cells for 72 hours in the presence or absence of 500 nM γ-secretase inhibitor XXI (n=3). After incubation, luciferase activity was measured and percent inhibition of infectivity compared to control calculated. Red and blue bars represent mucosal and cutaneous HPV types, respectively.

5) Neutralization of diverse HPV by antiserum to L2 antigen α11-88×8

The amino terminus of L2 contains a protective epitope and it is also evolutionarily highly conserved between types and even different species. We previously described that rabbit antiserum targeting the L2 antigen α11-88×8 can neutralize a broad spectrum of mucosal HPV types, notably those associated with cervical cancer and benign genital warts, and that passive transfer of this antiserum protects mice from genital HPV PsV challenge [78]. While this candidate vaccine antigen was designed to broadly protect against mucosal HPV that are associated with cervical cancer and genital warts, it may also protect against cutaneous HPV types as there is considerable sequence conservation in the 11-88 region. Cutaneous HPV types have been associated with non-melanoma skin cancers and benign skin warts that are costly to treat or a common nuisance, particularly for immunocompromised patients [55]. To examine the breadth of cross-neutralization against diverse HPV types, we tested antiserum from a rabbit vaccinated with the L2 antigen α11-88×8. PsV from each of the 34 HPV types was incubated with 2-fold serially diluted rabbit serum and the mixture was then transferred to 293TT cells to assess infectivity. The α11-88×8 antiserum detectably neutralized PsV of all alpha and beta HPV types tested (Table 1), whereas the pre-immune serum did not. Likewise, this antiserum reacted with L2 in PsV preprations of all 34 HPV types tested in a Western blot analysis (Figure 4B). These observations suggest the potential of this candidate vaccine antigen to protect against diverse beta types associated with non-melanoma skin cancer in immunocompromised and EDV patients.

d) DISCUSSION

The two licensed vaccines safely provide robust protection for men and women against acquiring infections by the two most potent oncogenic types, HPV16 and HPV18, and Gardasil also protects against the two most common types associated with genital warts, HPV6 and HPV11. In spite of their effectiveness, there is still a demand for new approaches for the prevention of HPV infection due to the high cost and type-restricted protection afforded by the licensed vaccines and also the need for immunization [96]. Aside from HPV16 and HPV18, about a dozen other oncogenic alpha HPV types are associated with cervical cancer, although each contributes only a minor fraction. Although the licensed vaccines provide varying degrees of cross-protection against closely related oncogenic HPV types, the extent and duration of cross-protection remain controversial [77]. Consequently, there are ongoing efforts to develop a nonavalent L1 VLP vaccine to target the seven oncogenic HPV types most commonly detected in cervical cancer, as well as HPV6 and HPV11. However, inclusion of 9 HPV VLP types into a single vaccine formulation will most likely increase the cost of vaccine production, which is one of the main limitations to global implementation of HPV immunization. Moreover, there has been little focus on vaccination against cutaneous HPV types, notably the beta species. While the beta HPVs typically have minimal clinical impact among patients with intact immune systems, immunocompromised patients (i.e. HIV-positive patients or those receiving immunosuppressive treatment following solid organ transplantation) and those with the rare hereditary syndrome EDV are vulnerable to non-melanoma skin cancers caused by these beta types, notably HPV5 and HPV8 [55]. It is

unlikely that the licensed vaccines or even the new nonavalent vaccine will impact infection by the diverse beta HPV types, and therefore there is interest in alternative approaches to provide coverage against beta HPV.

More than 120 HPV genotypes has been completely characterized so far, but studies of the mechanisms of infection have been focused only on a very limited number of HPV types of the greatest medical significance, mainly due to restricted availability of PsV of a few HPV types. Therefore to assist in understanding the biology of HPV as well as assessing the breadth of protection provided by new vaccine and microbicide candidates, we generated and tested a diverse set of 34 PsV including 29 alpha types and 5 beta types (Table 1). The mechanisms underlying the different tropism of HPV are not understood. The alpha HPV types, with the exception of alpha2 and alpha4 subgroups, preferentially infect the genital mucosa and are sexually transmitted, whereas the beta HPV types infect the keratinizing squamous epithelium of the skin, typically starting during childhood. The distinct tropism may be governed by differences in entry into the cell, or viral transcription or replication, or a combination of these functions. To initially examine whether there are differences in the uptake processes of the predominantly mucosatropic alpha and cutaneotropic beta HPVs we tested the potency of known inhibitors of infection against PsV of the diverse range of HPV types. Use of PsV delivering a luciferase reporter for these studies eliminates any influence of viral tropism that would be conferred by the native viral genome with respect to viral transcription or replication.

The initial studies focused on HSPG because it is the most studied cell surface receptor for HPV, and there is evidence of differential utilization by different genotypes

[62,80,81,93]. We verified that the two HSPG mimetics heparin and carrageenan are inhibitors of diverse mucosatropic HPV PsV infection. This is significant because of ongoing efforts to develop carrageenan as a vaginal microbicide and its use as an ingredient in many sexual lubricants. Both compounds completely inhibit infection by most alpha types at high and intermediate concentrations (Figure 7), although most alpha types demonstrated partial inhibition by 10 μg/mL heparin, the lowest concentration tested (Figures 7A and 7). However, while infection by HPV27 was blocked by heparin at 1000 μg/mL, carrageenan did not inhibit even at the highest concentration of 1000 μg/mL (Figure 7B), possibly reflecting their different structures and mechanism of particle binding [80,95]. Notably, the alpha4 viruses HPV27 and HPV57 are capable of infecting cutaneous sites. A previous study suggested that cutaneous types tended to have negatively charged L1 at pH 7.4 and lower surface net charge compared to mucosal types [80]. We calculated a net charge for L1 of 34 HPV types at pH 7.4 and beta types (Table 1). Interestingly HPV27 and HPV57 L1 display the lowest net charge at pH 7.4 among the 29 alpha types and they were not inhibited effectively by carrageenan at a concentration of 1000 μg/mL, 100 μg/mL and 10 μg/mL like most beta types and HPV57 was not inhibited by heparin at 100 μg/mL and 10 μg/mL. These net charge differences may contribute to the distinct response of HPV27 and 57 PsV to the HSPG mimetics as compared with the other alpha types [80,95].

When the concentration of heparin or carrageenan was reduced to 10 μg/mL, PsV of some cutaneous HPV types demonstrated enhanced infectivity (Figure 7A, B). This result was unexpected because heparin and carrageenan are inhibitors of mucosatropic HPV infection, although low amounts of heparin increased HPV infectivity in a recent

report [62,94,97]. It is possible that when virion surface is partially coated by heparin or carrageenan, it can trigger a conformational change which, in turn, enhances infectivity perhaps by rendering L2 more accessible to furin cleavage [94]. Conversely, if the virion surface is completely coated by heparin or carrageenan due to high affinity, and if the binding groove and charged residues on the capsid surface that interact with the HSPG cellular receptor are masked completely[58], this can potentially prevent cell binding and infection.

Despite efficiently blocking infection by the mucosatropic alpha types, neither heparin nor carrageenan were consistent inhibitors of the cutaneous types, and indeed frequently promoted infection. This failure of heparin to inhibit infection by one beta type was also observed previously for HPV5 [62]. Several studies suggest that key surface exposed positively charged lysine residues in L1 are important for interaction of HPV16 PsV with heparan sulfate, and mutation of these lysine residues resulted in reduced cell binding and infectivity. We aligned L1 of 34 HPV types tested and noted only one of these lysine residues, HPV16 L1 278K, is highly conserved in alpha types but not beta types (data not shown). Differences in interaction with HSPG-like molecules on the cell surface may account in part for the tropism of HPVs for cutaneous versus mucosal epithelia [62]. However, the relationship is not straightforward; heparin inhibited HPV3, HPV23 and HPV27, and carrageenan inhibited HPV3 and HPV23 infection (Figure 7). It is unclear whether heparin and carrageenan fail to inhibit most beta HPVs because they simply cannot bind to the virion, or they do bind but cannot block receptor interaction. A failure to bind beta HPV particles is unlikely since many PsV showed increased infectivity with addition of heparin and carrageenan. A potential concern with these

observations is that low numbers of infectious particles in the PsV preparation of the cutaneous HPV types could have led to an artifact, although L2 incorporation and encapsidation of reporter plasmid was similar to the alpha types. It is also important to recognize considerable variability in L2 incorporation, genome encapsidation and infectivity for PsV preparations of different HPV types when using them as a model for native virions assembled in warts [81].

We expected to observe broad spectrum blockade of HPV infection by furin inhibitor because the furin cleavage consensus sequence RX(R/K)R in L2 was conserved for all HPV types examined herein. This sequence and the requirement for furin cleavage is also conserved in bovine (BPV) and other animal papillomaviruses [50]. Furin inhibitor blocked infection by all HPV types tested although not completely for some beta PsV. This may reflect use by the beta types of protein convertases other than furin that are partially inhibited by furin inhibitor [50], or possibly an artifact relating to the lower signal to noise ratio for the less infectious types.

The γ-secretase inhibitor XXI also effectively inhibited PsV infection for all HPV types tested (Figure 9). Beta types, which tend to exhibit somewhat lower sensitivity than alpha types to the furin inhibitor, were strongly inhibited by XXI. The data suggest that all the HPVs follow a common entry pathway dependent upon both furin- and γ-secretase-dependent proteolysis, despite potential differences in interactions with cell surface HSPGs between the mucosal and cutaneous types. However, while several γ-secretase inhibitors that have been tested clinically for prevention of Alzheimer disease might be re-tasked for broad prevention of HPV infection, they have also been associated with squamous cell cancer [98].

Although the amino terminus of L2 is a weaker immunogen compared to L1 VLP, it is considered to provide broader protection [74]. In particular, the concatenated fusion protein L2 α11-88×8 comprising residues ~11-88 of L2 derived from eight alpha HPV types [78] has been shown to induce cross-neutralizing antibodies that broadly protect against the HPV types most commonly found in cervical cancer and benign genital warts. Here we also examined L2 α11-88×8 antiserum for its capacity to cross-neutralize cutaneous beta HPVs that are also associated with cancer, particularly among immunocompromised and EDV patients. Remarkably, the L2 α11-88×8 antiserum neutralized all beta HPV types tested (Table 1). The result implies that the neutralizing epitopes at the amino terminus of L2 are highly conserved and play an important role in both alpha and beta HPV infection, and that an L2 α11-88×8 vaccine might potentially provide broad protection against the diverse alpha and beta HPV family. Serologic studies suggest that beta HPV infections are acquired throughout life, beginning in childhood [55]. Therefore, optimally vaccination against beta HPV would need to begin early.

In conclusion, we have considered agents that block infection via inhibiting receptor interaction, furin cleavage or γ-secretase function as alternative approaches to vaccination to broadly prevent both mucosal and cutaneous HPV infections. At present, carrageenan shows promise as a vaginal microbicide to broadly prevent infection by mucosatropic high-risk papillomaviruses [65]. However our results show that it is unlikely to prevent cutaneous HPV transmission. Further, it would need to be regularly applied at high amounts to ensure full protection. As both furin and γ-secretase contribute to numerous cellular processes, systemic use of their inhibitors are also likely to have

significant side effects rendering them impractical despite broad anti-HPV activity, although topical application might be feasible. Nevertheless, our study with 34 medically significant HPV types indicates that there is a conserved infection pathway once the virus enters its host cell, and thus targeting conserved residues of the capsid proteins that interact with the key cellular entry factors has potential for broad protection. Indeed, rabbit antisera to the L2 α11-88×8 antigen provided broad protection against alpha HPV challenge via cross-neutralizing antibodies [78]. Here we find that L2 α11-88×8-specific antibodies also cross-neutralize diverse beta papillomaviruses, suggesting the potential to protect against types associated with non-melanoma skin cancer in immunocompromised or EDV patients (should they mount a sufficient response after vaccination) or the potential of herd immunity in the general population to limit their circulation. Taken together, this study supports the possibility of vaccination with L2 candidate vaccines for pan-prevention of HPV infection.

II) Multivalent human papillomavirus L1 DNA vaccination utilizing electroporation

a) Introduction

Persistent infection by oncogenic human papillomavirus (HPV) drives the development of cervical cancer [1]. HPV infection also causes subsets of other cancers such as vulvar, vaginal, penile, anal, and oropharyngeal cancers [3,100,101]. The importance of preventing HPV infection drove the development of two commercial viruslike particle-based (VLP) vaccines, Gardasil® by MSD and Cervarix® by GSK, respectively. These two L1 VLP-based vaccines elicit robust type-restricted neutralizing antibodies that effectively inhibit HPV infection [7,6,13,102-105]. However, Gardasil® and Cervarix® each contain L1 VLP derived from only two high risk genotypes, HPV16 and HPV18, although Gardasil also contains L1 VLP derived from the two most common genotypes causing benign genital warts, HPV6 and HPV11. Since HPV16 and HPV18 cause 50% and 20% of all cervical cancers [54,106], the two licensed vaccines are potentially able to prevent most but not all cases of cervical cancer because of the type-restricted immunity [11,107]. However, HPV16 causes, 90% of cases of HPV-associated vaginal, vulval, anal and oropharyngeal cancers, suggesting a distinct type distribution at these anatomic sites [3,100,101]. Passive transfer studies in animal models of HPV infection suggest that the type-restricted neutralizing antibodies induced by L1 VLP vaccination effect protection, although a role for cellular immunity has not been excluded [108]. The breadth of protection may be expanded by simply increasing the number of L1 VLP of different HPV genotypes, although this increases the cost and complexity of

production. Merck is currently testing a nonavalent L1 VLP vaccine that targets the seven most common HPV genotypes found in cervical cancer and two types that cause most cases of genital warts [109].

The minor capsid protein, L2, harbors several conserved neutralizing epitopes at its amino terminus that elicits cross-protection among diverse HPV types [44,46,110,111]. However, by comparison to L1 VLP, weaker immunogenicity is an obstacle L2 vaccine development [44,112]. Several attempts have been made to enhance immunogenicity of L2 conserved epitopes and create a single vaccine protective against most high-risk HPV types. For example, L2 epitopes have been displayed repetitively by generating L2 multimer fusion proteins, or insertion into the immunodominant neutralizing epitope of VLPs of HPV and other viruses [48,49,113,114].

Cost and the need for a cold chain are barriers to global implementation of HPV immunization. Unfortunately, 85% of cervical cancer cases occur in women in developing countries and even the tiered pricing for the two licensed vaccines is beyond the reach of many lower income countries [115]. The L2 multimer vaccine can be manufactured as a single protein in the *E. coli* system lowering its cost compared to multivalent L1 based vaccines produced in yeast or insect cells [34,116,117]. However, protein-based vaccines are prone to degradation at ambient temperature and typically require refrigeration such that development of heat-stable formulations is needed to facilitate implementation in low income and remote populations [117].

Naked DNA vaccines encoding vaccine antigens have several potential advantages. Production of DNA vaccines does not require culture, inactivation of infectious pathogens, and their purification from bacteria is well standardized and

comparatively inexpensive [118]. Importantly, naked DNA can be readily stored at ambient temperature. Moreover, the antigenic structure of the vaccine antigen produced by DNA vaccination likely closely resembles the appropriate native structure with the correct posttranslational modifications. Indeed, L1 expressed in *E. coli* does not form regular VLPs and requires *in vitro* disassembly and reassembly [34,119]. Furthermore, the DNA vector itself can have an adjuvant effect via its inherent immunostimulatory elements. Unmethylated CpG dinucleotide motifs can be sensed by Toll-like receptor (TLR)-9 [120], a microbial pattern recognition receptor (PRR), and trigger innate inflammatory responses [121-123]. DNA in the cytoplasm can be recognized and stimulate Absent In Melanoma 2 (AIM2) [124], and STimulator of IFN Genes (STING) pathways [125]. DNA vaccines also provide sustained antigen expression for a prolonged immune stimulation compared to the short half-life of protein antigens [126]. Despite many advantages over protein vaccines, low immunogenicity is a major shortcoming of DNA vaccines, and is believed to reflect inefficiency of delivery of the vaccine to the host nucleus.

There are several alternative modes of DNA administration that can overcome inefficient delivery. The gene gun provides ballistic delivery of gold particles coated with DNA to cells in the skin including professional antigen presenting cells, termed Langerhans cells [127,128]. While the method is more efficient than i.m. injection, only a limited amount of DNA can be used due to technical issues. A second improved method of DNA delivery via *in vivo* electroporation elicits robust immune responses as a consequence of increased transfection of somatic cells and inflammation caused by localized cell death [129-131]. The potential of electroporation in clinical trials has

recently been demonstrated with DNA vaccines targeting hepatitis B virus [132], HIV [133,134] and HPV oncoproteins, E6 and E7 [135].

Here we show the potential of L1-expressing DNA vaccines administered with electroporation as a prophylactic vaccine. In addition, our results also demonstrate interference between L1 DNA vaccines administered at the same site with electroporation, likely reflecting co-assembly of different L1 into chimeric particles rather than immunologic competition. Finally, we find that this interference can be eliminated if L1 DNA vaccines of different HPV genotypes are spatially separated upon administration, or ameliorated if the cognate L2 proteins are co-expressed.

b) Materials and Methods

1) Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (protocol MO08M19).

2) Vaccine Preparation

Codon optimized capsid genes, L1 and L2, of HPV6, HPV16, HPV18, HPV26, and HPV51 were sub-cloned into double expression vector, pVITRO1-neo-mcs (Invivogen, San Diego CA) or pcDNA (Invitrogen, Carlsbad CA), for DNA vaccination. HPV16 L2 multimer expression construct encompassing residues 11−88 of 8 HPV types (L2×8) was sub-cloned into pcDNA 3.1 for mammalian expression, and L2 a11-88×8 polypeptide was produced, purified, and dialyzed as previously described [79]. All plasmids employed for the immunization were purified and endotoxins were removed with UltraClean® endotoxin free kit (Mo Bio, Carlsbad CA).

3) Pseudovirus (PsV) Production

Codon optimized L1 and L2 capsid genes of HPV6, 11, 16, 18, 26, 31, 45, 51,

were sub-cloned into double expression vector, pVITRO1-neo-mcs (Invivogen, San Diego CA). Pseudovirions (PsVs) were generated in 293TT cells following the standard PsV production protocol (http://home.ccr.cancer.gov/Lco/pseudovirusproduction.htm). Firefly luciferase expression plasmid was employed as a reporter for PsV infection in neutralization assays and for vaginal challenge studies.

4) Immunoprecipitation

293TT cells grown in DMEM-10 medium were transfected with empty vector, a single expression vector for only HPV6, 16, or 18 L1, or mixture of L1 DNA of HPV6, 16, 18 in equal parts using transit 2020 (Mirus Bio LLC, Madison WI), and harvested in 48 hours. Cells were lysed with non-denaturing lysis buffer containing 20 mM Tris HCl pH 8, 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM EDTA, and a protease inhibitor cocktail (Roche). mAb H18.F8 [9] was added to whole cell lysate (0.5 mg) and tumbled end-over-end overnight at 4°C. Protein G Sepharose (GE healthcare, Waukesha WI) was added and mixed for an additional 4 hours at 4°C. Resins were harvested by centrifugation at 14,000 × g at 4°C for 1 min and supernatants were discarded. Resins were washed three times with 1 ml lysis buffer, resuspended in 2× sample loading buffer, and boiled for 5 min.

5) Western blot analysis

Western blotting was performed with standard protocols

(http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf). Primary antibodies used were H6.C6, H16.O7, and H18.E20 [9], and secondary antibody used was HRP-goat anti-mouse IgG light chain (Jackson ImmunoResearch, West Grove PA).

6) Vaccination

Groups (n=5) of 3–4 week old female Balb/c mice were vaccinated s.c. three times at two week intervals with 25 ug of L2×8 multimer polypeptide formulated with alum (50 ug), or 50 ul of Gardasil, or i.m. with 10 ug of L2 11-88×8 multimer expression plasmid, or i.m. utilizing *in vivo* electroporation with PBS, 10 ug of L2×8 multimer expression plasmid in pcDNA 3.1, 2 ug or 10 ug of HPV6, 16, 18, 26, 51 L1 expression plasmid in pVITRO1-neo-mcs, 20 ug of HPV6, 16, 18 L1 expression plasmid in pVITRO1-neo-mcs, 10 ug of HPV11, 16, 18, 26, 31, 45, 51 L1+L2 expression plasmid in pVITRO1-neo-mcs, 20 ug of HPV6, 16, 18 L1+L2 expression plasmid in pVITRO1-neo-mcs, 10 ug of mixed (HPV 6, 16, 18, 26, 51 L1, 2 ug each) plasmids in pVITRO1-neo-mcs, 60 ug of mixed (HPV6, 16, 18 L1, 20 ug each) plasmids in pVITRO1-neo-mcs, 60 ug of mixed (HPV6, 16, 18 L1+L2, 20 ug each) plasmids in pVITRO1-neo-mcs, or 20 ug of HPV6, 16, 18 L1 expression plasmids in pVITRO1-neo-mcs at different sites. Blood samples were collected two weeks and three months after the last vaccination. Serum was separated by centrifugation at 2,000 g for 10 min at 4°C after the blood had congealed overnight at room temperature.

7) Electroporation

Mice were injected with DNA in 30 ul water i.m. into the gastrocnemius muscle, or biceps femoris muscle of hind leg. A pair of electrode needles was inserted into the muscle flanking injection site and electrical pulses were delivered utilizing an ECM830 electroporation generator (BTX Harvard Apparatus company, Holliston MA). Eight pulses of 106 V each were delivered for 20 ms pulse duration at 200ms intervals.

8) ELISA

For analysis of antibody response against HPV16 L2 protein, microtiter plates were coated with L2 protein at 500 ng in 100 ul PBS/well overnight at 4°C, and blocked with PBS/1% BSA for 1 hour at 37°C. Plates were incubated with serum samples diluted 1:50 in PBS/1% BSA for 1 hour at 37°C. After 3 washes with washing buffer (0.01% v/v Tween 20 in PBS), HRP-sheep anti-mouse IgG diluted in 1% w/v BSA at 5000-fold was added to each well as a secondary antibody, and incubated for 1 hour at 37°C. After 3 further washes, 100 ul of ABTS solution, 2,2′ Azinobis [3-ethylbenzothiazoline-6-sulfonic acid], Roche, (Basel Switzerland) was added to each well for developing color and read by an ELISA reader, Benchmark Plus (Bio Rad, Hercules CA) at 405 nm.

9) *In vitro* Neutralization Assays

Serum samples (4 ul) were serially diluted two-fold in culture media, and mixed with 0.03 ul of HPV PsV carrying luciferase reporter plasmid. Mixtures were incubated

at 37°C for two hours, added to 3×10^4 of 293TT cells, and incubated at 37°C for 72 hours. Cells were washed with 1× PBS, lysed with 30 ul of Cell Culture Lysis Reagent (Promega, Madison WI) for 15 min at room temperature on a rocking platform. Lysates were transferred to 96-Well black plate, and luciferase activity was measured by GloMax®-Multi Detection System (Promega, Madison WI) after adding 50 ul of luciferin substrate (Promega, Madison WI) to each well.

10) Vaginal Challenge Studies

Mice were subcutaneously injected with 3 mg of medroxypro-gesterone (Depo-Provera, Pfizer, New York NY) four days before vaginal challenge to synchronize their estrus cycles. Viral challenge was performed by delivery of 10 ul of HPV PsV (HPV6: 135 billion, HPV16: 189 billion particles in total) mixed with 10 ul of 3% carboxymethyl cellulose (CMC) into the vagina before and after cytobrush treatment (15 rotations, alternating directions) while the mice were under isoflurane anesthesia. Three day after challenge, mice were anesthetized by isoflurane, and 20 ul of luciferin substrate (7.8 mg/ml, Promega, Madison WI) was delivered into the vaginal vault before imaging. Bioluminescence was acquired for 10 min with a Xenogen IVIS 100 (Caliper Life Sciences, Hopkinton MA) imager, and analysis was accomplished with Living Image 2.0 software. For imaging of passively immunized mouse groups, mice were injected i.v. with 20 ul of serum one day before vaginal challenge.

11) Statistical analysis

Exploratory statistical analyses are performed to analyze the observed titer data. Square-root data transformations were used to achieve normality in residuals for titer data. One-way ANOVA and pair wise multiple comparisons with Bonferroni adjustment were performed using SAS 9.3. When the overall significance test is not significant, the pair wise comparisons are not conducted. To claim significance, we use alpha level 0.1 for pair wise comparisons. The homogeneity of variances is examined with Levene's test.

c) Results

1) *In vivo* electroporation with HPV L1 DNA vaccines elicits robust type-restricted neutralizing antibody titers in mice

Vaccination of rabbits with naked DNA expressing CRPV L1 either via i.m. injection or ballistic delivery on gold beads (gene gun) protects rabbits from experimental viral challenge [136,137]. The initial enthusiasm for naked DNA vaccination based on animal data has been tempered by a low efficiency of delivery by i.m. injection of patients. However, recent clinical studies suggest that *in vivo* electroporation of naked DNA vaccines can induce robust humoral responses in patients [134,135,138-140]. Furthermore, codon optimization appears to be important for robust expression of the HPV capsid proteins [83,84,141]. Therefore we sought to determine whether a strong neutralizing antibody response could be elicited in mice with codon optimized HPV16 L1 capsid gene-expressing naked DNA constructs utilizing i.m. injection as compared to i.m. injection with *in vivo* electroporation, or i.d. delivery via gene gun. We utilized HPV pseudovirion infection of 293TT cells to examine neutralization by serum antibody as a surrogate of wart-derived virions infecting primary human keratinocytes [25]. Vaccination of mice three times at 2 week intervals with 40 ug of HPV16 L1 DNA by i.m. injection resulted in a weak HPV16 neutralizing antibody response as compared to s.c. administration of $1/10^{th}$ of a human dose of Gardasil for which the serum titer was 2 $\log_{10}$ greater (Figure 10A). The response was greater when this HPV16 L1 DNA vaccine was administered i.d. via gene gun at a dose of 2 ug (the maximum that can be applied by this method), but the titer was still a $\log_{10}$ below that obtained with Gardasil. When the

HPV16 L1 DNA vaccine was injected i.m. followed by *in vivo* electroporation, doses of 10 ug or greater elicited an HPV16 neutralizing antibody titer approaching that of Gardasil, whereas the response to the 2 ug dose was weak. A one-way analysis of variance (ANOVA) shows that there is significant difference among 2 ug, 10 ug, 20 ug and 40 ug HPV16 L1 electroporation (EP) groups (p-value = 0.0347). With Bonferroni multiple comparison adjustment, at a level 0.1, we find a significant difference between 2 ug and 10 ug HPV 16 L1-EP groups (adjusted p-value 0.0991) and between 2 ug and 40 ug HPV 16 L1-EP groups (adjusted p-value = 0.0582). A significant difference is also detected between 2 ug HPV16 L1-GG and 40 ug HPV 16 L1-EP groups (p-value = 0.0031).

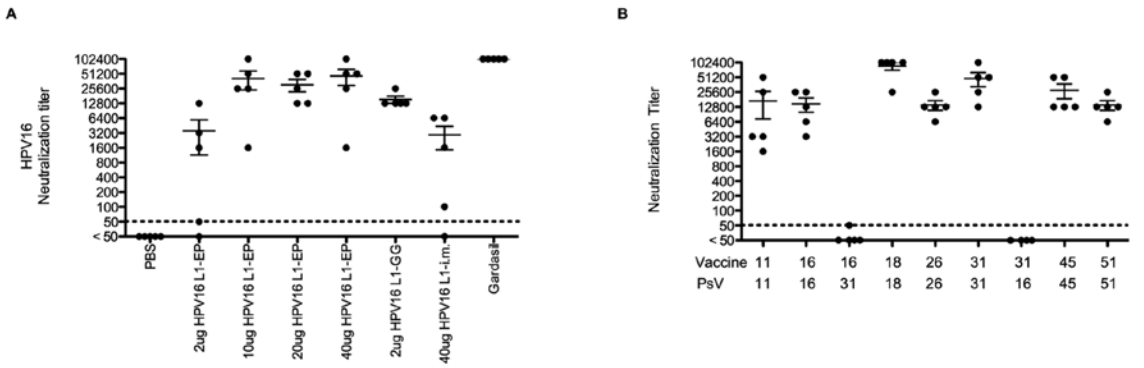


Figure 10. Impact of DNA vaccine dose and delivery method on the induction of type-restricted neutralizing antibodies.

(A) Balb/c mice were vaccinated three times at two week intervals with HPV16 L1 DNA expression vector via i.m. injection alone (40 mg)(i.m.), i.m. injection and *in vivo* electroporation (0, 2, 10, 20 or 40 mg)(EP) or i.d. ballistic delivery on gold particle via gene gun (2 mg)(GG). Vaccination with Gardasil s.c. was included as a positive control. Serum samples were collected two weeks after the third vaccination, and tested for *in vitro* neutralization assay against HPV16. (B) Mice were vaccinated with DNA vector expressing L1+L2 of the genotypes indicated (see line labeled 'Vaccine') i.m. utilizing electroporation. Neutralizing antibody titer against PsV of the indicated genotypes (see line labeled 'PsV') was measured for sera harvested two weeks after the last vaccination.

2) *In vivo* electroporation with L1 only or L1+L2 DNA vaccines elicits a similar antibody response

The co-expression of the minor capsid protein L2 with L1 can enhance the efficiency of VLP assembly [142]. Therefore, we explored whether a higher titer antibody response could be achieved upon vaccination with a DNA vaccine expressing both L1 and L2 versus L1 alone. However, vaccination three times i.m. using *in vivo* electroporation with 10 ug of a DNA vaccine expressing HPV16 L1+L2 elicited a similar neutralizing antibody titer to HPV16 L1 alone. Further, vaccination three times i.m. using *in vivo* electroporation to deliver 10 ug of a DNA vaccine expressing L1+L2 derived from HPV11, HPV18, HPV26, HPV31, HPV45 or HPV51 each elicited a similar neutralizing antibody titer against PsV of the same type utilized for vaccination (Figure 10B). The titers of neutralizing antibodies induced upon vaccination three times i.m. using *in vivo* electroporation to deliver 10 ug of a DNA vaccine expressing L1+L2 were similar to, but still lower than those elicited by Gardasil. Since the responses to electroporation of 10 ug of a DNA vaccine expressing L1+L2 appeared more variable than for Gardasil, the homogeneity of variances among L1 DNA EP, L2×8 Protein and Gardasil groups was examined with Levene's test. While the difference is not significant (p-value = 0.0556), this may reflect the rather limited sample size (5 mice in each group).

L2 contains cross-neutralizing epitopes, and therefore we tested whether *in vivo* electroporation of mice with 10 ug of the DNA vaccine expressing HPV16 L1+L2 induced antibodies that cross-neutralized PsV of HPV31, the genotype most closely related to HPV16, or vice versa. However, no significant cross-neutralization was observed between these two types (Figure 10B), and no L2-specific antibody response

was observed (despite robust L2 expression, Figure 11C) indicating that L2 is not immunogenic in the context of the capsid. These findings suggest that a multivalent L1-based DNA vaccine approach or vaccination with L2 (in the absence of L1) would be necessary to generate a broadly protective response.

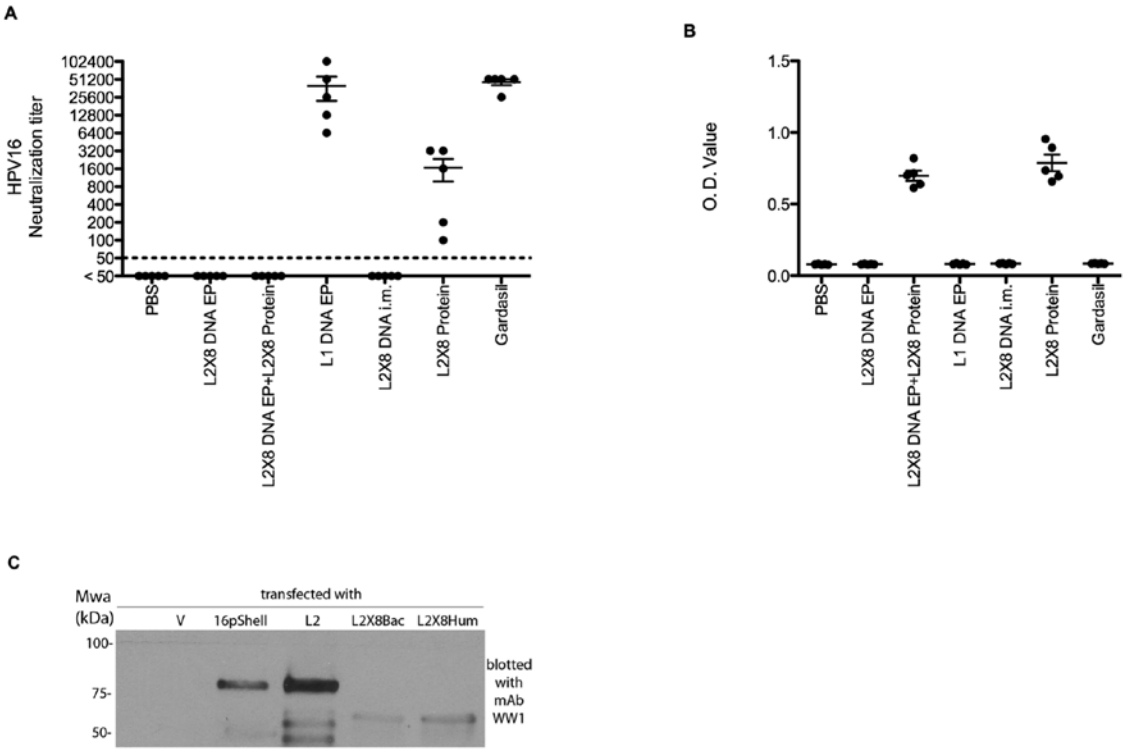


Figure 11. Neutralizing antibody titer and antibody response of sera from mice vaccinated with L1 or L2×8 delivered as protein or a DNA vaccine via electroporation.

Balb/c mice were vaccinated three times at two week intervals with PBS, 10 mg L2×8 DNA vaccine i.m. with electroporation three times, or twice utilizing 10 mg HPV16 L1 DNA vaccine i.m. with electroporation followed by a single boost with 25 ug L2×8 protein in alum s.c., 10 mg HPV16 L1 DNA vaccine i.m. with electroporation three times, three times with 10 mg L2×8 DNA vaccine i.m., or 25 ug L2×8 protein in alum s.c., or Gardasil s.c.,,. Serum samples were collected two weeks after the third vaccination, and were tested for *in vitro* HPV16 neutralization titer (A) and antibody response to HPV16 L2 (B) as measured by ELISA. (C) To assess relative levels of expression, 293TT cells were transfected with no plasmid, HPV16 L1+L2 DNA in pShell, full length HPV16 L2 DNA in p16L2h, bacterial codon optimized L2×8 DNA in pcDNA, or human codon

optimized L2×8 in pcDNA. 293TT cells were lysed two days after transfection. Western blotting was performed with lysate samples using a monoclonal antibody to HPV16 L2 17–36.

3) *In vivo* electroporation with an L2 multimer DNA vaccine failed to elicit neutralizing antibodies

Prior studies by Hitzeroth et al suggest that i.m. vaccination of mice with 100 ug DNA expressing codon-optimized full length HPV16 L2 induces a T cell response but only a very weak antibody titer, whereas vaccination with the amino terminus of L2 may be more immunogenic [143,144]. Vaccination of mice with a multimer peptide comprising L2 residues 11-88 amino acids derived from 8 HPV types (L2×8) induced broadly neutralizing antibodies, although at a titer, 30-fold lower than for L1 VLP [79]. Therefore we compared the HPV16 neutralizing antibody titers induced upon vaccination via *in vivo* electroporation with codon-optimized HPV16 L1 DNA versus L2×8 multimer DNA. While the L1 DNA construct elicited very high neutralization titers approaching those induced by Gardasil vaccination (Figure 11A), L2×8 multimer DNA vaccination failed to induce neutralizing antibody against HPV16 pseudovirions, even with a single L2×8 protein in alum boost (although the latter did induce an L2-specific response detectable by ELISA with a titer of 400). Vaccination with L2×8 multimer DNA failed to induce a neutralizing antibody response either with or without electroporation, and whether the codon optimization of the L2×8 construct was biased for bacterial expression or mammalian expression (not shown). Similar levels of expression of L2×8 protein were detected in 293TT cells 48 h post transfection with the L2×8 constructs codon optimized for bacterial or mammalian cell expression (Figure 11C). The levels of L2×8 were substantially lower than for full length codon-optimized HPV16 L2 and there was evidence of a greater extent of degradation, possibly contributing to the poor immune response (Figure 11C).

Vaccination with antigen delivered first via DNA vaccine and then boosting with protein has been suggested to enhance humoral immunity as compared to DNA vaccination or protein alone [145,146]. We tested the sera of mice vaccinated twice with L2×8 multimer DNA followed by a third immunization of L2×8 multimer peptide mixed with alum adjuvant. Surprisingly, we were not able to detect an HPV16 neutralizing antibody titer with the L2×8 multimer DNA vaccine prime and protein boost combination regimen (Figure 11A), although antibody response to full length HPV16 L2 was detected (Figure 11B) with an ELISA titer of 400. In contrast, vaccination three times with L2×8 protein in alum elicited L2-specific antibody (ELISA titer of 12,800) and significant titers of HPV16 neutralizing antibody (a mean titer of 1600, Figure 11A), although the latter is 30-fold lower than for L1 DNA vaccination or Gardasil. This suggests that even vaccination twice with L2×8 multimer DNA was inadequate for priming a neutralizing antibody response prior to a single L2×8 protein boost, and this may reflect poor expression, protein instability, insensitivity of the neutralization assay, weak immunogenicity for L2×8 vaccination via DNA vector, and/or the preferential induction of antibody to non-neutralizing L2 epitopes when using this immunization regimen.

4) Vaccination with a mixture of L1 DNAs of multiple HPV types exhibits interference

Since L1-based DNA vaccine electroporation successfully induced a robust but type-restricted neutralizing antibody response, we evaluated the potential for a multivalent vaccine in which L1 DNAs of several HPV types are mixed prior to i.m. injection and *in vivo* electroporation. Our initial study suggested that a 2 ug dose of HPV16 L1 DNA vaccine induces a suboptimal neutralizing antibody response, but 10 ug produces the maximal response. To examine if the 2 mg L1 DNA vaccine dose provided a consistent response regardless of HPV type, and whether the response to a pentavalent vaccine would be additive or synergistic, we performed a pilot experiment in which five different DNA vaccines expressing L1 of HPV6, HPV16, HPV18, HPV26, and HPV51 respectively were mixed (2 ug each) and delivered i.m. by electroporation for comparison to vaccination with each type singly at doses of 2 ug or 10 ug. HPV6, HPV16 and HPV18 are each members of different papillomavirus species (α10, α9, and α7 respectively), whereas HPV26 and HPV51 are both members of the α5 species. The latter two types were included to determine if interference in the response to mixtures of L1 expression constructs occurred intra-species or inter-species or both [12]. After three immunizations, mice vaccinated with the pentavalent L1 DNA mixture exhibited distinct neutralizing antibody responses compared to mice vaccinated with L1 DNA of single HPV type (Table 2). The 2 ug dose of a single L1 DNA vaccine gave an inconsistent response for HPV16 and between different HPV types, whereas the response to 10 ug was consistent across all 5 types tested. The variable response at the 2 ug dose of a single L1 DNA vaccine did not reflect L1 expression level alone. Indeed, the relative number of particles produced using these codon-optimized L1 genes to produce pseudovirions in 293TT

cells, as estimated by Coomassie-stained SDS-PAGE gel studies of purified pseudovirions and normalized to HPV16 are: HPV6 L1: 15.5%, HPV16 L1: 100%, HPV18 L1: 1%, HPV26 L1: 119%, HPV51 L1: 147%. Thus despite significantly lower production of particles by HPV6 and HPV18, both of these constructs elicit a robust neutralizing antibody response. Serum from the L1 DNA mixture group showed no detectable neutralizing titer against HPV6 and HPV16 PsVs, the expected titer for HPV51 but an increased titer against HPV18 and HPV26 PsVs (Table 2). This finding suggested that the DNA constructs may not act independently when mixed together and that increasing the dose of L1 DNA vaccine from 2 ug to 10 ug improves the level and consistency of the neutralizing antibody response.

Vaccine	Dose (μg)	HPV6 IVNT	HPV16 IVNT	HPV18 IVNT	HPV26 IVNT	HPV51 IVNT
vector	10	ND	ND	ND	ND	ND
HPV6 L1	2	ND				
	10	6,400				
HPV16 L1	2		ND			
	10		6,400			
HPV18 L1	2			50		
	10			51,200		
HPV26 L1	2				1,600	
	10				6,400	
HPV51 L1	2					50
	10					12,800
HPV6,16,18,26, and 51 L1	2,2,2,2,2	ND	ND	6,400	6,400	50
Gardasil	0.1 mL	102,400	102,400	102,400	ND	ND

Table 2. Neutralizing antibody titers induced upon vaccination of mice with a pentavalent HPV L1 DNA vaccine

Balb/c mice were vaccinated i.m. three times at two week intervals with a mixture of L1 vectors of HPV6, HPV16, HPV18, HPV26, and HPV51 (2 mg each), L1 vector of single HPV type (2 mg or 10 mg) utilizing electroporation. Gardasil vaccination was included as a positive control. Sera from 5 mice were pooled together, and *in vitro* neutralizing antibody titer (IVNT) was measured with HPV6, HPV16, HPV18, HPV26, and HPV51 PsVs. ND: Not detected at 1:50.

5) Spatial separation of L1 DNA vaccines of different types, but not L2 co-expression, fully restores independent neutralizing antibody responses upon multi-type vaccination

In a follow-up study (Figure 12), several changes were made in order to address interference when L1 DNA vaccines are mixed. First, the dose of each L1 DNA was increased from 2 ug to 20 ug to enhance the level and consistency of the neutralizing antibody response, and the number of HPV types decreased from five to three (HPV6, HPV16 and HPV18) to minimize the potential for interference and because vaccination with Gardasil suggests limited or no immunologic interference in the antibody responses to L1 VLPs these three types (Table 2). Furthermore, these types are the most common causing genital warts (HPV6) and squamous cell (HPV16) and adenocarcinoma (HPV18) of the uterine cervix, suggesting their likely inclusion in future vaccines as compared with HPV26 and HPV51 that are infrequently found in cervical cancer [147]. It is possible that the immunologic interactions between the L1 vaccines when administered as a mixture reflect heterotypic binding and inappropriate co-assembly and/or immunologic dominance of particular construct(s). Therefore, to prevent co-assembly of L1 of different types into a chimeric VLP, we tested the impact on the humoral response of administering the three L1 DNA constructs each at a different site (HPV6 L1 DNA was injected into the left biceps femoris muscle, HPV16 L1 DNA was injected into the right gastrocnemius muscle, and HPV18 L1 DNA was injected into the left gastrocnemius muscle) versus mixed together and delivered at the same site. In addition, L2 exhibits some type-restriction for interaction with L1 [148-150], and facilitates VLP assembly by ~4-fold [142]. Therefore, mice were vaccinated with a mixture of three DNA vaccines expressing L1+L2 of HPV6, HPV16 and HPV18 based upon the

hypothesis that the presence of the cognate L2 would both increase the assembly of VLPs four-fold and the specificity of co-assembly with the homotypic L1, thus limiting heterotypic VLP production. Two weeks after three vaccinations i.m. with the DNA constructs indicated, the *in vitro* neutralization titer of the serum of each mouse was tested against HPV6, HPV16 and HPV18 PsV. Vaccination with 20 ug of each L1 DNA vaccine individually induced a robust homotypic neutralizing antibody response and an equivalent titer was observed when utilizing DNA vaccines expressing L1+L2 (Figure 12A–C) as the Bonferroni adjusted pair wise comparisons are not significant in all cases at alpha level 0.1. Despite reducing the number of HPV L1 DNAs to three types, (HPV6, HPV16, and HPV18), there was still some interference in production of neutralizing antibody, most noticeably for HPV16 (Figure 12B) for which ANOVA analysis results show that there is significant difference among groups (p-value = 0.0030), to a lesser extent for HPV18 (Figure 12C) (p-value = 0.0243), but not significantly for HPV6 (Figure 12A) (p-value = 0.5577). As seen in figure 12A, the HPV16 neutralizing serum antibody titer induced by vaccination with HPV16 L1 alone was significantly higher than when the HPV6, HPV16 and HPV18 L1 constructs were mixed together and injected at the same site (the Bonferroni adjusted t- test p = 0.0038), but not when given at different sites (p = 0.6893) or when L2 was co-expressed in the constructs (p = 0.7567). This pattern was also observed for sera harvested 3 months post vaccination (Figures 12D-F) as ANOVA analysis results show that there is again significant difference among groups for HPV16 (Figure 12E, p-value = 0.0005), and HPV18 (Figure 12F, p-value = 0.0027), but not for HPV6 (Figure 12D, p-value = 0.6497). Specifically, in figure 12E, the HPV16 neutralizing serum antibody titer induced 3 months after vaccination with HPV16 L1

alone was significantly higher than when the HPV6, HPV16 and HPV18 L1 constructs were mixed together and injected at the same site (the Bonferroni adjusted t-test p = 0.0394), but not when given at different sites or when L2 was co-expressed in the constructs. Importantly, when the three L1 DNA vaccines were each administered at a physically separate site, robust homotypic neutralizing antibody titers were observed, consistent with those obtained when administering each construct alone for all three virus types. This observation suggests that the interference does not reflect immunologic competition, but rather suggests that co-expression of L1 of HPV6, HPV16 and HPV18 in the same cells might result in aberrant VLP assembly as a consequence of heterotypic binding. While vaccination at different sites resolved interference completely, electroporation of mice with a mixture of three DNA vaccines co-expressing both L1 and L2 of HPV6, HPV16 and HPV18 only partially reduced interference in the responses to HPV16 and HPV18 (Figure 12B and C). This phenomenon was preserved when analyzing sera harvested at 3 months after the final vaccination (Figures 12D–F). We also tested whether vaccination with L1+L2 DNA of a single type or a mixture of three types can generate an L2-specific antibody response but no consistent antibody response against HPV16 L2 was observed (Figure 12G). These results imply that formation of chimeric VLP still occurs when the valency of the L1 multitype vaccination is reduced, and this problem can be ameliorated by co-expression of the cognate L2 proteins, but is eliminated by vaccinating at different sites.

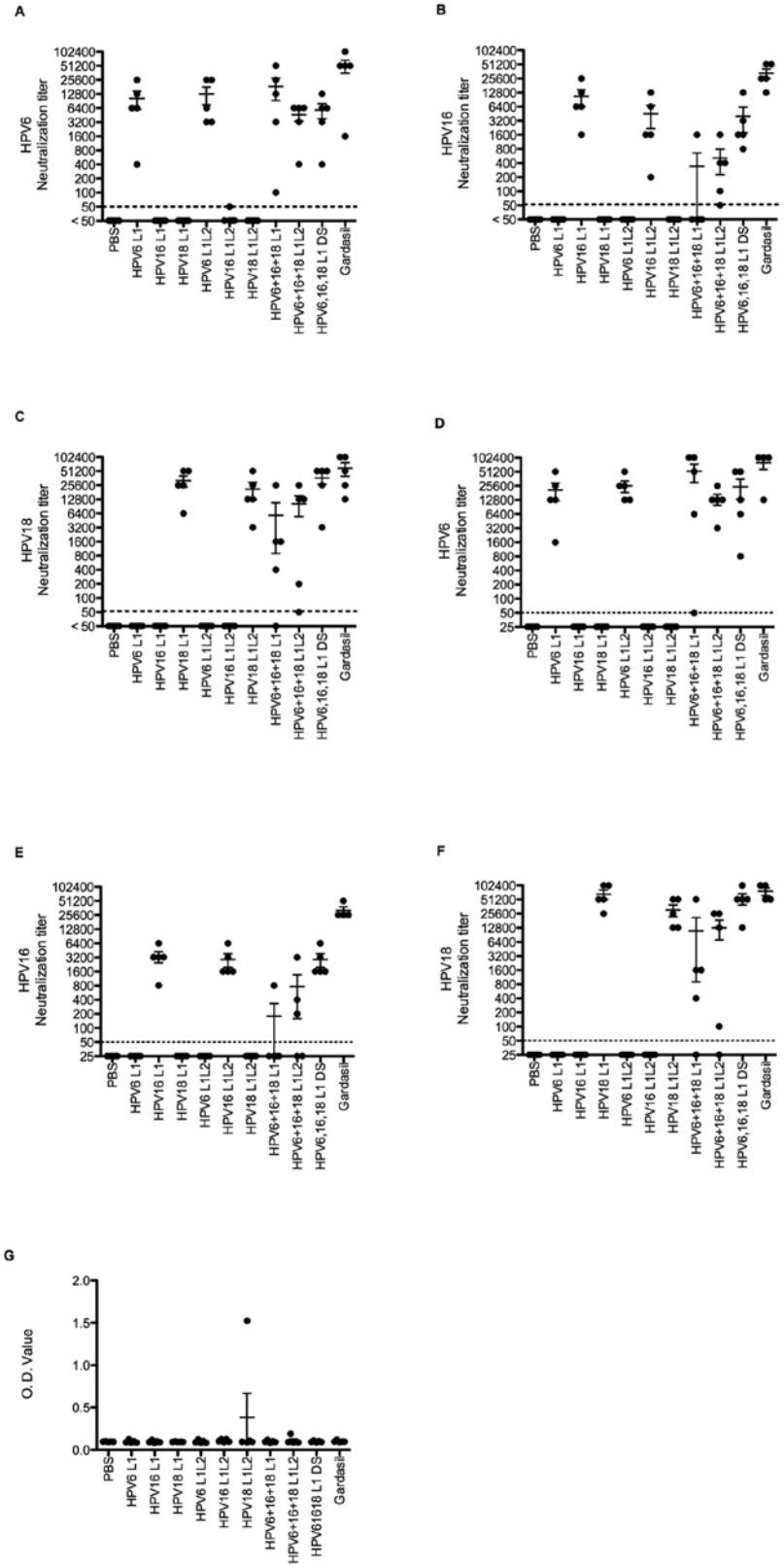

A
HPV6 Neutralization titer
102400
51200
25600
12800
6400
3200
1600
800
400
200
100
50
< 50
PBS
HPV6 L1
HPV16 L1
HPV18 L1
HPV6 L1L2
HPV16 L1L2
HPV18 L1L2
HPV6+16+18 L1
HPV6+16+18 L1L2
HPV6,16,18 L1 DS
Gardasil
B
HPV16 Neutralization titer
C
HPV18 Neutralization titer
D
HPV6 Neutralization titer
25
E
HPV16 Neutralization titer
F
HPV18 Neutralization titer
G
O.D. Value
2.0
1.5
1.0
0.5
0.0
HPV61618 L1 DS

Figure 12. A comparison of antibody responses of mice vaccinated with DNA expressing L1 or L1+L2 of HPV6, 16, or 18, either singly or together at the same or different sites.

Balb/c mice were vaccinated i.m. with electroporation three times at two week intervals with 20 mg each of DNA expressing L1 or L1+L2 of HPV6, HPV16, and HPV18, either individually, or together at the same site or each at a different site (DS), or s.c. with Gardasil. Serum samples were harvested at two weeks after the third vaccination (A–C) or 3 months after the third vaccination (D–F), and neutralizing antibody titers were measured with HPV6 (A,D), HPV16 (B,E), or HPV18 PsV (C,F). Antibody response to L2 was measured by ELISA against full length HPV16 L2 with serum samples collected two weeks after the third vaccination (G).

6) Electroporation of L1 DNA vaccines induces type-restricted protective antibody responses

As the titers of neutralizing antibodies induced upon vaccination three times i.m. using *in vivo* electroporation to deliver 20 ug of an L1 DNA vaccine were similar to, but still lower than those elicited by Gardasil, we therefore tested whether they are still sufficient to protect against vaginal challenge with the homologous type HPV. Although HPV does not produce disease in mice, the host restriction in HPV infection is determined after delivery of the viral genome to the nucleus, and thus infection of the vaginal tract of mice with HPV pseudovirions carrying a luciferase reporter can be detected by imaging bioluminescence [61]. Passive transfer of 20 ul of serum from mice vaccinated i.m. three times with *in vivo* electroporation to deliver 20 ug of DNA vaccine expressing either L1 only or L1+L2 completely protected na¨ive mice from experimental vaginal challenge with the homologous genotype (Figure 13A). This was also shown for HPV6 (Figure 13B), and the protection was maintained when using sera harvested at 3 months post vaccination for the passive transfer study (Figure 13C). Thus the data suggest that the neutralizing antibody titers induced by *in vivo* electroporation with an L1 DNA vaccine, although lower than for Gardasil, are sufficient for complete protection against vaginal challenge by the vaccine type.

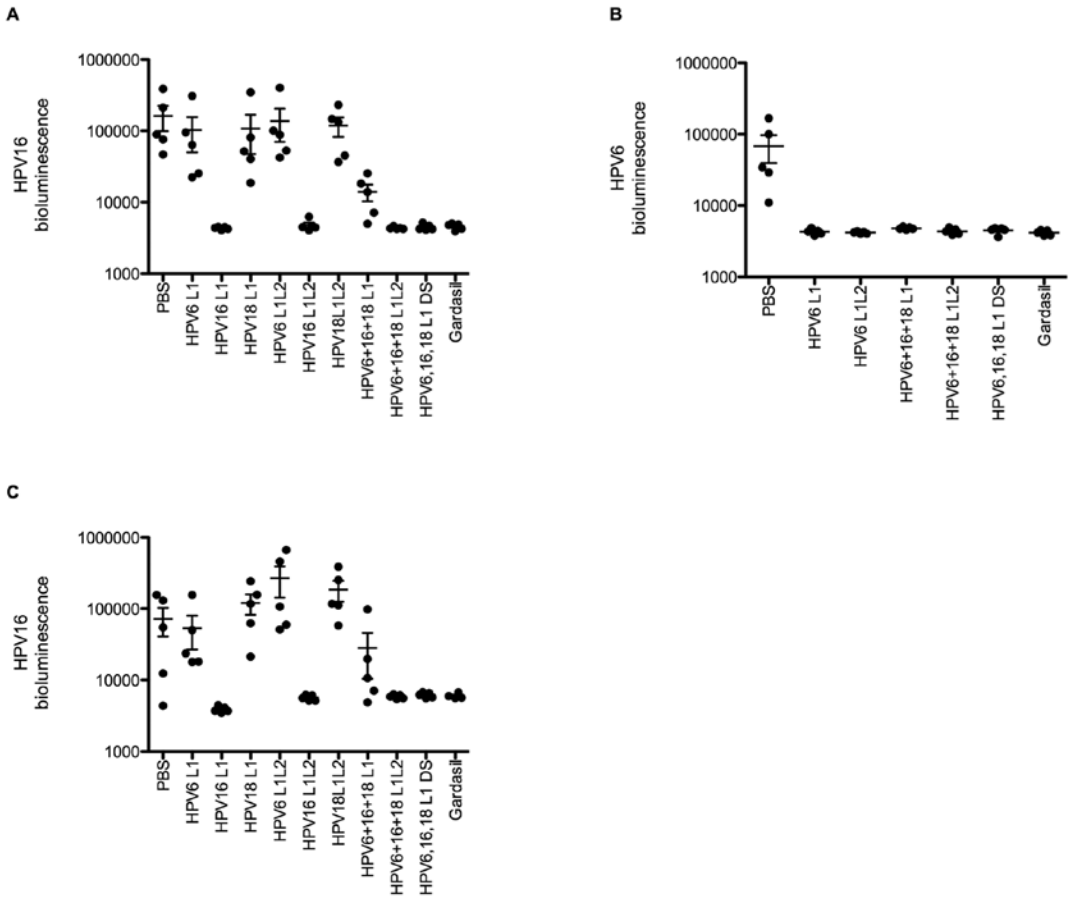


Figure13. Comparison of protective antibody responses.

Balb/c mice were vaccinated i.m. with electroporation three times at two week intervals with 20 mg each of DNA expressing L1 or L1+L2 of HPV6, HPV16, and HPV18, either individually, or together at the same site or each at a different site, or s.c. with Gardasil. Serum samples were collected two weeks (A,B) and three months (C) after the last vaccination to test their protective efficacy *in vivo* and *in vitro*. Na¨ive Balb/c mice (5 per group) were passively immunized i.v. with 20 ml of pooled serum. Mice were challenged intra-vaginally with HPV16 (A,C) or HPV6 (B) PsV carrying a luciferase reporter construct. Three days later, luciferin was administered intra vaginally, and bioluminescence imaging was performed.

7) L2 co-expression is sufficient to restore protection using multivalent L1-based DNA vaccination

The host restriction in HPV infection is determined after delivery of the viral genome to the nucleus, and thus infection of the vaginal tract of mice with HPV pseudovirions carrying a reporter gene such as luciferase whose expression can be detected by imaging bioluminescence [61]. This provides a useful model to examine *in vivo* protection of naïve mice after passive transfer of neutralizing antibodies [26,46]. Passive transfer of 20 ul of serum of mice electroporated i.m. with a mixture of three DNA vaccines expressing L1 of HPV6, HPV16, and HPV18 provided complete protection against intra-vaginal challenge with HPV6 PsV (Figure 13B). In contrast, when 20 ul of serum of mice electroporated i.m. with a mixture of three DNA vaccines expressing L1 of HPV6, HPV16, and HPV18 was injected into naive mice, significantly weaker protection against intra-vaginal challenge with HPV16 PsV was observed (Figure 13A). Interestingly, the sera from mice that were electroporated i.m. with a mixture of three DNA vaccines expressing both L1 and L2 of HPV6, HPV16, and HPV18, were completely protective by passive transfer. This finding suggests that although the co-expression of L2 does not completely recover the humoral response obtained by vaccination with a single L1 DNA vaccine, nevertheless the titers induced are sufficient for complete protection upon passive transfer of 20 ul of serum (which corresponds to ~1:50 dilution in the mouse). As expected, passive transfer of sera from mice electroporated i.m. at different sites with the three DNA vaccines expressing L1 of HPV6, HPV16, and HPV18, were also completely protective against HPV16 challenge. Importantly, these phenomena were consistent when testing for protective capacity

against vaginal HPV16 challenge of naïve mice after passive transfer of 20 ul of sera obtained three months after active vaccination (Figure 13C). These data suggest that the protective responses elicited by multivalent vaccination are durable if the L1 DNA vaccines expressing individual types are injected in different locations or L2 is co-expressed with each L1.

8) Immunologic interference is associated with heterotypic interactions between L1 proteins

The reduction in neutralizing responses observed when the DNA vaccines expressing L1 of different types are injected into the same site but not different sites suggests that it is direct interaction between L1 of different types rather than immunologic competition that this responsible for this interference. It has been previously described that HPV6 and HPV16 L1 subunits co-assemble together generating hybrid VLPs in yeast [73] and co-expression of either HPV11 or BPV1 L1 with HPV16 L1 reduces the assembly of HPV16 L1 VLPs [74]. In addition, it is known that neutralizing epitopes on VLPs are conformationally dependent and type-restricted [75,76,77]. Therefore, to test for direct interaction between L1 of different HPV types, 293TT cells were transfected with the three DNA vaccines expressing L1 of HPV6, HPV16, and HPV18 either individually or simultaneously, and immunoprecipitation experiments were performed on cell lysates using the H18.F8 monoclonal antibody that recognizes a conformational and type-specific HPV18 L1 epitope [78]. The presence of HPV6 L1, HPV16 L1 and HPV18 L1 in the immunoprecipitates was detected by Western blot analysis with type-restricted monoclonal antibodies H6.C6, H16.O7 and H18.E20 [78], respectively (Figure 14). H18.F8 immunoprecipitated HPV18 L1, but not HPV6 L1 or HPV16 L1 from lysates of cells transfected with only a single L1 DNA vaccine (Figure 14). By contrast, H18.F8 immunoprecipitated HPV6 L1, HPV16 L1 and HPV18 L1 from the lysates of cells co-transfected with the three DNA vaccines expressing L1 of HPV6, HPV16, and HPV18 L1. These results demonstrate that HPV18 L1 can bind to both HPV6 L1 and HPV16 L1.

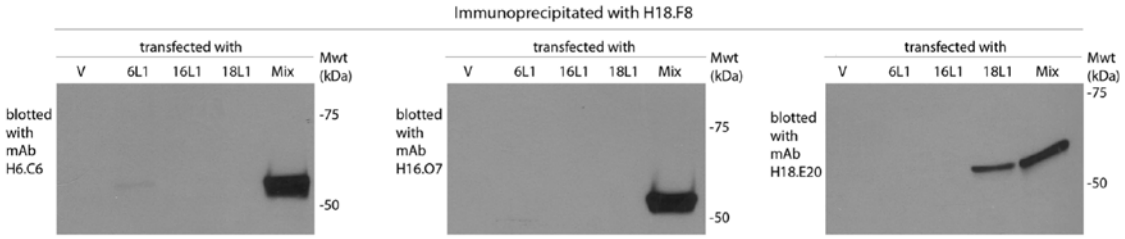


Figure14. Interaction between HPV18 L1 with L1 of both HPV6 and HPV16.

To test for interaction between L1 proteins of different HPV genotypes, 293TT cells were transfected with empty DNA vector (V), or vector expressing HPV6 L1, HPV16 L1, or HPV18 L1 individually (6L1, 16L1, 18L1, respectively), or all three together (Mix). Two days later, the cells were harvested and lysed. HPV18 L1 was immune-precipitated from lysates using the conformationally-dependent and neutralizing monoclonal antibody H18.F8. The presence of HPV6 L1, HPV16 L1 and HPV18 L1 in the immune-precipitates was detected by Western blotting with H6.C6 (left panel), H16.O7 (middle panel), and H18.E20 (right panel) respectively.

d) Discussion

L1 DNA vaccines have potential as an alternative prophylactic HPV vaccine that is simple and inexpensive to produce and heat stable, properties that would facilitate widespread immunization in low resource and remote settings. However, delivery of DNA vaccines *in vivo* has been limited by the efficiency of transfection *in vivo*. Several approaches including ballistic delivery via gene gun, *in vivo* electroporation devices, and tattooing have been developed to improve *in vivo* delivery. Here, we have tested two of these approaches previously tested clinically for DNA vaccine delivery; gene gun and *in vivo* electroporation. While gene gun vaccination demonstrated strong neutralizing antibody responses with only 2 mg of HPV16 L1 DNA vaccine, this was the highest amount of DNA that could be loaded onto the gold particles. The *in vivo* electroporation approach required 10 mg of HPV16 L1 DNA vaccine to achieve a consistent and robust neutralizing antibody response (that was not significantly higher with 20 mg or 40 mg doses), and these titers were higher than for 2 mg maximal dose of HPV16 L1 DNA vaccine delivered via gene gun. Although electroporation clearly enhances the efficiency of *in vivo* transfection of cells with the DNA vaccine, it may also trigger local inflammation, cell death and recruitment of immune cells to enhance the immune response [129-131]. While L1 DNA vaccination clearly has potential as a heat stable and low cost vaccine, a requirement for *in vivo* electroporation would raise the cost and complexity of vaccination because of the need for an electroporation device and access to electricity. Delivery via a self-contained gene gun device like PD-10 is another option, but the DNA dose is much more limited than for *in vivo* electroporation.

Hitzeroth et al observed a very weak (titer of 1:50) and non-neutralizing antibody response to vaccination of mice i.m. twice with 100 mg of DNA expressing full length codon-optimized HPV16 L2 [143]. The L2×8 DNA vaccination failed to elicit a detectable neutralizing antibody titer (Figure 11). A possible reason for the lack of response is poor expression by the L2×8 construct as compared with full length HPV16 L2 is incorrect codon optimization resulting in poor expression. However, when we re-optimized codon usage in the L2×8 construct for mammalian expression and compared expression levels to the previous bacterial codon optimized construct there was no significant improvement in expression. There are negative transcription and translation regulating sequences in the L2 gene, and the L2×8 DNA constructs may therefore still contain 8-fold more negative regulatory sequences than HPV16 L2, even after the nucleotide sequence changes resulting from codon optimization. In addition, recent studies suggest the presence of a transmembrane-like domain within this region of L2 that may limit expression and/or antigen release [157]. The L2×8 protein exhibited extensive degradation when expressed in mammalian cells (Figure 11C), which may also contribute to its low level and immunogenicity. Finally, it is clear that L2×8 is substantially less immunogenic than L1 VLP as the L2×8 protein vaccine induces, 30-fold lower neutralizing antibody titers than Gardasil [79].

Previously it was shown that HPV6 L1 and HPV16 L1 can co-assemble forming hybrid VLP in yeast [151] and that co-expression of either BPV1 or HPV11 L1 with HPV16 L1 reduces HPV16 L1 VLP assembly in 293T cells [152]. We also showed that HPV18 L1 binds to both HPV6 L1 and HPV16 L1 when co-expressed in 293TT cells (Figure 14). The interaction between L1 of different HPV types, i.e. heterotypic binding,

likely reflects the high degree of sequence conservation of the internal L1 structure, and has a deleterious effect upon multivalent L1 DNA vaccination. Indeed, mice vaccinated with a mixture of three DNA vaccines expressing L1 of HPV6, HPV16 and HPV18 showed relatively reduced protection against HPV16 compared to the group vaccinated with single HPV16 L1 DNA. This was also the case for HPV18 L1, but not for HPV6 L1. Thus the interference was inconsistent, possibly reflecting different L1 expression levels and affinity for heterotypic L1 interaction. Vaccinating at different sites completely restored the induced neutralization titer to the levels obtained when vaccinating with a single construct (Figures 12 and Figure 13). This observation suggests that spatial separation of individual type L1 DNA vaccines during vaccination prevents the formation of heterotypic L1 interactions and aberrant assembly of chimeric VLP. Three HPV types were used in our experiments, but more HPV types covering most of oncogenic types could potentially be delivered by a multi-microneedle injector to spare the patient from receiving multiple injections at each visit.

The co-expression of the cognate L2 in each construct is a possible alternative to spatial separation of the L1 DNA vaccines of different genotypes during multivalent vaccination. While this approach enhanced the neutralizing antibody responses relative to a mixed multivalent L1 DNA vaccine, it did not achieve in all cases the titers obtained upon vaccinating with a single type or spatial separation with multivalent L1 DNA vaccination (Figure 12). Nevertheless, the titers achieved with a mixed multivalent L1+L2 vaccine were completely protective against vaginal HPV16 challenge upon passive transfer to na¨ive mice with 20 ml of serum whereas antiserum from mice administered with the mixed multivalent L1 vaccine was only partially protective (Figure

13). This protection data is consistent with the *in vitro* neutralization titers and suggests that only a low neutralizing antibody titer is required for complete protection. Further the protective response to the electroporation of the mixed multivalent L1+L2 vaccine was durable at 3 months after the final vaccination, suggesting that co-expression of L2 might be an alternative to administering each construct at a different site.

Both of the licensed HPV vaccines utilize an alum-based adjuvant and Cervarix also includes a TLR4 agonist, MPL. Naked DNA vaccines, delivered appropriately, can potentially activate TLR9 via CpG islands as well as cytoplasmic DNA sensors such as DAI and AIM2 to enhance the immune response. However, we have not examined L1 DNA vaccination with alum and utilizing electroporation. Previously, it was found that mixing a hepatitis B DNA vaccine with aluminum phosphate improved the antibody titers, 10-fold relative to DNA alone injected i.m. without electroporation [158]. While the titers of neutralizing antibody induced by L1 DNA vaccines might be improved with adjuvant and utilizing electroporation to reach the levels produced by Gardasil, it is clear that the L1 DNA vaccines elicit completely protective responses. Passive transfer of 20 ml of serum from either L1 DNA or Gardasil vaccinated mice rendered na¨ive animals immune to vaginal challenge. This corresponds to a, 1:50 dilution in the mouse, indicating a robust response. Furthermore, while the studies demonstrate that neutralizing antibody is sufficient to mediate protection, it does not rule out a contribution of L1-specific T cell immune responses to protection in actively vaccinated mice. Further study is warranted to determine the relevance of L1-specific T cell immune responses to preventing HPV infections and the relative levels induced by L1 VLP protein and L1 DNA vaccination.

Safety issues and practical considerations surrounding the use of DNA as a

vaccine need further consideration. Although it has not been an issue in clinical studies of naked DNA vaccines to date, the potential for a low frequency of integration of the vaccine DNA into the host chromosome or the induction of anti-DNA antibodies to cause disease remains a concern. Standard i.m. injection of naked DNA is very inefficient because only a small fraction of DNA is taken up by cells and expressed, but advances in delivery technologies such as ballistic delivery and electroporation are beginning to overcome this barrier. Indeed, our results suggest that vaccinating at different sites with multi-type L1 or L1+L2 DNA vaccines and using electroporation to enhance delivery shows promise as a next generation HPV vaccine candidate.

III) Capsomer vaccines protect mice from vaginal challenge with human papillomavirus

a) Introduction

Persistent infection by high risk HPV types is a necessary, but not sufficient cause of cervical cancer, as well as a significant fraction of other anogenital cancers and subset of head and neck cancers [2]. Two licensed HPV vaccines, Gardasil® (Merck) and Cervarix® (GSK), are derived from the viral major capsid protein L1, which assembles into virus-like particles (VLPs) when expressed in eukaryotic cells [74]. Gardasil® is derived from L1 expression in yeast and Cervarix® from expression in baculovirus infected insect cells. In Gardasil®, L1 VLPs derived from HPV6 (20 mg), HPV11 (40 mg), HPV16 (40 mg) and HPV18 (20 mg) are formulated using an amorphous aluminum hydroxyphosphate sulphate adjuvant (225 mg). In Cervarix®, HPV16 and HPV18 L1 VLPs (20 mg each) are adjuvanted with aluminum hydroxide (500 mg) and 3-O-desacyl-4'-monophosphoryl lipid A (MPL, 50 mg). Both vaccines have demonstrated remarkable protective efficacy against infection by the specific HPV types present in each vaccine [8,10,75,159]. Importantly, efficacy has been demonstrated against the development of low and high grade intraepithelial neoplasia at the cervix, vaginal, vulva and anus that are associated with HPV16 and HPV18 infection [160]. Vaccination with HPV6 and HPV11 VLPs also prevents the occurrence of benign genital warts caused by these types [161]. Vaccination with L1 VLPs induces type-restricted immunity, i.e., near absolute protection against the type utilized to generate the vaccine, significant but weaker cross-

protection for the most closely phylogenetically-related types (e.g., HPV16 and HPV31, or HPV18 and HPV45), and partial to no protection against more distantly related types or types from other species [11,75]. HPV16 and HPV18 are associated with 50% and 20% of cervical cancer cases, respectively. Because more than a dozen high risk types are associated with cervical cancer and benign genital warts cause significant morbidity, there is an effort to develop a nonavalent L1 VLP vaccine targeting the seven most common oncogenic HPV types and the two most common types in genital warts, HPV6 and HPV11 [42,54].

High-risk HPV infection causes <5% of all cancer worldwide, but <85% of cervical cancer cases occur in low income countries, reflecting their lack of resources and infrastructure to support national cytologic screening efforts [115]. Therefore, the potential benefits of HPV vaccination are likely to be greatest in low resource settings, highlighting the need to develop inexpensive and broadly protective HPV vaccines that can be delivered globally [74].

VLPs are formed by the assembly of seventy two capsomers, and each capsomer is comprised of five L1 molecules [5,162]. L1 capsomers are potential low cost alternatives to VLPs because they can be purified after expression in bacteria in high yields [34,116,117]. Deletion of the N-terminal 9 amino acids and the C-terminal 29 amino acids from L1 (herein abbreviated as L1Δ) enhances the yield of capsomers [34,119].

Vaccination of dogs with GST-fused canine oral papillomavirus (COPV) L1 capsomers protects against experimental oral challenge with COPV [36]. Vaccination

with COPV L1 is completely protective without an adjuvant using only 400 ng capsomers or 50 ng VLPs. Passive transfer of serum immunoglobulins from COPV L1 VLP-vaccinated dogs to naïve recipients protected the latter from experimental COPV challenge, indicating the capacity of neutralizing antibody to mediate protection. Capsomers display comparable type-restricted neutralizing epitopes to L1 VLPs [37,163], but low avidity and broadly reactive L1 epitopes have been described [164]. Because they are not coordinated with other capsomers and are deleted at both termini, we hypothesized that L1Δ capsomers potentially may display cross-protective epitopes that are otherwise hidden in VLPs and trigger low avidity broadly protective antibodies difficult to detect by neutralization assays [165]. Herein we examine the potential of L1Δ capsomers to generate antibodies that are both broadly neutralizing and protective against vaginal challenge.

The need for broad protection against high-risk HPV types can potentially be provided by multivalent combinations of capsomers, but this formulation complicates manufacture. As an alternative, we explored the C-terminal fusion of the minor capsid protein L2 to L1Δ a site on the capsomer that tolerates such additions without adversely affecting its structure. Residues at the amino terminus of L2, including amino acids 13–47 [46,166,167], contain broadly protective epitopes [45,46,111], but they are weakly immunogenic in comparison to L1 [44,112]. Indeed, when L2 is co-assembled with L1 in a VLP, L2 is unrecognized by the immune system either because of its internal positioning or the context of L1 dominant epitopes [112]. We hypothesized that the tandem fusion of L2 residues 13–47 from three different and common high-risk HPV types (derived from HPV18, HPV31 and HPV45 that cause 17.2%, 2.9% and 6.7% of

cervical cancer cases respectively [54]) to the C-terminus of HPV16 L1Δ (the type responsible for 53.9% of cervical cancer cases) would enhance the immunogenicity of L2 by displaying a dense and regular array of epitopes [168], and thus provide broad protection (potentially >80%) against cervical cancer using a single antigen.

b) Materials and Methods

1) Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (protocol MO08M19).

2) Cells, plasmids, media and growth conditions

Plasmid pGEX-HPV16 L1Δ (encoding amino acids 36–502 from GenPept accession number NP_041332.1) was constructed for the expression of the deletion mutant HPV16 L1ΔN9ΔC29 with an N-terminal GST tag. The HPV16 L1 gene was codon optimized for expression in Escherichia coli and synthesized by Genscript with a BamHI site at its 59 end, and two stop codons and an XhoI site at its 39 end. It was then cloned into the BamHI/XhoI sites of pGEX-4T-2 (GE Healthcare) and verified by sequencing.

Plasmid pGEX-HPV16 L1Δ-L2×3 was constructed by amplifying HPV16 L1ΔN9ΔC29 from the aforementioned plasmid and reinserting the L1 segment into pGEX-4T-2 using the BamHI and EcoRI restriction sites. The L2 segment of this plasmid was created synthetically using overlapping oligonucleotides that were assembled and amplified using PCR. A sequence containing amino acids 12–46 from HPV18 L2, amino

acids 13–46 from HPV31 L2 and amino acids 12–46 from HPV45 L2 (GenPept accession numbers AAP20600.1, AAA92893.1 and AAY86493.1, respectively) in tandem were submitted to the online program DNAWorks (http://helixweb.nih.gov/dnaworks/). This sequence also contained five glycines preceding the HPV18 L2 sequence and two glycines in between the HPV18 and 31 and HPV31 and 45 sequences. The DNAWorks output included 16 oligonucleotides optimized for expression in *E. coli*. These oligonucletoides were assembled by PCR in a reaction containing 100 ng of each oligo, 200 mM of each dNTP, 1x PfuTurbo reaction buffer and 2.5 U PfuTurbo (Stratagene). The assembly reaction was performed as follows: 2 min at 95°C, 25 cycles of 30 sec at 95°C, 30 sec at 55– 65°C and 1 min at 72°C followed by 10 min at 72°C. Each of these reactions (1 ml as a template) was then amplified using the outermost oligonucleotides as forward and reverse primers (100 ng each). The amplification reaction was performed as follows: 2 min at 95°C, 25 cycles of 30 sec at 95°C, 30 sec at 62°C and 1 min at 72°C followed by 10 min at 72°C. The assembly reactions performed between 60 and 65°C resulted in the appropriately sized product. This product was digested and ligated into the pGEX-4T-2 vector containing HPV16 L1Δ between the EcoRI and NotI sites. The plasmid was verified by DNA sequencing.

3) Purification of capsomers

Truncated HPV16 L1 was expressed as a GST fusion protein either alone or with the tandem HPV L2 epitopes in BL21(DE3) *E. coli* (Stratagene). A single colony of

transformed bacteria was inoculated into 50 ml of Terrific Broth (TB) (1.2% Tryptone, 2.4% yeast extract, 0.5% glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4) containing 100 ug/ml ampicillin and grown overnight at 30°C. The 50 ml overnight culture was then used to inoculate 500 ml of fresh selection medium to an optical density $(OD)_{595}$ of 0.1, and this culture was grown at 37°C until the OD_{595} reached 4.0. The temperature was decreased to 25°C, 200 ul of 0.5 M IPTG was added and the culture was grown to an OD_{595} of 8. The cultures were then split into two bottles of 250 ml each, and the cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C. The cell pellets were frozen at -20°C until use.

The cells were lysed, and the fusion proteins were purified via affinity chromatography. Each cell pellet was resuspended in 100 ml of ice-cold buffer L (50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 1mM EDTA) with 5mM DTT, 1mM PMSF and 1x complete protease inhibitor cocktail (Roche). The cell suspension was homogenized using a French pressure cell (Thermo Scientific) at 2,000 psi to complete cell lysis. DNase I (40 U/ml), 2 mM ATP and 10 mM $MgCl_2$ were added to the lysed bacteria, and the lysate was rocked for 1 h at room temperature. Solid urea was then added to a final concentration of 2.3 M, and the lysate was again rocked for 1 h at room temperature. The lysate was dialyzed overnight at 4°C into two changes of buffer L/5 mM DTT without urea, centrifuged at 25,000 × g for 20 min at 4°C and passed through a 0.2 mm filter. A volume of 50 ml of clarified lysate was applied to a glutathione sepharose column (GSTrap FF 5 ml column, GE Healthcare) at 0.5 ml/min using an ÄKTA FPLC system (GE Healthcare). The column was washed with four column volumes each of buffer L with 2 mM DTT and buffer L with 2 mM DTT and 0.01% Tween 80. The GST-tagged

proteins were eluted with buffer L with 2 mM DTT, 0.01% Tween80 and 10 mM reduced glutathione. SDS-PAGE analysis of the eluted fusion protein demonstrated GST-HPV16L1Δ or GST-HPV16L1Δ-L2×3 and free GST as the major components. The concentration of the GST-16L1Δ and GST-16L1Δ-L2×3 proteins in the samples used for vaccination were estimated by SDS-PAGE in comparison to BSA standards. The conformation of these proteins was verified by ELISA with the H16.V5 antibody [156], which specifically reacts with a conformationally-dependent neutralizing epitope on the L1 surface.

4) Generation of HPV pseudoviruses (PsV)

Pseudoviruses were generated as previously described [82]. Briefly, plasmids pshellHPV16L1L2, pVitroHPV18L1L2, pVitroHPV31L1L2 or pVitroHPV45L1L2 were transfected with either a luciferase or alkaline phosphatase (SEAP) reporter gene plasmid into 293TT cells [82] using TransIT-2020 transfection reagent (Mirus). Three days after transfection, cell pellets were collected and rinsed with DMEM and Dulbecco's PBS. The pellets were resuspended in a small volume of DPBS-Mg (DPBS supplemented with 9.5 mM $MgCl_2$) and then transferred into siliconized tubes. Cells were pelleted by low speed centrifugation, and the supernatant was discarded. For PsV maturation, an equal volume of lysis buffer (DPBS-Mg supplemented with 0.5% of Brij 58 and 0.2% benzonase) was added to the cell pellet, which was allowed to incubate at 37°C for 24 h. After maturation, lysates were adjusted to 850 mM NaCl and extracted with high salt buffer (DPBS with 0.8 M NaCl). Lysates were clarified by centrifugation at 10,000 × g for 10

min, loaded onto an Optiprep step gradient (27, 33 and 39%) and spun at 40,000 rpm in a SW40 rotor for 16 h at 16°C. After centrifugation, 0.5 ml fractions were collected from the top of the gradient. A sample representing each fraction was diluted and tested on 293TT cultures [82] for reporter gene expression. Fractions with highest reporter gene expression were pooled and used for the serum neutralization assay and the *in vivo* challenge experiments.

5) Immunization of mice

Inbred 4- to 6-week old female BALB/c mice (NCI) in groups of five or 10 were immunized subcutaneously three times at two-week intervals. The immunogens used were 25 ug of GST-HPV16L1Δ, 25 ug of GST-HPV16L1Δ-L2×3 or the indicated doses of the 11-88×5 HPV L2 fusion peptide. Each dose of immunogen (50 ul) contained comparable adjuvant (50 ug aluminum hydroxide and 5 ug 3-O-desacyl-4'-monophosphoryl lipid A [MPL]) to $1/10^{th}$ of a human dose of the Cervarix® vaccine.

6) Measurement of neutralization antibody titers

Serum samples collected from each mouse two weeks after the final immunization were serially diluted two-fold in culture medium, mixed with an equal volume of HPV pseudovirions containing either luciferase or SEAP reporter genes, and incubated at 37°C for 2 h. These samples were each added to 293TT cell cultures (3×10^4 cells/well), which were then incubated for 72 h [82]. To measure luciferase expression,

culture media were removed from each well and 1x Cell Culture Lysis Reagent (CCRL, Promega) was added to lyse the cell monolayer for 15 min at room temperature on a rocking platform. A volume of 20 ul of the lysate from each well was transferred to a black microtiter plate followed by the addition of 50 ul/well of 1× luciferase substrate (Promega). The plates were scanned with the GloMax®-Multi Detection System (Promega). For SEAP measurement, 40 ul of cell-free supernatant was collected from each well, mixed with 20 ul of 0.05% CHAPS, heated at 65°C for 30 min and then cooled on ice. A volume of 200 ul/well of p-nitrophenyl phosphate (pNPP) substrate (2 M diethanolamine with 1 mg/ml of pNPP) was added to each well for color development at ambient temperature, and absorbance data was collected with an automatic ELISA plate reader (Bio-Rad). Data was collected when the absorbance from the wells incubated with only PsV (ODpsv) had an OD_{405}>1. The highest dilution of serum that resulted in 50% or more reduction in ODpsv was defined as the endpoint neutralization titer and expressed as its reciprocal value.

7) Determination of ELISA antibody titers

L2 antigens (HPV16 L2 amino acids 11–200 or full length HPV18 L2 or HPV31 L2 were generated in *E. coli* as 6×His fusions [170], or L2 amino acids 17–38 of HPV16, 17–36 of HPV31, 17– 36 of HPV35, 16–35 of HPV45 or 16–35 of HPV58 chemically synthesized as a peptide) were coated onto an ELISA plate (MaxiSorp; Thermo Scientific) at 100 ng of protein or 500 ng of peptide per well and incubated at 4°C overnight. Coated plates were blocked with PBS-BSA (1× PBS supplemented with 1%

BSA) at 37°C for 1 h and then incubated with 100 ul of the diluted serum samples (two-fold serial dilution from 1:50) at ambient temperature for 1 h. Plates were then washed three times with PBST (1× PBS with 0.01% Tween 20), and bound antibody was detected with horseradish peroxide-conjugated sheep anti-mouse IgG antibodies (GE Health) followed by further washes with PBST and development with the 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Roche). The cutoff OD value (OD_{cutoff}) for the ELISA assay was set at three times the average absorbance detected from five na¨ive mouse serum samples. The highest dilution of serum sample that had an OD value equal to or above the OD_{cutoff} was defined as the endpoint ELISA titer.

8) *In vivo* PsV vaginal challenge

Four days before challenge, female Balb/c mice were injected subcutaneously with 3 mg of medroxyprogesterone (Depo-Provera; Pfizer) to synchronize their estrus cycles [61]. Each challenge dose was comprised of 10 ul PsV mixed with 10 ul of 3% carboxymethyl cellulose (CMC). The dose was delivered twice into vaginal vault, the first 10 ml just prior to and the second 10 ul just after treatment with a cytobrush cell collector. The cytobrush cell collector was inserted into the vaginal vault and turned both counter-clockwise and clockwise 15 times while the mice were anesthetized. Three days after PsV delivery, the mice were again anesthetized and 20 ul of luciferin (7.8 mg/ml) was deposited in the vaginal vault. Luciferase signals were acquired for 10 min with a Xenogen IVIS 100 imager, and analysis was performed with Living Image 2.0 software.

9) Statistical analysis

Data were analyzed with one-way ANOVA, or the Kruskal-Wallis test and multiple comparison methods (GraphPad 4).

c) Results

1) Capsomer construction and vaccination

The generation of the HPV16 L1 deletion mutant (GST-HPV16L1Δ) and its purification after expression as a GST fusion protein in bacteria have been previously described [34]. A second construct (GST-HPV16L1Δ-L2×3) was prepared for this study by fusing a bacterial codon-optimized sequence encoding L2 amino acids 13–47 of HPV18 with the corresponding sequences of HPV31 and HPV45 in tandem to the C-terminus of GST-HPV16L1Δ. Both constructs were used for the expression of high levels of soluble protein in bacteria. These proteins were purified from lysates by affinity FPLC chromatography with glutathione sepharose. A ~5-fold lower dose of L1 as VLP compared to capsomers is sufficient to induce immunity [22,36]. Therefore, to assess the immunogenicity of the GST-HPV16L1Δ and GST-HPV16L1Δ-L2×3 capsomers, groups of Balb/c mice were immunized three times at two-week intervals with 25 ug of capsomers formulated in 50 ug alum and 5 ug MPL per dose. For comparison, an additional group of mice was vaccinated with 1/ 10^{th} of a human dose of Cervarix®, which was comprised of 2 ug each of HPV16 and HPV18 L1 VLPs formulated in 50 ug alum and 5 ug MPL.

2) Serum neutralization antibody titers

Two weeks after the third immunization, serum samples from each immunized group were tested for neutralizing antibody titers against the four HPV types most commonly found in cervical cancer [53]. HPV16-specific serum neutralization titers were detected in all groups of mice except for the na¨ive group (Figure 15). Animals immunized with Cervarix® showed a very high mean serum titer of HPV16 neutralizing antibodies (53,120), whereas the GST-HPV16-L1Δ and GST-HPV16-L1Δ-L2×3 capsomer-immunized groups exhibited mean titers of 7520 and 6160, respectively (Figure 15A), suggesting that the greater immunogenicity of VLP may provide dose sparing as compared with capsomers. However, the average anti-HPV18, HPV31 and HPV45 neutralization titers were below the limit of detection (<100) for both groups that received capsomer vaccines, which is consistent with type-restricted neutralization (Figures 15B-D). Serum samples from the Cervarix®-immunized group exhibited an average neutralization titer of 34,720 against HPV18, whereas average neutralization titers against HPV31 and HPV45 were below the limit of detection (<100). The latter finding is surprising because vaccination with Cervarix® protects patients against HPV31 and HPV45 [75] and modest titers of neutralizing antibodies for these types were detected in some vaccinated patients [169], indicating that even very low titers of neutralizing antibodies are protective.

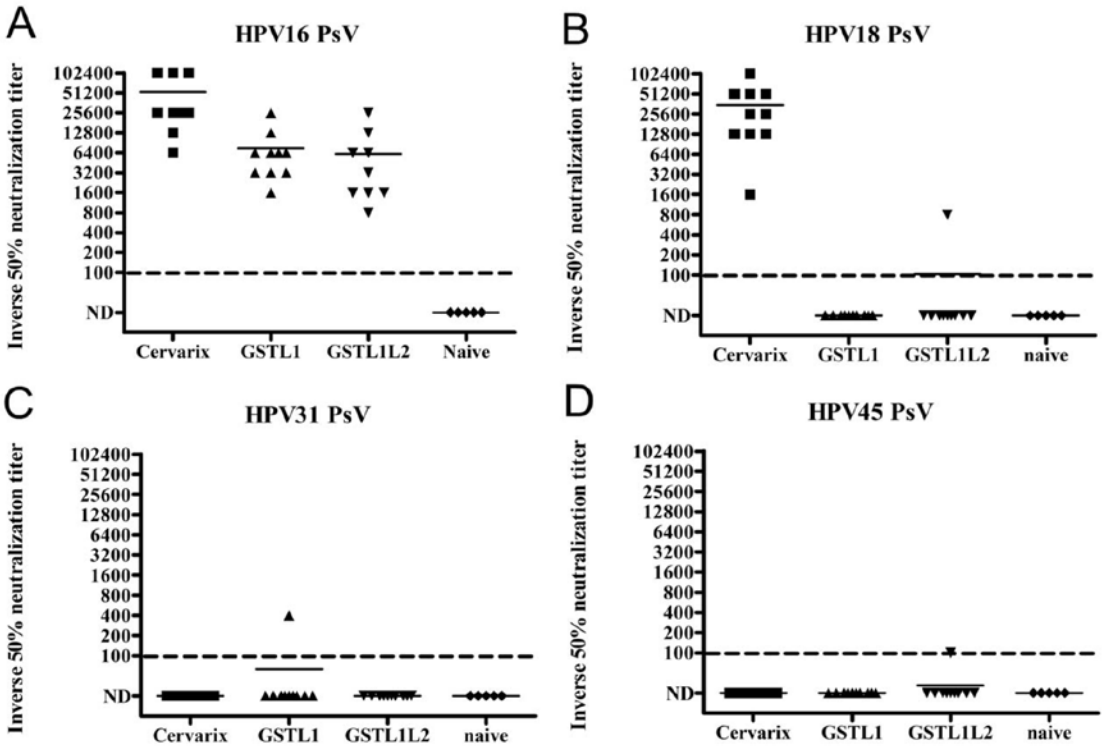


Figure 15. Neutralizing antibody responses of mice vaccinated with capsomers.

Two weeks after the third immunization with GST-HPV16L1Δ, GST-HPV16L1Δ-L2×3 or Cervarix®, two-fold dilutions of serum from na¨ive or immunized mice were tested for *in vitro* neutralization activity against pseudovirions derived from HPV16 (A), HPV18 (B), HPV31 (C) or HPV45 (D) starting at a dilution of 1:100. The reciprocal of the highest serum dilution with ≥50% reduction in reporter gene expression signal was defined as the serum virus neutralizing antibody titer, and <100 was considered not detected (ND) and arbitrarily plotted at 10.

3) Serum anti-HPV L2 ELISA titers

Serum samples from each immunized group taken two weeks after the third immunization were tested for L2 specific antibody titers by ELISA against either HPV16 L2 11–200 or HPV16 L2 17–36, conserved neutralizing and protective epitopes [46,170]. Sera of mice vaccinated with GST-HPV16L1Δ-L2×3 exhibited mean ELISA titers of 545 and 230, respectively, against HPV16 L2 11–200 and 17–38 (Figure 16). Since the GST-HPV16L1Δ-L2×3 contains L2 sequences derived from HPV18, 31 and 45, we examined the reactivity of the sera to full length HPV18 L2 protein, HPV31 full length L2 protein and 17–36 peptide, and HPV45 L2 16–35 peptide. The GST-HPV16L1Δ-L2×3 antisera reacted specifically with the HPV18 and HPV45 L2 polypeptides, but not with either the HPV31 full length protein or the 17–36 peptide. The GST-HPV16L1Δ-L2×3 antisera also failed to react with HPV35 L2 17–36 or HPV58 L2 16–35 peptides, two types that were not included in the vaccine construct.

We previously showed that the L1 and L2-specific antibody responses to vaccination with L1 capsomers mixed with multimeric L2 protein 11-88×5 (a fusion of L2 amino acid residues 11–88 derived from HPV1, 5, 6, 16 and 18) were independent (i.e., no interference was noted) [37]. Although fusion of L2 with L1 capsomers did not significantly impact the neutralizing antibody response, surprisingly, no neutralizing antibody titers were detected for HPV18, HPV31 or HPV45, indicating that the L1-specific response might dominate the L2 specific response to GST-16L1Δ-L2×3 vaccination. GST-HPV16L1Δ-L2×3 is a fusion peptide that contains GST, an L1 deletion mutant and three copies of L2 amino acids 13 to 47 derived from HPV types 18, 31 and 45, while 11-88×5 is a fusion of L2 amino acids 11 to 88 from five different HPV types

(HPV1, 5, 6, 16 and 18) [48]. Thus, vaccination with 4 ug of the latter would provide a similar amount of L2 for comparison. Serum collected from mice immunized with 4 ug of 11-88×5 had a mean HPV16 L2 17–36 peptide ELISA titer of 2960, which is ≈12-fold higher than the mean serum titer of the GST-HPV16L1Δ-L2×3-immunized group (230) (Figure 16B). As compared with GST-HPV16L1Δ-L2×3 antisera, the antisera to the 11-88×5 fusion was also more cross-reactive with L2 neutralizing epitopes of HPV types not utilized in making the 11-88×5 construct, notably HPV31, HPV35, HPV45, and HPV58.

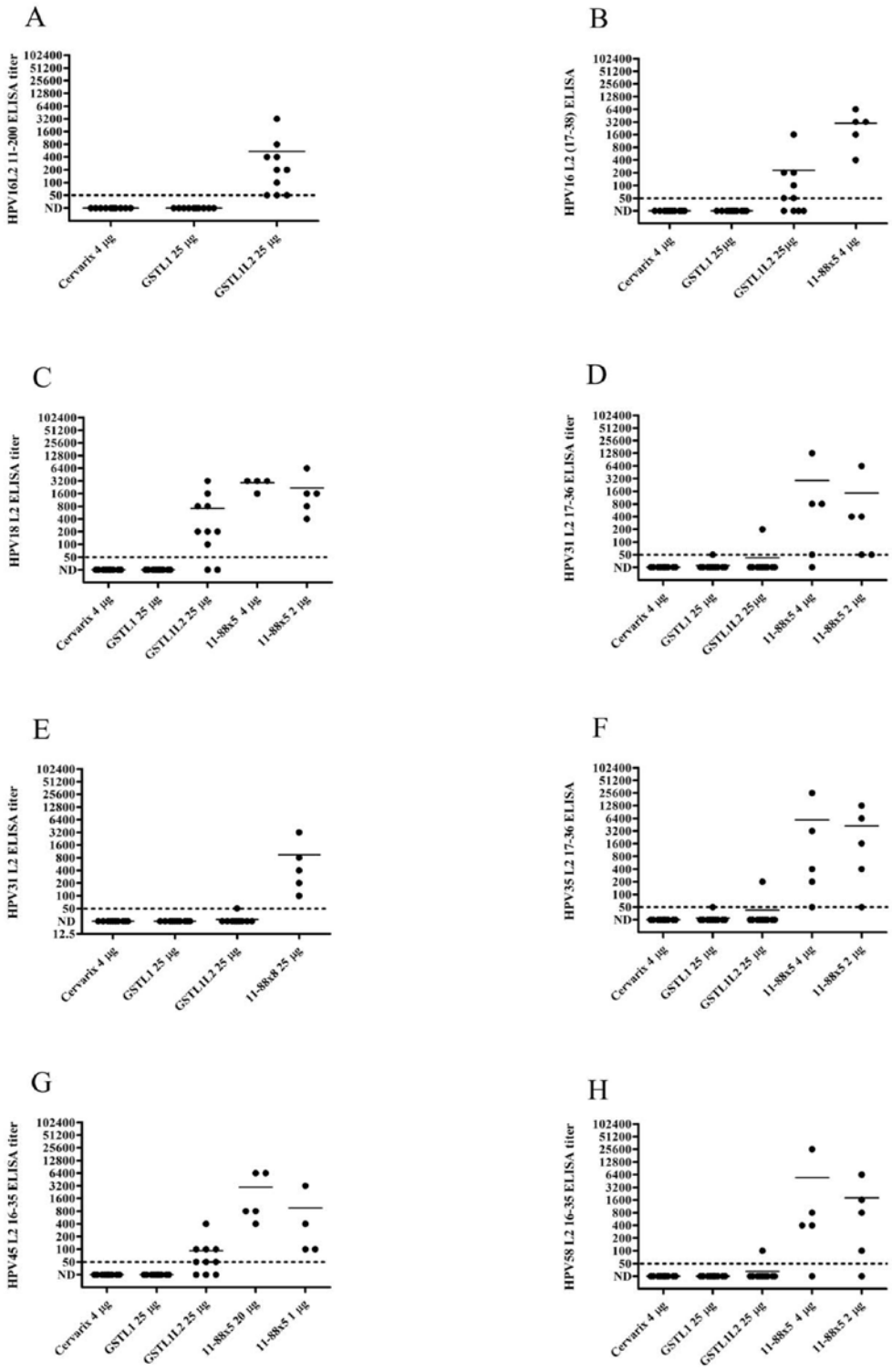

A
HPV16L2 11-200 ELISA titer
102400
51200
25600
12800
6400
3200
1600
800
400
200
100
50
ND
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
B
HPV16 L2 (17-38) ELISA
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25µg
11-88x5 4 µg
C
HPV18 L2 ELISA titer
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
11-88x5 4 µg
11-88x5 2 µg
D
HPV31 L2 17-36 ELISA titer
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25µg
11-88x5 4 µg
11-88x5 2 µg
E
HPV31 L2 ELISA titer
12.5
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
11-88x8 25 µg
F
HPV35 L2 17-36 ELISA
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
11-88x5 4 µg
11-88x5 2 µg
G
HPV45 L2 16-35 ELISA titer
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
11-88x5 20 µg
11-88x5 1 µg
H
HPV58 L2 16-35 ELISA titer
Cervarix 4 µg
GSTL1 25 µg
GSTL1L2 25 µg
11-88x5 4 µg
11-88x5 2 µg

Figure 16. L2-specific antibody titers induced by vaccination of mice with capsomers.

Two weeks after the third immunization with GST-HPV16L1Δ, GST-HPV16L1Δ-L2×3 or Cervarix®, two-fold dilutions of serum from immunized mice were tested by ELISA for L2 specific antibodies. Microtiter plates were coated with either HPV16 L2 amino acids 11–200 (A), or the 17–38 L2 peptide (B), or HPV18 L2 full length protein (C), or HPV31 L2 17–36 peptide (D), or HPV31 full length protein (E) or HPV35 L2 17–36 peptide (F), or HPV45 L2 16–35 peptide (G), or HPV58 L2 16–35 peptide (H), and then incubated with diluted serum samples (1:50 as the initial dilution; two-fold serial dilutions).

4) *In vivo* PsV vaginal challenge of capsomer-immunized mice

Because vaccination with both capsomer vaccines elicited robust HPV16 neutralizing antibody titers, the mice were subjected to vaginal challenge with HPV16 pseudovirus carrying a luciferase reporter to facilitate the measurement of infectivity *in vivo*. Three days after intravaginal challenge with HPV16 PsV, the non-immunized animals exhibited a high mean luciferase activity (Figure 17A). Conversely, mice immunized with either GST-HPV16L1Δ or GST-HPV16L1Δ-L2×3 exhibited background levels of luciferase reporter gene activity similar to that of Cervarix®-immunized mice, which is consistent with complete protection. The background luciferase levels in the mice immunized with GST-HPV16L1Δ, GST-HPV16L1Δ-L2×3 or Cervarix® were not significantly different (Figure 17A).

Vaccination with GST-16L1Δ-L2×3 elicited L2 specific antibody responses, but no cross-neutralizing antibody responses were detected for HPV18, HPV31 or HPV45. However, previous studies indicate that the antibody levels necessary for protection are low and that the current neutralization assay may lack sensitivity for L2 specific neutralizing antibodies [26]. In order to determine if vaccination with GST-HPV16L1Δ can induce cross-protection, or if fusion of L2×3 to the C-terminus of GST-HPV16L1Δ can enhance cross-protection, immunized mice were vaginally challenged with HPV18. Despite using a greater challenge dose of HPV18 PsV (8.3 ug of L1) than HPV16 PsV (1 ug of L1), it is noteworthy that HPV16 PsV are dramatically more infectious in mice than HPV18 PsV (Figure 17B). Nevertheless, na¨ive mice exhibited a clear signal after HPV18 PsV challenge, while mice vaccinated with Cervarix® that contains HPV18 L1 VLPs, exhibited background levels, which is consistent with complete protection (Figure 17B).

Vaccination with GST-HPV16L1Δ capsomers failed to provide significant protection against HPV18 challenge, suggesting that capsomers, like L1 VLPs, trigger a type-restricted protective immune response. Conversely, vaccination with GST-16L1Δ-L2×3 provided robust protection against HPV18 challenge, which was not significantly different from that achieved with Cervarix® (Figure 17B).

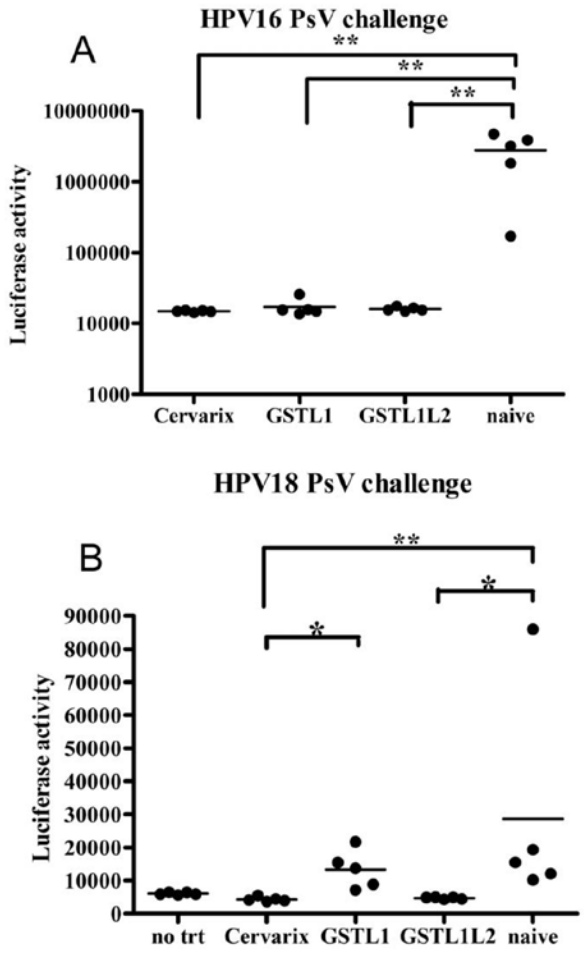


Figure 17. Vaginal challenge of mice with HPV PsV after immunization with capsomers.

Mice were subcutaneously immunized with GST-HPV16L1Δ, GST-HPV16L1Δ-L2×3 or Cervarix® three times at two-week intervals. Three and five weeks after the last immunization, the mice were challenged with either HPV16 (A) or HPV18 (B) PsV carrying a luciferase reporter, respectively. Three days after challenge, the mice received an intravaginal instillation of 20 ml of D-luciferin and were imaged with a Xenogen IVIS 100 system (image captured for 10 min post-installation of luciferin). Image analysis in each experiment was based on the average measurement (photons/s/cm^2) collected from each selected equally-sized area (A, 1.9 cm^2; B, 0.6 cm^2) in the vaginal tract.

d) Discussion

Vaccination with GST-HPV16L1Δ was as effective as Cervarix® in protecting mice against vaginal challenge with HPV16, although inducing neutralizing antibody titers approximately seven-fold lower than Cervarix®. However a more optimized capsomer formulation may reduce or negate this difference, and the difference with Gardasil® may be less [171]. It should be noted, however, that very low titers of antibodies appear to be sufficient to protect mice from vaginal challenge [26], as illustrated by the cross-protection of mice against HPV18 by vaccination with GST-16L1Δ-L2×3. In this case, the mean titer of HPV18 neutralizing antibodies was below the threshold of detection, yet a similar level of protection was observed in mice vaccinated with Cervarix®, which exhibited very high titers of antibodies. It is possible that the difficulty in detecting L2-specific protective antibodies reflects differences in the exposure of L2 during *in vitro* infection of 293TT cells as compared with *in vivo* infection of the genital mucosa, or dramatically lower levels and/or avidity as compared to L1-specific neutralizing antibodies. The presence of L2-reactive antibodies in the sera of mice immunized with GST-HPV16L1Δ-L2×3 was detectable at low titer by ELISA, suggesting the latter possibility. The antibody responses to the conserved L2 neutralizing epitope comprising residues 17–36 [46] were approximately 12-fold lower in mice immunized with GST-HPV16L1Δ-L2×3 as compared mice immunized with L2 11-88×5. This result suggests that fusion of L2 with L1 does not enhance, and may compromise the response to L2. When L2 is simply mixed with L1 capsomers, no interference is observed [37], whereas L2 is subdominant to L1 when they are co-assembled into VLPs [44]. Thus

there are several efforts to substitute the immunodominant L1 epitopes with a cross-neutralizing L2 epitope to render L2 more immunogenic like L1 [49,172,173]. Because vaccination with GST-16L1Δ protected against challenge with HPV16 but not HPV18 PsV, the protection provided by L1 capsomers appears to be type-restricted as seen for L1 VLPs, and exposure of the sides of the capsomers by preventing assembly does not uncover cross-protective L1-specific epitopes. Clearly, the alternate approach to achieve broad protection is to generate a highly multivalent L1 capsomer-based vaccine, and capsomers have already been produced for HPV11, HPV16, HPV18 and HPV35 [155]. Nevertheless, the fusion of an L2 multimer to the C-terminus of L1 did not negatively impact the L1 responses, and it provided broader protection.

The cost of manufacture remains a major, although not the only, barrier to global implementation of HPV vaccines [74]. The ability to produce very high yields of capsomers in a low cost bacterial expression system suggests the potential for cost reduction. The N-terminal deletion in L1 enhances production, and fusion to GST facilitates capsomer purification. The C- terminal deletion removes the arm that otherwise coordinates with adjacent capsomers within a VLP as well as the L1 DNA-binding domain, thereby obviating the need for disassembly/reassembly to remove contaminating host DNA during VLP purification [174-176].

Herein we examined the immunogenicity of L1 capsomers on which the GST was retained. Although a tag such as GST would typically be removed from an antigen being considered for clinical application, the GST was retained because it has potential as an anti-schistosome immunogen [177,178]. Schistosomiasis is the second major parasitic disease after malaria and is an important public health problem in many non-

industrialized countries. Furthermore urinary schistosomasis is a trigger for bladder cancer. The GST used in this study for affinity purification is derived from S. japonicum. GST derived from *S. hematobium* is currently in phase III testing (clinicaltrials.gov; identifier NCT00870649) to examine its value as a therapeutic vaccine (Bilhvax) in children exposed to urinary schistosomiasis [178]. Therefore, the inclusion of GST in the capsomer vaccine not only facilitates the purification process, it also confers the potential to trigger immunity at low cost against both papillomavirus and schistosome infections, two carcinogenic infections that cause significant mortality and morbidity in developing countries [115].

IV) Efficacy of RG1-VLP vaccination against infections with genital and cutaneous human papillomaviruses

a) Introduction

Human papillomaviruses (HPVs) are species-specific, epitheliotropic DNA viruses with over 120 types completely characterized today [12]. Infections are widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing 5% (Parkin et al., 2005) of the global cancer burden. Persistent infection with a subset of mucosal HPVs causes high-grade intraepithelial neoplasias that eventually progress to invasive cancer of the cervix (CxCa) and of other anogenital and oropharyngeal sites. More than 15 high-risk (HR) types are found in almost all CxCas, causing 250,000 deaths in women worldwide every year [54,179]. Low-risk (LR) mucosal HPVs cause anogenital warts in 30 million patients. Common cutaneous HPVs induce skin warts with substantial impact on high-prevalence groups such as children or immunocompromised individuals [180,181]. Moreover, a distinct group of cutaneous (genus b) types have been implicated as subsidiary causative factor of nonmelanoma skin cancers, although this remains controversial for those other than patients with the rare genodermatosis Epidermodysplasia verruciformis [182,183] Recombinantly expressed major capsid protein L1 of papillomaviruses self-assembles into virus-like particles (L1-VLPs) [7,22,102,142,184] that have been introduced as prophylactic vaccines. Both (bivalent Cervarix and quadrivalent Gardasil) licensed vaccines comprise VLPs of HR HPV16/18, which cause 70% of cervical disease. One

(quadrivalent) vaccine additionally contains VLPs of LR HPV6/11, causing 90% of genital warts. Prophylactic vaccination confers enduring yet predominantly vaccine type–restricted protection mediated by high-titer neutralizing antibodies. To target HPVs responsible for the remaining third of CxCas [54], a nonavalent vaccine including 7 HR types is in clinical trials (http://clinicaltrials.gov). However, the complexity of such a vaccine is unlikely to reduce the already very high costs of current HPV vaccines, which may impede delivery to the developing world with the highest CxCa burden. Furthermore, a vaccination strategy against the many types causing skin papillomas (warts) has not been established [153,185]. Papillomavirus minor capsid protein L2–based immunogens represent an alternative strategy to multivalent L1-VLP vaccines. The amino (N) terminus of L2 contains highly conserved motifs that are buried in native virions and become exposed only shortly during the infectious process. However, immunization with L2 peptides alone can induce low-titer antibodies, which mediate cross-neutralization [44,45,111,186] *in vitro* and cross-protection in animal models *in vivo* [170,187]. An HPV16 L2 peptide comprising amino acids (aa) 17–36 is a broadly cross-neutralization B-cell epitope recognized by mAb RG1 [46]. Owing to its essential role for viral infectivity and its high conservation within many types, the RG1 epitope may represent an attractive target to develop a broad-spectrum HPV vaccine. We have previously introduced chimeric RG1-VLP as a possible strategy to improve immunogenicity of the RG1 epitope by its genetic insertion into the immunogenic DE-surface loop of HPV16 L1 [49,173,188,189]. Upon expression as a recombinant fusion protein, assembly into capsids repetitively displaying RG1 epitopes on the capsid surface (RG1-VLP) is highly efficient. Vaccination induced high-titer neutralizing antibodies against HPV16 and

improved L2-specific antibodies. Then, available limited *in vitro* assays demonstrated cross-neutralization of mucosal HR HPV18/31/45/52/58, LR HPV HPV6/11, and a single genus β-type (HPV5) [49]. This study comprehensively examines RG1-VLP vaccine efficacy to cross-protect against all relevant mucosal HR HPVs *in vivo* and *in vitro*, endurance of protection (an important issue for L2-based vaccine development), and induction of cell-mediated immunity. Vaccine efficacy against natural infection was validated using authentic virion-based neutralization assays.

b) Materials and Methods

1) Chimeric RG1-VLP

Chimeric HPV16 L1–HPV16 L2 $_{(aa\ 17–36)}$ VLP (RG1-VLP) have been generated [49] by the insertion of HPV16 L2 peptide aa 17–36 (RG1) [46] into the DE-surface loop of HPV16 L1 and expressed in insect cells [7].

2) Immunization

NZW rabbits were immunized with 50 ug of RG1-VLP subcutaneously (weeks 0, 4, 6, and 8, n = 6; NZW nos. 1–6) or 20 ug of RG1-VLP (weeks 0, 3, and 6, n = 4; NZW nos. 7–10) (Charles River, Kisslegg, Germany) adjuvanted with alum-MPL (10:1) (Sigma Aldrich, St Louis, MO). Sera were drawn at weeks 10 (nos. 1–6) or 8 (nos. 7–10). Long-term antibody responses were determined for NZW nos. 1/2 kept at 10 months. Sera were drawn before and 14 days after final boost (week 52) and were stored at -20°C. For type-specific L1 antisera, a single rabbit each was immunized with 20 ug PsV of HPV6/26/34/35/39/43/44/51/52/53/56/ 58/59/66/68/69, or 70, respectively (weeks 0, 3, and 6), in complete/ incomplete Freund's adjuvant.

3) Native virion–based neutralization assays (reverse transcriptase– PCR)

Native virions HPV2/27/1/4 were extracted from plantar warts; HPV6 from

genital warts; HPV26 from a highly differentiated carcinoma [190]; and HPV40 virions were provided by Neil Christensen (Hershey, PA) [192]. After mechanical disruption of wart tissue by high-speed homogenizer (Fastprep-24, MP Biomedicals, Eschwege, Germany) and centrifugation (14,000 r.p.m./4°C/5 minutes), supernatants containing virions were used for neutralization assays [188,191]. In brief, 3×10^5 HaCaT keratinocytes were infected with virions that were either untreated or preincubated with rabbit antisera and incubated overnight. Cellular RNA was reverse transcribed, and spliced viral E1^E4 mRNA was identified by 30-cycle nested PCR (95 °C/1 minute, 60 °C/1 minute, and 72 °C/3 minutes).

4) Murine vaginal challenge

The intravaginal PsV challenge model (Roberts et al., 2007) was performed with slight modification [51]. Female Balb/C mice (groups of 5 or 10) were pretreated with 3mg of progesterone subcutaneously medroxyprogesterone (Depocou, Pfizer, Vienna, Austria), and on day 3 they were passively transferred with 20ml rabbit antiserum: RG1-VLP (pre) immune serum (rabbits 1, 6, and 10), or sera to L1-VLP of HPV16/31/33/45/6, to L1/L2-VLP of HPV18 (J Schiller, NIH, Bethesda, MD), to L1-DNA of HPV59/73, or to L1/L2-PSV of HPV26/34/35/39/51/52/53/56/58/66/ 68/69/ or 70, respectively. After 24hours, vaginal microtrauma was induced by cervical cytobrush, and mice were challenged with luciferase-encoding PsV in 3% carboxymethylcellulose. After 3 days, 20 ul of luciferin (Caliper, Waltham, MA; 7.5 mg ml^{-1}) was applied into the vagina, and infection was analyzed by bioluminescence imaging (IVIS 50, Caliper). Data are given

with background signal subtracted (mice challenged with carboxymethylcellulose).

5) Statistical methods

Statistical analysis was performed using Microsoft Excel (heteroscedastic two-tailed unpaired t-test to evaluate P-values).

6) Consent and approval

Human sera and tissue samples were collected after written informed consent of the patient or the patient's guardian in accordance with the Ethics Committee of the Medical University Vienna (ECS 1327/2012). The study was conducted according to the Declaration of Helsinki Principles.

7) Animal welfare

Animal studies have been approved (BMWF-66.009/0173-1I/3b/ 2011) and animal care was in accordance with the guidelines of the Austrian Federal Ministry for Science and Research.

c) Results

We have shown previously that vaccination of rabbits and mice with recombinant RG1-VLP plus alum-MPL (aluminum hydroxide plus 3-O-desacyl-4'-monophosphoryl lipid A) adjuvant [49] elicited high-titer neutralizing antibodies to HPV16 and cross-neutralizing antibodies to pseudovirion (PsV) of the limited number of then available mucosal HR HPV18/31/45/52/58, LR HPV6/11, and cutaneous b HPV5.

1) RG1-VLP vaccination induces a robust antibody response against the L2 epitope

To assess the robustness of the humoral immune responses to RG1-VLP, eight additional rabbits were vaccinated either 4 or 3 times (New Zealand White (NZW) nos. 1–6: 4 × 50 ug and nos. 7–10: 3 × 20 ug) and sera drawn 2 weeks after the last boost. Robust antibody responses to L2 (titers of 2,500– 12,500) were detected for both vaccination protocols using the 16L2 N-terminal peptide (aa 11–200) as ELISA antigen (Figure18). Conversely, reactivity was absent for rabbit antiserum to HPV16 wild-type L1-VLP as expected.

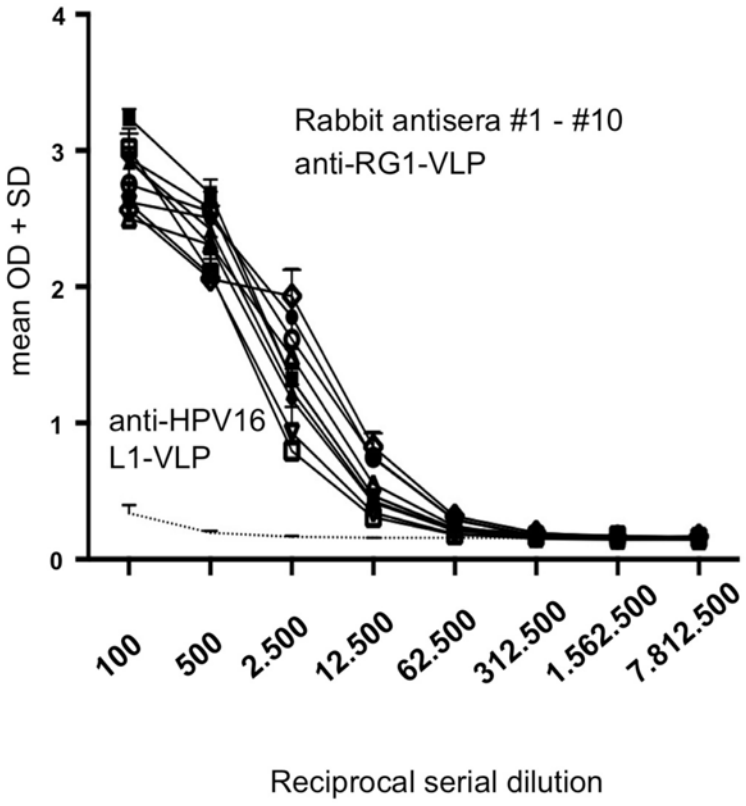


Figure 18. HPV16 L2 peptide ELISA

Ten NZW rabbits were immunized with either 50µg RG1-VLP, week 0, 4, 6 and 8 (n=6); or 20µg of RG1-VLP, week 0, 3, 6 (n=4) using Alum-MPL adjuvant. Antisera drawn two weeks after the last boost were serially diluted 5-fold and tested in triplicates by ELISA using bacterially expressed HPV16 L2-protein (aa 11-200) as antigen. End-point dilution revealed robust ELISA titers of 2,500-12,000 in all animals vaccinated with RG1-VLP, regardless of immunization scheme. In contrast, a serum from an HPV16 L1-VLP vaccinated animal showed no reactivity against L2. Data are shown as mean OD+SD.

2) Antisera to RG1-VLP neutralize distantly related mucosal HPV types *in vitro*

The spectrum of cross-neutralization induced by RG1-VLP vaccination was further explored by analyzing rabbit antisera (n = 10) in neutralization assays for a large panel of genus-a HPV using additional PsV types (Table 3). Similar to findings reported previously for rabbits 1/2 (*), the additional eight rabbits' immune sera contained high-titer neutralizing antibodies against HPV16 (titers of 10,000–100,000). Broad-spectrum cross-neutralization was found for species α9 HPV31, 52, 58, 33, 35 (in 2, 5, 9, 6, and 9 out of 10 sera), α7 HPV18, 45, 39, 59, 68, 70 (9, 6, 5, 1, 2, 7/10), α5 HPV51, 26, 69 (3, 10, 2/10), α11 HPV73 and 34 (10 and 8/10) (titers from 25 to 10,000), but not for α6 HPV 56, 53, and 66. Cross-neutralization beyond mucosal HR types was analyzed for the most potent sera nos. 1/2 (Table 4). Apart from LR HPV6/11, α1 HPV32 (causing Heck's disease) was neutralized (titers of 50/100), whereas HPV44 (α10) sporadically found in genital warts was not neutralized. To narrow the gap between PsV-based *in vitro* assays and natural HPV infection, RG1-VLP-induced neutralizing antibodies were also detected using infectious native virions [190]. As shown in Figure 19A, HaCaT cells infected with HPV26 virions (lane 2) revealed a specific band corresponding to spliced viral mRNA in nested reverse transcriptase–PCR, in contrast to uninfected control cells (lane 1). Preincubation of virions with RG1-VLP antiserum (1:400) completely abolished mRNA detection, indicating viral neutralization (lane 4), whereas preimmune serum (lane 3), mAb RG1 [46] (lane 5), or serum from a Gardasil-vaccinated individual (lane 6) had no effect. RG1-VLP antiserum also cross-neutralized LR HPV40 (Figure 19b; lanes 5 and 6) and HPV6 virions (Figure 20) at dilutions of 1:100–400. Taken together, the *in vitro* cross-neutralization spectrum of RG1-VLP vaccination includes almost all HR HPVs

causing CxCas, as well as LR mucosal types in PsV and native virion–based assays.

	Neutralizing titers against high-risk genital HPV pseudovirions									
	RG1-VLP plus alum-MPL (50 μg); weeks 0, 4, 6, and 8 (NZW; $n=6$)						RG1-VLP plus alum-MPL (20 μg); weeks 0, 3, and 6 (NZW; $n=4$)			
	1	2	3	4	5	6	7	8	9	10
HPV16	100,000 *	100,000 *	100,000	100,000	10,000	10,000	10,000	10,000	10,000	10,000
HPV18	1,000*	1,000 *	100	100	1,000	100	100	100	100	<25
HPV45	1,000 *	100 *	<25	50	1,000	<25	<25	50	100	<25
HPV31	10,000 *	1,000 *	<25	<25	<25	<25	<25	<25	<25	<25
HPV52	100 *	50 *	50	<25	25	<25	<25	<25	25	<25
HPV58	1,000 *	1,000 *	100	100	100	25	50	100	1,000	<25
HPV33	100	100	50	25	100	<25	<25	<25	100	<25
HPV35	1,000	1,000	100	100	1,000	100	50	100	1,000	<25
HPV39	500	100	<25	25	100	<25	<25	<25	50	<25
HPV56	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
HPV59	25	<25	<25	<25	<25	<25	<25	<25	<25	<25
HPV68	1,000	100	<25	<25	<25	<25	<25	<25	<25	<25
HPV73	1,000	1,000	1,000	100	100	100	50	25	100	25
HPV51	25	<25	<25	<25	<25	<25	<25	50	<25	50
HPV26	100	100	100	100	100	50	25	1,000	100	100
HPV53	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
HPV69	<25	<25	<25	<25	100	<25	<25	<25	50	<25
HPV66	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
HPV70	100	50	<25	100	1,000	50	<25	50	100	<25
HPV34	1,000	1,000	100	100	1,000	50	<25	<25	100	25

Table 3. Cross-neutralization of mucosal HR HPV by RG1-VLP antisera in PBNA *in vitro*

Abbreviations: alum-MPL, aluminum hydroxide plus 3-O-desacyl-4’-monophosphoryl lipid A; HPV, human papillomavirus; HR, high risk; NZW, New Zealand White; PBNA, pseudovirion-based neutralization assay; VLP, virus-like particle. Antisera of 10 NZW rabbits raised against RG1-VLP were analyzed for cross-neutralization of 20 mucosal HR HPV pseudovirions in duplicates using end point serial dilutions of 1:25–1:100,000. Neutralization titers were determined as described earlier [49]. Boxed titers indicate sera also tested for cross-neutralization *in vivo* (Figure 21a–c).

Pseudovirions	Neutralizing titer	
	1	2
Low-risk mucosal		
HPV6 *	100	50
HPV11 *	100	<25
HPV32	50	100
HPV44	<25	<25
Genus β		
HPV5 *	100	50
HPV38	<25	<25
Common cutaneous		
HPV3	1,000	1,000
HPV4	<25	<25
HPV76	100	100
Nonhuman		
BPV1	100	100

Table 4. Cross-neutralization of mucosal LR and cutaneous HPV by RG1-VLP antisera *in vitro*

Abbreviations: BPV1, bovine papillomavirus type 1; HPV, human papillomavirus; LR, low risk; VLP, virus-like particle. Antisera of two rabbits (nos. 1 and 2) raised against RG1-VLP were tested for cross-neutralization of 4 LR mucosal, 2 genus β cutaneous, 3 common cutaneous HPVs, and nonhuman BPV1 as indicated. Neutralizing titers were determined as described in Table 3.

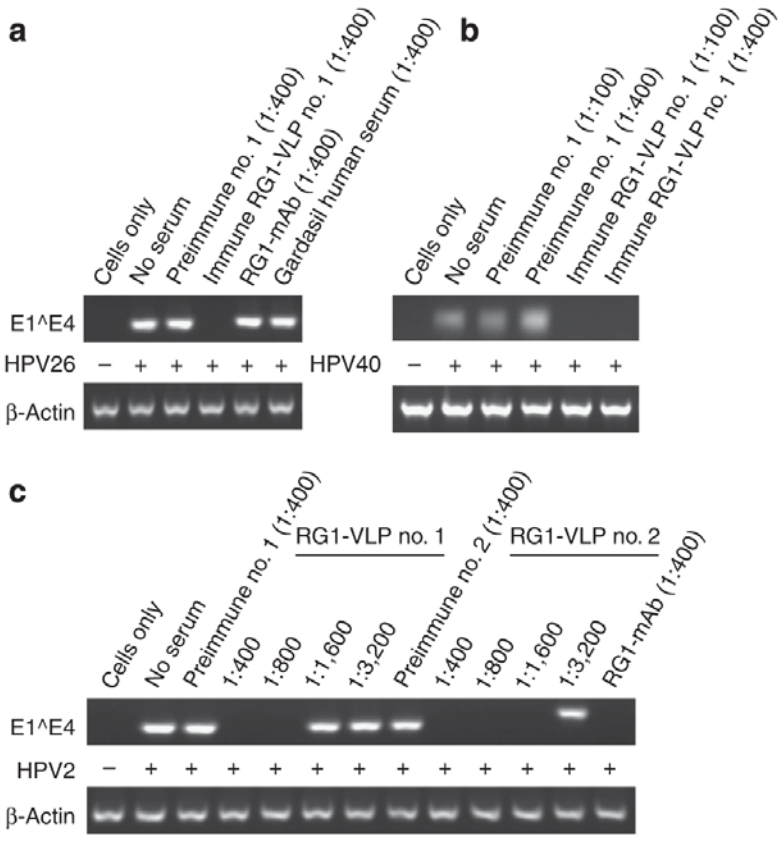


Figure 19. *In vitro* reverse transcriptase–PCR (RT–PCR) neutralization assays using native cutaneous and mucosal high-risk (HR) and low-risk (LR) human papillomavirus (HPV) virions.

HaCaT cells were incubated with native virions of (a) mucosal HR HPV26, (b) mucosal LR HPV40, and (c) cutaneous HPV2. Infection was detected by amplification of spliced viral mRNA. Cells were either mock treated (cells only) or infected with native virus (þ) in the absence of serum (no serum), or with virions preincubated with (pre)immune sera at indicated dilutions. Neither mAb RG1 nor a human antiserum from a Gardasil-immunized individual neutralized infection with HPV26.

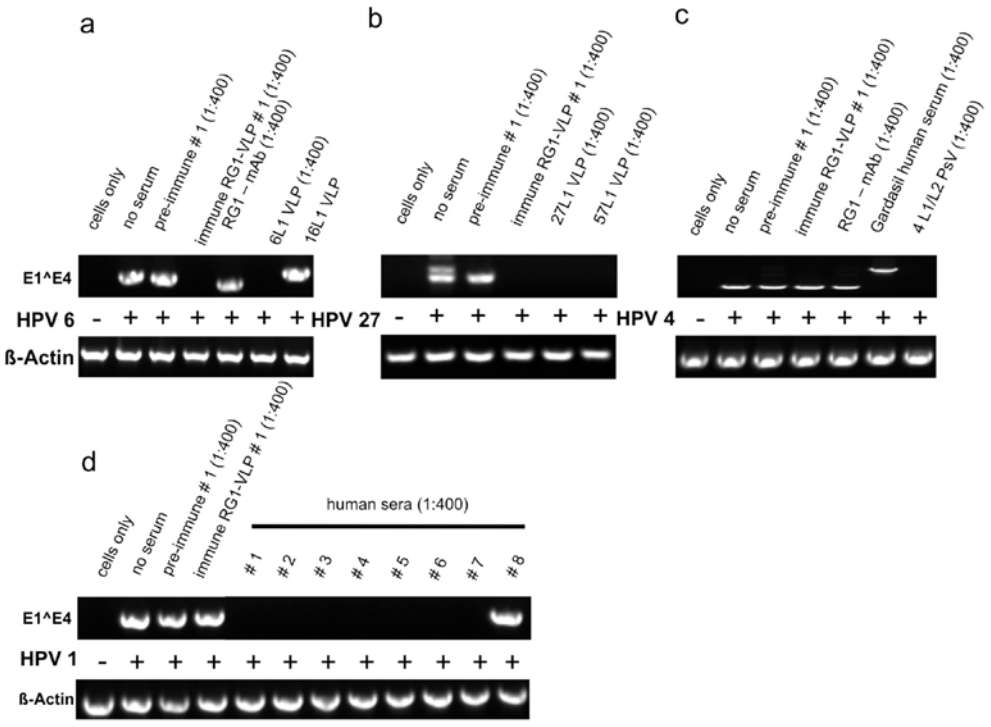


Figure 20. *In vitro* RT-PCR neutralization assays using native mucosal lr HPV6, or cutaneous HPV27, HPV4, or HPV1 virions

Type-specific neutralizing anti-L1 VLP antisera (HPV6, 27) or anti L1-L2 PsV antiserum (HPV4), were used as respective controls. Sera of 8 pre-pubertal girls (#1-#8) were used as controls in the HPV1 assay, as a type-specific neutralizing serum was not available.

3) Antisera to RG1-VLP cross-neutralize prevalent common cutaneous and HR b HPVs

In immunocompetent individuals with palmoplantar and plane warts, the most frequently detected types are genus α HPV2/27/57 [193], γ HPV4, m HPV1, and α HPV3/10. Genus β HPV5/8 have been first identified in patients suffering from Epidermodysplasia verruciformis, whereas a possible role of β papillomavirus in non-melanoma skin cancer pathogenesis is still controversial. Thus, rabbit antisera nos. 1/2 were screened for cross-neutralization against available cutaneous HPVs in pseudovirion-based neutralization assay (PBNA; Table 4), showing previously reported cross-neutralization of HPV5(*), and additionally of HPV3 (α2) and HPV76 (β4) (titers of 1,000/100), but not of HPV4 (γ1) or HPV38 (β2). Furthermore, antisera nos. 1 and 2 cross-neutralized native virions of HPV2 with titers of 800 and 1,600 (Figure 19c, lanes 5 and 11), and HPV27 at dilution 1:400 (Figure 20). Interestingly, antiserum to HPV2 L1-VLP neutralized HPV27, as did antiserum to HPV27 L1-VLP (1:400), indicating that closely related genotypes HPV2 and HPV27 (α4) may represent common serotypes (antisera to HPV2/27 L1-VLP were a kind gift from L Gissmann, DKFZ, Heidelberg, Germany). Neither HPV1 nor HPV4 virions (Figure 20) were cross-neutralized by RG1-VLP antiserum. Sera of 7/8 prepubertal girls were neutralizing against highly prevalent cutaneous HPV1 (Figure 20), indicating robust seroconversion after natural infection, whereas serum from a Gardasil-vaccinated individual did not cross-neutralize cutaneous HPV4. Moreover, the cross-neutralization spectrum of RG1-VLP vaccine even extends to bovine papillomavirus type 1 in PBNA (Table 4).

4) RG1-VLP vaccination induces cellular immune responses

The enzyme-linked immunospot analysis of splenocytes from mice vaccinated with RG1-VLP or similarly HPV16 L1-VLP showed IFN-γ-producing cells when stimulated with a previously described HPV16 L1 cytotoxic T lymphocyte epitope (Figure 21), indicating the induction of a strong cellular immune response [194]. In contrast, stimulation of cells with the RG1 peptide did not result in significant IFN-γ production.

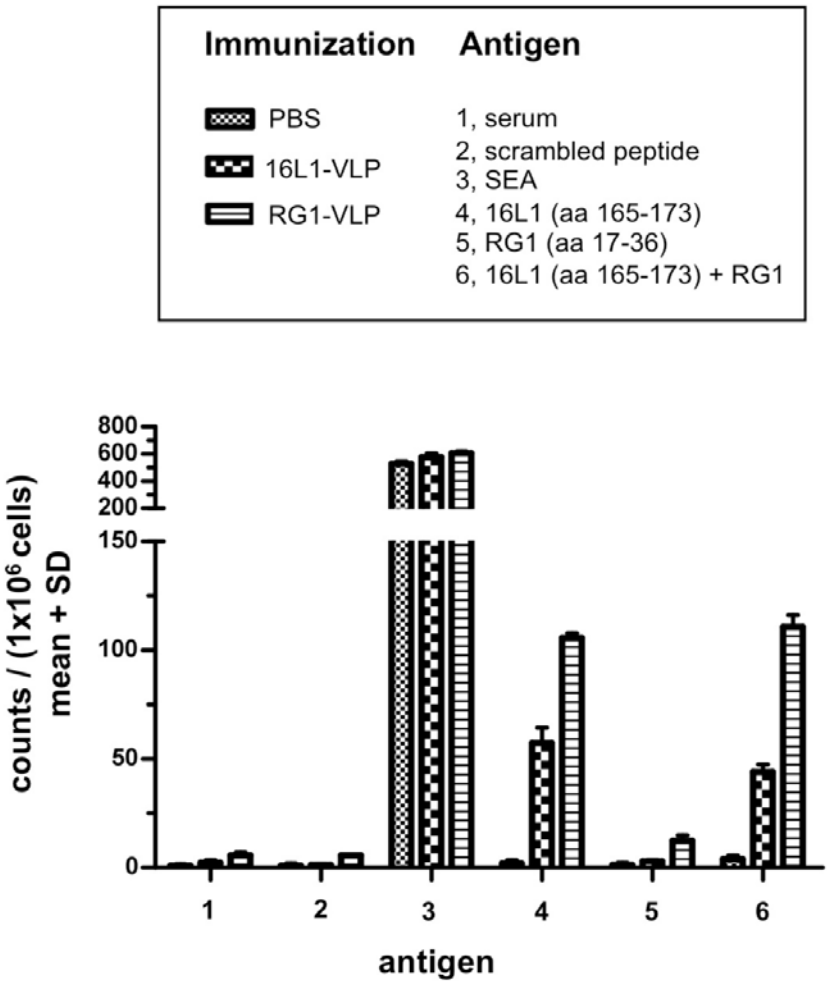


Figure 21. INF-γ ELISPOT assay detecting cytotoxic T-cell responses to vaccination with RG1-VLP. Female C57/BL6 mice (3 x 3) were vaccinated with 2μg of HPV16 L1-VLP, RG1-VLP, or PBS, respectively. Isolated splenocytes were stimulated with serum (1), scrambled peptide (2), SEA (3), HPV16 L1 peptide (aa 165-173) (4), HPV16 L2 RG1 peptide (aa 17-36) (5), or HPV16 L1 plus RG1 peptides combined (6). Spots representing IFN-γ producing cells were counted and results expressed as mean number/10^6 cells + SD of triplicate cultures.

5) RG1-VLP antisera efficiently protect mice against experimental vaginal challenge with mucosal HPV *in vivo*

Given the broad spectrum of HR HPVs cross-neutralized *in vitro*, vaccine efficacy *in vivo* was determined using an experimental mouse model [61]. As expected, passive transfer of RG1-VLP antiserum reduced vaginal infection in mice challenged with HPV16 PsV to background levels, similar to HPV16 L1 VLP antiserum (Figure 22a). Importantly, mice were also cross-protected from infection with phylogenetically divergent types HPV31/33/35/ 52/58 (α9), HPV18/45/39/59/68 (α7), HPV34/73 (α11), HPV53/56/66 (α6), and HPV26/51 (α5), and LR HPV6 (α10) and HPV43 (α8) (Figure 22b and c). Indicative, yet statistically unsignificant, results were obtained for LR HPV44 (α10). The level of cross-protection by RG1-VLP antiserum was indistinguishable from that induced by type-specific antisera to the homologous L1-VLP, respectively, except for HPV53/ 43/44, for which protection was recognizably lower. In contrast, HPV16 L1-VLP antiserum showed predominantly type-restricted protection against homologous HPV16, with significant cross-protection against very closely related HPV31/33/35 and to a smaller extent against more divergent HPV56/59/6. Detectable *in vitro* cross-neutralization titers (≥25) were always accompanied by cross-protection *in vivo* (Tables 3 and 4). However, RG1-VLP antisera also conferred complete (HPV56) or at least partial cross-protection (HPV53/66) *in vivo* (Figure 22a and b), for which types cross-neutralization was not detected *in vitro*, suggesting low sensitivity of current assays. To further demonstrate vaccine efficacy irrespective of subtle cross-neutralization titers *in vitro*, antisera nos. 6/10 were selected for *in vivo* assays. Antisera nos. 6/10 demonstrated a narrow cross-neutralization spectrum *in vitro* for only 6/19 or 3/19 mucosal HR types

(Table 3). Although neither of the two sera cross-neutralized HPV31/45 *in vitro*, both effectively cross-protected against infection with HPV31/45 *in vivo* (Figure 22c).

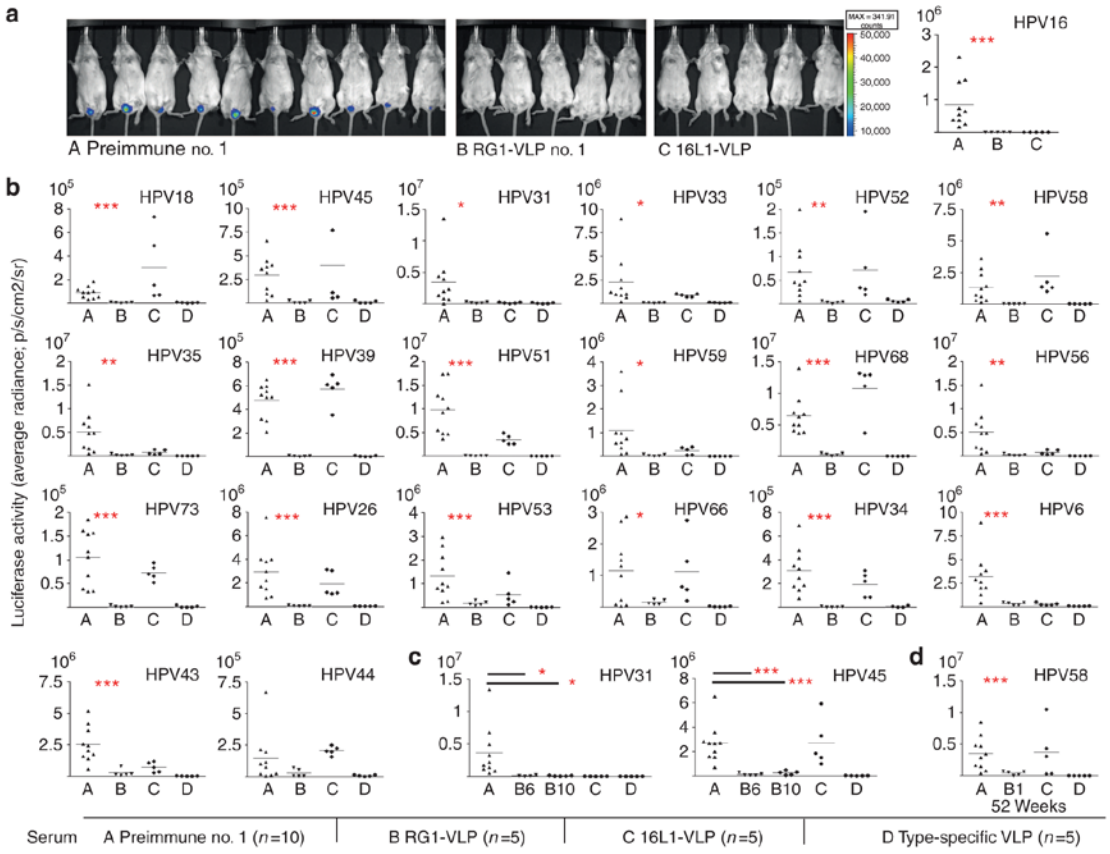


Figure 22. Cross-protection against vaginal challenge with mucosal high-risk (HR) and low-risk (LR) human papillomavirus (HPV) *in vivo*.

(a–d) *In vivo* vaginal challenge with mucosal HPV in mice, passively transferred with RG1-VLP antiserum. Female Balb/c mice were injected intraperitoneally (i.p.) with 20 ml of rabbit (A) preimmune serum; (B) RG1-VLP immune serum (serum no. 1) (a, b); serum no. 6, no. 10 (c); (C) HPV16 L1-VLP antiserum; or (D) L1 antiserum to the respective homologous type. Mice were challenged with pseudovirion (PsV) of (a) HPV16; (b) the next 11 most prevalent HR types HPV18, 45, 31, 33, 52, 58, 35, 39, 51, 59, and 68, and less frequent (<1%) types in cervical cancer (CxCa) HPV56, 73, 26, 53, 66, 34; LR HPV6, 43, and 44; or (c) high-risk HPV31 and 45. (d) An RG1-VLP rabbit

antiserum (no. 1) drawn at week 52 (10 months following the third boost with *in vitro* titer of <25 to HPV58) was analyzed for cross-protection against challenge with HPV58 (B no. 1, week 52). Antisera to (d, C) HPV16 L1-VLP and (d, D) HPV58 L1/L2 PsV drawn 2 weeks after the third boost were used as controls. Luciferase activity in bioluminescence imaging (Y axis: average radiance; p/s/cm^2/sr) is given with the respective background luminescence (unvaccinated mice challenged with carboxymethylcellulose (CMC) only) subtracted from all data points. P-values comparing groups A and B are indicated by *Pp0.05, **P≤0.01, and ***P≤0.005.

5) Induction of long-lasting B-cell memory and cross-protection

To examine long-term B-cell memory after RG1-VLP vaccination, rabbits 1/2 were housed for a further 10 months after the fourth immunization and boosted at week 52 with 50 ug of RG1-VLP (Figure 23). When compared with sera drawn at week 10, cross-neutralization titers had declined 1–2 logs (HPV18/31) or beneath the level of detection (HPV45/52/58/6/5) in week 52. A similar 2-log decline was observed for neutralizing antibodies against HPV16, which are predominantly induced by the L1 scaffold of RG1-VLP. Importantly, boosting with RG1-VLP raised antibody titers to former levels or beyond. To determine whether cross-protection *in vivo* is also long lasting, antiserum drawn at week 52 was analyzed for cross-protection against *in vivo* challenge with HPV58. Although neutralization of HPV58 was no longer detectable *in vitro* at week 52, cross-protection was still conferred *in vivo* (Figure 22d), yet to a slightly lesser extent as compared with sera drawn at week 10 (Figure 22b).

	Neutralizing titer (over time)					
	# 1			# 2		
Week (Immunizations)	**10** (4)	**52** (4)	**54** (5)	**10** (4)	**52** (4)	**54** (5)
Pseudovirions						
HPV 16	100,000	1,000	100,000	100,000	1,000	100,000
HPV 18	1,000	50	1,000	1,000	100	1,000
HPV 31	10,000	100	1,000	1,000	100	10,000
HPV 45	1,000	<25	1,000	100	100	1,000
HPV 52	100	<25	1,000	50	<25	50
HPV 58	1,000	<25	10,000	1,000	100	1,000
HPV 6	100	<25	1000	50	<25	100
HPV 5	100	<25	1,000	50	<25	100

Figure 23. RG1-VLP induced cross-neutralizing antibody titers over time (12 months)

Two NZW rabbits (#1 and #2) were kept for additional 10 months following the 4th immunization with RG1-VLP in Alum-MPL. Sera were drawn before and two weeks after a 5th injection at week 52 with 50μg of antigen plus Alum-MPL, and tested by PBNA for indicated types. Serum titers are shown and compared to sera obtained at week 10 as indicated.

d) Discussion

The search for second-generation HPV vaccines is driven by the need to protect against the plurality of carcinogenic genital HPV by safe and affordable formulations. Because licensed vaccines do not target HR HPVs other than HPV16/18, causing 30% of CxCas, cytological screening programs cannot be superseded. This major limitation in particular affects developing countries, which bear >85% of the global CxCa burden. Moreover, precancerous cervical neoplasia with a substantial disease prevalence and morbidity in younger women is even more strongly associated with types other than HPV16/18 [195]. To extend protection to the most common oncogenic HPVs, a nonavalent L1-VLP vaccine (comprising seven HR and two LR types) is in clinical trials. Although such a highly multivalent L1 vaccine is expected to significantly increase the breadth of coverage, L2-based vaccines are attractive as single-antigen formulations targeting even more mucosal and, additionally, nongenital types. The spectrum of L2-mediated cross-neutralization by chimeric RG1-VLP has been analyzed by a limited number of *in vitro* assays, whereas the vaccine's efficacy to confer protection in mammals against infection with a broad range of HPVs has not been substantiated so far. In a very extensive comparison of HPV16 L1 VLP versus RG1-VLP (plus alum-MPL adjuvant) provided herein, RG1-VLP vaccination (cross-) protects with high efficacy *in vivo* against infection with 18 divergent HR types out of 4 different species (a5/7/9/11), which cause more than 95% of CxCas worldwide, and also partially protects against the remaining four types tested [179]. Mice were protected from vaginal challenge with large doses of mucosal PsV despite considerable antiserum dilution (B1:50) into the mouse

circulation. Interestingly, undetectable cross-neutralization *in vitro* did not stringently imply incomplete protection *in vivo*. In line with the recently reported insufficient sensitivity of current *in vitro* assays to detect anti-L2 antibodies, *in vivo* testing has become the gold standard in detecting neutralizing HPV antibodies [108] and documenting vaccine efficacy. The results imply that the actual immunogenicity is substantially greater than that reflected by the standard *in vitro* neutralization assay, and suggest that this vaccine should have a significant impact not only on reducing the risk of CxCa but also on the overall incidence of genital HPV infection, thus providing a major advantage to HPV-based screening programs when compared with the current vaccines. Complementary *in vitro* assays were used to more extensively characterize the spectrum of cross- neutralization by RG1-VLP vaccination, which was not predicted by L1-based classification of papillomaviruses, the site of infection, or host species. In PBNA, variable (cross)-neutralization by RG1-VLP antiserum was detected for 17 mucosal HR types, whereas cross-protection *in vivo* was intact for the serum with least potency *in vitro*. Although technically demanding and limited to those few types available from productive lesions, neutralization assays using native virions may reflect viral infection more authentically than PBNA [196]. Reassuringly, the RG1-VLP antisera neutralized lesion-derived virions as well. The RG1-VLP antisera also cross-neutralized LR types HPV6/11 (α10), HPV32 (α1), HPV40 (α8), and cutaneous HPV2/27 (α4), HPV3 (α1), and HPV76 (β3), but not HPV1 (μ), HPV4 (γ), and HPV38 (β). On comparing the RG1 motifs of all types cross-neutralized, sequence homology to HPV16 RG1 was at least 60%. Whether this lower threshold of homology necessary for inducing cross-neutralization holds out in more sensitive *in vivo* assays needs to be demonstrated.

Therefore, RG1-VLP vaccination is expected to protect against a substantial proportion of benign papillomas, including both sexually transmitted genital and nongenital skin warts. The latter is not only of tremendous clinical importance for the growing group of immunocompromised patients (e.g., organ-transplant recipients, HIV patients), but may have expanded relevance if these types contribute to the genesis of nonmelanoma skin cancers. Because the incidence of common skin warts peaks in early childhood, this would provide a rationale for implementing the vaccine into existing childhood programs. Various approaches to enhance cross-neutralizing L2 antibodies have been presented over the past years, including conjugation of L2 peptides to a T-helper epitope and a Toll-like receptor 2 ligand [197], concatenated multimeric L2 peptide vaccination [48], or display on bacteriophage [198], adeno-associated virus [113], or HPV L1-VLP [49,173,188,189,199]. Favorably, RG1-VLP vaccination plus alum-MPL (as in Cervarix) not only elicits robust antibody responses against RG1 in ELISA but also retains high-level protection against the most important HPV16. Therefore, RG1-VLP vaccination should provide strong protection not just against CxCas but also against the even higher percentage of noncervical cancers attributable to HPV16. In contrast, in a comprehensive direct comparison, HPV16 L1-VLP vaccination protected against HPV16 and 31 and only to a lower extent against 35, 39, 56, and 6. Licensed vaccines provide long-term protection of at least 8.5 years, and the antibody levels required are still unknown. Importantly, sera drawn 12 months after RG1-VLP vaccination completely protected mice against vaginal challenge with HR HPV58 *in vivo*. RG1-VLP holds promise as next-generation vaccine with broad efficacy against the vast majority of relevant mucosal and additional cutaneous HPVs, provides a rationale for childhood vaccination, and adds

economic advantage of a single antigen formulation compared with multivalent L1-VLP formulations. As post-licensure data confirm excellent safety profiles for HPV L1-VLP vaccines, we infer that RG1-VLP vaccination may prove similarly safe, offering the possibility to evaluate vaccine immunogenicity in early-phase human trials.

V) Optimization of multimeric human papillomavirus L2 vaccines

a) Introduction

The efficacy of vaccination with HPV L1 virus-like particles (VLP) for the prevention of new infections provides an opportunity to reduce the incidence of HPV-associated cancers globally if these vaccines can be widely utilized [2,8,10,75,159]. This opportunity is particularly dramatic for women who currently lack access to effective cytologic screening and intervention programs. Indeed, 85% of the global burden of disease occurs in such low income countries [3]. Unfortunately, the current cost of the licensed L1 VLP vaccines has proven a significant barrier to their sustained global implementation, and this has driven an effort to create a second generation of low cost HPV vaccines that require fewer doses to improve access for under-served populations [74]. The licensed HPV vaccines target only the two types most commonly found in cervical cancer, HPV16 and HPV18 that cause 70% of cases, but there are a dozen other types responsible for remaining, 30% of cervical cancer cases [54]. The L1 VLP vaccines provide type-restricted protection and, while a variable degree of cross-protection against highly related types has been described, there is concern that it is incomplete and may wane [11,75]. This has triggered an ongoing clinical effort to develop a nonavalent L1 VLP vaccine, but its potential to further increase the cost of vaccination against HPV has encouraged the development of alternate vaccines based on the more cross-protective capsid antigen L2 [74].

L2 can be produced at high levels in bacteria and numerous studies demonstrate it is a protective antigen although it does not form a VLP [170,187,200,201]. Vaccination of rabbits with the N-terminus (residues 94–122, 11–200 or 1–88) of L2 prevents papilloma development after experimental challenge with virions but not viral DNA, suggesting that protection is mediated by neutralizing antibodies [170,202]. Indeed, neutralizing antibodies binding to linear epitopes in HPV16 L2 17–36, 65–81 and 108–120 have been described [46,111,167]. The development of HPV pseudovirion (PsV) technology in which a reporter gene is encapsidated within the papillomavirus L1 and L2 capsid has greatly facilitated the measurement of neutralizing antibodies, and recently has been utilized in a mouse challenge model [61,203]. Passive transfer of the HPV16 L2 17–36 specific neutralizing antibody RG-1 protected na¨ive mice from cutaneous challenge with HPV16 PsVs suggesting that L2-specific neutralizing IgG is sufficient to mediate protection [46].

Antisera to the N-terminus of L2 broadly cross-neutralizes HPV, although it is most effective against the virus type from which the vaccine was derived, and the titers induced are significantly lower than those produced by L1 VLP vaccines [44,45]. The induction of sustained neutralizing antibody titers for durable/lifetime protection is a critical goal and might offer an opportunity to move from an adolescent to childhood vaccination schedule to further improve vaccine access. To potentially enhance the level, durability and breadth of cross-protection by reinforcing the most conserved epitopes, we designed concatenated fusion proteins consisting of the N-terminal protective region of L2 derived from multiple medically significant HPV genotypes [48]. This study suggested that a pentameric fusion of L2 residues of 11–88 from divergent HPV types

could induce a robust humoral response, but another study suggested that inclusion of more repeats might be beneficial [204]. Herein we define HPV16 L2 residues 17–36, 32–51 and 65–81 as protective epitopes, and show an unexpected enhancement of the neutralizing antibody response to the amino terminus of L2 by residues 45–67.

b) Materials and methods

1) Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (protocol MO08M19).

2) Antigen preparation

The L2 multimer constructs 11–88×8, 13–47×15, 11–88×8Δ, and 11–88×8ΔPADRE were codon optimized for expression in *E. coli* by lowest free energy calculation and synthesized by Blue Heron Inc. The 11–88×8 family constructs were cloned with BamHI sites at their N-terminus and XhoI sites at their C terminus into the pET28a vector (Novagen), whereas NheI and XhoI were used for 13–47×15. The N-terminal hexahistidine-tagged recombinant polypeptides were expressed in *E. coli* BL21 (Rosetta cells, Novagen) [45]. The recombinant L2 polypeptides were affinity purified by binding to a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) in 8 M urea (using the QiaExpressionist standard purification protocol for denaturing conditions) and then dialyzed in cassettes (Pierce) against Dulbecco's phosphate buffered saline (PBS). Purity was monitored by SDS-PAGE and protein concentration determined by bicinchoninic acid test (Pierce) using a bovine serum albumen standard. L2 peptides were synthesized with a C-terminal cysteine to >90% purity, sequence validated by mass spectrometry and

10 mg conjugated with 5 mg keyhole limpet hemocyanin (KLH) protein carrier using maleimide (Proteintech) to enhance their immunogenicity [24].

Two rabbits were immunized with each KLH-coupled peptide in Freund's adjuvant and boosted on days 14, 28, 35 and 76 using incomplete Freund's adjuvant and exsanguinated at day 56 (Proteintech).

3) Neutralization assays

The HPV pseudovirion *in vitro* neutralization assays were performed as described earlier and the secreted alkaline phosphatase content in the clarified supernatant was determined using the p-Nitrophenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in diethanolamine and absorbance measured at 405 nm. Constructs and detailed protocols for the preparation of the pseudovirions can be found at http://home.ccr.cancer.gov/lco/. Titers were defined as the reciprocal of the highest dilution that caused a 50% reduction in A_{405}, and a titer <50 was not considered significant.

4) Animal Studies

Female Balb/c mice, 4–6 weeks age (NCI Frederick) were vaccinated in groups of 5 animals three times at two week intervals s.c with 25 ug of antigen (11–88×8, 13–47×15, 11–88×8Δ, 11–88×8ΔPADRE) either with Alum alone, or Alum+MPL, Alum+CpG 1018 (Dynavax Inc.) or GPI-0100 (Hawaii Biotech Inc.). Serum samples

were obtained by tail vein bleeds two weeks and four months after the final immunization.

5) Passive transfer of mice and vaginal challenge with HPV pseudovirus

Five days before the challenge, female Balb/c mice were injected s.c. with 3 mg of medroxyprogesterone (Depo-Provera; Pfizer) to synchronize their estrus cycles. For those mice receiving the passively transferred rabbit antiserum, 100 ul of serum was administered intraperitoneally 24 hours prior to infection. Each challenge dose comprised of 20 ul PsV mixed with 20 ul of 3% carboxymethyl cellulose (CMC). The dose was delivered twice into vaginal vault, the first 20 ul just prior to and the second 20 ul just after treatment with a cytobrush cell collector. The cytobrush cell collector was inserted into the vaginal vault and turned both counter-clockwise and clockwise 15 times while the mice were anesthetized. Three days after PsV delivery, the mice were again anesthetized and 20 ul of luciferin (7.8 mg/ml) was deposited in the vaginal vault. Luciferase signals were acquired for 10 min with a Xenogen IVIS 100 imager, and analysis was performed with Living Image 2.5 software.

6) Statistical analysis

One-way ANOVA and Bonferroni's multiple comparison tests were performed with GraphPad 4.00 (GraphPad Software, San Diego, CA).

c) Results

1) Passive transfer with L2 peptide anti-sera protects mice from vaginal HPV16 challenge

To define protective epitopes within the amino terminus of L2, rabbits were vaccinated with KLH-coupled synthetic HPV16 L2 peptides comprising residues 17–36, 32–51, 47–66, 65–81, 108–120 or as a negative control C-terminal peptide 373–392, each formulated initially in CFA and boosted in IFA. The pre- and hyper-immune antisera were each tested for reactivity with full length HPV16 L2 protein by ELISA. None of the pre-immune sera were reactive whereas the immune sera to HPV16 L2 17–36, 32–51, 47–66, 65–81, 108–120, and 373–392 exhibited ELISA titers of 6400, 400, 100, 800, 800, and 6400 respectively. Passive transfer of 0.1 ml/mouse rabbit anti-sera generated against HPV16 L2 residues 17–36, 32–51 and 65–81 protected na¨ive Balb/c mice (n=5) against intra-vaginal challenge with HPV16 PsV one day later ($p<0.01$, $p<0.05$ and $p<0.05$ respectively, Figure 24). Conversely, passive transfer of 0.1 ml/mouse of antisera to HPV16 L2 residues 47–66 (n=10), 108–120 (n=5) or 373–392 (n=5) was not significantly protective. A second rabbit immunized with the HPV16 L2 47–66 peptide did not produce a detectable titer in the HPV16 L2 protein ELISA and also was not protective (not shown), suggesting that this peptide is either poorly immunogenic and/or was not presented in the appropriate context for immunization. Passive transfer of rabbit antiserum to HPV16 L1 VLP also protected mice from vaginal challenge (n=5, Figure 24).

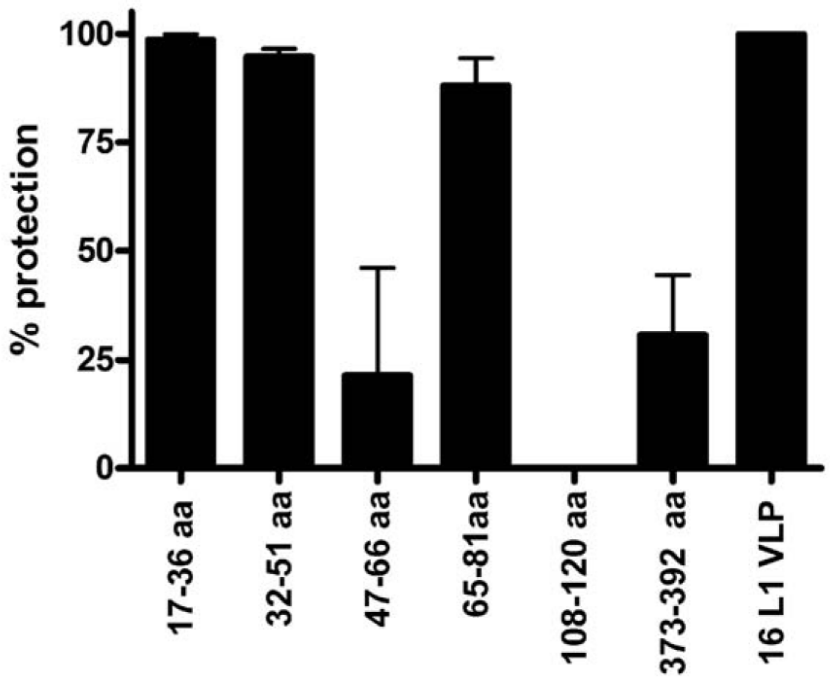


Figure 24. Passive transfer of HPV16 L2 peptide antisera protects mice against vaginal challenge with HPV16.

Mice were injected i.p. with 0.1 ml buffer or rabbit antiserum to KLH-coupled HPV16 L2 peptides encompassing residues 17–36, residues 32–51, residues 47–66, residues 65–81, 108–120, residues 373–392, or antiserum to HPV16 L1 VLP. One day later the mice were challenged intravaginally with HPV16 pseudovirion encoding luciferase.

2) Antibody responses to different adjuvant formulations of 11–88×8 and 13–47×15

Recent studies have shown that vaccination with polymeric fusions of L2, each unit being derived from a different HPV type, produces a more broadly neutralizing response than the monomer, and that increasing the numbers of the fusion epitopes can enhance immunogenicity [48,204]. To potentially enhance cross-protection by reinforcing the responses to conserved neutralizing epitopes within the medically significant α7, α9 and α10 clades [12], we generated two multitype L2 fusion proteins, 11–88×8 and 13–47×15, by recombinant expression in *E. coli* [48]. The first, 11–88×8, comprised L2 residues, 11–88 of HPV6, HPV16, HPV18, HPV31, HPV39, HPV51, HPV56 and HPV73 L2 concatenated to form the '11–88×8' antigen, and the second, 13–45×15, comprised L2 residues, 13–47 of the fifteen HPV types (HPV6, HPV11, HPV 16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV73) that encompass the two most common types in genital warts and the thirteen most common oncogenic HPV types. Both proteins were expressed in bacteria and affinity purified under denaturing conditions utilizing an N-terminal 6His tag. The expression level of 13–45×15 was noticeably higher than 11– 88×8.

In order to compare the immunogenicity of the 11–88×8 and 13–45×15 antigens, mice were immunized three times at two week intervals with either 13–47×15 or 11–88×8 protein, alone or formulated with alum alone, alum+MPL, alum+CpG 1018, or the saponin-based adjuvant GPI-0100. Both the 11–88×8 and 13– 45×15 polypeptides were immunogenic and induced antibodies that neutralize key papillomavirus types, including HPV16, HPV18, HPV45 and HPV58 (Figure 25). However, the 13– 47×15 protein was not as immunogenic as 11–88×8, regardless of the adjuvant used. Amongst the adjuvants

tested, GPI-0100 was the most effective, producing titers a log higher than alum and either MPL or CpG. The HPV16 neutralization titers at 3 months post vaccination were robust and similar to those measured at 2 weeks post immunization for 11–88×8, indicating that the titers were stable, whereas the response to 13–45×15 remained weak.

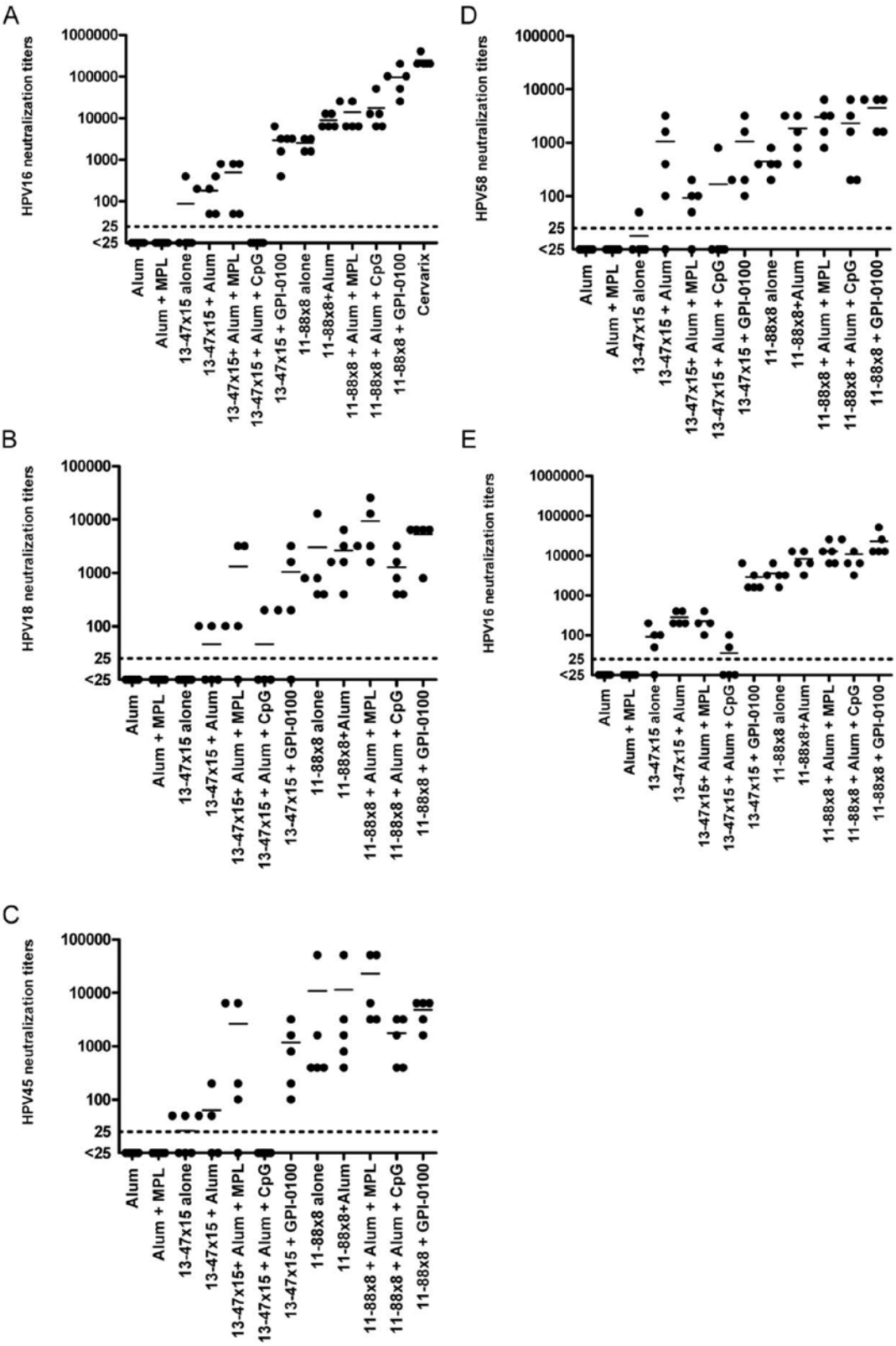

A
HPV16 neutralization titers
1000000
100000
10000
1000
100
25
<25
Alum
Alum + MPL
13-47x15 alone
13-47x15 + Alum
13-47x15+ Alum + MPL
13-47x15 + Alum + CpG
13-47x15 + GPI-0100
11-88x8 alone
11-88x8+Alum
11-88x8 + Alum + MPL
11-88x8 + Alum + CpG
11-88x8 + GPI-0100
Cervarix
B
HPV18 neutralization titers
100000
10000
1000
100
25
<25
Alum
Alum + MPL
13-47x15 alone
13-47x15 + Alum
13-47x15+ Alum + MPL
13-47x15 + Alum + CpG
13-47x15 + GPI-0100
11-88x8 alone
11-88x8+Alum
11-88x8 + Alum + MPL
11-88x8 + Alum + CpG
11-88x8 + GPI-0100
C
HPV45 neutralization titers
100000
10000
1000
100
25
<25
Alum
Alum + MPL
13-47x15 alone
13-47x15 + Alum
13-47x15+ Alum + MPL
13-47x15 + Alum + CpG
13-47x15 + GPI-0100
11-88x8 alone
11-88x8+Alum
11-88x8 + Alum + MPL
11-88x8 + Alum + CpG
11-88x8 + GPI-0100
D
HPV58 neutralization titers
100000
10000
1000
100
25
<25
Alum
Alum + MPL
13-47x15 alone
13-47x15 + Alum
13-47x15+ Alum + MPL
13-47x15 + Alum + CpG
13-47x15 + GPI-0100
11-88x8 alone
11-88x8+Alum
11-88x8 + Alum + MPL
11-88x8 + Alum + CpG
11-88x8 + GPI-0100
E
HPV16 neutralization titers
1000000
100000
10000
1000
100
25
<25
Alum
Alum + MPL
13-47x15 alone
13-47x15 + Alum
13-47x15+ Alum + MPL
13-47x15 + Alum + CpG
13-47x15 + GPI-0100
11-88x8 alone
11-88x8+Alum
11-88x8 + Alum + MPL
11-88x8 + Alum + CpG
11-88x8 + GPI-0100

Figure 25. HPV *in vitro* neutralization titers of sera of mice vaccinated with 13–47×15 and 11–88×8 in different adjuvants.

Balb/c mice were vaccinated three times at 2 week intervals with the indicated 13–47×15 and 11–88×8 proteins formulated in alum, alum+MPL, alum+CpG or GPI-0100. Sera were harvested two weeks later for testing *in vitro* neutralization titers against HPV16 (A), HPV18 (B), HPV45 (C) and HPV58 (D) pseudovirions, or HPV16 at 3 months after the final vaccination (E).

3) Elimination of residues 45–67 compromises 11–88×8 immunogenicity

These findings suggested that residues 45–88 contribute significantly to the immunogenicity of the construct, consistent with the protective epitope described between residues 65–81 (Figure 24). However, residues 47–66 do not appear to be immunogenic and antisera to this peptide were not significantly protective (Figure 24), suggesting that it might be dispensable from the vaccine construct. Furthermore, while the 13–47×15 protein was highly expressed, the 11–88×8 protein was produced at a significantly lower level (data not shown) possibly reflecting the hydrophobicity of L2 between residues 45–67 (IL- QYGSMGVFFGGLGIGTGSGTG). In an effort to focus the antibody response on the key protective epitopes by removing potentially competing non-protective regions, we designed 11– 88×8Δ by removing this region from each unit of the 11–88×8 protein. The expression level of 11–88×8Δ was dramatically higher than 11–88×8 and, unlike the 13–45×15, it still contains the neutralizing epitopes residing with residues 65–81.

While CD4 T cell epitopes are found within E2, E6, E7 and L1 and at this point it is unknown where within the L2 capsid protein a potential epitope would be located, if any, we postulated the existence of a key T helper epitope might possibly overlap or lie within the 45–67 region as a reason for the greater immunogenicity of the 11–88×8 as compared with the 11–88×8Δ polypeptide. Interestingly, an in silico analysis of the 11–88Δ8 sequence using ProPred predicts that the 45–67 region contains promiscuous MHC class II epitopes [206-208]. Thus, in removing this 45–67 region from each unit of 11–88Δ8, we were concerned that this would also eliminate potentially important epitopes recognized by CD4 T helper cells. Therefore, we generated another construct in which

the potent and conserved CD4 T helper epitope PADRE (AKFVAAWTLKAAA) was fused to the 11–88×8Δ protein, forming '11–88×8ΔPADRE'. The PADRE epitope was chosen because it is recognized in C57BL/6 mice, but not Balb/c mice, and it is broadly recognized by human HLA-DR [209,210]. Both the 11–88×8Δ protein and the 11–88×8ΔPADRE proteins were expressed in *E. coli* at noticeably higher levels than 11–88×8.

To determine the impact of deleting the 45–67 region upon the immunogenicity of 11–88×8, immunization studies were performed in Balb/c and C57BL/6 mice with 11–88×8, 11–88×8Δ, or 11–88×8ΔPADRE using alum+MPL as an adjuvant. The mice were immunized three times at two week intervals and sera were obtained two weeks after the final immunization. Neutralization assays for HPV16, HPV45, and HPV58 indicated that the titers were consistently lower in the 11–88×8Δ and 11–88×8ΔPADRE immunized mice as compared to those vaccinated with 11–88×8. This phenomenon was observed in both strains of mice (Figure 3) while the PADRE epitope is only recognized by the C57BL/6 mice. This suggests that the reduction in immunogenicity does not reflect a loss of a key T helper epitope upon elimination of the 45– 67 region in each subunit of 11–88×8. Nevertheless, all three immunogens were able to protect both strains of mice from vaginal challenge with HPV16 pseudovirions (Figure 4).

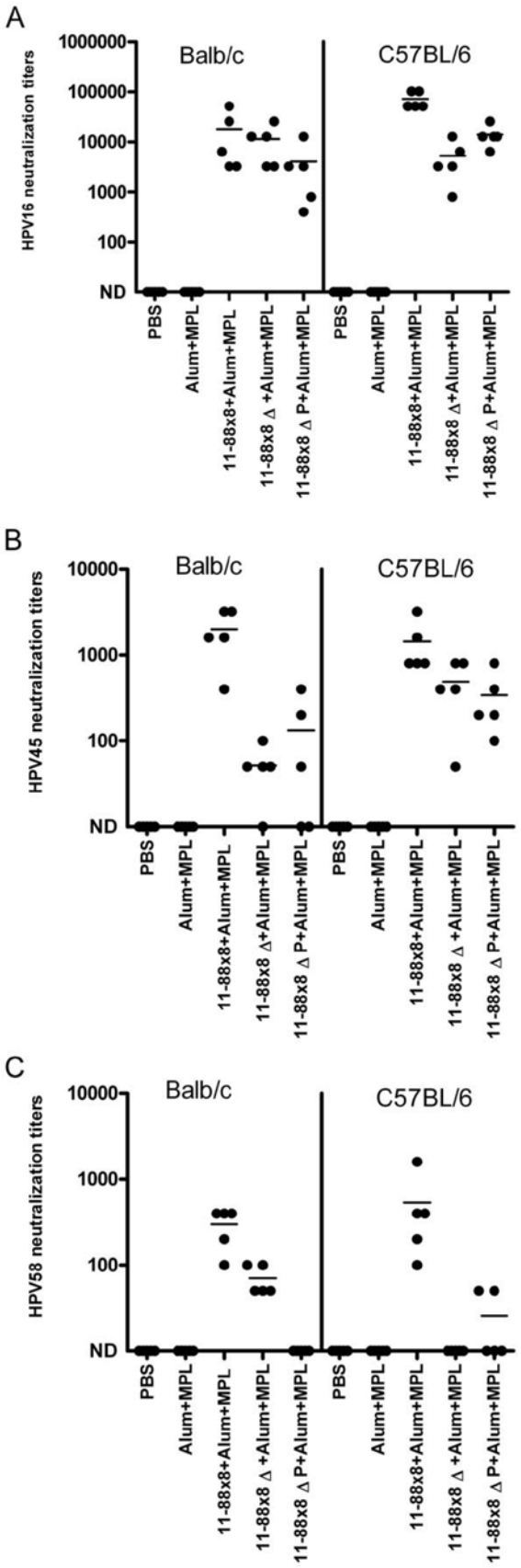

A
HPV16 neutralization titers
1000000
100000
10000
1000
100
ND
Balb/c
C57BL/6
PBS
Alum+MPL
11-88x8+Alum+MPL
11-88x8 Δ +Alum+MPL
11-88x8 Δ P+Alum+MPL
B
HPV45 neutralization titers
10000
1000
100
ND
C
HPV58 neutralization titers

Figure 26. HPV *in vitro* neutralization titers of sera of Balb/c and C57BL/6 mice vaccinated with 11–88×8, 11–88×8Δ and 11– 88×8ΔPADRE using Alum+MPL as adjuvant.

Balb/c or C57BL/6 mice were vaccinated three times at 2 week intervals with 11–88×8, 11– 88×8Δ or 11–88×8ΔPADRE (11–88×8ΔP) using Alum+MPL as adjuvant. Sera were harvested two weeks later and tested for *in vitro* neutralization titers against HPV16 (A), HPV45 (B) and HPV58 (C) pseudovirions.

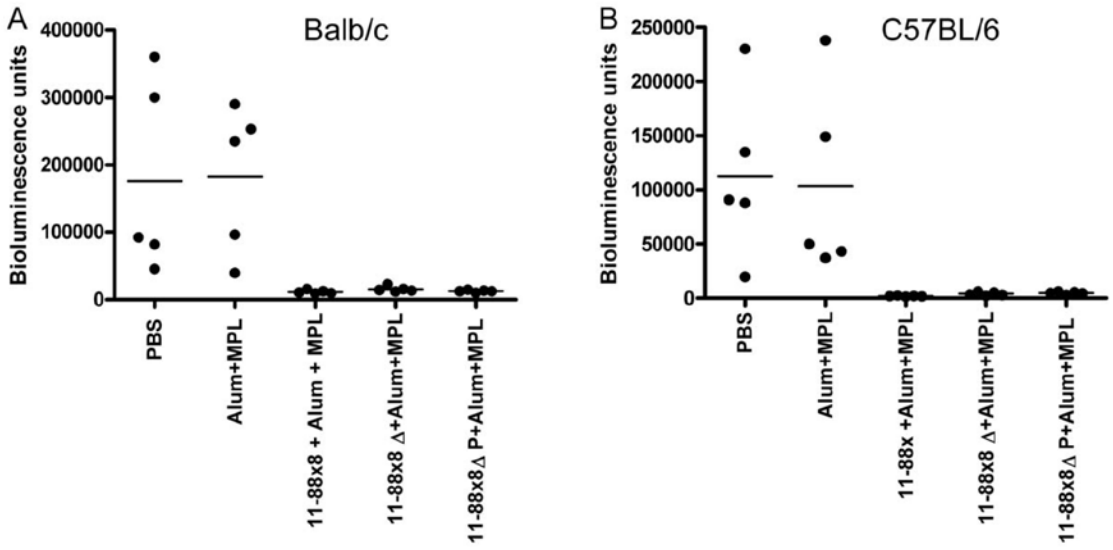


Figure 27. Vaginal Challenge of Balb/c and C57BL/6 mice vaccinated with 11–88×8, 11–88×8Δ and 11–88×8ΔPADRE (11–88×8ΔP) in Alum+MPL.

Balb/c or C57BL/6 mice were vaccinated three times at 2 week intervals with the indicated 11–88×8, 11–88×8Δ and 11–88×8ΔPADRE using Alum+MPL as adjuvant. The mice were vaginally challenged one month later using HPV16 pseudovirions carrying a luciferase reporter. Infection is measured as bioluminescence.

4) Antibody to residues 45–67 is not required for protection by 11–88×8 antiserum

To determine if antibody to residues 45–67 plays a role in the protective response elicited by vaccination with 11–88×8, we first sought to confirm that pooled mouse antisera to 11–88×8 does contain antibody specific to the 43–67 region. Using ELISA, we observed that vaccination with 11–88×8 does induce antibody reactive with 43–67 peptide at 1:100 dilution, whereas the 11– 88×8Δ antigen does not (as expected since this epitope is deleted from this construct) as it exhibits background reactivity (Figure 28A). Next, we sought to define which epitopes are critical by blocking neutralization of HPV16 pseudovirus by 11–88×8 antisera with overlapping peptides (20mer) that together span the entire HPV16 L2 13–90 region. We observe that only mixing of the 11–88×8 antisera with excess of the first two peptides, corresponding to residues 13–32 and 18–37, block the neutralizing response, whereas peptide 23–42 and peptides thereafter had minimal impact on *in vitro* neutralization. This again supports the central role of the 17–36 region in neutralization and protection. To further rule out a role for the 47–66 region in protection, we mixed the 11–88×8 antiserum with excess peptides encompassing the 47–66 region and compared its protective capacity against HPV16 challenge. Thus 20 ul, 5 ul or 2 ul of 11–88×8 mouse antiserum was mixed with excess 47–66 peptide, or not, and then administered i.p to na¨ive mice (5 mice/group) prior to challenge. As controls, additional groups of mice received 200 ug of antibody purified from the rabbit antisera to HPV16 L2 17–36, 47–66 or 373–392 (approximately 20-fold higher doses than utilized in Figure 24). The pre-incubation of the 47–66 peptide with the 11– 88×8 mouse antiserum had no significant impact upon its protective capacity in this model, further supporting the notion that antibodies to 47–66 are not effecting protection after

vaccination of mice with 11–88×8. Furthermore, the antibody to 17–36 but not that to 47–66 or 373–392, was strongly protective.

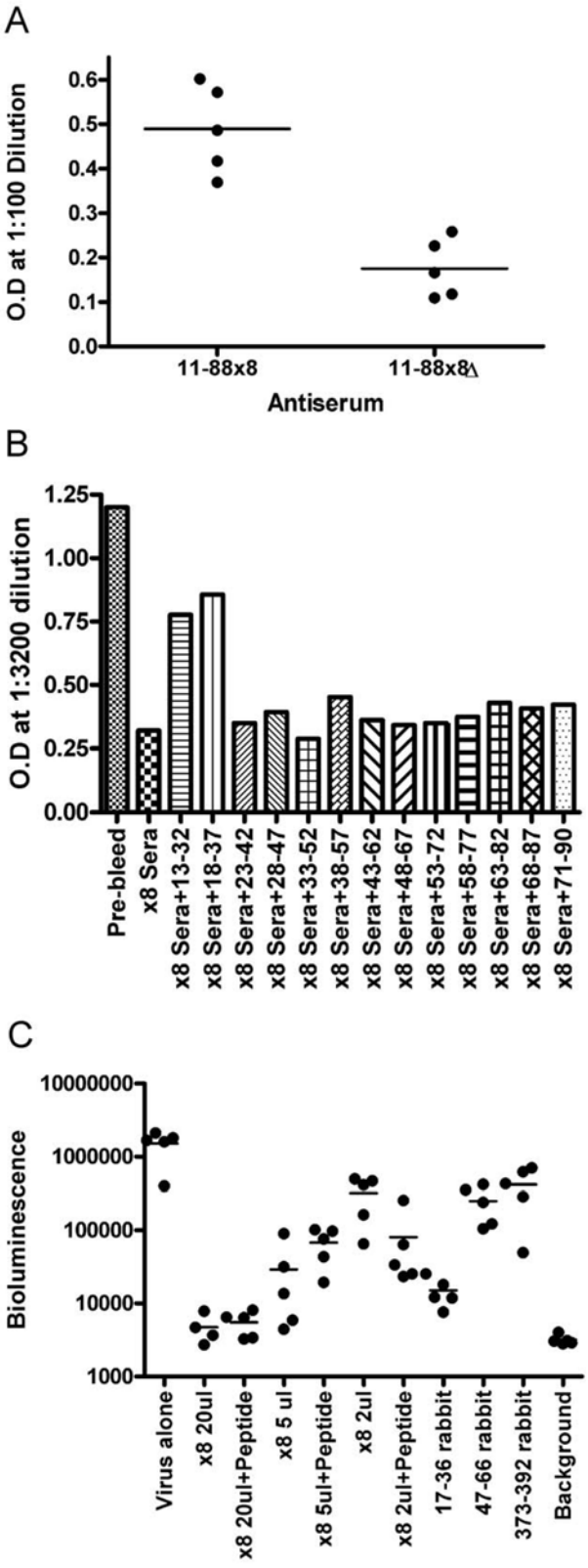

A
O.D at 1:100 Dilution
0.6
0.5
0.4
0.3
0.2
0.1
0.0
11-88x8
11-88x8Δ
Antiserum
B
O.D at 1:3200 dilution
1.25
1.00
0.75
0.50
0.25
0.00
Pre-bleed
x8 Sera
x8 Sera+13-32
x8 Sera+18-37
x8 Sera+23-42
x8 Sera+28-47
x8 Sera+33-52
x8 Sera+38-57
x8 Sera+43-62
x8 Sera+48-67
x8 Sera+53-72
x8 Sera+58-77
x8 Sera+63-82
x8 Sera+68-87
x8 Sera+71-90
C
Bioluminescence
10000000
1000000
100000
10000
1000
Virus alone
x8 20ul
x8 20ul+Peptide
x8 5 ul
x8 5ul+Peptide
x8 2ul
x8 2ul+Peptide
17-36 rabbit
47-66 rabbit
373-392 rabbit
Background

Figure 28. Peptide blockade of 11–88×8 antiserum.

A. Sera of Balb/c mice immunized three times with 11–88×8 or 11–88×8Δ using Alum+MPL as adjuvant were harvested two weeks after the final boost. The antisera diluted 1:100 were reacted with microtiter plates coated with 1 mg of two HPV16 L2 peptides encompassing residues 43–62 and 48–67. After washing, specific reactivity was measured by ELISA using peroxidase-linked anti-mouse IgG. B. Pooled sera of Balb/c mice immunized three times with 11–88×8 using Alum+MPL as adjuvant were harvested either pre-immunization (Pre-bleed) or two weeks after the final boost (68 Sera). The antiserum to 11–88×8 was diluted 1:50 in a total volume of 200 ml and incubated with 7 mg of HPV16 L2 peptide encompassing residues 13–32, 18–37, 23–42, 28–47, 33–52, 38–57, 43– 62, 48–67, 53–72, 58–77, 63–82 or 71–90 for an hour prior to mixing with HPV16 pseudovirions carrying a SEAP reporter for a further hour at ambient temperature and subsequent infection of 293TT cells. Infection was measured as optical density, and only HPV16 L2 pepitdes 13–32 and 18–37 substantially blocked neutralization by 1:3200 dilution of antiserum to 11–88×8. C. Sera of Balb/c mice immunized three times with 11–88×8 using Alum+MPL as adjuvant were harvested two weeks after the final boost and pooled (68). The 11–88×8 antiserum was mixed with two HPV16 L2 peptides encompassing residues 43–62 and 48–67 (+Peptide) in the same ratios as in B. The 11–88×8 antiserum was administered i.p. either alone or pre-mixed with peptide in volumes of 20 ml, 5 ml or 2 ml to na¨ive mice (in groups of 5). Separate groups of mice received 200 mg each of antibody affinity purified with protein G columns from the sera of rabbits hyper-immunized with HPV16 L2 peptides 17–36, 47–66 or 373–392. All groups of mice (except the Background group) were subsequently challenged intra-vaginally with

HPV16 pseudovirions carrying a luciferase reporter. Infection was assessed by measuring bioluminescence three days later.

d) Discussion

Vaccination studies in several challenge models indicate that the amino terminus of L2 has potential as a protective antigen [10]. There are ongoing efforts to map potentially protective epitopes by the generation of antisera to synthetic L2 peptides or the generation of monoclonal antibodies and testing of their *in vitro* neutralizing activity [45,46,111,167,211,212]. We extend these studies by showing that passive transfer of antisera to HPV16 L2 residues 17–36, 32–51, and 65–81 is protective, whereas an antiserum to HPV16 L2 108–120 was not protective. These findings are generally consistent with prior *in vitro* neutralization studies, with the exception of the antiserum to HPV16 L2 108–120 [212-214]. However, we note that Rubio et al showed that both neutralizing and non-neutralizing antibodies can bind to the HPV16 L2 18–38 epitope, and thus it is possible that the HPV16 L2 108–120 peptide coupled to KLH utilized here for immunization induced only non-neutralizing antibodies despite containing a neutralizing epitope. Alternatively, the avidity of the neutralizing antibodies induced in the rabbit antiserum to HPV16 L2 108–120 peptide coupled to KLH was insufficient to afford protection *in vivo* at the dilution used (100 ul of serum in ,2 ml plasma volume of a mouse suggests ,1:20). We favor the latter possibility since we previously showed that this antiserum to HPV16 L2 108–120 peptide at 1:50 can partially neutralize native HPV11 virions [188].

Vaccination with the 11–88×8 protein induced a robust and broadly neutralizing antibody response (11–88×8 antiserum neutralizes eleven HPV genotypes, Kwak K et al,

International Papillomavirus Meeting, Montreal, Canada, July 2010, Abstract P-201, http://hpv2010.org/main/images/stories/hpv2010_abstracts. pdf) and protected mice from vaginal challenge with HPV16. Surprisingly, the 13–47×15 was significantly less immunogenic than 11–88×8, and this likely reflects in part the absence of the second neutralizing epitope within residues 65–81. Similarly, we previously observed that 17–36×22 was less immunogenic than 11–88×5 or 11–200×3 [48]. However, 11–88×8Δ (from which the 45–67 equivalent region has been eliminated from each unit of L2) was also less immunogenic than 11–88×8. Since passive transfer of mice with rabbit HPV16 L2 47–66 antiserum was not significantly protective against HPV16 challenge and the peptide was poorly immunogenic, this implies that 11–88×8Δ is not less immunogenic than 11–88×8 because a potent protective epitope within the 45–67 region was deleted. However, a recent study defined the epitope for a neutralizing monoclonal antibody (Mab24B) between residues 58– 64 [215]. Since the Mab24B antibody was generated in Balb/c mice, and our failure to detect neutralizing activity in the L2 47–66 peptide antiserum likely reflects its low immunogenicity in rabbits. Nevertheless, when we performed blockade studies by mixing individual overlapping 20mer peptides encompassing the N-terminus of HPV16 L2 with the mouse antiserum to 11–88×8, only peptides encompassing residues 13–32 and 18–37 impacted the neutralizing activity, suggesting the protective response is focused on this region in mice.

An in silico analysis using ProPred suggested that the reduction of immunogenicity by elimination of the 45–67 region in each unit of L2 within 11–88×8 might reflect the loss of CD4 T helper epitope(s). The C57BL/6 mouse strain is known to generate strong helper T cell response to PADRE, whereas Balb/c mice do not. Therefore

a PADRE epitope was included in the 11– 88×8ΔPADRE protein to determine if it could complement the loss of T helper epitope(s) in the 45–67 region and restore the immunogicity to that of 11–88×8 in C57BL/6 mice [30]. Since the 11–88×8ΔPADRE and 11–88×8Δ were both similarly less immunogenic than the 11–88×8 in either C57BL/6 or Balb/c mice, this suggests that loss of critical T help is not responsible for their lower immunogenicity than 11–88×8.

Although we note that the L2 multimers are purified under denaturing conditions and evidence to date suggests that the L2 neutralizing epitopes are linear, another possibility is that the 45–67 region within each unit of L2 contributes to the immunogenicity of 11–88×8 by maintaining the appropriate spacing, structure or conformation of L2 neutralizing epitopes. Thus, while the immunogens are initially purified under denaturing conditions in urea, we speculate that the 45–67 region may facilitate the folding or association of the L2 multimers under more physiologic conditions (as the urea is removed during dialysis) into a structure that better presents the neutralizing epitopes to the immune system. Indeed, recent work by Bronnimann et al suggests a structural role for the GxxxG motifs in this 45–67 region [216].

The observations herein support the use of the, 11–88 region of L2 as an appropriate subunit of a multimeric L2 vaccine, but do not fully address the optimal number of subunits to include within the construct. Prior studies by Rubio with repeated units of HPV16 L2 epitopes and our own work with repeated L2 units derived from different HPV types, suggest that more than 3 subunits is optimal, and that there is little difference between 5–9 subunits and that greater numbers of repeats are not necessary, and possibly detrimental [48,204]. Indeed, the 13–45×15 was less immunogenic than 11–

88×8Δ, but it is not clear whether it reflects too many subunits, perhaps too closely spaced (17–36×22 was also poorly immunogenic), or the loss of a key neutralization epitope between residues 65–81 [111]. An alternative approach to enhance immunogenicity is to display these protective L2 epitopes on the surface of virus-like particles in immunodominant locations [16,217]. While this complicates the use of sequences derived from multiple HPV types to enhance the breadth of cross-protective responses, virus display may enhance the strength and duration of the immune response to appropriately presented sequences, and several groups are pursuing this strategy [49,114,172,173].

VI) Phylogenetic considerations in designing a broadly protective multimeric L2 vaccine

a) Introduction

Persistent infection with oncogenic types of human papilloma-virus (HPV) is the cause of 5% of cancers worldwide [3]. Therefore, these HPV-associated cancers are potentially prevent- able through global implementation of a vaccine that provides durable protection against infection by all oncogenic HPVs [54], of which at least a dozen types have been identified from within the α7, α9, α5, α6, and α11 papillomavirus species [12]. The licensed HPV vaccines, Cervarix (GSK) and Gardasil (Merck), provide protection over at least a decade against the two most common oncogenic HPV types in cancer, HPV16 and HPV18, but their efficacy against other oncogenic types is variable and of less-certain duration [11,75], and no therapeutic benefit has been demonstrated for preexisting infection [218]. Gardasil also targets HPV6 and HPV11 to protect against genital warts that, while benign, are associated with significant morbidity and treatment costs [159]. The efficacious but type-restricted protection provided by these L1 virus-like particle (VLP) vaccines has driven ongoing development of a nonavalent formulation (NCT00543543). While this nonavalent vaccine has potential to provide broad protection against oncogenic HPV infections, the complexity of its manufacture is likely to further drive up costs. Unfortunately, cost remains the principal impediment to broad implementation of HPV vaccines, particularly in developing countries which also lack the

resources for an effective national cytologic screening infrastructure and thus bear ~85% of cervical cancer cases globally [74].

The twin requirements for inexpensive and broadly protective HPV vaccines have propelled interest in the minor capsid protein, L2. Vaccination with the amino terminus of L2 produced in bacteria protects animals from experimental challenge with either animal papillomaviruses or HPV pseudovirions that carry a reporter plasmid [46,110,170,187,197]. Passive transfer of either L1 VLP antiserum or L2-specific neutralizing antibody is sufficient to protect naive animals from experimental viral challenge [21,22,26,46], whereas vaccination with L2 failed to impact existing disease or protect against challenge with viral DNA [170,219]. While L1 VLP vaccination induces antibodies against conformation-dependent, type-restricted neutralizing epitopes [6,7], L2-specific antibodies recognize linear epitopes and can be broadly neutralizing [44,45,112]. L1 vaccines are produced in insect cells or *Schizosaccharomyces cerevisiae* yeasts that allow VLP assembly [8,220], whereas L2 can be expressed at high levels in bacteria, potentially reducing the cost of manufacture [110,187].

Vaccination with L2 induces more broadly neutralizing but lower titer antibodies than L1 VLP. Furthermore, L2-induced neutralizing antibody titers are generally higher against papillomaviruses most closely related to the type(s) from which the L2 vaccine was derived [44]. Therefore, to broaden and enhance the antibody response against conserved neutralizing epitopes, we developed polypeptide vaccines comprising concatenated protective regions of L2 derived from multiple medically significant HPV genotypes [48]. Here we construct a concatenated L2 vaccine consisting of the amino acids 11 to 88 of five or eight α HPV types and examine whether there is immunologic

competition between the subunits with respect to the generation of protective immunity, if the lower neutralization titers generally seen with L2 vaccine formulated in alum adjuvant are sufficient to render protection against diverse HPV types, whether it is necessary to include an L2 unit derived from each phylogenetic clade to achieve broad immunity against human papillomaviruses [12], and how the spectrum of L2-based immunity compares to that of the two licensed HPV vaccines.

b) Materials and Methods

1) Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mouse studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University, and rabbit studies were performed with the approval of the Animal Ethics committee of Shantha Biotechnics, Hyderabad, India. Research using neonatal foreskin keratinocytes was performed with the approval of the institutional review board of the University of Alabama, Birmingham.

2) Generation and neutralization of HPV18 virions

Primary human keratinocytes (PHKs) were recovered from neonatal foreskins [221] that were collected from UAB Hospital (Birmingham, AL). PHKs were cultured in serum-free medium (K-SFM; Invitrogen, Carlsbad, CA) used at passage one or two for the neutralization experiments. HPV18 virions were partially purified from organotypic cultures of PHKs containing HPV18 genomic plasmid, and titers were determined by using real-time quantitative PCR (qPCR) to measure packaged viral genomes [222]. For neutralization studies, 4×10^4 PHKs/well were seeded in 12-well plates and cultured for 2 days. On day three, after the cell numbers were determined, PHKs were then infected with HPV18 virions at a multiplicity of infection (MOI) of 500. Prior to infection, the

virions were mixed with different dilutions (1:62,500, 1:12,500, 1:2,500, or 1:500) of polyclonal antisera described in Table 5 and raised to α11-88×8 (animals 1a, 2a), α11-88×5 L2, Gardasil (animals 1d, 2d), and L1/L2 VLPs of HPV6, HPV11, HPV16, and HPV18 formulated in Freund's adjuvant anti-L1L2 [13]. Similarly, HPV18 virions were mixed with undiluted or diluted (1: 1,250, 1:250, or 1:50) monoclonal antibody RG-1 or A-1 prior to addition to PHKs at an MOI of 500. In the nonneutralizing control, the virions were mixed with K-SFM prior to infecting PHKs. The cells were incubated for 24 h at 37°C. The medium was changed, and PHKs were cultured for an additional 3 to 4 days until 100% confluence. In parallel, uninfected PHKs were similarly cultured as a negative control. Total RNAs were extracted with TRIzol (Invitrogen) and treated with DNase (Applied Bio-systems, Carlsbad, CA). Five micrograms of total RNA was reverse transcribed (Promega, Madison, WI) in a 75 ul reaction mixture. Two microliters of the cDNA products was used for 20 cycles of PCR in a 35 ul reaction mixture to amplify the β-actin cDNA fragment (642 bp) [223] or for 30 cycles for the spliced HPV18 E1Ê4 cDNA fragment (211 bp) [223].

The primers for the HPV18 E1^E4 transcript are as follows: forward, GGTGTGCATCCCAGCAGTAAG (nucleotides [nt] 888 to 908); reverse, AGGTCCACAATGCTGCTTCT (nt 3583 to 3602). Fifteen microliters of PCR products was used for electrophoresis in 2% agarose gel. The gels were stained with ethidium bromide for imaging.

3) Generation of HPV pseudoviruses

Pseudoviruses (PsV) were generated as previously described [82]. Briefly, plasmid double expression vectors for codon-optimized L1 and L2 genes were transfected with either a luciferase or alkaline phosphatase (SEAP) reporter gene plasmid into 293TT cells [82] using TransIT-2020 transfection reagent (Mirus). Three days after transfection, cell pellets were collected and rinsed with Dulbecco's modified Eagle medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS). The pellets were resuspended in a small volume of DPBS-Mg (DPBS supplemented with 9.5 mM $MgCl_2$) and then transferred into siliconized tubes. Cells were pelleted by low-speed centrifugation, and the supernatant was discarded. For PsV maturation, an equal volume of lysis buffer (DPBS-Mg supplemented with 0.5% of Brij 58 and 0.2% benzonase) was added to the cell pellet, which was incubated at 37°C for 24 h. After maturation, lysates were adjusted to 850 mM NaCl and extracted with high-salt buffer (DPBS with 0.8 M NaCl). Lysates were clarified by centrifugation at 10,000 × *g* for 10 min, loaded onto an Optiprep step gradient (27, 33, and 39%), and spun at 40,000 rpm in an SW40 rotor for 16 h at 16°C. After centrifugation, 0.5-ml fractions were collected from the top of the gradient. A sample representing each fraction was diluted and tested on 293TT cultures [82] for reporter gene expression. Fractions with the highest reporter gene expression were pooled and used for the serum neutralization assay and the *in vivo* challenge experiments.

4) Preparation of vaccine antigen

We fused L2 sequences corresponding to residues ~11 to 88 of HPV16 L2 [48] of 5 or 8 different α HPV types and expressed each L2 multimer protein in a bacterial system facilitating inexpensive, robust, and reproducible production. Briefly, the multitype L2 nucleotide sequence of α11-88×8 was codon optimized for *Escherichia coli* expression by lowest free-energy calculation and synthesized at Blue Heron Biotechnology, Inc. (Bothell, WA), with 5′ = NcoI and 3′ = XhoI sites to facilitate cloning (detailed description in reference 79). The L2 gene was subcloned into the pET28a vector (Novagen, San Diego, CA) for expression in *Escherichia coli* BL21 (Rosetta cells; Novagen). The L2 gene for α11-88×5 was PCR amplified using the above-mentioned plasmid and similarly subcloned into the pET28a vector and transformed to BL21 Rosetta cells [79]. The manufacturing process for the production of bulk proteins was initiated with a batch fermentation of the recombinant bacteria. Protein expression was induced by adding 1 mM isopropyl-D-thiogalactopyranoside to the transformed bacterial cells. Following cell harvest, a primary recovery process was performed to purify inclusion bodies. Solubilized inclusion bodies were subjected to chromatographic separations, and the purified proteins were refolded *in vitro*. Both vaccine antigens were >90% pure when analyzed by SDS-PAGE. Protein estimation was done using absorbance at 280 nm, and 250 ug/ml protein was adsorbed onto 1 mg aluminum adjuvant before being placed in glass vials. Bulk proteins were tested for impurities arising from the host cell, including DNA and bacterial protein levels, and to confirm that bacterial endotoxin was <10 EU/ml using the Limulus amebocyte lysate assay.

5) Immunization of mice

Inbred 4- to 6-week-old female BALB/c mice (NCI) in groups of 5 or 10 were immunized subcutaneously three times at 2-week intervals. The immunogens used were 25 ug of α11-88×5 in alum or 11-88×8 in alum or one-tenth of the human dose of Cervarix or Gardasil. Alum alone and PBS were used as controls for the study. Mice were bled 2 weeks after final immunization.

6) Measurement of HPV pseudovirion titers and neutralizing antibodies

Serum samples collected from each mouse 2 weeks after the final immunization were serially diluted 2-fold in culture medium, mixed with an equal volume of HPV pseudovirions containing either luciferase or SEAP reporter genes, and incubated at 37°C for 2 h. These samples were each added to 293TT cell cultures (3×10^4 cells/well), which were then incubated for 72 h [82]. To measure luciferase expression after infection of cells with titrated challenge virus preparations, culture media were removed from each well and 1× cell culture lysis reagent (CCRL; Promega) was added to lyse the cell monolayer for 15 min at room temperature on a rocking platform. A volume of 20 ul of the lysate from each well was transferred to a black microtiter plate, followed by the addition of 50 ul/well of 1× luciferase substrate (Promega). The plates were scanned with the GloMax-Multi detection system (Promega). For SEAP measurement, 40 ul of cell-free supernatant was collected from each well, mixed with 20 ul of 0.05% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, heated at 65°C for 30 min, and then cooled on ice. A volume of 200 ul/well of *p*-nitrophenyl phosphate (pNPP) substrate (2 M diethanolamine with 1 mg/ml of pNPP) was added to each well for color

development at ambient temperature, and absorbance data were collected with an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad). Data were collected when the absorbance from the wells incubated with only PsV (ODpsv) had an optical density at 405 nm (OD_{405}) of >1. The highest dilution of serum that resulted in 50% or more reduction in ODpsv was defined as the endpoint neutralization titer and expressed as its reciprocal value.

7) Vaginal challenge of mice with HPV pseudovirions

Four days before challenge, female BALB/c mice were injected subcutaneously with 3 mg of medroxyprogesterone (Depo-Provera; Pfizer) to synchronize their estrus cycles [61]. Each challenge dose was comprised of 20 ul PsV mixed with 20 ul of 3% carboxymethyl cellulose (CMC). The dose was delivered twice into the vaginal vault, with the first 20 ul injected just prior to treatment and the second 20 ul injected just after treatment with a cytobrush cell collector. The cytobrush cell collector was inserted into the vaginal vault and turned both counterclockwise and clockwise 15 times while the mice were anesthetized. Three days after PsV delivery, the mice were again anesthetized and 20 ul of luciferin (7.8 mg/ml) was deposited in the vaginal vault. Luciferase signals were acquired for 10 min with a Xenogen IVIS 100 imager, and analyses were performed with Living Image 2.5 software. Each mouse received only a single HPV challenge.

8) Statistical analysis

Exploratory statistical analyses are performed to analyze the observed titer data. Log transformations were used to achieve normality in residuals for titer data. One-way analysis of variance (ANOVA) and pairwise multiple comparisons with the Bonferroni adjustment were performed using SAS 9.3.

c) Results

1) Design of L2 vaccines

The licensed HPV vaccines and the nonavalent vaccine under development (NCT00543543) target exclusively the oncogenic HPV types within the α9 and α7 species, selected from the most prevalent types found in cervical cancer [54]. Nevertheless, a significant fraction of cervical cancers are caused by α5, α6, and α11 papillomaviruses, suggesting the value of developing a more broadly targeted HPV vaccine based upon L2 multimers. To evaluate the importance of having at least one unit derived from each species upon the breadth of protection, we generated a single protein (termed α11-88×5) comprising five different units of L2 (amino acid residues ~11 to 88) derived from three papillomavirus species (α10, α7, and α9) (Figure 29) and a second protein (termed α11-88×8) containing L2 residues 11 to 88 derived from six species (α10, α7, α9, α5, α6, and α11) (Figure 29A). Rather than selecting only the most common HPV types present in cervical cancer, the sequences included were intended to cover as much of the sequence diversity as possible within two key neutralization epitopes of L2 residing between residues 17 to 36 and 65 to 81 (Figure 29B). The two multimeric L2 fusion proteins were expressed without tags in *E. coli* and purified using conventional chromatography techniques to homogeneity (Figure 29C).

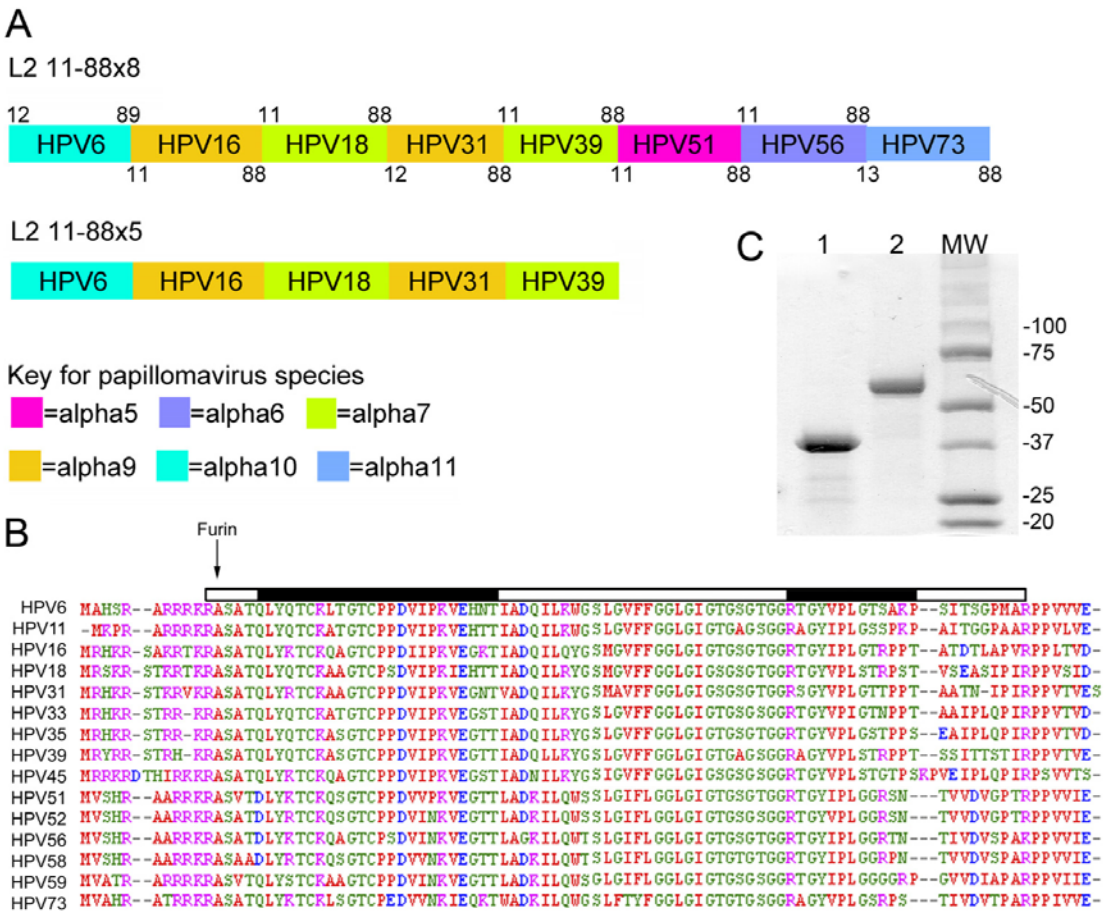


Figure 29. Design of L2 vaccines.

(A) Diagram showing the composition of α11-88×8 and α11-88×5 candidate HPV L2 vaccines with individual subunits derived from different medically significant genotypes and several papillomavirus species (color coded). (B) Comparison of sequences within the amino terminus of medically significant alpha papillomaviruses by Clustal W with color coding by a functional amino acid side chain. The location of the putative furin cleavage sites at the amino terminus of each is indicated. The bar shows the region utilized in the multimer vaccines, and the two filled regions show neutralizing epitopes defined by monoclonal antibodies specific for HPV16 L2, notably residues 17 to 36 by RG1 [46], residues 20 to 38 by K4L2 and K18L2, residues 28 to 42 by K8L2, and

residues 72 to 78 by K1L2 [167], and residues 69 to 81 by MAb6 [111]. (C) Coomassie-stained SDS-PAGE gel of the α11-88×5 (lane 1) and α11-88×8 (lane 2) antigens. MW, molecular weight markers.

2) Neutralizing and protective capacity of L2 multimer antisera

Two rabbits each were primed with either 50 ug or 200 ug of α11-88×8 in complete Freund's adjuvant (CFA) and boosted twice with the same dose in incomplete Freund's adjuvant (IFA). The immune sera were tested for HPV16, HPV18, HPV31, HPV45, and HPV58 pseudovirion neutralizing titers (Table 5), and all were broadly neutralizing. Similarly, serum of a rabbit immunized with α11-88×5 was able to broadly neutralize these types, whereas antisera from two rabbits vaccinated with Gardasil strongly neutralized HPV18 (which is targeted by the vaccine) and weakly neutralized HPV31 and HPV45 (the two types most closely related to HPV16 and HPV18 that are included in the vaccine), but no neutralizing activity against HPV58 was detected. Passive transfer of α11-88×8 antiserum completely protected naive mice from vaginal challenge with HPV16 and HPV58 pseudovirions (Figure 30A and B), whereas infection was robust in the presence of preimmune serum, confirming that the L2-specific neutralizing antibodies are sufficient to mediate protection of mice against vaginal challenge with HPV pseudovirions, even when those of a type not used to make the α11-88×8 vaccine.

Antigen	Rb ID	Dose (μg)	Titer against: HPV16 PsV	HPV18 PsV	HPV31 PsV	HPV45 PsV	HPV58 PsV	HPV18 native
α11-88x8	1a	50	204,800	25,600	12,800	6,400	3,276,800	2,500
α11-88x8	1b	50	25,600	6,400	1,600	6,400	3,200	Untested
α11-88x8	2a	200	409,600	51,200	6,400	51,200	25,600	2,500
α11-88x8	2b	200	204,800	25,600	12,800	25,600	12,800	Untested
α11-88x5	2a	200	1,638,400	204,800	102,400	204,800	409,600	12,500
Gardasil	2d	12	102,400	102,400	200	200	<50	2,500
Gardasil	1d	30	51,200	204,800	100	800	<50	2,500

Table 5. *In vitro* neutralization titers of rabbit antisera to candidate HPV vaccines and Gardasil[a]

[a] Rabbits were vaccinated on days 1, 28, and 42 with the indicated dose of polymeric L2 constructs in complete Freund's adjuvant (CFA) with the initial dose and incomplete Freund's adjuvant (IFA) thereafter as described previously [48]. Two additional rabbits were vaccinated with 12 or 30 μg of Gardasil on days 1, 21, and 35, and blood samples were collected 1 week after the final vaccination. Individual rabbit identifiers (Rb ID) are provided in the second column. Blood was allowed to congeal overnight, and the serum was separated by centrifugation and stored at ~20°C until testing for *in vitro* neutralization titers against HPV16, HPV18, HPV31, HPV45, and HPV58 pseudovirions (PsV) as described previously [48] or against organotypic raft-derived HPV18 (native) virions (last column).

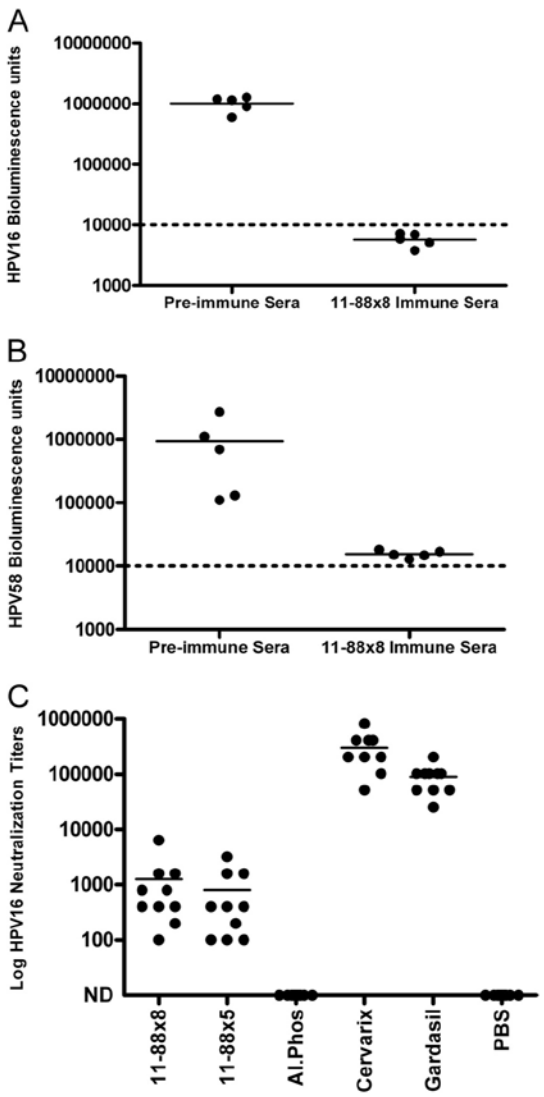


Figure 30. Neutralizing and protective capacity of L2 multimer antisera.

Passive transfer of rabbit α11-88×8 antiserum, but not preimmune serum, protects mice from vaginal challenge with HPV16 (A) or HPV58 (B) pseudovirus. The dashed line indicates the background bioluminescence level without pseudovirion challenge. (C) *In vitro* HPV16 neutralization titers of 6 groups of 10 BALB/c mice that were immunized three times at 2-week intervals with PBS alone, alum alone, α11-88×5 in alum, α11-88×8 in alum, or one-tenth of a human dose of either Gardasil or Cervarix for comparison.

3) Neutralization of raft-derived HPV18 by L2 and L1 VLP-specific antibodies

It has been suggested that neutralization of organotypic raft culture-derived HPV virions differs from that of HPV pseudovirions [224]. Therefore, we wished to determine if α11-88×5 and α11-88×8 antisera were able to neutralize infection of primary human keratinocytes by HPV18 derived from organotypic raft culture. HPV18 virions were partially purified from organotypic raft cultures, incubated with dilutions of sera from vaccinated rabbits, monoclonal antibody RG-1, or an isotype-matched non-neutralizing L2-specific monoclonal antibody A1, and then used to infect primary human keratinocytes at an MOI of 500. Infection was determined by the detection of early spliced E1^E4 viral mRNA by reverse transcription-PCR (RT-PCR). The L2-specific neutralizing monoclonal antibody RG-1 reduced HPV18 infection (Figure 31A), whereas the isotype-matched control monoclonal antibody A1 had no effect (Figure 31B). Sera from rabbits vaccinated with HPV6, -11, -16, or -18 L1 VLP in alum (Gardasil) neutralized infection at a dilution of 2,500, and sera from a rabbit that was vaccinated with these VLPs in CFA/IFA neutralized HPV18 at a titer of 12,500 [13] (Figure 31B). Sera of two rabbits vaccinated with α11-88×8 in CFA/IFA neutralized HPV18 at a titer of 2,500, and sera of a rabbit immunized with α11-88×5 in CFA/IFA neutralized at 12,500 (Figure 31A and Table 5). For comparison, neutralizing antibody titers of the same sera as determined using *in vitro* infection with HPV18 pseudovirions carrying a reporter construct are also shown in Table 5. The differences in titer likely reflect distinct methodologies and cell types used in these neutralization assays.

For a direct comparison of the immunogenicities of α11-88×5, α11-88×8, and the licensed HPV vaccines using an adjuvant already licensed for human use, groups of 10

BALB/c mice were immunized three times at 2-week intervals with either alum alone, α11-88×5 in alum, α11-88×8 in alum, or one-tenth of a human dose of either Gardasil or Cervarix. Sera obtained 2 weeks after the final immunization were tested for *in vitro* neutralization titers against HPV16 pseudovirions. The titers in sera of mice vaccinated with Cervarix were higher than those vaccinated with Gardasil ($P = 0.0022$). The HPV16 neutralization titers induced by α11-88×5 and α11-88×8 were similar ($P = 0.3870$) and two logs lower than those for the licensed HPV vaccines (Figure 30C). There was no significant difference between ELISA titers 2 weeks after the final immunization with α11-88×5 in alum (2,250 ± 380 [$n = 20$]) and those at 4 months (1,779 ± 221 [$n = 19$]; short- versus long-term; $P = 0.5586$).

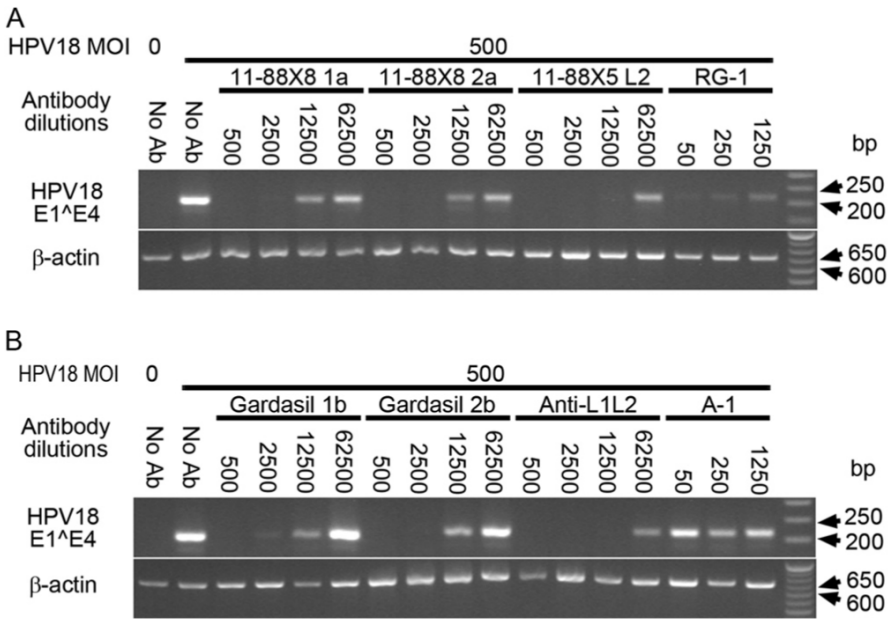


Figure 31. Neutralization of raft-derived HPV18 by L2 and L1 VLP-specific antibodies.

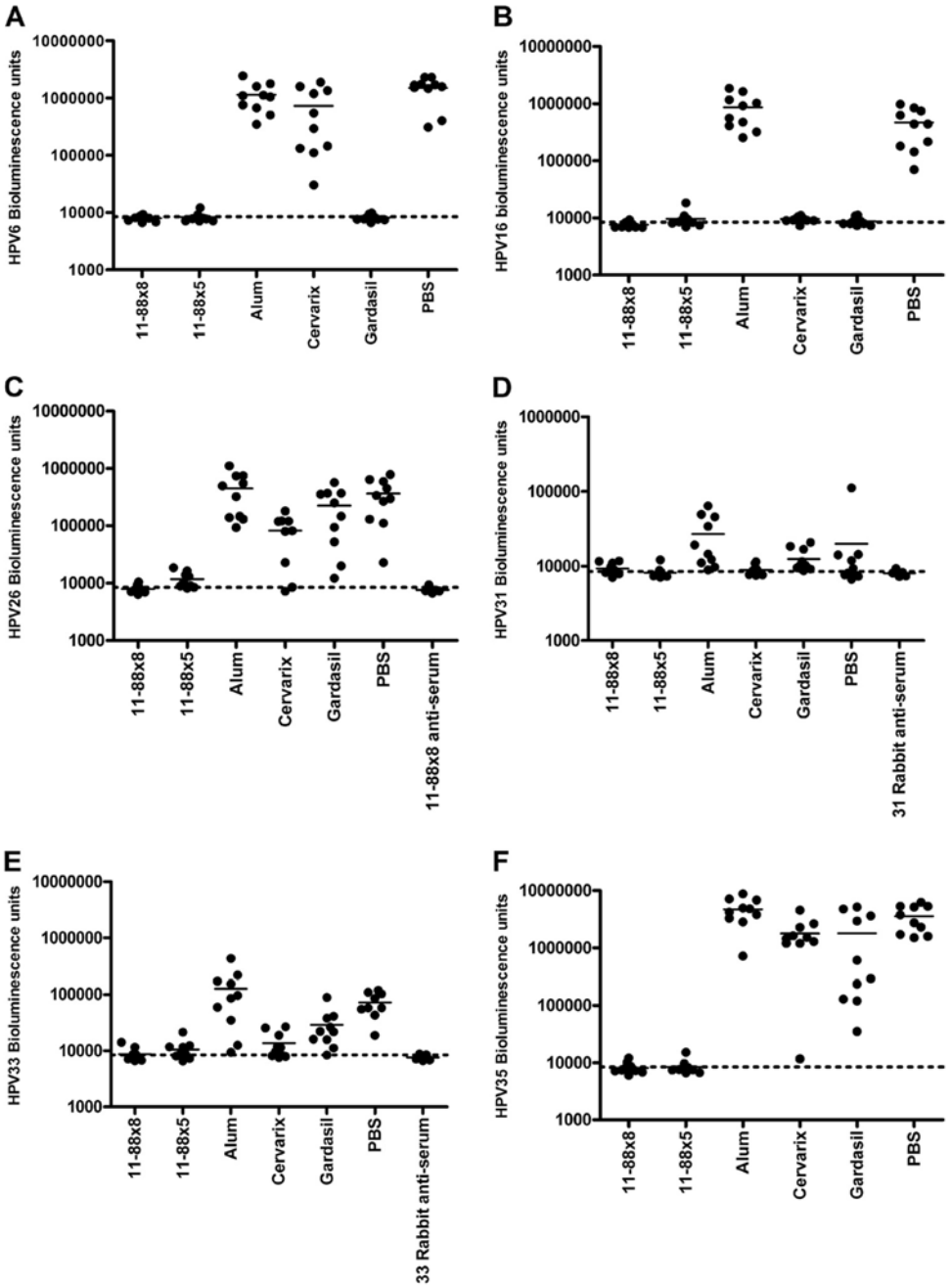
(A and B) HPV18 virions were partially purified from organotypic cultures of PHKs containing the HPV18 genomic plasmids [222]. Primary human keratinocytes (PHKs at 4 × 10^4/well of twelve-well plates) were seeded in K-SFM and cultured for 3 days. HPV18 virions were mixed with undiluted or diluted (1:1,250, 1:250, or 1:50) RG-1 or A1 hybridoma supernatant or different dilutions (1:62,500, 1:12,500, 1:2,500, or 1:500) of polyclonal antiserum α11-88×8 1a and 2a, α11-88×5 2a, Gardasil 1b and 2b, or anti-L1L2, and then added to PHKs at an MOI of 500. The cells were cultured for 4 days until reaching confluence. In parallel, uninfected PHKs were similarly cultured as a negative control. Purified total RNAs were extracted with TRIzol, treated with DNase, and reverse transcribed, and a β-actin cDNA fragment (642 bp) [223] or the spliced HPV18 E1Ê4 cDNA fragment (211 bp) was amplified by PCR. The PCR products were analyzed by electrophoresis in 2% agarose gel and stained with ethidium bromide for imaging.

4) Protection of mice from vaginal HPV challenge after vaccination with L2 based and licensed L1 VLP vaccines

To differentiate the breadth of protection elicited by the L2 based and licensed HPV VLP vaccines, 10 groups of 10 mice were immunized three times at 2-week intervals with PBS alone, alum alone, α11-88×5 in alum, α11-88×8 in alum, or one-tenth of a human dose of either Gardasil or Cervarix for comparison. The mice were intravaginally challenged with HPV16 pseudovirus, and 3 days later, infection was assessed by measuring luciferase activity. The level of infection in mice vaccinated with alum alone was the same as that in mice vaccinated with buffer, whereas mice vaccinated with either Gardasil or Cervarix were completely protected, as were mice vaccinated with either α11-88×5 or α11-88×8 (Figure 32B).

These studies were extended by vaccinating additional groups of mice as described above, and for a positive control, another group of 5 naive mice received passive transfer of protective antisera intraperitoneally (i.p.) 1 day prior to challenge, as indicated. These groups of vaccinated mice were challenged intravaginally with pseudovirions derived from HPV6, HPV26, HPV31, HPV33, HPV35, HPV45, HPV51, HPV56, HPV58, or HPV59 (Figure 32). Gardasil, but not Cervarix, provided complete protection against HPV6 challenge, because only the former contains HPV6 L1 VLP. Both α11-88×5 and α11-88×8 contain HPV6 L2 residues 11 to 88, and the vaccines demonstrated the same level of protection against HPV6 as Gardasil in the mouse challenge model. Likewise, α11-88×5 and α11-88×8 both contain HPV31 L2 and were strongly protective against HPV31. α11-88×8 contains L2 units derived from HPV51 and HPV56, whereas α11-88×5 does not. While α11-88×8 appeared to provide slightly better

protection against HPV51 than α11-88×5, the difference was not significant for either HPV51 (P = 0.31) or HPV56 (P = 1.0). For the remaining challenges with virus types whose L2 is not represented in either α11-88×5 or α11-88×8, both vaccines provided similarly strong protection (no significant difference for HPV26, HPV33, HPV35, HPV45, or HPV59 challenges)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HPV6 Bioluminescence units
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11-88x8
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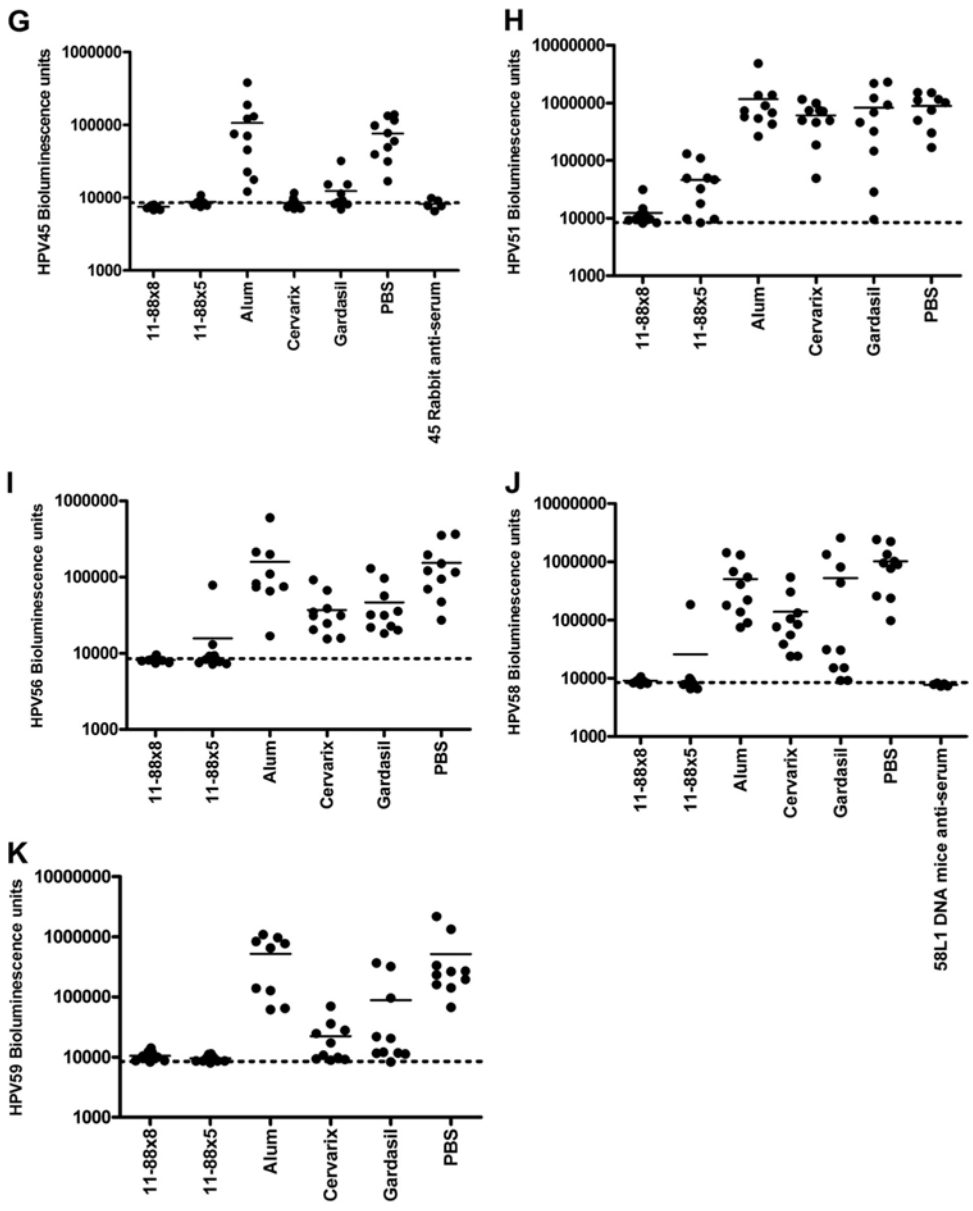

Figure 32. Protection of mice from vaginal HPV challenge after vaccination with L2-based and licensed L1 VLP vaccines.

For each HPV challenge study, six groups of 10 mice were vaccinated three times at 2-week intervals with PBS alone, alum alone, α11-88×5 in alum, α11-88×8 in alum, or one-tenth of a human dose of either Gardasil or Cervarix for comparison. If needed as a positive control, another group of 5 naive mice received passive transfer of 20 ul antisera

i.p. 1 day prior to challenge, including rabbit antisera to HPV31 L1 VLP (D), HPV33 L1 VLP (E), and HPV45 L1 VLP (G), generated as previously described [13], α11-88×8 formulated in Freund's adjuvant (C), or antisera pooled from mice vaccinated intramuscularly (i.m.) with HPV58 L1 expression vector (J). New groups of vaccinated mice were challenged intravaginally with HPV6 at 2 months after the final immunization (A), HPV16 at 1 month after the final immunization (B), HPV26 at 2 months after the final immunization (C), HPV31 at 3 months after the final immunization (D), HPV33 at 3 months after the final immunization (E), HPV35 at 4 months after the final immunization (F), HPV45 at 4 months after the final immunization (G), HPV51 at 2 months after the final immunization (H), HPV56 at 4 months after the final immunization (I), HPV58 at 4 months after the final immunization (J), or HPV59 pseudovirions at 7 months after the final immunization (K).

5) Summary of the protection of mice from vaginal HPV challenge obtained after vaccination with L2-based and licensed L1 VLP vaccines

Figure 33 shows that a very high percentage of mice had >90% reduction in luciferase signal, demonstrating that both the α11-88×5 and α11-88×8 vaccines strongly protect against members of five medically significant papillomavirus species in the mouse vaginal challenge model: α5 (HPV26, HPV51), α6 (HPV56), α7 (HPV39, HPV45, HPV59), α9 (HPV16, HPV31, HPV35, HPV33, HPV58), and α10 (HPV6). Gardasil and Cervarix provided some degree of cross-protection against nonvaccine types, notably HPV45 and HPV59 (Figure 32 and 33). For several genotypes, vaccination with α11-88×5 provided significantly better protection than Cervarix (HPV26, $P = 0.0062$; HPV35, $P < 0.0001$; HPV51, $P < 0.0001$; HPV56, $P = 0.0007$; HPV58, $P = 0.0075$; HPV6, $P < 0.0001$) or Gardasil (HPV26, $P < 0.0001$; HPV33, $P = 0.0002$; HPV35, $P < 0.0001$; HPV51, $P < 0.0001$; HPV56, $P = 0.0001$; HPV58, $P = 0.0069$), and similar results were obtained with the α11-88×8 immunogen. Importantly, the protection afforded by the L2 vaccines persisted at least several months postvaccination in all cases tested, including 7 months after the final vaccination for HPV59 (Figure 32J).

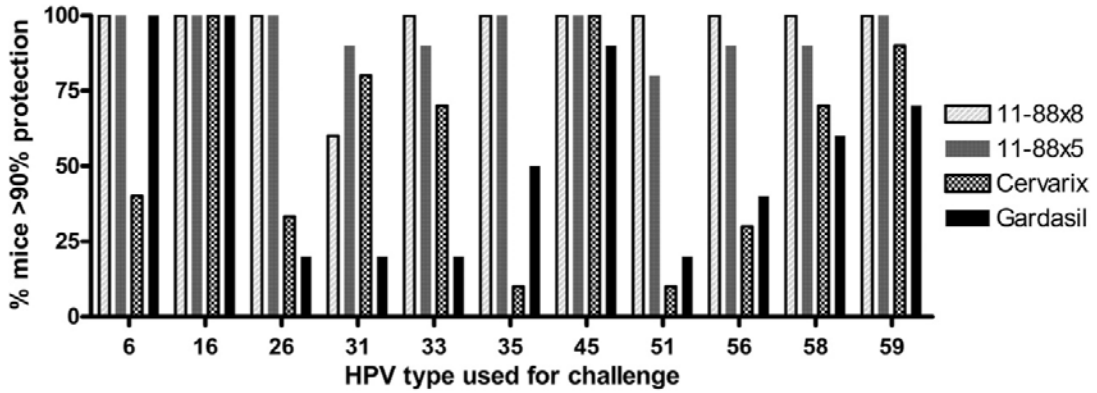

Figure 33. Summary of the protection of mice from vaginal HPV challenge obtained after vaccination with L2-based and licensed L1 VLP vaccines.

The percentage of mice in each vaccine group (n = 10) that exhibited a >90% reduction in luciferase signal upon challenge compared to control mice in studies shown in Figure 32 is plotted for each HPV challenge virus.

d) Discussion

Studies of Cervarix and Gardasil demonstrated strong protection against the two vaccine-targeted types but only limited protection against persistent infection by most other oncogenic HPV types, illustrating the need for broader coverage [11,225]. Indeed, clinical efficacy, as measured by persistent infection with an L1-based vaccine like Cervarix, against any nonvaccine oncogenic HPV type at 6 and 12 months is 11.3% (96.1% confidence interval [CI], 3.7% to 18.3%) and 10.9% (96.1% CI, -0.9% to 21.4%), respectively [75], and Malagón et al. suggest that there is some evidence for waning of cross-protection with increased follow-up [76,77]. Gardasil exhibits 23.4% (95% CI, 7.8% to 36.4%) efficacy for protection against CIN1-3 or AIS associated with 10 nonvaccine oncogenic HPV types, although this is not directly comparable to the above-mentioned data for Cervarix [11,75]. Both L2-based vaccines provide broad protection in mice against vaginal challenge with diverse members of the genus α papillomaviruses, notably at least nine oncogenic HPV types that are responsible for cervical cancer. An HPV vaccine that is broadly effective against all members of the α HPV genus is likely to reduce the number of patients with atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL) that require colposcopy and more-intensive follow-up, potentially providing significant cost savings.

There was no difference in the HPV16 pseudovirion-neutralizing antibody titers induced by vaccination with α11-88×5 or α11-88×8, but the titers were two orders of

magnitude lower than those observed for the licensed HPV vaccines. However, the currently used *in vitro* neutralization assays were recently shown to underestimate the protective capacity of L2 antibodies because of differences in the infection process in cervicovaginal tissues versus cultured cells [226]. Consistent with this observation, robust protection from experimental pseudovirus challenge was observed in mice even 5 to 7 months postimmunization with the L2 vaccines formulated in alum despite low titers, as measured in the conventional neutralization assay used herein, and the use of higher doses of pseudovirions in the *in vivo* assays. These observations suggest that the new neutralization assay described by Day et al. [226], or a higher-throughput version, might better be used to assay the serum titers of vaccinated patients during the clinical development of the L2 multimer vaccine.

Although vaccination of healthy volunteers with an HPV16 L2E6E7 protein (without an adjuvant) has been shown to elicit L2-specific (cross-) neutralizing serum antibody responses [227,228], the ability of an alum-formulated L2 vaccine to protect patients from natural acquisition of HPV and the duration of protective immune responses remain an open question. However, vaccination of healthy volunteers with HPV6 L2E7 formulated in alum produced robust serum IgG responses and antigen-specific T cell proliferative responses, apparently stable at least 6 months after vaccination [229], and our mouse challenge studies indicate that protection is maintained at least this long. Likewise, cattle maintained protection for 1 year after vaccination with L2 [230]. Durability of protection is a critical issue for assessing the need for booster immunizations and cost/benefit analyses since HPV vaccines are ideally given several years prior to sexual debut and protection should be maintained at least through

midadulthood.

Like Gardasil, the L2-based vaccines were also highly effective for protection of mice against vaginal challenge with HPV6. HPV6 and HPV11 are the major types found in genital warts, but HPV40, HPV42, HPV43, HPV44, etc., also contribute a significant fraction of genital warts [231], and rabbit antisera to α11-88×8 effectively neutralize these types (data not shown). While genital warts are benign, they cause significant morbidity, and approximately $200 million is spent annually on their treatment in the United States alone [232].

A broadly protective HPV vaccine may be particularly beneficial to patients with HIV infections or who are immunosuppressed following organ transplantation. These patients are more prone to infection associated with unusual HPV genotypes not targeted by the current HPV vaccines, and the lesions can be more persistent and recalcitrant to treatment [233].

Based on passive-transfer experiments, protection in the vaginal challenge model is mediated by neutralizing antibodies. Importantly, antisera to the L2 vaccines effectively neutralized native HPV18 virions derived from organotypic rafts, suggesting that the protection does not represent an artifact of the pseudovirion system utilized in these studies. Although comparable in endpoint neutralization titers to Gardasil in this assay, it is important to note that the L2 vaccines were administered in CFA/IFA rather than alum (Table 5). It should also be noted that several animal studies using wart-derived virus for challenge have demonstrated the capacity of L2-based vaccines to protect against BPV4 in the soft palate of cattle, rabbit oral papillomavirus (ROPV) in the

oral mucosa of rabbits, and cottontail rabbit papillomavirus (CRPV) in skin [110,187].

The α11-88×5 and α11-88×8 vaccines induced indistinguishable titers of HPV16-neutralizing antibodies, suggesting that immunologic competition between units is not a significant issue. There was no statistically significant difference in the protection against vaginal challenge with any of the 11 different virus types tested that was induced by vaccination with α11-88×5 or α11- 88×8. These findings suggest that it is not necessary to include a unit of L2 derived from each species to achieve broader protection against diverse medically significant HPV types than is achieved with the licensed HPV vaccines in the mouse vaginal challenge model.

The current HPV vaccines do not overcome the need for continuing cervical cancer screening programs because they do not protect against many oncogenic HPV types. The substantial burden to health care systems of maintaining both primary and secondary prevention programs might be reduced by a vaccine that provides effective protection against all oncogenic HPV types. Further, the introduction of a broadly protective vaccine is particularly important in low-resource settings that lack cytologic screening, and low cost is an important factor in reaching these populations [74]. The findings herein using the mouse vaginal challenge model suggest that α11-88 L2 multimer vaccines produced by bacterial expression and formulated in alum (a low-cost and well-accepted adjuvant) show promise in this regard.

C. Conclusion

As there are billions of people who need HPV vaccines in developing countries but cannot afford the current licensed vaccines due to their high cost, and because current vaccines do not provide complete protection against the 15 known oncogenic HPV types, next generation HPV vaccines should be both cost-effective and broadly protective. We have generated several vaccine candidates including naked DNA expression vectors expressing L1(±L2), and engineered protein-based L2 vaccines as candidate second generation vaccines to address this need. DNA vaccines can be manufactured simply and are stable at ambient temperature. Immunization with L1-expressing DNA utilizing in vivo electroporation induced antibody responses with high neutralization titers approaching those elicited by Cervarix and Gardasil. We mixed L1 DNAs from multiple different HPV types and immunized mice with the DNA mixture expecting broad protection against diverse HPV types. However, when L1 DNAs were mixed together reduced neutralization titers were observed with certain combination of types, likely resulting from heterotypic L1 interactions that can be observed in co-immunoprecipitation studies. Vaccination with L1 DNA vaccines of different types at different sites resolved this interference. Thus, multivalent vaccination via *in vivo* electroporation optimally requires spatial separation of individual type L1 DNA vaccines, although this issue could be ameliorated by inclusion of L2 in each construct.

Although L1 DNA vectors have potential as an inexpensive and heat stable approach to HPV vaccination, injecting L1 DNA of different types at different sites and the use of electroporation make this approach complex and costly to apply clinically, and

electroporation is also associated with significant local pain. Thus, we next examined the potential of the L2 minor capsid protein as an immunogen because it is known to elicit broad protection, although L2 immunity is associated with low antibody titers compared to L1 VLP vaccines. In an effort to enhance immune presentation of L2, we engineered key L2 protective epitopes into diverse antigen forms. Initially, a chimeric L1 capsomer was utilized as a platform to display L2 protective epitopes in which the N-terminal residues of HPV18, HPV31, and HPV45 L2 were fused in tandem with the C-terminus of L1 without compromising its self-assembly into a capsomer. Vaccination with capsomer-L2 elicited complete protection against HPV16 and HPV18 intravaginal challenge. However we could not measure detectable neutralization titers against HPV18, HPV31, and HPV45, suggesting that the *in vivo* intravaginal challenge assay is more sensitive than the *in vitro* neutralization assay and that L2 antibody response elicited by capsomer-L2 vaccination is weak.

Next, a key conserved and protective epitope of HPV16 L2 (recognized by the RG1 neutralizing monoclonal antibody) was linked to DE surface loop of HPV16 VLP in order to display the RG1 epitope (aa 17-36) on the surface of the VLP. We hypothesized this would enhance the L2 antibody response as VLP is a more potent platform than capsomer, and the epitope was inserted into an immunodominant loop that contributes to the structure of a conformational and neutralizing L1 epitope. RG1-VLP generated detectable, but somewhat varied, cross-neutralizing antibody titers and complete protection against diverse HPV types. Titers were higher against HPV types phylogenetically close to HPV16 but low against other types possibly because single HPV16 L2 was used as an immunogen. However, it is important to note that the L2-

specific response was still far weaker than the corresponding L1-specific antibody titers. This indicates that the RG1-VLP conformation kept most L1 neutralizing epitopes intact, but the presentation of the linear L2 neutralizing epitope was not as efficient as the conformational L1 epitope that it replaced.

We hypothesized from previous results that high neutralization titers to diverse HPV types can be achieved when the N-terminus of L2 from multiple HPV types is administered fused together as a concatemer, i.e. a linear array as opposed to the three dimensional display of L2 in a chimeric VLP. Thus, we generated L2×8 fusing protective domains comprising L2 residues 11-88 derived from eight different HPV subtypes. Neutralization titers elicited by L2×8 given in alum+MPL adjuvant were robust against vaccine and even non-vaccine HPV types, although still lower seen with L1 VLP vaccines. Complete protection was also observed against numerous HPV types including high and low risk types, suggesting L2×8 has potential as a candidate prophylactic HPV vaccine to provide broad immunity. However, the yield of L2×8 upon recombinant expression and column chromatography was low because of llow expression levels and substantial degradation during production in *E. coli*.

We eliminated the hydrophobic region at N-terminus of L2 (L2×8Δ) to enhance the yield, as consecutive hydrophobic residues appeared contribute to protein aggregation and degradation. Although the yield was considerably increased after removal, neutralization titers elicited by vaccination with L2×8Δ were significantly decreased (by about ten-fold) compared to L2x8. Since this might reflect the deletion of a key CD4 T helper epitope (and because *in silico* analysis predicted that the deleted hydrophobic region contains promiscuous MHC class II epitopes), we attempted to complement this

loss via fusion of the known promiscuous CD4 T helper epitope PADRE to L2×8Δ protein and thus rescue immunogenicity. Neutralization assays of antisera indicated that titers were not recovered after addition of PADRE to L2×8Δ construct, implying that reduction of immunogenicity by elimination of hydrophobic region was not because of loss of CD4 helper epitope but possibly because of other reasons, such as changes in the structure of the epitope.

We postulated that decreasing the number of L2 repeats in the L2 multimer would improve yield while maintaining immunogenicity, as there would be a lesser number of hydrophobic regions in the L2 multimer in this case. A truncated derivatives of L2×8 with the last three units deleted (L2×5) was produced at a significantly higher yield and with less evidence of degradation. Further vaccine efficacy was measured to examine if the L2×5 multimer elicits comparable neutralization titers and protection to the L2×8 vaccine. Fortunately, a significant increase in protein yield in *E. coli* was observed after truncation, and L2×5 elicited robust antibody responses with high neutralization titers and potent protection against diverse HPV types with similar efficacy to the L2×8 vaccine. These results suggest that L2×5 is a promising candidate second generation HPV vaccine.

In conclusion, the current prophylactic HPV vaccines do not overcome the need for continued pap-smear screening because of the type-restricted protection properties of these vaccines, and their high price limits access of the vaccine to people in developing countries. To overcome the disadvantages of current vaccines, we examined several inexpensive and broadly protective next generation HPV vaccine candidates including L1 DNA and engineered L2 protein vaccines. After thorough evaluation, we concluded that the L2×5 and RG1-VLP are the best candidates and suggest that they warrant further

testing in early phase trials. It will be important to optimize the immunogenicity of L2 in patients by use of an appropriate adjuvant because of the need for durable protection, but given the priority for safety, initial efforts will focus on these L2 vaccines formulated in alum or alum+MPL.

D. References

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E. Curriculum Vitae

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Research and Professional Experiences

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- "Understanding the mechanism of human papillomavirus infection for rational development of prophylactic human papillomavirus vaccines"

2007 – 2008 **Research Technician, Salk Institute, San Diego, CA**

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- "Investigation of the mechanisms through which acute or long-term alcohol exposure alters the activity of the HPA axis"

2007 **Research Technician, University of Utah, Salt Lake City, UT**

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- "Cre recombinase expression controlled by the hematopoietic regulatory domain of Gata-1 is erythroid-specific"

Honors and Awards

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Publications

1. **Kwak K**, Jiang R, Wang JW, Jagu S, Kirnbauer R, Roden RB. (2014). Impact of inhibitors and L2 antibodies upon the infectivity of diverse alpha and beta human papillomavirus types. PLos One., 9;9(5):e97232.

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Presentations

i) **Invited Talk**

1. **International papillomavirus conference**, Berlin, Germany

ii) **Poster presentation**

1. **International papillomavirus conference 2012**, Puerto Rico, USA
2. **International papillomavirus conference 2011**, Berlin, Germany
3. **International papillomavirus conference 2010**, Montreal, Canada