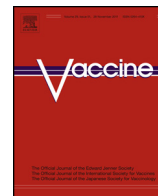


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A universal virus-like particle-based vaccine for human papillomavirus: Longevity of protection and role of endogenous and exogenous adjuvants



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ARTICLE INFO

Article history:

Received 18 April 2013

Received in revised form 1 July 2013

Accepted 6 July 2013

Available online 9 August 2013

Keywords:

PP7 bacteriophages

Virus-like particles

HPV vaccine

Adjuvants

ABSTRACT

Antibodies targeting epitopes within the amino terminus of the minor capsid protein L2 of human papillomavirus (HPV) are broadly neutralizing against diverse HPV isolates. We have constructed bacteriophage virus-like particle (VLP)-based vaccines that display short L2 peptides and elicit high-titer and broadly protective antibody responses. Here, we further characterize two additional features of these VLP-based vaccines; the longevity of protection and the role of endogenous and exogenous adjuvants on the magnitude and characteristics of the antibody response. We show that vaccinated mice have long-lived antibody responses against L2, persisting over 18 months after vaccination. Vaccinated mice were strongly protected against infection by diverse HPV pseudoviruses over a year after immunization. We also show that exogenous and endogenous adjuvants (LPS and encapsidated single-stranded RNA) have minor effects on antibody titers. Immunization with VLPs containing encapsidated ssRNA predominantly shifts the response to a Th1, rather than a Th2-like response. Importantly, immunization with L2-VLPs (without endogenous and exogenous adjuvants) in the presence of alum hydroxide elicited a robust antibody response.

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1. Introduction

Human papillomavirus (HPV) infection is estimated to be responsible for about 5% of human cancers worldwide [1]. The two most carcinogenic HPV types, HPV16 and HPV18, cause approximately 70% of cervical cancer cases [2,3]. The current HPV vaccines (Gardasil[®] and Cervarix[®]) are comprised of virus-like particles (VLPs) derived from the HPV major capsid protein, L1. Both vaccines are highly immunogenic and strongly protect immunized individuals against infection with the HPV types included in the vaccines (both Gardasil[®] and Cervarix[®] include HPV16 and 18 VLPs, Gardasil[®] also includes HPV6 and HPV11 VLPs). However, these vaccines largely provide little cross-protection against other HPV types not included in the vaccines, although there is evidence for some cross-protection against other closely related types, such as HPV31 [4–8]. The partial protection that these vaccines provide against the spectrum of HPV types associated with cancer has provoked the development of second-generation HPV vaccines. It has been suggested that a L1-VLP vaccine that offers more than 90%

protection from cervical cancers should include L1 VLPs from HPV types 31, 33, 45, 52 and 58 in addition to HPV types 16 and 18 [9]. Merck, the manufacturer of Gardasil[®], currently has a nonavalent vaccine (V503) in phase III clinical trials that is comprised of L1-VLPs derived from seven carcinogenic HPVs as well as HPV6 and 11 [10]. This nonavalent vaccine is likely to increase the breadth of HPV protection; however, the cost of production and formulation will likely be high, particularly given the fact that the current HPV vaccines are already very expensive [11]. Thus, the nonavalent vaccine may not be affordable in underdeveloped countries where ~85% of cervical cancer cases occur.

As an alternative, we and others have targeted a highly conserved broadly neutralizing epitope from the HPV minor capsid protein, L2. Although L2 is not required for the formation of HPV VLPs, it plays essential roles in viral entry and assembly. During natural HPV infection L2 is poorly immunogenic, probably reflecting the fact that L2 is only transiently exposed on the surface of the virus particle during the infectious process [12]. Nevertheless, antibodies raised against L2 have the ability to broadly neutralize diverse HPV types [13–15]. We have developed vaccines that display a short peptide derived from L2 in a highly multivalent and immunogenic format on the surface of bacteriophage VLPs [16–18]. L2-VLP vaccines elicit robust anti-L2 IgG responses in mice and vaccination provides broad protection from genital and cutaneous HPV pseudovirus challenge in animal models of infection. L2-VLPs can

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be produced easily using a bacterial expression system. Given the ease of production and breadth of protection, L2-VLPs may be a potential second generation HPV vaccine suitable for use in the developing world.

HPV L1-VLPs are remarkably immunogenic; they elicit high-titer neutralizing antibody responses that provide long-lasting protection from viral infection [19]. Even when HPV16 L1-VLPs are administered without adjuvant nearly 100% of individuals produce high-titer anti-HPV antibody responses [20]. The strong immunogenicity and protection provided by L1-VLPs is likely due to several factors. First, L1-VLPs, like all VLPs, have a highly repetitive structure that strongly activates B cells through multivalent, cross-linking interactions with B cell receptors [21]. This interaction results in high-titer antibody production. Second, HPV L1-VLPs have the ability to activate Dendritic Cells through a MyD88-dependent process, which likely plays a role in strongly priming adaptive immune responses and may help explain the strong antibody responses induced even in the absence of exogenous adjuvant [22]. Third, L1-VLPs elicit a polyclonal neutralizing antibody response against HPV that recognizes multiple epitopes of the virus and leads to strong neutralization of HPV virions [23].

L2-displaying bacteriophage VLPs have some features that are analogous to L1-VLPs. Ninety copies of L2 are displayed on the surface of the VLP, ensuring that the targeted peptide is displayed in a highly multivalent and stimulatory fashion. Bacteriophage VLPs also encapsidate ssRNA, which can serve as an adjuvant by interacting with toll-like receptors (TLRs) expressed by B cells and other antigen-presenting cells. This endogenous adjuvant potentially augments the immunogenicity of the particles. However, the relative roles of multivalency and endogenous adjuvant on the immunogenicity of bacteriophage VLPs have not been examined in detail. In contrast to the L1-VLP vaccines, L2-displaying bacteriophage VLPs only target a single viral epitope. One potential concern with a single-epitope vaccine is that it will not elicit long-lived, protective antibody responses. In this manuscript, we investigate the potency, longevity, and correlates of immunogenicity of L2-displaying bacteriophage VLP vaccines. We show that L2-VLP vaccines elicit long-lasting protective antibody responses in mice and that endogenous adjuvants contribute to their immunogenicity.

2. Materials and methods

2.1. Expression and purification of PP7 L2-VLPs

PP7 L2-VLPs, which contain HPV1, 5, 6, 11, 16, 18, 45, or 58 L2 sequences (epitope 17–31) genetically inserted into the AB-loop of the coat protein of bacteriophage PP7 VLPs were described previously [17]. The PP7 L2-VLPs were made by transforming C41 cells (Lucigen) with pET2P7K32 plasmids that contain the HPV L2 sequence insertions. PP7 L2-VLPs were expressed and purified as previously described [17]. Briefly, transformed C41 cells were grown at 37°C until they reached an optical density of 0.6 after which they were induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside for 3 h. Cultures were harvested by centrifugation and pellets were lysed using lysozyme solution (0.1 g egg lysozyme, in 50 ml of 50 mM Tris–HCl, pH 8.5, 100 mM NaCl, 10 mM EDTA, 10 mM DTT). Lysates were cleared by centrifugation and the supernatant purified by size-exclusion chromatography using Sepharose CL-4B as previously described [16].

2.2. Removal of LPS from VLPs

LPS was removed by phase extraction using triton X-114 [24]. PP7 L2-VLPs were mixed with 1% Triton X-114 (final

concentration), vortexed and incubated on ice for 5 min. The mixture was then incubated at 37°C for 5 min and was spun at 13,000 rpm (in a microfuge) for 1 min at the same temperature. The aqueous phase was transferred using pyrogen-free tips to pyrogen-free microcentrifuge tubes. The extraction process was then repeated to ensure optimal LPS depletion. LPS levels in VLPs (before and after LPS removal) were determined using endosafe limulus amoebocyte lysate (LAL)-based kinetic-turbidimetric assay (Charles River) according to the manufacturer's instructions.

2.3. Removal of RNA from VLPs

RNAs from PP7 16L2-VLPs were removed as previously described [25] with a few modifications. VLPs were exposed to a pH of 11.5 at 37°C overnight followed by 3X PBS buffer exchanges in Amicon 100 K MWCO centrifugal filters (Millipore). PP7 16L2-VLPs including RNA-free VLPs were run on a 1% agarose gel stained with ethidium bromide to check for the presence or absence of coat protein-encapsidated RNAs; the same gel was then stained with Coomassie blue to check for the presence of recombinant coat proteins at the same position of encapsidated RNA.

2.4. Transmission electron microscopy (TEM)

PP7 16L2-VLPs, RNA-free PP7 16L2-VLPs and LPS-free PP7 16L2-VLPs were adsorbed on carbon-coated glow-discharged copper grids for 2 min and were stained for 2 min with 2% uranyl acetate. Grids were analyzed on a Hitachi H7500 transmission electron microscope and pictures were taken at a magnification of 70,000 \times .

2.5. Immunizations

All animal work was done in accordance with the National Institutes of Health and the University of New Mexico Institutional Animal Care and Use Committee (UNM IACUC) guidelines and was approved by the University of New Mexico IACUC (protocol 12-100827-HSC). Groups of mice (Balb/c, C57BL/6, or B6.B10ScN-Tlr4^{lps-del}/JthJ; TLR4^{−/−} C57BL/6) were immunized intramuscularly two or three times at two-week intervals with 5 μ g of individual VLPs or 10 μ g of a mixture of PP7 VLPs displaying L2 peptides derived from eight different HPV types. The mixture consisted of 2 μ g each of 1L2 and 5L2-VLPs, 2.5 μ g of 16L2-VLPs, 0.7 μ g of 6L2, 11L2, 18L2, 45L2, and 58L2-VLPs. In some cases VLPs were mixed with adjuvant (Incomplete Freund's Adjuvant, IFA; Sigma, or 2% alhydrogel, alum hydroxide; Invivogen) according to manufacturers recommendations. Sera were collected at two weeks after the final immunization. For long-term immunogenicity studies, sera were taken monthly up to 18 months post-immunization.

2.6. Determination of antibody titers

A peptide-based ELISA was used to measure anti-L2 IgG titers. The ELISA was conducted as described in [16,17]. Briefly, ELISA plates were coated overnight with 500 ng of the appropriate target peptide (representing L2 amino acids 14–40 from HPV1, 5, 6, 16, and 18; synthesized by Designer Bioscience) conjugated to streptavidin using a bifunctional cross-linker (SMPH; Thermo Scientific). Wells were blocked for 2 h at room temperature with 0.5% non-fat dry milk in PBS buffer and four-fold serial dilutions of mice sera in blocking buffer were added and plates were incubate at room temperature for two additional hours. Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, (Jackson ImmunoResearch, West Grove) at a dilution of 1:5000 was applied for 1 h as a secondary antibody. The plates were developed

with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; ABTS) and reactivity was determined by measuring the mean optical density (OD) values at 405 nm. In some cases, IgG subclass antibody levels were also determined by ELISA. Sera was diluted (1:40) and then ELISA were performed using a 16L2 peptide (14–40) as target the antigen. HRP-labeled goat anti-mouse total IgG, IgG1, IgG2a, IgG2b, or IgG2c (1:1000 dilution; Jackson ImmunoResearch) were used as secondary antibodies.

2.7. Assessing protection from cervicovaginal infection with HPV pseudoviruses

HPV pseudoviruses (PsV; types 16, 31, and 45) encapsidating a reporter plasmid (pClucf) encoding both luciferase and green fluorescence protein (GFP) genes were produced and purified from 293TT cells as previously described [17,26,27]. L1/L2 expression and reporter plasmids were generously provided by Chris Buck, Susana Pang, John Schiller, Martin Muller, and Tadahito Kanda. Vaccinated and control mice were challenged with HPV PsV16, 12

months after immunization; the same mice were re-challenged at 17.5 and 18.5 months after immunization with PsV31 and PsV45, respectively. All challenges were done as described [17,28,29]. Briefly, mice were treated with 3 mg of Depo-Provera (Pharmacia Corp) and five days post-Depo-Provera treatment, mice were challenged cervicovaginally with 3.9×10^5 – 3.0×10^6 infectious units of PsV. Forty-eight hours post-PsV challenge, mice were vaginally instilled with 0.4 mg of luciferin (Caliper Life Sciences) and three minutes later their images were taken (with a five minute exposure) using Caliper IVIS Lumina II (Caliper Life Sciences). Average radiance (p/s/cm²/sr) was determined from the images by drawing equally sized regions of interests surrounding the site of PsV instillation.

2.8. Statistical analysis

Statistical significance was determined by one-tailed (pseudovirus protection assays) and two-tailed (immune response assays) unpaired *t*-test.

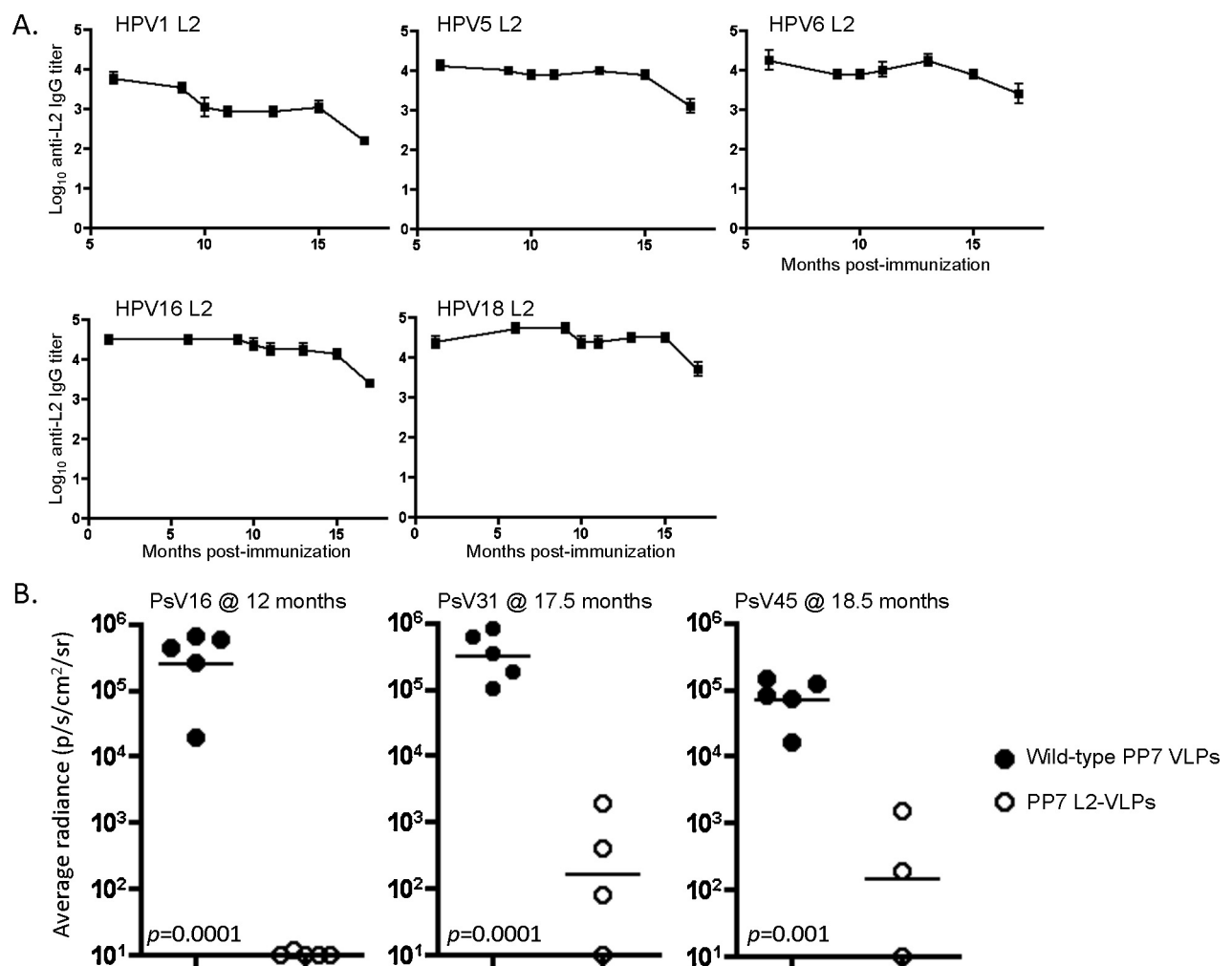


Fig. 1. Longevity of antibody responses in mice immunized with PP7 L2-VLPs. Groups of five Balb/c mice were immunized with a mixture of PP7 VLPs displaying L2 peptides (epitope 17–31) from eight diverse HPV types (HPV1, 5, 6, 11, 16, 18, 45, and 58) or wild-type PP7 VLPs. (A) Sera was collected at the indicated time points and IgG titers were determined by end-point titration ELISA using disulfide-constrained L2 peptides (amino acid 14–40) from HPV1, 5, 6, 16, and HPV18 as target antigens. (B) Mice were vaginally challenged with HPV PsV16, 12 months after immunization and with HPV PsV31 and PsV45, 17.5 months and 18.5 months, respectively, after immunization. Luciferase activity was measured 48 h post challenge and average radiance (p/s/cm²/sr) values for each mouse was determined using Living Image 3.2 software. Each circle represents the average radiance of an individual mouse and each line represents the geometric mean radiance for each group. Error bars signify standard error of mean (SEM). All levels of protection are statistically significant ($p < 0.01$) as calculated by one-tailed unpaired *t*-test. Note: one of the mice immunized with L2-VLPs died at 15 months and a second mouse in this group was euthanized at 18 months due to a wound on an area where depo-provera was administered.

3. Results

3.1. Longevity of antibody responses and protection from infection

We previously reported that a mixture of PP7 VLPs displaying L2 sequences (amino acids 17–31; PP7 L2-VLPs) derived from eight different HPV strains (HPV1, 5, 6, 11, 16, 18, 45, and 58) elicits high-titer antibody responses that provide broad protection from genital challenge with diverse HPV pseudovirions [17]. To determine the duration of the antibody responses induced by PP7 L2-VLPs, we immunized mice three times with the mixture of eight PP7 L2-VLPs (without any exogenous adjuvant) and monitored their anti-L2 IgG antibody titers over 18 months after immunization. As we have previously described [17], vaccinated mice had high-titer antibody levels capable of reacting with synthetic disulfide-constrained L2 peptides representing amino acids 14–40 from diverse HPV types (HPV 1, 5, 6, 16, and 18). The antibody titers declined only minimally over a fifteen-month period post-immunization (Fig. 1A). At 17 months post-immunization, antibody titers began to decline; levels at this time-point were approximately 10-fold lower than the peak titers we observed.

To investigate whether these mice were still protected from HPV infection, we challenged the mice (vaginally) with three different HPV pseudoviruses (PsVs) beginning one year after the initial immunization. Compared to the control group, mice that were

immunized with the mixed PP7 L2-VLPs were completely protected from HPV16 PsV infection one year after infection (Fig. 1B). At 17.5 months and 18.5 months post-immunization, vaccinated mice and control mice were vaginally challenged with two additional HPV PsV types, PsV31 and PsV45, respectively. As shown in Fig. 1B, robust and statistically significant protection from both PsV types was still observed. Thus, immunization with PP7 L2-VLPs elicits durable, protective antibody responses.

3.2. LPS does not contribute to the immunogenicity of L2-VLPs

Because PP7 VLPs are produced in *E. coli*, there is contaminating bacterial lipopolysaccharide (LPS) in our lab-grade VLP preparations (~200,000 EU/mg). LPS is known to exert an immune-stimulatory effect via its interaction with TLR4, so we were concerned that contaminating LPS may have contributed to the immunogenicity of our VLP preparations. In this regard, we evaluated the immunogenicity of PP7 L2-VLPs independent of the TLR4-LPS stimulatory effect. TLR4^{-/-} C57BL/6 and wild type C57BL/6 mice were immunized with PP7 VLPs displaying the HPV16 L2 peptide. Both groups of mice had similar antibody levels (Fig. 2A), suggesting that contaminating LPS did not play a role in the immunogenicity of the VLPs. We also challenged the TLR4^{-/-} mice with PsV16 four months after immunization. As shown in Fig. 2B, TLR4^{-/-} mice were strongly and significantly protected from infection, in comparison to the TLR4^{-/-} mice immunized with wild-type

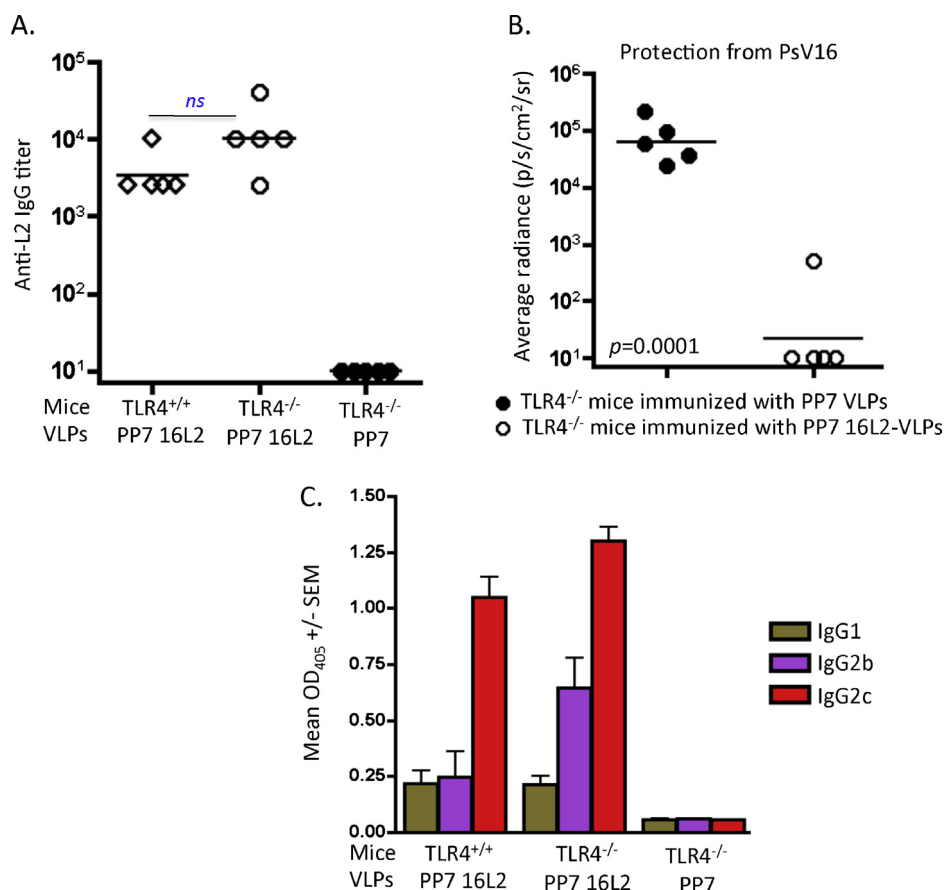


Fig. 2. Immunogenicity of PP7 16L2-VLPs is independent of TLR4 stimulation. (A) Groups of five TLR4^{-/-} C57BL/6 mice or wild type C57BL/6 mice were immunized twice (without IFA) intramuscularly at two-weeks interval with PP7 16L2 VLPs. Two weeks after the second immunization sera was collected and total IgG titers were determined by end-point dilution ELISA using an HPV 16L2 peptide (a.a. 14–40) as a target antigen. (B) Mice were vaginally challenged with HPV PsV16, four months after immunization and infection was measured by in vivo imaging as described in Fig. 1. (A and B) Each circle or tetragon represents an individual mouse and each line represents the geometric means for each group. (C) IgG subclasses were determined by ELISA using isotype-specific secondary antibodies and serum from immunized mice at a 1:40 dilution. Level of protection is statistically significant ($p < 0.001$) as calculated by one-tailed unpaired *t*-test; ns, no significant difference in antibody titers as calculated by two-tailed unpaired *t*-test. Error bars signify SEM.

VLPs. Finally, we measured the anti-L2 IgG subclasses in the immunized mice. The predominant isotype in both groups of mice was IgG2c, indicative of a Th1 response. However, detectable levels of IgG1 and IgG2b (indicative of a Th2 response) were present in both groups of mice (Fig. 2C).

As a complementary approach, we also assessed the immunogenicity of VLPs in which contaminating LPS had been removed. We explored five different reagents to remove LPS from PP7 L2-VLPs; EndoTrap Red, Hydroxyapatite type II, Detoxi-Gel (with and without octyl- β -D-glucopyranoside), and Triton X-114. Use of EndoTrap Red and Hydroxyapatite resulted in dramatic losses of PP7 L2-VLPs (data not shown), so these methods were not pursued further. About two-thirds of L2-PP7 VLPs were recovered using Detoxi-Gel alone or in combination with octyl- β -D-glucopyranoside. However these techniques were not highly effective at removing LPS from the VLP preparations (Detoxi-Gel alone decreased contaminating LPS levels by 2.7-fold, Detoxi-Gel plus octyl- β -D-glucopyranoside decreased LPS levels by about 40-fold). Phase extraction using the nonionic detergent Triton X-114 [24] was the most effective method for removing LPS. This technique resulted only in a little loss of VLPs (~92% of VLPs recovered; data not shown), and a dramatic decrease in LPS levels (to <750 EU/mg). The morphology and the antigenic integrity of Triton X-114 extracted VLPs were assessed on an agarose gel and further confirmed by TEM and ELISA; treatment of PP7 L2-VLPs with Triton X-114 did not affect

the encapsidated RNA/coat proteins (Fig. 3A) nor did it change the morphology of the VLPs (Fig. 3B). Similarly, the binding of an anti-L2 monoclonal antibody (RG-1) with PP7 16L2-VLPs was not affected by Triton X-114 treatment (Fig. 3C).

Balb/c mice were immunized with LPS-free PP7 16L2-VLPs and anti-L2 antibody titers were compared with mice immunized with VLPs containing contaminating LPS. As shown in Fig. 3D, there was no difference in anti-L2 IgG levels in mice immunized with LPS-free PP7 16L2-VLPs compared to mice immunized with PP7 16L2-VLPs containing LPS. Taken together, these data indicate that LPS does not substantially contribute to VLP immunogenicity.

3.3. Encapsidated RNA skews the antibody isotypes induced by L2-VLPs

Next, we investigated the role of encapsidated RNA in the immunogenicity of PP7 L2-VLPs. RNA was removed from the VLPs by incubation at pH 11.5, which allows hydrolysis of the encapsidated RNA [25]. As shown in Fig. 4A, agarose gel electrophoresis analysis indicates that VLPs exposed to basic conditions migrate through the gel similarly to untreated VLPs (indicated by the Coomassie-blue stained protein band), but no longer encapsidate RNA (i.e. there is no RNA band on the ethidium bromide-stained gel). VLPs incubated at pH 11.5 migrated a little slower than untreated VLPs. TEM analysis indicates that exposure to basic

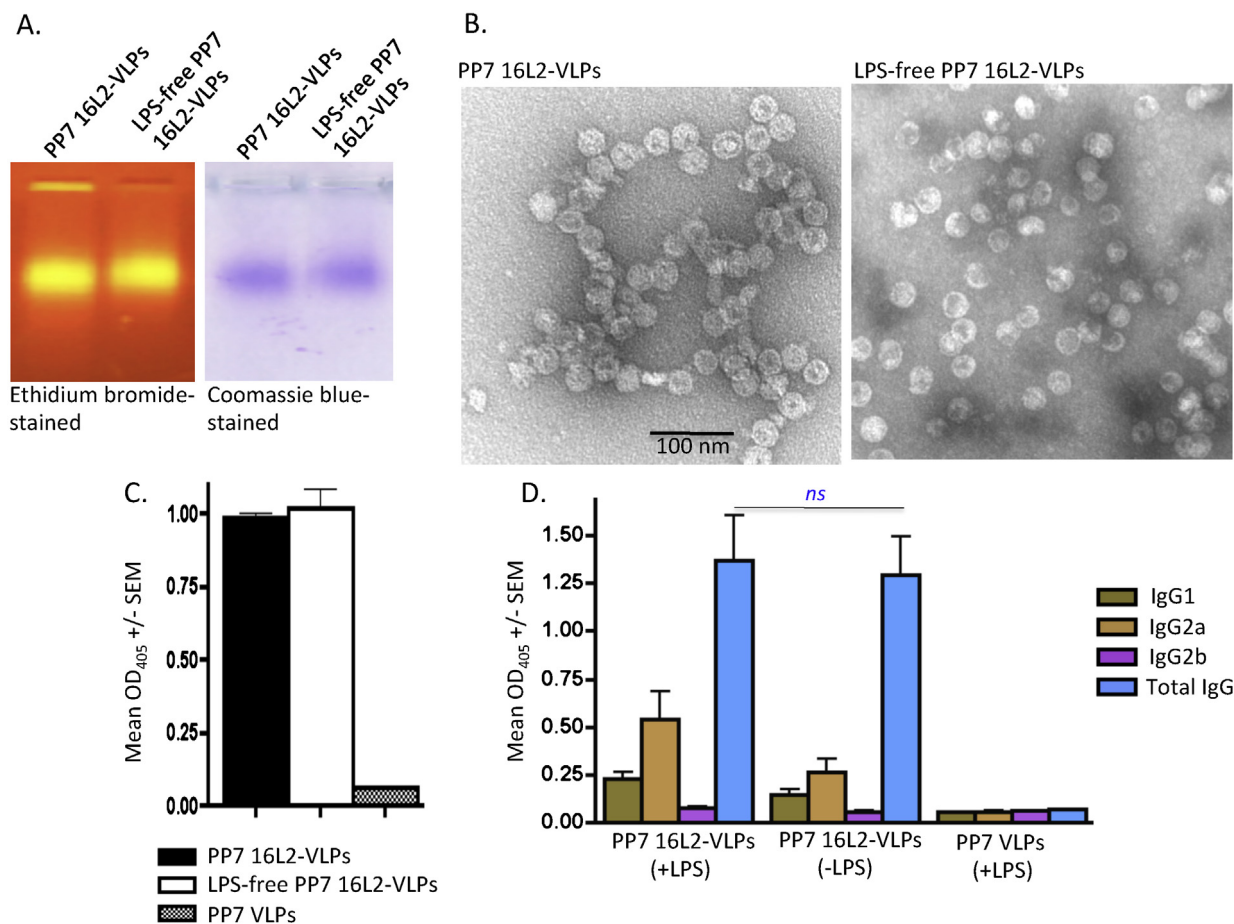


Fig. 3. Immunogenicity of PP7 16L2-VLPs in the absence of bacterial LPS. LPS was removed by Triton X-114 (twice). VLPs were analyzed (A) on a 1% agarose gel stained with ethidium bromide (left panel) or coomassie blue (right panel) and (B) by electron microscopy. (C) Binding of the anti-L2 monoclonal antibody RG-1 to LPS-free VLPs. (D) Immunogenicity of LPS-free VLPs. Groups of four Balb/c mice were immunized twice intramuscularly at two-weeks interval with PP7 16L2-VLPs (+LPS), LPS-free PP7 16L2-VLPs (-LPS), or wild-type PP7 VLPs (+LPS). Sera was collected two weeks after the last immunization and the levels of IgG subclasses (and total IgG) were determined by ELISA using 1:40 dilution of sera and HPV 16L2 peptides (14–40) as target antigen. Statistical analysis was calculated by two-tailed unpaired *t*-test; ns, not significant. Error bars signify SEM.

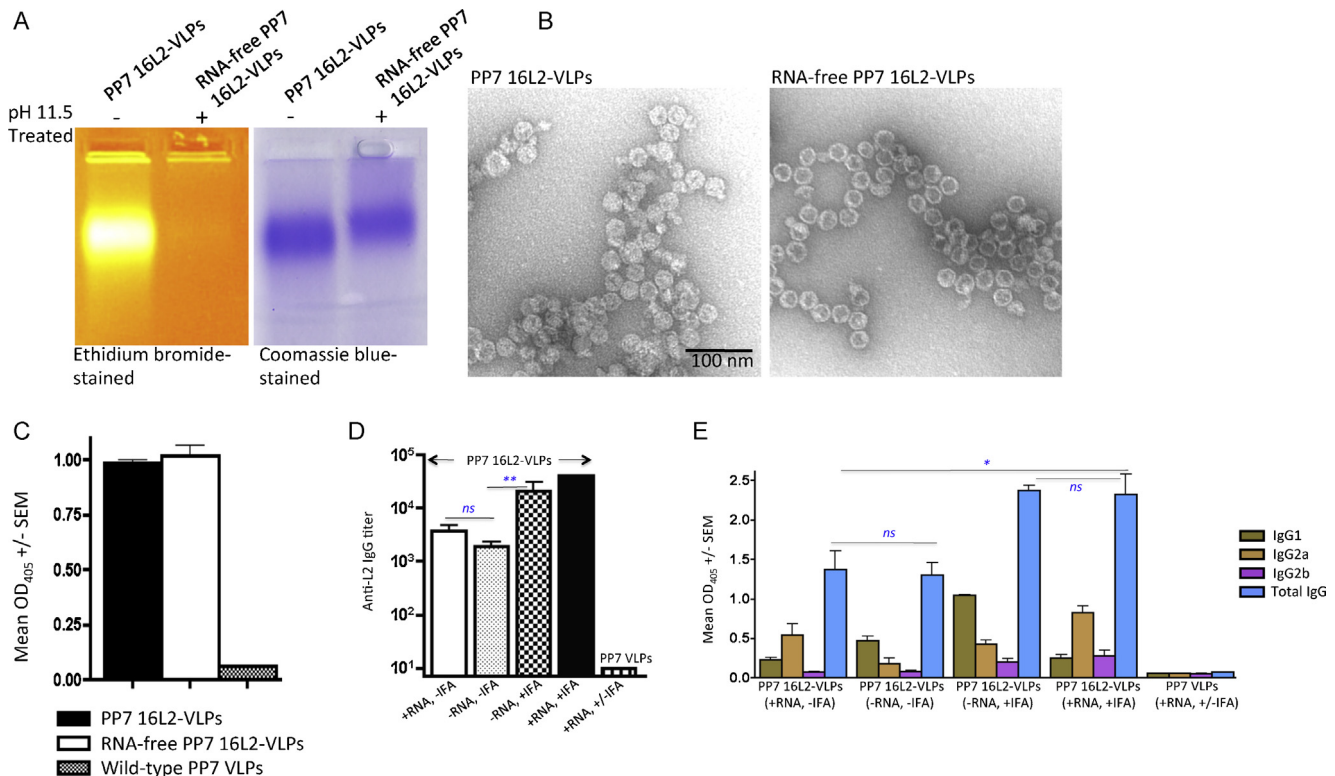


Fig. 4. Immunogenicity of VLPs lacking encapsulated RNA. PP7 16L2-VLPs were exposed overnight to a pH of 11.5 at 37 °C and the integrity of the VLPs were analyzed (A) on a 1% agarose gel stained with ethidium bromide (left panel) or Coomassie blue (right panel), and by (B) electron microscopy. (C) Binding of the anti-L2 monoclonal antibody RG-1 to RNA-free VLPs. (D) Immunogenicity of RNA-free VLPs. Groups of three to six Balb/c mice were immunized twice intramuscularly at two-weeks interval with PP7 16L2-VLPs (+RNA), RNA-free PP7 16L2-VLPs (-RNA) or PP7 VLPs (+RNA) with or without IFA. Two weeks after the last immunization, sera was collected and IgG titers determined by ELISA using HPV 16L2 peptide (14–40) as target antigen. (E) IgG subclasses were measured by ELISA using 1:40 dilution of sera. Statistical analysis was calculated by two-tailed unpaired *t*-test; *ns*, not significant, **p* < 0.05, and ***p* < 0.01. Error bars signify SEM.

conditions does not cause disassembly of the VLPs or changes in their morphology (Fig. 4B). Further, the lack of an electron-dense core is consistent with RNA-depletion. Similarly, the pH 11.5-exposed 16L2-VLPs (RNA-free) had the same level of reactivity with RG-1 monoclonal antibody when compared to non-exposed PP7 16L2-VLPs (Fig. 4C).

To assess the immunogenicity of the RNA-free PP7 16L2-VLPs, mice were immunized twice with the VLPs in the presence or absence of IFA. As shown in Fig. 4D, similar levels in anti-L2 IgG levels were observed in mice immunized with either RNA-free 16L2-VLPs (-RNA, -IFA) or those immunized with 16L2-VLPs (+RNA, -IFA), which has encapsulated ssRNA. However, there was a difference in IgG subclasses elicited; immunization with VLPs containing encapsulated RNA led predominantly to an IgG2a response (indicative of a Th1 response) whereas immunization in the absence of the encapsulated RNA switched the immune response to IgG1 subclass (Fig. 4E). This data suggests that in the absence of RNA, VLPs largely elicits a Th2-type response. When mice were immunized with the two VLPs, 16L2-VLPs (-RNA) and 16L2-VLPs (+RNA), in the presence of IFA (+IFA), the antibody response in both groups of mice were increased by greater than 1.5-fold (Fig. 4E). The use of IFA did not affect the isotype profiles.

3.4. Alum effectively adjuvants VLPs

Removal of both LPS and RNA from PP7 L2-VLPs leads to an approximately 9.1-fold reduction of anti-L2 antibody titers (Fig. 5). Given that aluminum salts are the most common adjuvants currently used in approved human vaccines, we assessed the ability of aluminum hydroxide (Alhydrogel) to boost antibody titers in

VLP-immunized mice. As shown in Fig. 5, Alum substantially boosted the anti-L2 antibody titers elicited by both LPS-free VLPs and RNA/LPS-free VLPs. Thus, Alum may be a suitable adjuvant for clinical development of L2-displaying bacteriophage VLP vaccines.

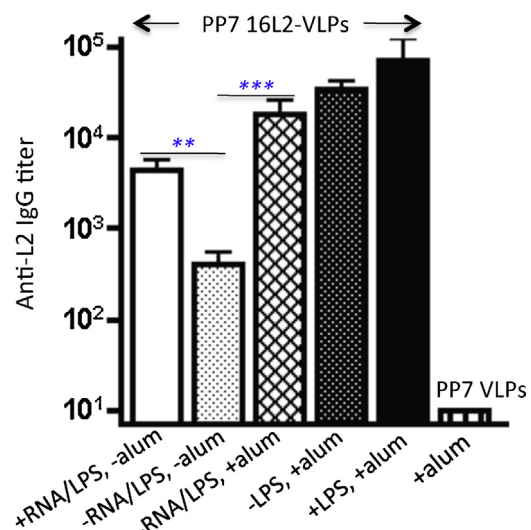


Fig. 5. Alum enhances the immunogenicity of PP7 L2-VLPs. Groups of four Balb/c mice were immunized twice intramuscularly at two-weeks interval with PP7 16L2-VLPs (without alum), RNA and LPS-free PP7 16L2-VLPs (with or without alum), LPS-free PP7 16L2-VLPs (with alum), PP7 16L2-VLPs (with alum), or wild-type PP7 VLPs (with alum). Sera were collected two weeks after the last immunization and IgG titers were determined by end-point dilution ELISA using HPV 16L2 peptides (14–40) as target antigen. Statistical analysis was calculated by two-tailed unpaired *t*-test; ***p* < 0.01, and ****p* < 0.001. Error bars signify SEM.

4. Discussion

There is a need to develop a cost-effective broadly protective second-generation HPV vaccine that will benefit resource-poor countries [11]. Various groups have been working on developing second-generation candidate HPV vaccines targeting broadly cross-neutralizing epitopes in the minor capsid protein, L2 [17,30–34]. However, it is unclear whether L2-directed vaccines that largely target a single neutralizing epitope will elicit antibody responses that are as long-lasting as the current HPV vaccine.

Here, we show that a mixture of PP7 VLPs displaying L2 peptides from diverse HPV types are highly immunogenic and induce durable antibody responses; sera from immunized mice still had high-titer antibodies that were reactive with L2 peptides representing diverse HPV types over a year after immunization. Antibody titers were only observed to decline beginning 18 months following immunization (when the mice were approximately 20 months old). Importantly, we show that mice immunized with PP7 L2-VLPs were completely protected from PsV16, 1 year after immunization. Additionally, mice were protected from PsV31 and PsV45 in the presence of diminishing antibodies, more than 17 months after immunization. Only a few previous studies have looked at immune responses to candidate HPV L2 vaccines for any length of time following vaccination (typically 2–4 months following vaccination) [13,32]. Thus, these data are encouraging that L2 vaccines can provide long-term protection from infection.

Due to the robust immunogenicity of PP7 L2-VLPs in the absence of exogenous adjuvants, we asked if endogenous adjuvants in our VLP preparations were contributing to their immunogenicity. Use of TLR4^{−/−} C57BL/6 mice as well as LPS depletion from our VLP preparations indicated that *Escherichia coli* LPS contamination does not strongly contribute to the immunogenicity of the VLPs. We cannot rule out the possibility that other contaminating *E. coli* proteins may be exerting an adjuvant effect through a different TLR. Moreover, removal of LPS did not affect the anti-L2 IgG isotype profile. PP7 16L2-VLPs largely elicited IgG2a/2c, indicative of a Th1 immune response.

Previous studies have shown that interaction of viral ssRNA with TLR7 and TLR8 enhances innate immune responses [35,36]. To determine the contribution of encapsidated ssRNA in immunogenicity of the PP7 L2-VLPs, we removed the RNA from these particles. RNA-free VLPs elicited slightly lower (2-fold) antibody titers than RNA+ VLPs. More dramatically, VLPs without encapsidated RNA predominantly elicited IgG1, rather than IgG2a. These results are consistent with a recent study by Schmitz et al. [37], who reported that C57BL/6 mice immunized with RNA-free M2e-AP205 VLPs had a switch from IgG2c (the analog of IgG2a expressed by C57BL/6 mice) to IgG1. In this study there was no difference in overall immune responses (total IgG levels) in mice immunized with RNA-free VLPs or those immunized with RNA containing VLPs (+RNA) [37]. The ability to modulate between Th1 and Th2 dominated response may be useful depending on the IgG isotypes desired. We believe that it is unlikely that modulation of IgG isotypes is critical for providing protection from HPV infection, but the ability to modulate Th1 versus Th2 responses may be valuable for other vaccine targets.

Importantly, the immunogenicity of the RNA/LPS-free PP7 L2-VLPs can be enhanced with aluminum hydroxide (a licensed vaccine adjuvant); immunization with RNA/LPS-free PP7 16L2-VLPs in the presence of Alum elicited a robust antibody response. The combination of strong and long-lasting immunogenicity, broad protection against diverse HPV types, robust bacterial production, and compatibility with approved adjuvants make bacteriophage VLPs displaying L2 peptides an attractive candidate for clinical development, particularly for use in the developing world.

Acknowledgements

We would like to thank Michelle Ozbun, Cosette Wheeler, and other members of the University of New Mexico Interdisciplinary HPV Prevention Center for helpful discussions. We also like to thank Nicole Patterson for help with the production of pseudoviruses, Stephen Jett for help with transmission electron microscopy, and Sandra Lobo for help with endotoxin detection. This study was supported by a grant from the US National Institutes of Health (U19 AI084081) to the University of New Mexico Sexually Transmitted Infections Cooperative Research Center (STI-CRC). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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