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Vaccine synergy with virus-like particle and immune complex platforms for delivery of human papillomavirus L2 antigen

Andrew G. Diamos [a], Dalia Larios [a], Lauren Brown [a], Jacquelyn Kilbourne [a], Hyun Soon Kim [b], Divyasha Saxena [c], Kenneth E. Palmer [c], Hugh S. Mason [a,*]

[a] Center for Immunotherapy, Vaccines, & Virotherapy, Biodesign Institute at ASU; and School of Life Sciences, Arizona State University, Tempe, AZ 85287, United States
[b] Plant Systems Engineering Research Center, KRIBB, Gwahang-ro 125, Yuseong-gu, Daejeon 34141, Republic of Korea
[c] Center for Predictive Medicine for Emerging Infectious Diseases and Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, KY, United States, Center for Predictive Medicine, Louisville, KY 40202, United States

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ABSTRACT

Diverse HPV subtypes are responsible for considerable disease burden worldwide, necessitating safe, cheap, and effective vaccines. The HPV minor capsid protein L2 is a promising candidate to create broadly protective HPV vaccines, though it is poorly immunogenic by itself. To create highly immunogenic and safe vaccine candidates targeting L2, we employed a plant-based recombinant protein expression system to produce two different vaccine candidates: L2 displayed on the surface of hepatitis B core (HBc) virus-like particles (VLPs) or L2 genetically fused to an immunoglobulin capable of forming recombinant immune complexes (RIC). Both vaccine candidates were potently immunogenic in mice, but were especially so when delivered together, generating very consistent and high antibody titers directed against HPV L2 (>1,000,000) that correlated with virus neutralization. These data indicate a novel immune response synergy upon co-delivery of VLP and RIC platforms, a strategy that can be adapted generally for many different antigens.

1. Introduction

Papillomaviruses are an ancient and diverse group of viruses containing over 200 subtypes currently known to infect humans [1]. HPV is the most common sexually transmitted disease, with >15 HPV oncogenic types responsible for oropharyngeal and anogenital cancers that result in significant morbidity and mortality worldwide [2]. Currently available prophylactic HPV vaccines target the L1 capsid protein, which self-assembles into highly immunogenic virus-like particles (VLPs) [3]. Because neutralizing epitopes found on L1 are not broadly conserved among HPV types, multiple L1 proteins must be included in vaccine preparations to protect against multiple HPV types. The most broadly protective vaccine approved to date, Garadasil-9, provides protection against HPV types 6, 11, 18, 31, 33, 45, 52, 58, however cross-protection with other HPV types is minimal, and the complex formulation of the vaccine makes it cost-prohibitive for much of the world [4–6]. The HPV minor capsid protein L2 is an attractive candidate for improved HPV vaccines because, unlike L1, neutralizing epitopes on the N-terminus of L2 are broadly conserved, and L2 antibodies can provide protection against multiple HPV types [7–10]. However, as L2 is unable to form VLPs, it is poorly immunogenic by itself, necessitating strategies to enhance L2 antibody production.

Recombinant immune complexes (RIC) have been explored as versatile and potent vaccine platforms to enhance antigen immunogenicity [11]. RIC consist of an antibody fused to its cognate antigen, which form antigen-antibody clusters that efficiently bind C1q and Fcγ receptors, allowing increased antigen uptake and processing by antigen-presenting cells [12–14]. RIC have been used to produce vaccine candidates for *Clostridium tetani* [15], HIV [16], Ebola virus [17,18], *Mycobacterium tuberculosis* [19], and dengue virus [20]. Immune complexes based on poly-immunoglubulin scaffolds have also shown promise as a strategy to improve antigen immunogenicity [21,22].

Antigen display on the surface of hepadnavirus VLPs is another promising strategy to enhance antigen immunogenicity [23]. The hepatitis B core antigen (HBc) dimerizes to form α-helical spikes which project from the surface of the virus particle and thus are

Abbreviations: VLP, virus-like particle; RIC, recombinant immune complex; HPV, human papillomavirus; HBc, hepatitis B core antigen.

* Corresponding author.
E-mail address: Hugh.Mason@asu.edu (H.S. Mason).

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attractive targets for displaying foreign antigens [24]. However, direct fusion of an antigen to the HBc spike would, upon dimerization, result in close proximity of the antigens, potentially destabilizing the VLP [25,26]. To circumvent this issue, tandem-fusion of two HBc genes with a foreign antigen inserted into only one of the two HBc copies allowed the generation of plant-made HBc VLPs with enhanced capacity to accommodate foreign antigens [27].

To address the challenges of creating low-cost, highly immunogenic vaccine candidates targeting HPV L2, we produced RIC or HBc VLP targeting the N-terminal region of HPV16 L2 in plants. Plants are a highly scalable platform capable of producing safe, effective, and low-cost recombinant proteins [28]. We have developed a plant-based expression system based on bean yellow dwarf virus (BeYDV) which allows rapid, high-yield production of vaccine antigens and other biopharmaceuticals in the leaves of *Nicotiana benthamiana* [29–31]. Here, we show that this system can produce very high levels of HBc VLPs displaying an HPV L2 antigen, as well as properly assembled L2 RIC. Immunization of mice with VLP, RIC, or both combined, consistently elicit very high titers (>1,000,000) of L2 antibody which neutralized HPV infectivity. Co-delivery of VLP and RIC together resulted in a strong synergistic enhancement of anti-L2 antibody titers and neutralization activity.

2. Materials and methods

2.1. Vector construction

A dicot plant-optimized HPV-16 L2 coding sequence was designed based upon the sequence of GenBank accession CAC51368.1, and synthesized *in vitro* using synthetic oligonucleotides by the method described [32]. The plant-optimized L2 nucleotide sequence encoding residues 1-473 is posted at GenBank accession KC330735. PCR end-tailoring was used to insert XbaI and SpeI sites flanking the L2 aa 14–122 using primers L2-14-Xba-F (5′-CGTCTAGAGTCCGCAACCCAACTTTACAAG) and L2-122-Spe-R (5′-GGGACTAGTTGGGGCACCAGCATC). The SpeI site was fused to a sequence encoding a 6His tag, and the resulting fusion was cloned into a geminiviral replicon vector [31] to produce pBYe3R2K2Mc-L2(14-122)6H.

The HBche heterodimer VLP system was adapted from [27]. Using the plant optimized HBc gene [30], we constructed a DNA sequence encoding a dimer comprising HBc aa 1-149, a linker $(G_2S)_5G$, HBc aa 1-77, a linker $GT(G_4S)_2$, HPV-16 L2 aa 14-122, a linker $(GGS)_2GSSGGSGG$, and HBc aa 78-176. The dimer sequence was generated using multiple PCR steps including overlap extensions and insertion of BamHI and SpeI restriction sites flanking the L2 aa 14-122, using primers L2-14-Bam-F (5′-CAGGATCCGCAACC CAACTTTACAAGAC) and L2-122-Spe-R. The HBche-L2 and HBche (HBc dimer without insert) coding sequences were inserted into a geminiviral replicon binary vector pBYR2eK2M (Fig. 1A), to make pBYR2eK2M-HBcheL2ic and pBYR2eK2M-HBche, respectively. The vectors include the following elements: CaMV 35S promoter with duplicated enhancer [30], 5′ UTR of *N. benthamiana* psaK2 gene [31], intron-containing 3′ UTR and terminator of tobacco extensin [33], CaMV 35S 3′ terminator[33], and Rb7 matrix attachment region [31].

The recombinant immune complex (RIC) vector was adapted from [20]. The HPV-16 L2 (aa 14-122) segment was inserted into the BamHI and SpeI sites of the gene encoding humanized mAb 6D8 heavy chain, resulting in 6D8 epitope-tagged L2. The heavy chain fusion was inserted into an expression cassette linked to a 6D8 kappa chain expression cassette, all inserted into a geminiviral replicon binary vector to make pBYR11eMa-h6D8-L2 (Fig. 1A, RIC vector). Both cassettes contain CaMV 35S promoter with duplicated enhancer [30], 5′ UTR of *N. benthamiana* psaK2 gene [31], intron-containing 3′ UTR and terminator of tobacco extensin [33], and Rb7 matrix attachment region [31]. Details of the original h6D8 mAb vector construction are available in reference [17] and upon request from the authors.

2.2. Agroinfiltration of Nicotiana benthamiana leaves

Binary vectors were separately introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. The resulting strains were verified by restriction digestion or PCR, grown overnight at 30 °C, and used to infiltrate leaves of 5- to 6-week-old *N. benthamiana* maintained at 23–25 °C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000*g* and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to OD600 = 0.2, unless otherwise described. The resulting bacterial suspensions were injected by using a syringe without needle into leaves through a small puncture [34]. For RIC production, transgenic plants silenced for xylosyltransferase and fucosyltransferase were employed [35]. Plant tissue was harvested at 5 DPI, or as stated for each experiment.

2.3. Protein extraction and purification

L2 and HBche VLPs were purified by sucrose gradient centrifugation. Total protein extract was obtained by homogenizing agroinfiltrated leaf samples with 1:5 (*w:v*) ice cold extraction buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mg/mLsodium ascorbate, 0.3 mg/mL PMSF) using a Bullet Blender machine (Next Advance, Averill Park, NY) following the manufacturer's instruction. To enhance solubility, homogenized tissue was rotated at room temperature or 4 °C for 30 min. The crude plant extract was clarified by centrifugation at 13,000*g* for 15 min at 4 °C. 6 ml of clarified extract from one agroinfiltrated leaf was loaded onto a discontinuous sucrose gradient (1.5 ml layers of 10, 20, 30, 40, and 50% in PBS) and centrifuged at 148,000*g* for 2.5 h (Beckman, SW 28.1 rotor) at 4 °C. Peak fractions were pooled and dialyzed against PBS.

L2 RIC expressed from pBYR11eMa-h6D8-L2 was purified by protein G affinity chromatography. Agroinfiltrated leaves were blended with 1:3 (w:v) ice cold extraction buffer (100 mM Tris-HCl, pH 8.2, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mg/mL sodium ascorbate, 0.3 mg/mL phenylmethylsulfonyl fluoride), stirred for 30 min at 4 °C, and filtered through miracloth. To precipitate endogenous plant proteins, the pH was lowered to 4.5 with 1 M phosphoric acid for 5 min while stirring, then raised to 7.6 with 2 M tris base. Following centrifugation for 20 min at 16,000*g*, the clarified extract was passed through a 0.22 μm filter and loaded onto a Pierce Protein G column (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The RIC was eluted with 100 mM glycine, pH 2.5, directly into collection tubes containing 1 M Tris-HCl pH 8.0 to neutralize the elution buffer. To evaluate solubility of RIC in the original homogenate, the crude extract was centrifuged for 20 min at 16,000*g*, and the pellet was designated the insoluble fraction and treated with SDS sample buffer at 100C for 10 min before loading on SDS-PAGE (below).

L2-His expressed from pBYe3R2K2Mc-L2(14-122)6H was purified by metal affinity chromatography. Protein was extracted as described for L2 RIC, but without acid precipitation. The clarified extract was loaded onto a column containing TALON Metal Affinity Resin (BD Clontech, Mountain View, CA) according to the manufacturer's instructions. The column was washed with PBS and eluted with elution buffer (PBS, 150 mM imidazole, pH 7.4). Peak L2 elutions were pooled and dialyzed against PBS.

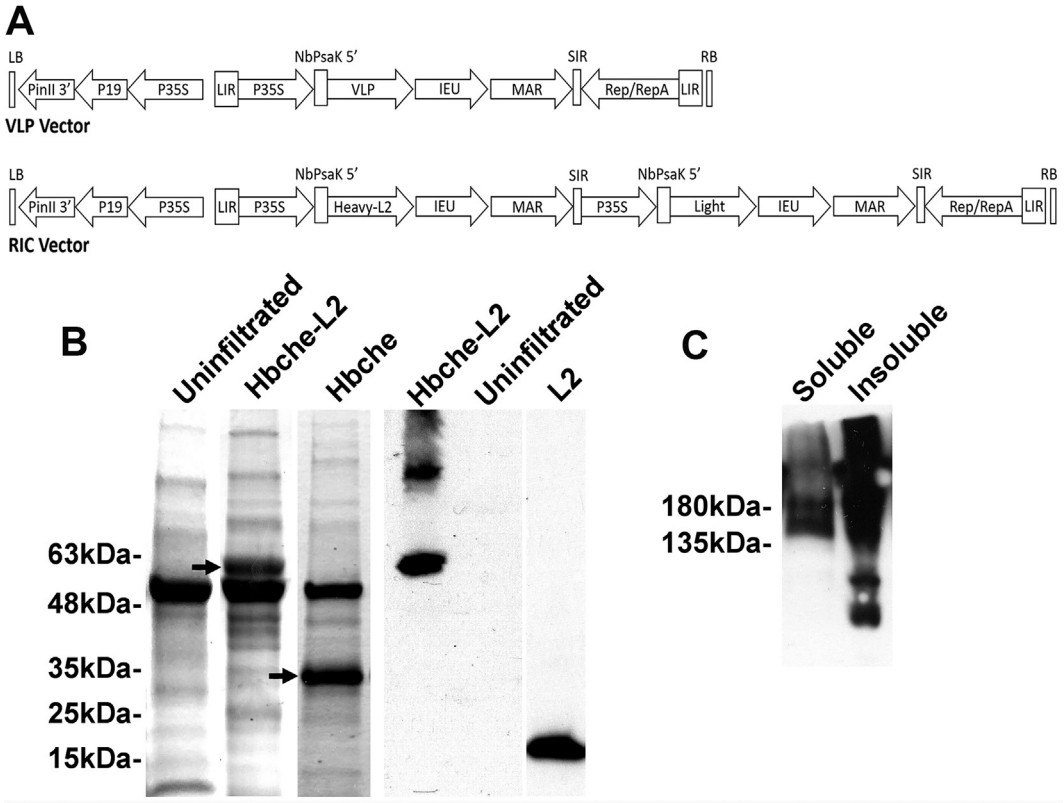

Fig. 1. Expression of VLP and RIC that display L2 antigen. (A) Schematic representation of BeYDV plant expression vectors used in this study. The region between the two LIR segments is circularized and amplified to high copy number in the plant nucleus, serving as transcription templates for the VLP and RIC. Abbreviations: LB, the agrobacterium T-DNA left border region; PinII3′, the terminator from the potato proteinase inhibitor II gene; P19, the RNA silencing suppressor from tomato bushy stunt virus; P35S, the 35S promoter from cauliflower mosaic virus; LIR, the long intergenic region from BeYDV; NbPsaK 5′, the truncated 5′ UTR from NbPsaK [31]; VLP, either L2-fused or unfused hepatitis B core antigen; Heavy-L2, the mAb 6D8 gamma (heavy) chain with C-terminal L2 fusion; Light, the 6D8 kappa (light) chain; IEU, the tobacco extensin terminator with intron [33]; MAR, the Rb7 matrix attachment region; SIR, the short intergenic region from BeYDV; Rep/RepA, the replication proteins from BeYDV; RB, the agrobacterium T-DNA right border region. (B) Expression of VLP: Agroinfiltrated leaf tissue was homogenized, clarified, and analyzed by SDS-PAGE and Coomassie staining (first 3 lanes) and western blot probed with anti-L2 antiserum (last 3 lanes). Arrows indicate HBche and HBche-L2 bands. (C) Expression of RIC: Western blot of soluble and insoluble fractions of crude extracts from RIC agroinfiltrated leaves, probed with anti-L2 antiserum.

2.4. SDS-PAGE and western blot

Clarified plant protein extract or purified protein samples were mixed with sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue) and separated on 4-15% polyacrylamide gels (Bio-Rad, Hercules, CA, USA). For reducing conditions, 0.5 M DTT was added, and the samples were boiled for 10 min prior to loading. Polyacrylamide gels were either transferred to a PVDF membrane or stained with Coomassie stain (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. For L2 detection, the protein transferred membranes were blocked with 5% dry milk in PBST (PBS with 0.05% tween-20) overnight at 4 °C and probed with polyclonal guinea pig anti-L2 (raised against *E. coli*-expressed 6His-tagged L2 amino acids 11–200 cloned into pET28) diluted 1:5000 in 1% PBSTM, followed by goat anti-guinea pig horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA). Bound antibody was detected with ECL reagent (Amersham, Little Chalfont, United Kingdom).

2.5. C1q binding

96-well high-binding polystyrene plates (Corning Inc, Corning, NY, USA) were coated with 15 μg/ml human complement C1q in PBS for 2 h at 37 °C. The plate was washed 3 times with PBST, and then blocked with 5% dry milk in PBST for 1 h. After washing 3 times with PBST, purified human IgG (Southern Biotech, Birmingham, AL, USA) and purified L2 RIC were added at 0.5 mg/ml with 3-fold serial dilutions and incubated for 1 h at 37 °C. After washing 3 times with PBST, bound IgG was detected by incubating with 1:4000 polyclonal goat anti human IgG-HRP (Southern Biotech, Birmingham, AL, USA) for 1 h at 37 °C. The plate was washed 4 times with PBST, developed with TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA), and the absorbance was read at 450 nm.

2.6. Immunization of mice and sample collection

All animals were handled in accordance to the Animal Welfare Act and Arizona State University IACUC. Female BALB/C mice, 6–8 weeks old, were immunized subcutaneously with purified plant-expressed L2 (14-122), HBche-L2 VLP, L2 RIC, or PBS, all mixed 1:1 with Imject Alum (Thermo Fisher Scientific, Waltham, MA, USA). In all treatment groups, the total weight of antigen was set to deliver an equivalent 5 μg of L2. Doses were given on days 0, 21, and 42. Serum collection was done as described [36] by submandibular bleed on days 0, 21, 42, and 63.

2.7. Antibody measurements

Mouse antibody titers were measured by ELISA. *E. coli*-expressed L2 (amino acids 11-200, cloned into pET28), 50 ng/well was bound to 96-well high-binding polystyrene plates (Corning Inc, Corning, NY, USA), and the plates were blocked with 5% nonfat dry milk in PBST. After washing the wells with PBST (PBS with

0.05% Tween 20), the mouse sera were diluted with 1% PBSTM (PBST with 1% nonfat dry milk) added and incubated. Mouse antibodies were detected by incubation with polyclonal goat anti-mouse IgG-horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA). The plate was developed with TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA) and the absorbance was read at 450 nm. Endpoint titers were taken as the reciprocal of the lowest dilution which produced an OD450 reading twice the background produced using PBS as the sample. IgG1 and IgG2a antibodies were measured from sera diluted 1:1000 or 1:50 respectively in 1% PBSTM with goat-anti mouse IgG1 or IgG2a horseradish peroxidase conjugate (Santa Cruz Biotechnology, Dallas, TX, USA). The ratios for the OD values (IgG2a:IgG1) were calculated, and the value obtained for L2 antigen was arbitrarily defined as 1.0. The ratios for VLP, RIC, and VLP/RIC were calculated as values relative to that of L2.

2.8. In vitro neutralization assay

293FT cells were plated at 15,000 cells/well in a 96-well assay plate and propagated for 24 h. Serum samples were serially diluted two-fold in 100 μL volume of culture media, starting from 1:20 dilutions and mixed with 3×10^7 IU of HPV16 pseudovirion [37]. The mixture was incubated at 37° C for 2 h and then added to the 293FT cells. After 72 h, cells were washed with 1X PBS and lysed with 30 μL of Cell Culture Lysis Reagent (Promega, WI, USA) for 15 min at RT on a rocking platform. The lysates were transferred to a black 96-well assay plate and 50 μL of luciferin substrate was added to each well to measure the luciferase activity.

2.9. Electron microscopy

Purified samples of HBche or HBche-L2 VLP were initially incubated on 75/300 mesh grids coated with formvar. Following incubation, samples were briefly washed twice with deionized water then negatively stained with 2% aqueous uranyl acetate. Transmission electron microscopy was performed with a Phillips CM-12 microscope, and images were acquired with a Gatan model 791 CCD camera.

2.10. Statistical analysis

The significance of vaccine treatments and virus neutralization was measured by non-parametric Mann-Whitney test using GraphPad prism software. Two asterisks (**) indicates p values < 0.05. Three asterisks (***) indicates p values < 0.001.

3. Results

3.1. Design and expression of HBc VLPs and RIC displaying HPV16 L2

As most broadly neutralizing anti-HPV antibodies are derived from the highly conserved N-terminal region of L2, amino acids 14-122 of HPV16 L2 were used to create HBche-L2 VLPs. L2 with flanking linkers was inserted into the tip of the α-helical spike of one HBc gene copy, which was fused to another copy of HBc lacking the L2 insert. This arrangement allows the formation of HBc dimers that contain only a single copy of L2, increasing VLP stability [27]. This heterodimer is referred to as HBche-L2. BeYDV plant expression vectors (Fig. 1A) expressing HBche-L2 VLP, HBche VLP, or L2-6His were agroinfiltrated into the leaves of *N. benthamiana* and analyzed for VLP and L2 production. After 4–5 days post infiltration (DPI), leaves displayed only minor signs of tissue necrosis, indicating that the VLP was well-tolerated by the plants. Leaf extracts analyzed by reducing SDS-PAGE and Coomassie staining showed an abundant band near the predicted size of 51 kDa for HBche-L2, just above the Rubisco large subunit (RbcL). HBche was detected at the predicted size of 38 kDa (Fig. 1B). Western blot probed with anti-L2 polyclonal serum detected a band for HBche-L2 at ~51 kDa and at ~12 kDa for L2-6His (Fig. 1B). These results indicate that this plant system produced high levels of L2-containing HBc VLP.

To express L2-containing RIC using our previously described RIC universal platform, amino acids 14-122 of HPV16 L2 were fused with linker to the C-terminus of the 6D8 antibody heavy chain and tagged with the 6D8 epitope [20]. A BeYDV vector (Fig. 1A) expressing both the L2-fused 6D8 heavy chain and the light chain was agroinfiltrated into leaves of *N. benthamiana* and analyzed for RIC production. To produce human-like N-glycosylation, which has been shown to improve antibody Fc receptor binding *in vivo*, transgenic plants silenced for xylosyltransferase and fucosyltransferase were employed [35]. By western blot, high molecular weight bands >150 kDa suggestive of RIC formation were observed (Fig. 1C). Expression of soluble L2 RIC was lower than HBche-L2 due to relatively poor solubility of the RIC (Fig. 1C).

3.2. Purification and characterization of HBche-L2 and L2 RIC

To assess the assembly of VLP, clarified plant extracts containing either HBche-L2 or HBche were analyzed by sucrose gradient sedimentation. HBche-L2 sedimented largely with HBche, which is known to form VLP, though a small increase in density was observed with HBche-L2, perhaps due to the incorporation of L2 into the virus particle (Fig. 2A). As most plant proteins do not sediment with VLP, pooling peak sucrose gradient fractions resulted in >95% pure HBche-L2 (Fig. 2B), yielding sufficient antigen (>3 mg) for vaccination from a single plant leaf. To directly demonstrate particle formation, sucrose gradient fractions were examined by electron microscopy. Both HBche and HBche-L2 formed ~30 nm particles, although the appearance of HBche-L2 VLP suggested slightly larger, fuller particles (Fig. 2C,D).

L2 RIC was purified from plant tissue by protein G affinity chromatography. By SDS-PAGE, an appropriately sized band was visible >150 kDa that was highly pure (Fig. 2B). Western blot confirmed the presence of L2 in this band (Fig. 2B). L2 RIC bound to human complement C1q receptor with substantially higher affinity compared to free human IgG standard, suggesting proper immune complex formation (Fig. 2E).

3.3. Mouse immunization with HBche-L2 and L2 RIC

Groups of Balb/c mice (n = 8) were immunized, using alum as adjuvant, with three doses each of 5 μg L2 delivered as either L2-6His, HBche-L2 VLP, L2 RIC, or a combination of half VLP and half RIC. VLP and RIC, alone or combined, greatly enhanced antibody titers compared to L2-6His by more than an order of magnitude at all time points tested (Fig. 3). After one or two doses, the combined VLP/RIC treatment group outperformed both the VLP or RIC groups, reaching mean endpoint titers of > 200,000, which represent a 700-fold increase over immunization with L2-6His (Fig. 3, $p < 0.001$). After the third dose, both the VLP and combined VLP/RIC groups reached endpoint titers > 1,300,000, a 2-fold increase over the RIC alone group. To determine the antibody subtypes produced by each treatment group, sera were assayed for L2-binding IgG1 and IgG2a. RIC and especially VLP-containing groups had an elevated ratio of IgG2a:IgG1 (>3-fold) compared to L2 alone (Fig. 4).

In vitro neutralization of HPV16 pseudovirions showed that the VLP and RIC groups greatly enhanced neutralization compared to L2 alone (Fig. 5, $p < 0.001$). Additionally, VLP and RIC combined further enhanced neutralization activity (~5-fold, $p < 0.05$) compared

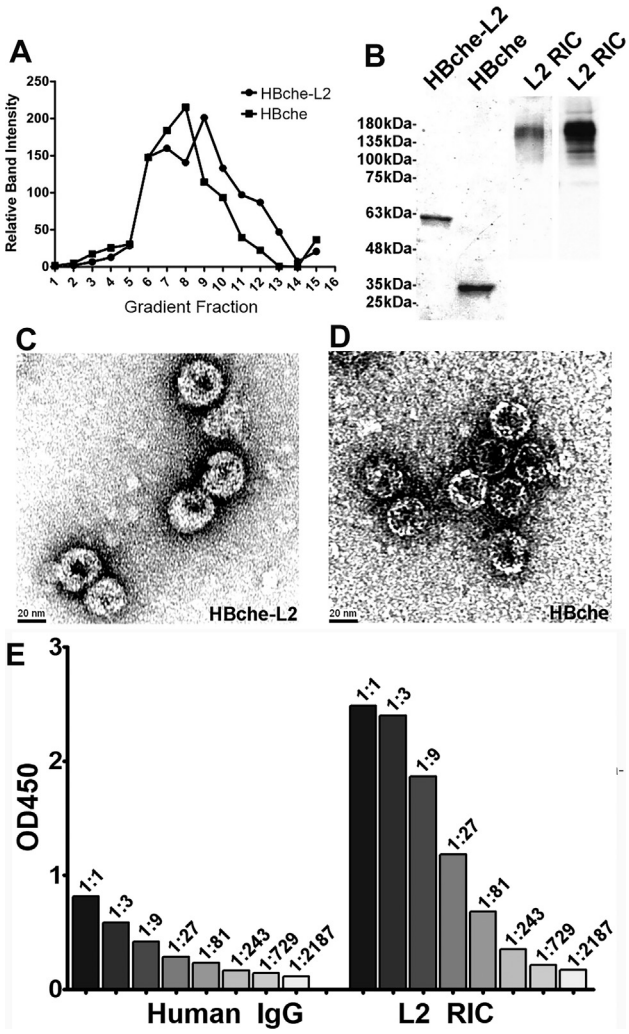

Fig. 2. Purification of fully assembled L2 VLP and RIC. (A) Sucrose gradient sedimentation profiles of crude extracts of leaf tissue agroinfiltrated with either HBche-L2 or HBche. Following sedimentation, fractions were analyzed by SDS-PAGE, using ImageJ software to quantify the band intensity. (B) SDS-PAGE of pooled material from either sucrose gradient purified VLP or protein G affinity purified RIC. Far right lane shows western blot of purified RIC with anti-L2 as probe. (C) Electron microscopy of dialyzed peak sucrose fractions from HBche-L2 gradient after negative staining with 0.5% uranyl acetate; bar = 20 nm. (D) Electron microscopy of dialyzed peak sucrose fractions from HBche gradient after negative staining with 0.5% uranyl acetate; bar = 20 nm. (E) C1q binding of HBche-L2 RIC. Purified human IgG (left) or purified HBche-L2 RIC (right) were used at 0.5 mg/ml (1:1) and 3-fold serial dilutions.

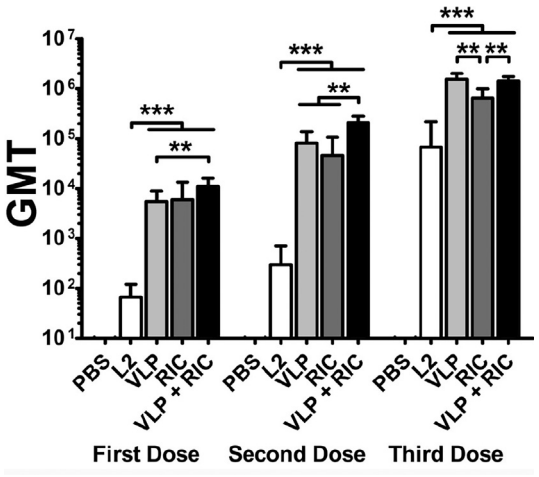

Fig. 3. L2-specific IgG titers in mice immunized with L2 antigens. BALB/c mice (8 per group) were immunized subcutaneously with three doses of either PBS, L2, VLP, RIC, or VLP/RIC mixed 1:1. Except for the PBS control group, each dose delivered 5 μg total L2 and was administered in three-week intervals. Blood samples were collected after each dose and analyzed for L2-specific antibodies by endpoint titer ELISA. The y-axis shows the geometric mean titers (GMT) and error bars show 95% confidence intervals. (**) indicates p values < 0.05; (***) indicates p values < 0.001.

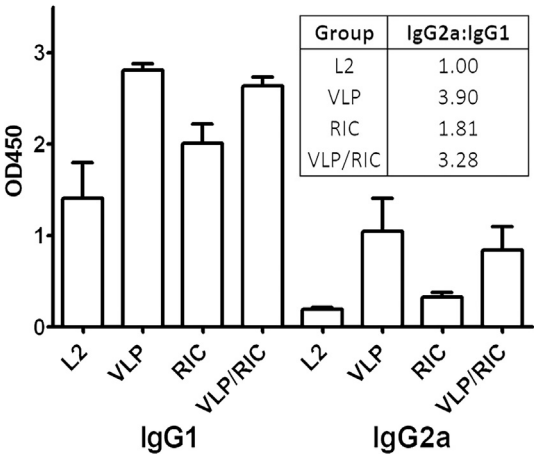

Fig. 4. Comparison of IgG1 and IgG2a Production. Serum samples after the final dose were analyzed by ELISA specific for IgG1 or IgG2a antibodies. Data are means ± the standard error from 8 mice. The table shows the ratio of IgG2a to IgG1 for each group, calculated as relative to L2, with the L2 value set arbitrarily at 1.0.

to either antigen alone, supporting the strong synergistic effect of delivering L2 by both platforms simultaneously.

4. Discussion

Substantial evidence demonstrates the excellent potential of L2 as a target for broadly-protective HPV vaccines. During HPV infection, the capsid undergoes a conformational change at the epithelial basal membrane exposing a furin cleavage site. Upon cleavage, cross-neutralizing epitopes of L2 are transiently exposed [38]. Epithelial damage during the initial phases of infection allows neutralizing serum IgG to access the genital tract [39]. Cross-neutralizing epitopes have been found throughout the N-terminus of L2, including amino acids 17–36, 56–75, 65–85, 96–115, though they vary in their cross-neutralization potential between HPV types [8,40–43]. L2 is minimally exposed on the virion and lacks the highly repetitive, dense arrangement of L1, rendering it poorly immunogenic. While the neutralization potential of even low titers of L2 antibodies have been shown against a large number of HPV types, strategies to improve the immunogenicity of L2 have often been unable to reach the high titers produced by L1 vaccines [10]. In this study, by displaying amino acids 14-122 of HPV16 L2 on the surface of plant-produced HBc VLPs or in recombinant immune complexes, we have generated very high titers of L2 antibody (Fig. 3).

We observed significant synergy of VLP and RIC systems when delivered together (Fig. 3). Since equivalent amounts of L2 were delivered with each dose, the enhanced antibody titer did not result from higher L2 doses. Rather, these data suggest that higher L2-specific antibody production may be attributed to VLP or RIC platform effects. While we did not produce data to show this, a possible explanation for the mechanism of RIC enhancement could be the augmented stimulation of L2-specific B cells by T-helper cells that were primed by RIC-induced antigen presenting cells

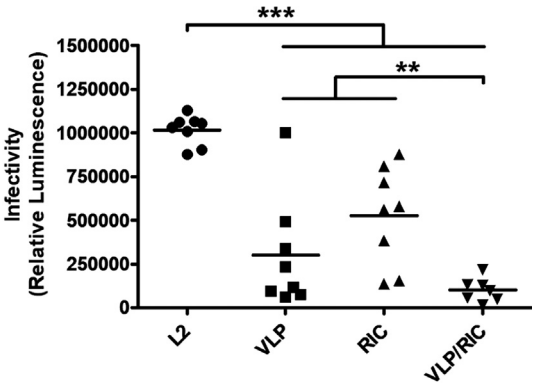

Fig. 5. In Vitro Neutralization of HPV Pseudovirions. Sera of mice immunized with L2, VLP, RIC, or VLP/RIC were diluted and used to neutralize HPV16 pseudovirions before infection of 293FT cells. Infectivity is shown as relative luminescence; diminished luminescence is evidence of impaired infection of 293FT cells. Horizontal lines indicate the group mean. (**) indicates p value < 0.05; (***) indicates p value < 0.001.

[15]. Furthermore, although treatment with VLP and RIC alone reached similar endpoint titers as the combined VLP/RIC group after 3 doses, virus neutralization was substantially higher (>5-fold) in the combined group (Fig. 5). Together, these data indicate unique synergy exists when VLP and RIC are delivered together. We have observed similarly significant synergistic enhancement of immunogenicity for a variety of other antigens (data to be published elsewhere). Further studies are needed to characterize the immune response elicited by antigens delivered by VLP and RIC together.

Mice immunized with L2 alone had highly variable antibody titers, with titers spanning two orders of magnitude. By contrast, the VLP and VLP/RIC groups had much more homogenous antibody responses, with no animals below an endpoint titer of 1:1,000,000 (Fig. 3). These results underscore the potential of HBc VLP and RIC to provide consistently potent immune responses against L2.

Fc gamma receptors are present on immune cells and strongly impact antibody effector functions such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity [44]. In mice, these interactions are controlled in part by IgG subtypes. IgG1 is associated with a Th2 response and has limited effector functions. By contrast, IgG2a is associated with a Th1 response and more strongly binds complement components [45] and Fc receptors [46], enhancing effector functions and opsonophagocytosis by macrophages [47]. We found that immunization with L2 alone produced low levels of IgG2a, however immunization with RIC and VLP produced significant increases in IgG2a titers. VLP-containing groups in particular showed a 3-fold increase in the ratio of IgG2a to IgG1 antibodies (Fig. 4). Importantly, production of IgG2a is associated with successful clearance of a plethora of viral pathogens [48–51]. It should be noted that we used alum adjuvant for these studies, because we wanted to compare with our previous work with the L2 antigen that used alum (data not shown). Since alum may skew the antibody response towards IgG1 at the expense of IgG2a, further work should test the RIC effect without alum adjuvant. However, alum-adjuvanted RIC and VLP did enhance the IgG2a responses compared to L2 alone.

HPV has a tremendous global disease burden, with HPV-derived cervical, anogenital, and oropharyngeal cancers accounting for 5% of all cancer cases worldwide. To address the need to produce safe, effective, and affordable HPV vaccines, we have employed a plant-based system capable of producing a variety of recombinant vaccine antigens. Plants can be grown abundantly and cheaply, providing a large source of inexpensive biomass without the need for costly bioreactors used by traditional fermentation-based systems [28]. Recent economic analyses have found substantial cost reductions for biological products made in plant-based systems compared to traditional systems [52,53]. Furthermore, unlike mammalian systems, plants do not harbor animal pathogens. We have developed a plant-based transient expression system based on bean yellow dwarf virus. In this system, the viral replication machinery is used to amplify the target gene to high copy number in the plant nucleus [30]. After rigorous genetic optimization, the system is capable of producing very high levels of recombinant protein, up to 30–50% of the total soluble plant protein, in 4–5 days [31]. Using this system, we produced and purified milligram quantities of fully assembled and potently immunogenic HBc VLPs displaying HPV L2 through a simple one-step purification process (Fig. 1, Fig. 3). Remarkably, all of the VLPs used for immunization in this study were derived from a single plant leaf, demonstrating the promise of plant-based recombinant expression systems.

The glycosylation state of the Fc receptor also plays an important role in antibody function. Advances in glycoengineering have led to the development of transgenic plants with silenced fucosyl- and xylosyl-transferase genes capable of producing recombinant proteins with authentic human N-glycosylation [54]. Antibodies produced in this manner have more homogenous glycoforms, resulting in improved interaction with Fc gamma and complement receptors compared to the otherwise identical antibodies produced in mammalian cell culture systems [55–58]. As the known mechanisms by which RIC vaccines increase immunogenicity of an antigen depend in part on Fc and complement receptor binding, we produced HPV L2 RIC in transgenic plants with silenced fucosyl- and xylosyl-transferase. Consistent with these data, we found that L2 RIC strongly enhanced the immunogenicity of L2 (Fig. 3). However, yield suffered from insolubility of the RIC (Fig. 1C). We found that the 11-128 segment of L2 expresses very poorly on its own in plants (data not shown) and may be a contributing factor to poor L2 RIC yield. Importantly, we have produced very high yields of RIC with different antigen fusions (data to be published elsewhere). Therefore, we suspect that antibody fusion with a shorter segment of L2 may substantially improve the yield of L2 RIC. Studies are underway to address these questions.

In summary, VLP and RIC vaccines targeting HPV L2 are highly immunogenic, eliciting very high antibody titers, which correlated with virus neutralization. These studies demonstrate the potential of VLP and RIC platforms as a highly effective, synergistic vaccine delivery system to increase antigen immunogenicity, as well as the productivity of the plant-based system for safe, affordable vaccines against HPV. Further, the modular nature of the VLP and RIC systems will provide convenient platforms for the delivery of many other antigens in order to produce high-titer antibody responses.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

AGD, DL, and HSM designed experiments and analyzed data; AGD, DL,and LB performed antigen expression and antiserum titer

experiments; HSK designed and synthesized the plant codon-optimized L2 gene; JK performed mouse immunization and bleeds; DS and KEP designed, performed, and analyzed the virus neutralization studies; AGD, DL, DS, and HSM wrote the manuscript.

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