Phylogenetic Considerations in Designing a Broadly Protective Multimeric L2 Vaccine

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While the oncogenic human papillomavirus (HPV) types with the greatest medical impact are clustered within the $\alpha 9$ and $\alpha 7$ species, a significant fraction of cervical cancers are caused by $\alpha 5$, $\alpha 6$, and $\alpha 11$ viruses. Benign genital warts are caused principally by the $\alpha 10$ viruses HPV6 and HPV11. In an effort to achieve broad protection against both cervical cancer- and genital wart-associated types, we produced at high levels in bacteria a multimeric protein ($\alpha 11$ -88x8) fusing eight polypeptides corresponding to a protective domain comprising L2 residues ~ 11 to 88 derived from HPV6 ($\alpha 10$), HPV16 ($\alpha 9$), HPV18 ($\alpha 7$), HPV31 ($\alpha 9$), HPV39 ($\alpha 7$), HPV51 ($\alpha 5$), HPV56 ($\alpha 6$), and HPV73 ($\alpha 11$) and a truncated derivative with the last three units deleted ($\alpha 11$ -88x5). Mice were immunized three times with $\alpha 11$ -88x8 or $\alpha 11$ -88x5 adjuvanted with alum or the licensed HPV vaccines and challenged intravaginally with HPV6, HPV16, HPV26, HPV31, HPV33, HPV35, HPV45, HPV51, HPV56, HPV58, or HPV59 pseudovirions. The $\alpha 11$ -88x5 and $\alpha 11$ -88x8 vaccines induced similarly robust protection against each HPV type tested and indistinguishable HPV16-neutralizing antibody titers. Passive transfer of $\alpha 11$ -88x8 antisera was protective. Further, rabbit antisera to $\alpha 11$ -88x8 and $\alpha 11$ -88x5 similarly neutralized native HPV18 virions. These findings suggest that immunologic competition between units is not a significant issue and 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.

Persistent infection with oncogenic types of human papillomavirus (HPV) is the cause of 5% of cancers worldwide (1). Therefore, these HPV-associated cancers are potentially preventable through global implementation of a vaccine that provides durable protection against infection by all oncogenic HPVs (2), of which at least a dozen types have been identified from within the $\alpha 7$, $\alpha 9$, $\alpha 5$, $\alpha 6$, and $\alpha 11$ papillomavirus species (3). 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 (4, 5), and no therapeutic benefit has been demonstrated for preexisting infection (6). Gardasil also targets HPV6 and HPV11 to protect against genital warts that, while benign, are associated with significant morbidity and treatment costs (7). 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 $\sim 85\%$ of cervical cancer cases globally (8).

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 (9–13). Passive transfer of either L1 VLP antiserum

or L2-specific neutralizing antibody is sufficient to protect naive animals from experimental viral challenge (12, 14–16), whereas vaccination with L2 failed to impact existing disease or protect against challenge with viral DNA (11, 17). While L1 VLP vaccination induces antibodies against conformation-dependent, type-restricted neutralizing epitopes (18, 19), L2-specific antibodies recognize linear epitopes and can be broadly neutralizing (20–22). L1 vaccines are produced in insect cells or *Schizosaccharomyces cerevisiae* yeasts that allow VLP assembly (18, 23), whereas L2 can be expressed at high levels in bacteria, potentially reducing the cost of manufacture (9, 10).

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 (21). 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 (24). 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

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TABLE 1 *In vitro* neutralization titers of rabbit antisera to candidate HPV vaccines and Gardasil^a

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

^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 (24). 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 coagulate 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 (24) or against organotypic raft-derived HPV18 (native) virions (last column).

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 (3), and how the spectrum of L2-based immunity compares to that of the two licensed HPV vaccines.

MATERIALS AND METHODS

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.

Generation and neutralization of HPV18 virions. Primary human keratinocytes (PHKs) were recovered from neonatal foreskins (25) 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 (26). 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 1 and raised to α11-88x8 (animals 1a, 2a), α11-88x5 L2, Gardasil (animals 1d, 2d), and L1/L2 VLPs of HPV6, HPV11, HPV16, and HPV18 formulated in Freund's adjuvant anti-L1L2 (27). 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 Biosystems, Carlsbad, CA). Five micrograms of total RNA was reverse transcribed (Promega, Madison, WI) in a 75-μl reaction mixture. Two micrograms of the cDNA products was used for 20 cycles of PCR in a 35-μl reaction mixture to amplify the β-actin cDNA fragment (642 bp) (28) or for 30 cycles for the spliced HPV18 E1E4 cDNA fragment (211 bp) (28).

The primers for the HPV18 E1E4 transcript are as follows: forward, GGTGTGCATCCCAGCAGTAAG (nucleotides [nt] 888 to 908); reverse, AGGTCACAAATGCTGCTTCT (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.

Generation of HPV pseudoviruses. Pseudoviruses (PsV) were generated as previously described (29). 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 (29) 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 MgCl₂) 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 $10,000 \times g$ 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 (29) 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.

Preparation of vaccine antigen. We fused L2 sequences corresponding to residues ~11 to 88 of HPV16 L2 (24) 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 α11-88x8 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 30). 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 α11-88x5 was PCR amplified using the above-mentioned plasmid and similarly subcloned into the pET28a vector and transformed to BL21 Rosetta cells (30). 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-β-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 µg/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 amoebocyte lysate assay.

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 µg of α 11-88x5 in alum or 11-88x8 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.

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 (29). To measure luciferase expression after infection of cells with titrated challenge virus preparations, culture media were removed from each well and $1 \times$ 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 µl of the lysate from each well was transferred to a black microtiter plate, followed by the addition of 50 µl/well of $1 \times$ luciferase substrate (Promega). The plates were scanned with the GloMax-Multi detection system (Promega). For SEAP measurement, 40 µl of cell-free supernatant was collected from each well, mixed with 20 µl 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 µl/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 (OD_{psv}) had an optical density at 405 nm (OD₄₀₅) of >1. The highest dilution of serum that resulted in 50% or more reduction in OD_{psv} was defined as the endpoint neutralization titer and expressed as its reciprocal value.

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 (31). Each challenge dose was comprised of 20 µl PsV mixed with 20 µl of 3% carboxymethyl cellulose (CMC). The dose was delivered twice into the vaginal vault, with the first 20 µl injected just prior to treatment and the second 20 µl 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 µl 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.

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.

RESULTS

The licensed HPV vaccines and the nonavalent vaccine under development (NCT00543543) target exclusively the oncogenic HPV types within the α 9 and α 7 species, selected from the most prevalent types found in cervical cancer (2). Nevertheless, a significant fraction of cervical cancers are caused by α 5, α 6, and α 11 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 α 11-88x5) comprising five different units of L2 (amino acid residues ~11 to 88) derived from three papillomavirus species (α 10, α 7, and α 9) (Fig. 1) and a second protein (termed α 11-88x8) containing L2 residues 11 to 88 derived from six species (α 10, α 7, α 9, α 5, α 6, and α 11) (Fig. 1A). 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 (Fig. 1B). The two multimeric L2 fusion proteins were expressed without tags in *E. coli* and purified using conventional chromatography techniques to homogeneity (Fig. 1C).

Two rabbits each were primed with either 50 µg or 200 µg of α 11-88x8 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 1), and all were broadly neutralizing. Similarly, serum of a rabbit immunized with α 11-88x5 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 α 11-88x8 antiserum completely protected naive mice from vaginal challenge with HPV16 and HPV58 pseudovirions (Fig. 2A 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 α 11-88x8 vaccine.

It has been suggested that neutralization of organotypic raft culture-derived HPV virions differs from that of HPV pseudovirions (32). Therefore, we wished to determine if α 11-88x5 and α 11-88x8 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 reverse transcription-PCR (RT-PCR). The L2-specific neutralizing monoclonal antibody RG-1 reduced HPV18 infection (Fig. 3A), whereas the isotype-matched control monoclonal antibody A1 had no effect (Fig. 3B). 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 (27) (Fig. 3B). Sera of two rabbits vaccinated with α 11-88x8 in CFA/IFA neutralized HPV18 at a titer of 2,500, and sera of a rabbit immunized with α 11-88x5 in CFA/IFA neutralized at 12,500 (Fig. 3A and Table 1). 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 1. The differences in titer likely reflect distinct methodologies and cell types used in these neutralization assays.

For a direct comparison of the immunogenicities of α 11-88x5,

FIG 1 Design of L2 vaccines. (A) Diagram showing the composition of α 11-88x8 and α 11-88x5 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 (12), residues 20 to 38 by K4L2 and K18L2, residues 28 to 42 by K8L2, and residues 72 to 78 by K1L2 (44), and residues 69 to 81 by MAb6 (45). (C) Coomassie-stained SDS-PAGE gel of the α 11-88x5 (lane 1) and α 11-88x8 (lane 2) antigens. MW, molecular weight markers.

α 11-88x8, 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, α 11-88x5 in alum, α 11-88x8 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 α 11-88x5 and α 11-88x8 were similar ($P = 0.3870$) and two logs lower than those for the licensed HPV vaccines (Fig. 2C). There was no significant difference between ELISA titers 2 weeks after the final immunization with α 11-88x5 in alum ($2,250 \pm 380$ [$n = 20$]) and those at 4 months ($1,779 \pm 221$ [$n = 19$]; short- versus long-term; $P = 0.5586$).

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, α 11-88x5 in alum, α 11-88x8 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 α 11-88x5 or α 11-88x8 (Fig. 4B).

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 anti-sera 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 (Fig. 4). Gardasil, but not Cervarix, provided complete protection against HPV6 challenge, because only the former contains HPV6 L1 VLP. Both α 11-88x5 and α 11-88x8 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, α 11-88x5 and α 11-88x8 both contain HPV31 L2 and were strongly protective against HPV31. α 11-88x8 contains L2 units derived from HPV51 and HPV56, whereas α 11-88x5 does not. While α 11-88x8 appeared to provide slightly better protection against HPV51 than α 11-88x5, 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 α 11-88x5 or α 11-88x8, both vaccines provided similarly strong protection (no significant difference for HPV26, HPV33,

FIG 2 Neutralizing and protective capacity of L2 multimer antisera. Passive transfer of rabbit α 11-88x8 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 pseudovirus 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, α 11-88x5 in alum, α 11-88x8 in alum, or one-tenth of a human dose of either Gardasil or Cervarix for comparison.

HPV35, HPV45, or HPV59 challenges). Figure 5 shows that a very high percentage of mice had $>90\%$ reduction in luciferase signal, demonstrating that both the α 11-88x5 and α 11-88x8 vaccines strongly protect against members of five medically significant papillomavirus species in the mouse vaginal challenge model: α 5 (HPV26, HPV51), α 6 (HPV56), α 7 (HPV39, HPV45, HPV59), α 9 (HPV16, HPV31, HPV35, HPV33, HPV58), and α 10 (HPV6). Gardasil and Cervarix provided some degree of cross-protection against nonvaccine types, notably HPV45 and HPV59 (Fig. 4 and 5). For several genotypes, vaccination with α 11-88x5 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, $P <$

FIG 3 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 (26). 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 α 11-88X8 1a and 2a, α 11-88X5 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) (28) or the spliced HPV18 E1E4 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.

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 α 11-88x8 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 (Fig. 4J).

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 (4, 33). 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 (5), and Malagón et al. suggest that there is some evidence for waning of cross-protection with increased follow-up (34, 35). 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 (4, 5). 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 col-

FIG 4 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, α 11-88x5 in alum, α 11-88x8 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 μ l 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 (27), α 11-88x8 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).

poscopy 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 α 11-88x5 or

α 11-88x8, 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

FIG 4 continued

differences in the infection process in cervicovaginal tissues versus cultured cells (36). 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. (36), 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 (37, 38), the ability of an alum-formulated L2 vaccine to protect patients from natural acquisition of HPV and the duration of pro-

TECTIVE 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 (39), 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 (40). 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 (41), and rabbit antisera to α 11-88x8

FIG 5 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 Fig. 4 is plotted for each HPV challenge virus.

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 (42).

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 (43).

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 1). 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 (9, 11).

The $\alpha 11-88x5$ and $\alpha 11-88x8$ 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 $\alpha 11-88x5$ or $\alpha 11-88x8$. 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 (8). The findings herein using the mouse vaginal challenge model suggest that $\alpha 11-88$ L2 multimer vaccines produced by bacterial expression and formulated in alum (a low-cost and well-accepted adjuvant) show promise in this regard.

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