Published in final edited form as:

Vaccine. 2015 June 26; 33(29): 3346–3353. doi:10.1016/j.vaccine.2015.05.016.

Preclinical Refinements of a Broadly Protective VLP-based HPV Vaccine Targeting the Minor Capsid Protein, L2

Ebenezer Tumban^{1,4,*}, Pavan Muttill², Carolina Andrea A. Escobar³, Julianne Peabody¹, Denis Wafula², David S. Peabody¹, and Bryce Chackerian^{1,*}

¹Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

²Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

³Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

Abstract

An ideal prophylactic human papillomavirus (HPV) vaccine would provide broadly protective and long-lasting immune responses against all high-risk HPV types, would be effective after a single dose, and would be formulated in such a manner to allow for long-term storage without the necessity for refrigeration. We have developed candidate HPV vaccines consisting of bacteriophage virus-like particles (VLPs) that display a broadly neutralizing epitope derived from the HPV16 minor capsid protein, L2. Immunization with 16L2 VLPs elicited high titer and broadly cross-reactive and cross-neutralizing antibodies against diverse HPV types. In this study we introduce two refinements for our candidate vaccines, with an eye towards enhancing efficacy and clinical applicability in the developing world. First, we assessed the role of antigen dose and boosting on immunogenicity. Mice immunized with 16L2-MS2 VLPs at doses ranging from 2–25 µg with or without alum were highly immunogenic at all doses; alum appeared to have an adjuvant effect at the lowest dose. Although boosting enhanced antibody titers, even a single immunization could elicit strong and long-lasting antibody responses. We also developed a method to enhance vaccine stability. Using a spray dry apparatus and a combination of sugars & an amino acid as protein stabilizers, we generated dry powder vaccine formulations of our L2 VLPs. Spray drying of our L2 VLPs did not affect the integrity or immunogenicity of VLPs upon reconstitution. Spray dried VLPs were stable at room temperature and at 37°C for over one month and the VLPs were highly immunogenic. Taken together, these enhancements are designed to facilitate implementation of a next-generation VLP-based HPV vaccine which addresses U.S. and global disparities in vaccine affordability and access in rural/remote populations.

© 2015 Published by Elsevier Ltd.

*Corresponding authors: Tel: +1 505 272 0269; fax: +1 505 272 6029, bchackerian@salud.unm.edu (B. Chackerian) and Tel: +1 906 487 2256; fax: +1 906 487 3167; etumban@mtu.edu (E. Tumban).

⁴Current address: Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931, USA

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

Keywords

MS2 and PP7 bacteriophages; Virus-like Particles; HPV vaccine; Adjuvants; Formulation

1. Introduction

Human papillomavirus (HPV) infection is a necessary cause of nearly all cases of cervical cancer; it is also a significant cause of other anogenital carcinomas as well as a growing percentage of oropharyngeal cancers [1, 2]. The current HPV vaccines (Gardasil and Cervarix) are comprised of virus-like particles (VLPs) derived from the HPV major capsid protein, L1 [3-5]. Both vaccines are highly immunogenic and elicit high titer and long-lasting neutralizing antibody responses. Although these vaccines provide strong protection against the oncogenic HPV types included in the vaccines (HPV16 and HPV18), they provide very little cross-protection against the other 13-16 “high-risk” HPV types associated with ~30% of cervical cancer cases [6-11]. More recently a nonavalent HPV vaccine, called Gardasil-9 (which is also based on L1 VLPs), was approved by the Food and Drug Administration [12]. While the nonavalent vaccine is likely to increase the breadth of HPV protection (it includes VLPs derived from HPV types that cause about 90% of cervical cancer cases), the cost of production and formulation will likely be high, particularly given the fact that the current HPV vaccines are already very expensive [13]. Thus, the nonavalent vaccine may not be affordable in underdeveloped countries where ~85% of cervical cancer cases occur. Another limitation of all current HPV vaccines is that they require cold-chain for transportation and storage. This requirement is a barrier for implementation in the developing world where refrigerated facilities for transportation and storage are often inadequate [14].

As an alternative to the current type-specific HPV vaccines, we have developed vaccines that target highly conserved, broadly neutralizing epitopes from the HPV minor capsid protein, L2 [9, 15-18]. Immunization with L2-displaying VLPs elicits high-titer and broadly neutralizing antibodies against HPV. For example, an RNA bacteriophage MS2-based vaccine displaying a short peptide representing amino acids 17-31 from HPV16 L2 induces antibodies that strongly protect mice from genital infection with HPV pseudoviruses representing eleven diverse HPV types [17].

The goal of this study was to develop techniques to enhance the clinical applicability of VLP-based vaccines targeting HPV L2, particularly in resource-poor settings. In these studies we asked whether 1) VLP-based vaccines targeting HPV L2 could elicit high titer antibodies responses after a single immunization, and 2) we could develop highly stable formulations of these VLP-based vaccines that were suitable for low-resource settings. We assessed the impact of antigen dose and boosts on antibody responses to L2 and also assessed the longevity of antibody responses. To create a more thermostable vaccine, we spray dried (SD) L2-VLPs into a dry powder formulation [19] and assessed its stability, immunogenicity, and ability to protect from HPV pseudovirus infection after storage at different temperatures and over different periods of time. Taken together these data indicate that bacteriophage VLP-based vaccines targeting HPV L2 are potentially immunogenic and can be formulated in a highly thermostable dry powder.

2. Materials and Methods

2.1. Production of L2 VLPs

Plasmids encoding recombinant MS2 and PP7 VLPs displaying HPV16 L2 peptides (amino acids 17-31) were used to express recombinant VLPs in bacteria which were then purified using our previously published standard protocols [20]. Plasmid pDSP62-16L2 encodes a single-chain dimer version of the MS2 coat protein and displays the HPV16 L2 sequence at the N-terminus of coat protein [17]. Plasmid pET2P7K32-16L2 encodes a single-chain dimer version of the PP7 coat protein and displays the HPV16 L2 sequence in an exposed loop structure in the downstream copy of coat protein [15]. VLPs were expressed in *E. coli* [C41(DE3)] and then purified from soluble lysates using our previously published techniques [20]. Endotoxin was removed from purified VLPs by phase extraction using Triton X114 [16].

2.2. Formulation of VLPs to SD powder

To prepare VLPs for spray-drying, purified VLPs were first dialyzed against a mixture of sugars and an amino acid based on the excipients used for spray drying the VLPs into the dry powder. These excipients consisted of a combined solution (%w/w) of: 85.4% mannitol (M); 1.71% trehalose (T); 0.85% dextran (D); 7.85% L-leucine and 4.27% inositol (I). The final spray drying solution (MTDLI) consisted of a total solids concentration of 2.05% w/v solution. VLPs were then diluted to 1.2 mg/ml into the MTDLI solution. A Buchi Mini Spray Dryer B-290 with a standard two-fluid nozzle (0.7 mm diameter; Buchi Corporation) was used for spray drying VLPs (or control MTDLI solution) using compressed nitrogen gas as the inert drying gas. Spray drying was conducted according to protocols that had been reviewed and approved by the Institutional Biosafety Committee at the University of New Mexico. The parameters for spray drying were: inlet temperature 140 °C, outlet temperature of 47±2 °C, nitrogen flow rate of 742 L/h, aspirator rate of 100% and the filter (PTFE) pressure gauge reading at -56 ± 4 mbar. The feed rate was set at 10%. A 0.2 µm EMFLON Filter (Pall Life Sciences) was attached to the distal end of the exhaust of the spray dryer in addition to the PTFE membrane filter supplied with the spray dryer. The spray dryer was housed in a BioPROtect III Jr.® BSC (Baker Corporation) in order to provide protection to the dry powder vaccine (from contamination) and the operator. All subsequent handling of the powders were performed in a biosafety cabinet (BSC) according to the approved protocol. The morphology of the SD powders with and without VLPs was examined using a scanning electron microscope (SEM, JEOL 5800LV); secondary electron signals at low voltages and currents were used in order to prevent the collapse of dry powders.

2.3. Stability studies

SD 16L2-MS2 and 16L2-PP7 VLPs powder were aliquoted into glass vials with tight sealed caps and stored at 4°C or at room temperature for 1, 2 and 3 months, or at 37°C for 1 month. As a comparison, liquid VLPs (in phosphate buffered saline, PBS) were stored at 4°C or room temperature for 1 month. After the storage period, spray-dried VLPs were reconstituted in PBS buffer and then the integrity of VLPs was assessed by agarose gel electrophoresis and/or by transmission electron microscopy. VLPs were run on a 1% agarose gel and then stained with ethidium bromide (to check for the presence of coat protein-

encapsidated RNAs) and with Coomassie blue (to check for the presence of recombinant coat proteins). Transmission electron microscopy (TEM) was conducted as previously described [17].

2.4. Immunizations and Characterization of Antibody Responses

All animal work was performed 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 UNM IACUC (protocol 12-100827-HSC). Groups of five female Balb/c mice were immunized intramuscularly with purified VLPs as described below. Anti-L2 IgG antibodies in sera were measured by peptide (16L2 amino acid 14-40) ELISA as described in Tumban et al. [15].

2.4.1 Dosing and Adjuvant Studies—Groups of mice were immunized intramuscularly with different doses of 16L2-MS2 VLPs (2-25 µg) with or without alum hydroxide adjuvant (alhydrogel; Invivogen) at 1% final concentration. Control mice were immunized with wild-type MS2 VLPs. Mice were boosted twice, 1 and 2 months after the initial immunization. Sera were collected 1 month after the first immunization and monthly thereafter. The groups of mice that received 10 µg doses were followed for more than 13 months after the initial immunization.

2.4.2 Single Dose Study—A group of mice was immunized with a single dose of 10 µg of 16L2-MS2 VLPs (without alum hydroxide). Sera were collected at 1, 3, and 6 months following this immunization.

2.4.3 Immunogenicity of SD VLPs—Groups of mice were immunized intramuscularly with 5 µg of reconstituted spray-dried VLPs mixed with alum hydroxide adjuvant; SD VLPs (at 0, 1, 2, 7.5 months after spray drying) were reconstituted using PBS. Another group of mice was immunized with 5 µg (total VLP concentration) of original Gardasil. Control mice were immunized with wild-type MS2 or PP7 VLPs diluted in MTDLI solution plus alum hydroxide. Two weeks after immunization, mice were boosted with the same dose and alum hydroxide adjuvant formulation. Serum was collected two weeks after the second immunization.

2.5 Protection from HPV Pseudovirion (PsV) infection

Mice were genitally challenged with HPV pseudovirions (PsVs; 1×10^5 – 7×10^5 infectious units of PsVs representing HPV types 6, 16, 18, 31, and 45) as previously described [15, 21, 22].

3. Results

3.1. Effect of dose and adjuvant on immunogenicity of liquid 16L2-MS2 VLPs

Previously we had shown that recombinant MS2 bacteriophage VLPs displaying a short peptide from HPV16 L2 could elicit broadly neutralizing antibodies against diverse HPV types [17]. To more thoroughly assess the effects of antigen dose, the use of adjuvant, and the role of booster immunizations on the immunogenicity of 16L2-MS2 VLPs, we

immunized groups of five Balb/c mice with doses ranging from 2-25 µg of VLPs (with or without alum hydroxide) and then assessed anti-L2 IgG antibody responses after one or two immunizations. As shown in Figure 1A, 16L2-VLPs were highly immunogenic at all doses, even after just one immunization. We only observed a slight, non-statistically significant, effect of dose on antibody titers. Mice given a dose of 2 µg (without Alum) had somewhat lower antibody titers after a single dose of vaccine. After two doses, there were no statistical differences in titer between any of the vaccinated groups. The use of alum hydroxide as an adjuvant slightly boosted antibody titers at the lowest doses, but this difference was also not statistically significant. To assess the longevity of antibody responses, we boosted the groups immunized with 10 µg of 16L2-MS2 a second time and then followed these groups for over 13 months from the initial immunization. In both groups of mice (immunized with or without alum hydroxide), there was only a minimal decline in anti-L2 IgG titers over this period (Figure 1B). Over one year after immunization, we genitally challenged mice with diverse HPV PsV types. As shown in Figure 1C, immunized mice were strongly protected from infection with HPV PsV16 (13 months after immunization), HPV PsV6 (15 months after immunization), and HPV PsV18 (18.5 months after immunization).

Because 16L2-MS2 VLPs were so highly immunogenic after a single dose (Figure 1A), we next asked whether a single dose of vaccine also elicited long-lasting antibody responses. A group of mice was given a single dose 10 µg of 16L2-MS2 VLPs (without alum hydroxide) and then antibody levels were monitored for six months. As shown in Figure 1D, anti-L2 antibody levels were high (end-point dilution titers of $\sim 10^4$) and stable 6 months after the immunization.

3.2. Formulation of VLPs into a thermostable dry powder product

One of the most common methods used to stabilize vaccine components is to prepare them in a dry powder form [23]. To determine whether two different L2-displaying bacteriophage VLPs were compatible with this technique, we dialyzed VLPs (16L2-MS2 and 16L2-PP7, described in [15, 17]) into an excipient solution (MTDLI) consisting of a mixture of four stabilizing sugars and an amino acid. The mixture was SD into a dry powder (Figure 2A) using a Mini Spray Dryer B-290. By scanning electron microscopy the VLP-containing powder showed distinct wrinkled particles as compared to blank MTDLI powder (Figure 2B). The size of the SD powder roughly ranged between 2 and 4 µm in diameter. SD VLPs were then reconstituted in PBS buffer. To assess the stability of SD VLPs, we subjected the resuspended VLPs to agarose gel electrophoresis. VLPs migrate through an agarose gel with a mobility that is determined by its overall surface charge. Co-migration of a Coomassie-staining band (indicating protein) and an ethidium bromide-staining band (for encapsidated RNA) indicates that particles are intact. Using VLPs not subjected to the SD process as a control (i.e. liquid VLPs), this analysis indicated that the structural integrity of the SD VLPs was maintained through the SD process. As shown in Figure 3, SD had no effect on the co-migration of 16L2-MS2 or 16L2-PP7 VLP protein and nucleic acid through an agarose gel. SD 16L2-MS2 VLPs showed some minor differences in mobility on the agarose gel (Figure 3A); the slower migrating species may reflect some clumping of VLPs. TEM analysis of SD 16L2-MS2 and 16L2-PP7 VLPs indicated that both VLPs had similar morphology/integrity as VLPs that were not subjected to the SD process (Figure 3).

3.3. Thermostability of SD VLPs at different temperatures

To assess whether the formulated VLPs powder were stable at different temperatures for different lengths of time, aliquots from SD VLPs were stored at 4°C, room temperature, or at 37°C for 1 month. For comparison, liquid L2 VLPs were also stored for the same length of time at 4°C and at room temperature. As shown in Figure 4A, both L2 VLPs stored in solution (i.e. liquid) at 4°C were stable (although we sometimes see superficial change in MS2-16L2 VLPs at this temperature); 16L2-MS2 VLPs stored at room temperature for 1 month disintegrated (Figure 4A), whereas liquid 16L2-PP7 VLPs stored at the same condition did not. SD 16L2-MS2 VLPs and SD 16L2-PP7 VLPs were stable after one month of storage at either room temperature (Figure 4B) or 37°C (Figure 4C). In addition to this, the integrity of the VLPs (stored at room temperature) only changed superficially after 2-3 months of storage at room temperature (Figure 4D). Agarose gel electrophoresis analysis of SD VLPs confirmed particle integrity under these storage conditions.

3.4. Immunogenicity of SD VLPs

To assess whether spray-drying affected the immunogenicity of the L2 VLPs, groups of female mice were immunized with reconstituted SD L2 VLPs (immediately after spray drying) and antibody titers against L2 were assessed by peptide ELISA. As shown in Figure 5A, rehydrated SD VLPs elicited high titer ($>10^4$) anti-L2 antibody responses. To determine whether immune responses elicited by reconstituted SD L2 VLPs were protective, mice immunized with the more broadly protective 16L2-MS2 SD VLPs were challenged with three different HPV PsVs, and protection was compared to mice immunized with the licensed HPV vaccine Gardasil. We chose 16L2-MS2 VLPs-immunized mice for further characterization because we have observed (in previous studies) that 16L2-MS2 VLPs elicit better cross-protective antibodies compared to 16L2-PP7 VLPs [17]. As shown in Figure 5B, reconstituted SD 16L2-MS2 VLP-immunized and Gardasil-immunized mice were similarly protected from infection by PsV16. Mice immunized with reconstituted SD 16L2-MS2 VLPs were also protected from infection with two heterologous PsV types (PsV31 and PsV45). Protection against HPV PsV31 was somewhat less complete, similar to what we observed previously [17]. In contrast, Gardasil only provided (partial) protection against PsV31 infection and failed to protect mice against PsV45 challenge. Taken together, these data indicate that the spray-drying process does not affect either the immunogenicity of 16L2-MS2 VLPs or their ability to elicit HPV neutralizing antibodies.

To test whether the storage of the SD 16L2-MS2 VLPs and SD 16L2-PP7 VLPs for over a month at different temperatures would affect the immunogenicity of the VLPs, mice were immunized with reconstituted SD VLPs that had been stored for either 1 month at 37°C or stored for 2 or 7.5 months at room temperature. These VLPs were still highly immunogenic and provided protection from HPV PsV16 infection. As shown in Figure 6A, mice immunized with SD VLPs stored at 37°C for 1 month or at room temperature for 2 months have geometric mean anti-L2 IgG antibody titers of $>10^4$, albeit slightly lower than frozen stock of liquid 16L2-MS2 VLPs (Figure 6B); mice immunized with SD 16L2-PP7 VLPs also had geometric mean anti-L2 IgG antibody titers of $>10^4$ (data not shown). Moreover, mice immunized with SD 16L2-MS2 VLPs stored at room temperature for 7.5 months were equally immunogenic (Figure 6B). Both groups of mice [immunized with 16L2-MS2 VLPs

stored at 37°C for 1 month and at room temperature for 2 (Figure 6C) or stored at room temperature for 7.5 months (Figure 6D)] were strongly protected from infection with HPV PsV16, and this protection was similar to that observed in mice immunized with Gardasil.

4. Discussion

Vaccines based on VLPs consisting of the HPV L1 major capsid protein are highly immunogenic and provide strong protection from HPV infection. However, there are >150 different HPV types, and the neutralizing antibodies that are induced by immunization with L1 VLPs derived from one HPV type are rarely neutralizing against others. An ideal next-generation HPV vaccine would offer protection against all 15 (or so) HPV types associated with cervical cancer, and would provide potential protection against other HPV-associated neoplasias (which include genital and cutaneous warts). In addition, since most of the cases of cervical cancer occur in the developing world, an ideal HPV vaccine would be (1) formulated in such a manner to allow for long-term storage without the necessity for refrigeration, (2) inexpensive, and (3) effective after a single dose. Merck's nonavalent vaccine, Gardasil-9 (which contains a mixture of nine L1 VLPs), has just been approved for use by the FDA [12]. It is likely that Gardasil-9 will provide expanded protection against HPV infection; it is predicted that this vaccine will provide protection against the HPV types associated with ~90% of cervical cancer cases [5, 12]. However, it is likely that Gardasil-9 will have many of the less optimal features of the current HPV vaccines; the vaccine is likely to be expensive (initial estimates are that the vaccine will cost ~US \$146/dose in the developed world [24]), three doses are recommended, and the vaccine requires refrigeration. Agreements brokered by the GAVI Alliance have lowered the price of Cervarix and Gardasil to a little less than US \$5 in as many as 40 developing countries, but it remains to be seen whether these agreements will also apply to Gardasil-9.

As an alternative to vaccines based on L1 VLPs, we and others have focused on developing broadly protective cost-effective next-generation HPV vaccines targeting highly conserved epitopes in the HPV minor capsid protein, L2 [9, 16, 17, 25-29]. Our group has developed candidate next-generation HPV vaccines based on bacteriophage VLPs displaying conserved L2 epitopes from HPVs and has shown that these vaccines provide broad protection from infection by diverse HPV types [9, 15-18]. In this study, we explored the effect of dose and adjuvant on the immunogenicity of one of our candidate HPV vaccines, 16L2-MS2 VLPs. In mice, 16L2-MS2 VLPs, with or without alum hydroxide, were highly immunogenic over a wide range of doses. Antibody responses were long-lasting and protected mice against diverse HPV types over 1 year after immunization. Strikingly, even a single dose of vaccine elicited high-titer and long-lasting antibody responses. Although licensed subunit vaccines are routinely administered using a prime/boost strategy of two or more doses, a recent clinical study showed that Cervarix can induce long-lasting and protective antibody responses after a single immunization [30, 31]. These data support the model [32] that antigens that strongly stimulate B cells (such as highly multivalent VLPs) can preferentially lead to the generation of long-lived antibody-secreting plasma cells.

To address thermostability issues, L2 VLPs were formulated in a combination of generally regarded as safe (GRAS) sugars and an amino acid [19] and SD into a dry powder vaccine.

The sugars used to encapsulate VLPs included Mannitol (resists moisture reabsorption during storage and prevents powder clumping), Trehalose (which has a high glass transition temperature [T_g] of ~120 °C and provides overall stability to the powder), Dextran (that decreases the crystallization process during long-term storage at extreme conditions), L-Leucine (an amino acid which provides flow properties to the dry powder, increasing product yield and dispersion of the powder), and Inositol (which has a stabilizing affect during the SD process) [33]. This excipient combination is desiccant-protectant and was predicted to stabilize the VLPs by forming an amorphous glassy matrix and providing hydrogen bonding to the VLPs, therefore limiting its molecular mobility. We showed that spray drying did not affect the integrity or immunogenicity of VLPs upon reconstitution, or their ability to provide protection against challenge by diverse HPV types. Moreover, SD VLPs were stable at 37°C and at room temperature for over one month. SD VLPs stored at room temperature for over 7 months were highly immunogenic and protected mice from genital challenge with HPV16 PsV as well as mice immunized with Gardasil.

In summary, spray drying is a one-step, easily scalable pharmaceutical process that can be used to transform candidate L2 VLP vaccines from liquid solution to a dry powder that does not require refrigeration. These results demonstrate that bacteriophage VLP-based vaccines targeting HPV L2 can be formulated to a dry powder that is stable for at least 7 months at room temperature. These vaccines can then be reconstituted in a buffer just prior to immunization. This formulation may be particularly advantageous in the developing world where maintaining a cold-chain is a major logistical issue in vaccine delivery. In the future, it would be interesting to determine whether SD VLPs are compatible with needle-free vaccination approaches. Vaccine delivery without reconstitution may be an additional enhancement that could improve vaccine implementation in the developing world.

Acknowledgments

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 Stephen Jett for help with transmission electron microscopy. L1/L2 expression and reporter plasmids were generously provided by Chris Buck, Susana Pang, John Schiller, Martin Muller, and Tadahito Kanda. This work was supported by a cooperative agreement from the US National Institutes of Health (National Institute of Allergy and Infectious Diseases) establishing the Epidemiology and Prevention Interdisciplinary Center for Sexually Transmitted Diseases (U19 AI113187) and the University of New Mexico Interdisciplinary HPV Prevention Center (U19 AI084081). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruze S, Anderson WF, Rosenberg PS, Gillison ML. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011; 29:4294–301. [PubMed: 21969503]
2. McLaughlin-Drubin ME, Meyers J, Munger K. Cancer associated human papillomaviruses. *Curr Opin Virol*. 2012; 2:459–66. [PubMed: 22658985]
3. Kim KS, Park SA, Ko KN, Yi S, Cho YJ. Current status of human papillomavirus vaccines. *Clin Exp Vaccine Res*. 2014; 3:168–75. [PubMed: 25003090]
4. Schiller JT, Lowy DR. Understanding and learning from the success of prophylactic human papillomavirus vaccines. *Nat Rev Microbiol*. 2012; 10:681–92. [PubMed: 22961341]

5. Tyler M, Tumban E, Chackerian B. Second-generation prophylactic HPV vaccines: successes and challenges. *Expert Rev Vaccines*. 2014; 13:247–55. [PubMed: 24350614]
6. Brown DR, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Wheeler CM, Perez G, Koutsky LA, Tay EH, Garcia P, Ault KA, Garland SM, Leodolter S, Olsson SE, Tang GW, Ferris DG, Paavonen J, Steben M, Bosch FX, Dillner J, Joura EA, Kurman RJ, Majewski S, Munoz N, Myers ER, Villa LL, Taddeo FJ, Roberts C, Tadesse A, Bryan J, Lupinacci LC, Giacoletti KE, Sings HL, James M, Hesley TM, Barr E. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naïve women aged 16–26 years. *J Infect Dis*. 2009; 199:926–35. [PubMed: 19236279]
7. Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, Zahaf T, Innis B, Naud P, De Carvalho NS, Roteli-Martins CM, Teixeira J, Blatter MM, Korn AP, Quint W, Dubin G. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet*. 2004; 364:1757–65. [PubMed: 15541448]
8. Smith JF, Brownlow M, Brown M, Kowalski R, Esser MT, Ruiz W, Barr E, Brown DR, Bryan JT. Antibodies from women immunized with Gardasil cross-neutralize HPV 45 pseudovirions. *Hum Vaccin*. 2007; 3:109–15. [PubMed: 17611417]
9. Tyler M, Tumban E, Dziduszko A, Ozbun MA, Peabody DS, Chackerian B. Immunization with a consensus epitope from human papillomavirus L2 induces antibodies that are broadly neutralizing. *Vaccine*. 2014; 32:4267–74. [PubMed: 24962748]
10. Wheeler CM, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Perez G, Brown DR, Koutsky LA, Tay EH, Garcia P, Ault KA, Garland SM, Leodolter S, Olsson SE, Tang GW, Ferris DG, Paavonen J, Steben M, Bosch FX, Dillner J, Joura EA, Kurman RJ, Majewski S, Munoz N, Myers ER, Villa LL, Taddeo FJ, Roberts C, Tadesse A, Bryan J, Lupinacci LC, Giacoletti KE, James M, Vuocolo S, Hesley TM, Barr E. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in sexually active women aged 16–26 years. *J Infect Dis*. 2009; 199:936–44. [PubMed: 19236277]
11. Schiller JT, Castellsague X, Garland SM. A review of clinical trials of human papillomavirus prophylactic vaccines. *Vaccine*. 2012; 30(Suppl 5):F123–38. [PubMed: 2319956]
12. Merck. [Jan. 15, 2015] Merck Newsroom. [Updated Dec. 11, 2014]; Available from: <http://www.mercknewsroom.com/news-release/prescription-medicine-news/fda-approves-mercks-hpv-vaccine-gardasil9-prevent-cancers-an>
13. Schiller JT, Nardelli-Haeffliger D. Chapter 17: Second generation HPV vaccines to prevent cervical cancer. *Vaccine*. 2006; 24(Suppl 3):S3/147–53.
14. Chen D, Kristensen D. Opportunities and challenges of developing thermostable vaccines. *Expert Rev Vaccines*. 2009; 8:547–57. [PubMed: 19397412]
15. Tumban E, Peabody J, Peabody DS, Chackerian B. A pan-HPV vaccine based on bacteriophage PP7 VLPs displaying broadly cross-neutralizing epitopes from the HPV minor capsid protein, L2. *PLoS One*. 2011; 6:e23310. [PubMed: 21858066]
16. Tumban E, Peabody J, Peabody DS, Chackerian B. A universal virus-like particle-based vaccine for human papillomavirus: Longevity of protection and role of endogenous and exogenous adjuvants. *Vaccine*. 2013
17. Tumban E, Peabody J, Tyler M, Peabody DS, Chackerian B. VLPs displaying a single L2 epitope induce broadly cross-neutralizing antibodies against human papillomavirus. *PLoS One*. 2012; 7:e49751. [PubMed: 23185426]
18. Tyler M, Tumban E, Peabody DS, Chackerian B. The use of hybrid virus-like particles to enhance the immunogenicity of a broadly protective HPV vaccine. *Biotechnol Bioeng*. 2014; 111:2398–406. [PubMed: 24917327]
19. Muttill P, Pulliam B, Garcia-Contreras L, Fallon JK, Wang C, Hickey AJ, Edwards DA. Pulmonary immunization of guinea pigs with diphtheria CRM-197 antigen as nanoparticle aggregate dry powders enhance local and systemic immune responses. *AAPS J*. 2010; 12:699–707. [PubMed: 20878294]

20. Caldeira Jdo C, Medford A, Kines RC, Lino CA, Schiller JT, Chackerian B, Peabody DS. Immunogenic display of diverse peptides, including a broadly cross-type neutralizing human papillomavirus L2 epitope, on virus-like particles of the RNA bacteriophage PP7. *Vaccine*. 2010; 28:4384–93. [PubMed: 20434554]
21. Buck CB, Thompson CD, Pang YY, Lowy DR, Schiller JT. Maturation of papillomavirus capsids. *J Virol*. 2005; 79:2839–46. [PubMed: 15709003]
22. Johnson KM, Kines RC, Roberts JN, Lowy DR, Schiller JT, Day PM. Role of heparan sulfate in attachment to and infection of the murine female genital tract by human papillomavirus. *J Virol*. 2009; 83:2067–74. [PubMed: 19073722]
23. Wang W. Lyophilization and development of solid protein pharmaceuticals. *Int J Pharm*. 2000; 203:1–60. [PubMed: 10967427]
24. Loftus, P. [Jan 16, 2015] FDA Clears New Version of Merck's Gardasil. Dec 10. 2014 Available from: <http://www.wsj.com/articles/fda-clears-mercks-gardasil-to-treat-broader-range-of-cancers-1418243098>
25. Schellenbacher C, Kwak K, Fink D, Shafti-Keramat S, Huber B, Jindra C, Faust H, Dillner J, Roden RB, Kirnbauer R. Efficacy of RG1-VLP vaccination against infections with genital and cutaneous human papillomaviruses. *J Invest Dermatol*. 2013; 133:2706–13. [PubMed: 23752042]
26. Jagu S, Karanam B, Gambhira R, Chivukula SV, Chaganti RJ, Lowy DR, Schiller JT, Roden RB. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. *J Natl Cancer Inst*. 2009; 101:782–92. [PubMed: 19470949]
27. Kondo K, Ishii Y, Ochi H, Matsumoto T, Yoshikawa H, Kanda T. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology*. 2007; 358:266–72. [PubMed: 17010405]
28. Pastrana DV, Gambhira R, Buck CB, Pang YY, Thompson CD, Culp TD, Christensen ND, Lowy DR, Schiller JT, Roden RB. Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2. *Virology*. 2005; 337:365–72. [PubMed: 15885736]
29. Kalnin K, Tibbitts T, Yan Y, Stegalkina S, Shen L, Costa V, Sabharwal R, Anderson SF, Day PM, Christensen N, Schiller JT, Jagu S, Roden RB, Almond J, Kleanthous H. Low doses of flagellin-L2 multimer vaccines protect against challenge with diverse papillomavirus genotypes. *Vaccine*. 2014; 32:3540–7. [PubMed: 24780250]
30. Safaeian M, Porras C, Pan Y, Kreimer A, Schiller JT, Gonzalez P, Lowy DR, Wacholder S, Schiffman M, Rodriguez AC, Herrero R, Kemp T, Shelton G, Quint W, van Doorn LJ, Hildesheim A, Pinto LA, Group CVT. Durable antibody responses following one dose of the bivalent human papillomavirus L1 virus-like particle vaccine in the Costa Rica Vaccine Trial. *Cancer Prev Res (Phila)*. 2013; 6:1242–50. [PubMed: 24189371]
31. Schiller JT, Lowy DR. Raising Expectations For Subunit Vaccine. *J Infect Dis*. 2014
32. Amanna IJ, Slifka MK. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunol Rev*. 2010; 236:125–38. [PubMed: 20636813]
33. Jin TH, Tsao E, Goudsmit J, Dheenadhayalan V, Sadoff J. Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). *Vaccine*. 2010; 28:4369–75. [PubMed: 20444437]

Highlights

Virus-like Particles displaying an HPV16 L2 peptide (16L2-MS2 VLPs) are highly immunogenic over a range of doses.

Immune responses to 16L2-MS2 VLPs last over 18 months.

Spray-drying L2 VLPs enhances their stability.

Spray dried L2 VLPs stored over 7 months at room temperature are highly immunogenic and protective against HPV infections

D.

Figure 1.

Effects of dose and adjuvant on antibody responses. A) Groups of 5 Balb/c mice were immunized once or twice with 16L2-MS2 VLPs (with or without alum hydroxide) at the indicated doses at weeks 0 and 4. Control mice were immunized with 10 µg of MS2 VLPs (in alum hydroxide). Sera were collected 4 weeks after the first or second immunization and anti-L2 IgG antibody titers were determined by ELISA using HPV 16L2 (amino acid 14-40) as a target peptide. B) Mice immunized with 10 µg of 16L2-MS2 VLPs (from Fig 1A above) received an additional boost of 10 µg (2 months after the initial immunization). Anti-L2 IgG antibody titers were followed (by ELISA) for over 13 months after immunization. C) Mice from Figure 1B above were later challenged with PsVs16 (13 months after immunization), PsV6 (15 months after immunization), and PsV18 (18.5 months after immunization; two control mice died prior to assay). Luciferase activity was measured forty-eight hours post challenge and average radiance (p/s/cm²/sr) values for each mouse was determined using Living Image 3.2 software. Each datum represents the average radiance of an individual mouse and each line represents the geometric mean radiance for each group. D) Mice were immunized with 1 dose of 10 µg of 16L2-MS2 VLPs (without alum) and their anti-L2 antibody titers were monitored for over 6 months.

A.

B.

Spray dried L2 VLPs powder

Spray dried MTDLI powder

Figure 2.

The formulation of L2 VLPs to dry powder. A) VLPs were diluted to 1.2 mg/ml using MTDLI solution, SD using Buchi Mini Spray Dryer, and bottled in glass vials. B) Scanning electron microscopy of L2 VLPs powder, SD with MTDLI solution (left) and MTDLI powder (right) SD alone.

A. 16L2-MS2 VLPs

Liquid VLPs

Reconstituted SD VLPs

B. 16L2-PP7 VLPs

Figure 3.

Integrity of 16L2-MS2 VLPs and 16L2-PP7 VLPs immediately after spray drying (at 0 month). A) Liquid 16L2-MS2 VLPs and SD 16L2-MS2 VLPs powder (reconstituted in PBS buffer) were analyzed on a 1% agarose gel (top panel) stained with ethidium bromide (left image) followed by Coomassie blue staining (right image). Electron microscopy of VLPs (bottom panel; 70,000x).

B): Liquid 16L2-PP7 VLPs or SD 16L2-PP7 VLPs powder (reconstituted in PBS buffer) were analyzed on 1% agarose gel (top panel) stained with ethidium bromide (left image) followed by Coomassie blue staining (right image). Electron microscopy of VLPs (bottom panel; 70,000x).

Figure 4.

Thermostability and integrity of L2 VLPs after 1 and 2 months at different temperatures. A) 1% agarose gel showing liquid 16L2-MS2 VLPs (left) and liquid 16L2-PP7 VLPs (right) each stained with ethidium bromide followed by Coomassie blue staining; liquid VLPs were stored at 4°C or room temperature (RT) for 1 month. Electron microscopy images and 1% agarose gels of reconstituted L2 VLPs from VLPs powder stored at B) RT and C) 37°C for 1 month. D) Electron microscopy images and 1% agarose gels of reconstituted L2 VLPs from VLPs powder stored at room temperature for 2 months. In all cases, SD L2 VLPs were reconstituted in PBS buffer.

Figure 5.

Immunogenicity of reconstituted SD L2 VLPs (at 0 month) and protection from infection with HPV PsVs. A) Groups of Balb/c mice were immunized twice intramuscularly with 16L2-MS2 VLPs (left panel; liquid and reconstituted powder or control MS2 VLPs) and 16L2-PP7 VLPs (right panel; liquid, reconstituted powder or control PP7 VLPs) plus alum hydroxide. Sera were collected 2 weeks after the last immunization and anti-L2 IgG antibody titers were determined by ELISA using HPV 16L2 (amino acid 14-40) as a target peptide. Each circle or tetragon represents an individual mouse and each line represents the geometric means for each group. B). Mice were immunized as described in Figure 6A and were later challenged with PsVs16, PsV31, and PsV45. Luciferase activity was measured forty-eight hours post challenge and average radiance (p/s/cm²/sr) values for each mouse was determined using Living Image 3.2 software. Each datum represents the average radiance of an individual mouse and each line represents the geometric mean radiance for each group. Statistical analysis was done using one-tailed unpaired t-test.

Figure 6.

Immunogenicity and protection of reconstituted SD L2 VLPs after 1, 2, and 7.5 months. A) SD VLPs stored for 1 month at 37°C or 2 months at room temperature (RT) were reconstituted in PBS buffer. Groups of Balb/c mice were immunized with reconstituted VLPs or control MS2 VLPs and immune responses were tested as described in Figure 5A legend above. B) SD VLPs stored for 7.5 months (room temperature) were also reconstituted in PBS buffer. Groups of Balb/c mice were immunized with either the reconstituted 16L2-MS2 VLPs, frozen stock of liquid 16L2-MS2 VLPs or Gardasil. Immune responses were tested as described in Figure 5A legend above. C) and D) Immunized mice (shown in Figures 6A and 6B, respectively) were infected with PsV16 (five weeks after second immunization) as described in Figure 5B legend above. Statistical analysis was done using one-tailed unpaired t-test.