Published in final edited form as:
Expert Rev Vaccines. 2013 February ; 12(2): . doi:10.1586/erv.12.151.

Virus-like particles for the prevention of human papillomavirus-associated malignancies

Joshua W. Wang[1] and **Richard B.S. Roden**[1,2,3,*]

[1]Departments of Pathology, The Johns Hopkins University, Baltimore, MD 21287-0014 USA

[2]Oncology, The Johns Hopkins University, Baltimore, MD 21287-0014 USA

[3]Gynecology and Obstetrics, The Johns Hopkins University, Baltimore, MD 21287-0014 USA

Abstract

As compared to peptide/protein-based vaccines, naked DNA vectors and even traditional attenuated or inactived virus vaccines, virus-like particles (VLPs) are an attractive vaccine platform because they offer a combination of safety, ease of production, and both high density B cell epitope display and intracellular presentation of T cell epitopes that induce potent humoral and cellular immune responses respectively. Indeed, human papillomavirus (HPV) vaccines based on VLP production by recombinant expression of major capsid antigen L1 in yeast (Gardasil®, Merck & Co.) or insect cells (Cervarix®, GlaxoSmithKline) have been licensed for the prevention of cervical and anogenital infection and disease associated with the genotypes targeted by each vaccine. These HPV vaccines however have not been demonstrated as effective to treat existing infections, and efforts to develop a therapeutic HPV vaccine continue. Furthermore, current HPV L1-VLP vaccines provide type-restricted protection, requiring highly multivalent formulations to broaden coverage to the dozen or more oncogenic HPV genotypes. This raises the complexity and cost of vaccine production. The lack of access to screening and high disease burden in developing countries has spurred efforts to develop second generation HPV vaccines that are more affordable, induce wider protective coverage and offer therapeutic coverage against HPV-associated malignancies. Given the previous success with L1 VLP-based vaccines against HPV, VLPs have been also adopted as platforms for many second generation HPV and non-HPV vaccine candidates with both prophylactic and therapeutic intent. Here we examine the progress and challenges of these efforts, with a focus on how they inform VLP vaccine design.

Keywords

Human Papillomavirus; Cervical cancer; Virus-like particles; L1; L2; minor capsid protein; HPV Prophylactic vaccines; VLPs

Introduction: The case for Virus-like particle vaccine systems

It is estimated that 16.1% of all cancers worldwide are caused by known infectious agents[1]. This knowledge that certain infectious agents can trigger cancer has driven the

*Corresponding author: Richard Roden, Department of Pathology, The Johns Hopkins University, Room 308, CRB2, 1550 Orleans St, Baltimore, MD 21287-0014 USA, Tel: 410 502 5161, Fax: 443 287 4295, roden@jhmi.edu.

Financial Disclosure: The study was funded by Public Health Service grantP50 CA098252.

Competing Interests: Richard Roden is an inventor of L2-related patents licensed to Shantha Biotechnics Ltd., GlaxoSmithKline, PaxVax, Inc. and Acambis, Inc. Richard Roden has received research funding from Sanofi Pasteur, Shantha Biotechnic and GlaxoSmithKline and is a founder of Papivax LLC. The terms of these arrangements are managed by Johns Hopkins University in accordance with its conflict of interest policies.

development and licensure of prophylactic vaccines. Two small DNA viruses, hepatitis B virus (HBV) and human papillomavirus (HPV) are each responsible for 5% of cancer worldwide. The concept of preventing cancer via vaccination was demonstrated within the last thirty years with the widespread use of the Hepatitis B virus (HBV) vaccines beginning in the late 1980s with Recombivax-HB® (Merck & Co.) and Engerix-B® (GlaxoSmithKline, GSK). A similar approach is being taken with Human Papillomavirus (HPV) vaccines, beginning with Gardasil® (Merck & Co.) and Cervarix® (GSK) in the late 2000s. These HPV vaccines are remarkably efficacious in protecting healthy individuals from acquiring new infections for at least a decade and exhibit an excellent safety profile[2-5]. Both the HBV and HPV vaccines are based on recombinant virus-like particles (VLPs) [6-9]. As a result of their success in terms of reductions in HBV and HPV related diseases and safety, the focus on VLPs has intensified in the vaccine development field.

VLPs offer several advantages over other types of vaccine platforms. Firstly, VLPs are very safe compared to live or attenuated vaccines. The major structural protein(s) of many viruses when expressed alone are able to self-assemble into highly immunogenic VLPs that mimic the structure of their respective native virion. VLPs however lack the infectious viral genome and this distinction makes VLPs safer vaccine candidates compared to their traditional counterparts such as attenuated or inactive viruses which could revert back into infectious form[10]. It also overcomes many issues of manufacture as viruses generally are prepared in mammalian cells lines, whereas VLPs can be produced in bacteria, yeast or insect cells, greatly facilitating scale up and reducing safety concerns. Secondly, compared to linear peptide vaccines, VLP vaccines are typically highly immunogenic; most VLPs are able to induce very high titers of neutralizing antibodies, often in the absence of adjuvants, and these responses are durable. This is primarily due to VLPs having a highly ordered structure which allows for presentation of repetitive epitopes to B-cells for potent activation or to dendritic cell for stimulation of cell-mediated immunity [11-13]. Thirdly, using molecular biology, one can also use VLPs as scaffolds for heterologous antigens against other infectious diseases. While this approach is promising, it is still unproven in the clinic. Nevertheless, the potential of this approach has led numerous vaccine research groups to continue to focus their efforts on VLP display platforms using either bacterial, plant or mammalian viruses. Fourthly, VLPs are able to break B-cell tolerance in many instances, and this may offer a way to induce antibody to self antigens e.g. to block their deleterious effects by active vaccination rather than using passive therapy with monoclonal antibodies [7]. Fifthly, VLP are taken up by dendritic cells and can present epitopes along the MHC class 1 pathway [8]. This offers an avenue to attach therapeutic T cell epitopes to VLP and develop vaccines to treat pre-existing, established infectious disease or cancer. As our review focuses on VLPs in the context of HPV, our discussion will first give a brief overview on the HPV vaccine field before touching on the successes and failures of recent efforts to create a second generation widely protective HPV vaccine using VLP platforms in both the prophylactic and therapeutic setting. HPV VLPs containing heterologous epitopes are being tested as a platform against other diseases, and technical considerations and key challenges with this approach will be discussed.

An overview of HPV biology

The Human Papillomaviruses are a family of non-enveloped viruses with a T=7*d* icosahedral capsid of approximately 60nm in diameter. Within the HPV capsid is a double-stranded, covalently-closed, circular DNA genome that is approximately 7800 base pairs. The genome encodes six early genes which regulate viral transcription and genome replication (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2) which are involved in the viral capsid structure. L1 is the major capsid protein and it forms the capsid by self-assembly of72 pentamers of L1 (termed capsomers). The other capsid protein, L2, is present

in the capsid at a ratio as high as 1 L2 per 5 L1, i.e. 72 L2 proteins within each HPV virion, although a ratio as low as 1:30 has been described [14,15]. Understanding HPV virology and its role in tumorgenesis is important for facilitating second generation HPV vaccine development, however due to space limitations, this will not be discussed in full detail in this article [reviewed in [14,16]]. Briefly, during intercourse, the cervicovaginal epithelium may experience micro-abrasions which can give rise to the exposure of HPV to their host cells-basal undifferentiated keratinocyte cells of the cervix. Here in the basal cells the viral early genes are expressed and the genome is replicated as an episome at approximately 10^2 copies/cell [14]. After establishing the virus infection in the basal cells, the HPV late gene expression program is triggered by differentiation of the infected basal cell as it divides and leaves the basement membrane. Late gene expression is associated with vegetative over-replication of the viral genome, E4 and capsid gene expression and virion formation. During neoplastic transformation, HPV DNA typically integrates into the host cell genome and loses its episomal form. Integration normally leads to disruption of E2 and loss of late gene expression, i.e. L1 and L2. The inactivation of E2 de-represses the expression of E6 and E7 whose expression drives, and is necessary to maintain, the transformed state. E6 compromises TP53 while E7 inactivates retinoblastoma (most prominent among many other growth regulatory pathways impacted by these two viral oncoproteins) and together, their synergistic effects lead to genomic instability and subsequently, cellular transformation and loss of differentiation, and advancing from high-grade squamous intraepithelial lesions (HSIL) into metastatic disease [10-14].

There are over 100 HPV genotypes with distinct tissue tropism for either cutaneous and mucosal tissue regions[17]. Most HPV infections induce benign and self-limited tumors in the skin (e.g. plantar and planar warts), or the genitals (genital warts). However, certain ‘high risk’ or ‘oncogenic’ types of HPV are a necessary cause of cancers of the cervix, and well as a subset of cancers of the anus, vaginal, vulva, penis and oropharyngeal (head and neck) regions. The vast majority (85-99%) of cervical cancers contain HPV[1,18]. However, while HPV infection is a necessary cause, fortunately most HPV infections do not lead to cancer because they are controlled or eliminated by the host's immune system [19,20]. There are over a dozen high risk types HPV types with the propensity to initiate cancers; however HPV16 is the most potent by far, causing50% of cervical cancer, whereas HPV18 casues another 20%, with the remainder each contributing relatively small fractions of cases. Notably, in the cancers of sites other than the cervix, HPV16 is even more predominant, causing over 90% of other HPV-related anogenital cancers and head and neck cancers [14]. As mentioned earlier, two prophylactic vaccines have been licensed on the basis of preventing persistent HPV infection and the precursors of HPV-related anogenital malignancies, high grade CIN, VIN, VaIN and AIN. The clinical trials also demontrate that Gardasil is effective in preventing genital warts[21]. Both HPV vaccines prevent for at least a decade the acquisition of the precursor lesions of cervical cancer associated with the types targeted by the vaccines, HPV16 and HPV18 [2-5,18]. Data also suggest that HPV vaccination prevents other HPV-associated neoplasia in the vagina, vulvar and anus, as well as HPV16 detection in oral rinses[22]

Current Licensed HPV Vaccines and their recognized limitations

Gardasil® and Cervarix® are based on VLP technology derived by expressing L1, the major protein capsid of the HPV virus, in yeast and insect cells respectively. L1 is able to self-assemble and form immunogenic but non-infectious VLPs that are able to induce similarly high titers of neutralizing antibodies as vaccination with native virions [23]. Administration of L1-based VLPs allows the immune system to generate antibody titers that are 100 fold greater than occur upon natural infection [2-4,9,24-27]. In addition, passive transfer studies using L1-VLP vaccinated animal serum into naïve animals confers protection [24,28,29].

These studies strongly suggest that the HPV VLP induced protection is mediated by humoral immunity but roles for innate immunity and cell-mediated immunity cannot be ruled out.

Despite the recent availability of two commercially licensed HPV vaccines, cervical cancer remains the third most common global cancer killer among women for several reasons (Table 1) [30]. Firstly, the availability of the vaccine is too recent to impact cervical cancer rates, because the cancers evolve over more than a decade and thus the impact on cancer incidence and associated deaths will most likely be felt in 2-3 decades and beyond from its introduction. Secondly, vaccination programs have not reached many low income regions of the world, partly reflecting the relatively high cost of the vaccine. Indeed, 85% of the global burden of cervical cancer occurs in developing countries where most are unable to afford this vaccine (~$360 for full regimen in the US, although tiered pricing has been introduced) [31,32]. However, there are ongoing efforts at regional production of generic vaccines that will likely drive down costs and greatly improve availability.

Current licensed HPV vaccines elicit type-restricted protection, i.e. the vaccines confer protection primarily against the genotypes from which the VLPs in the vaccine were derived [8,33,34]. While there is some clinical evidence for cross-protection against the most closely related types, the levels of cross-protective antibodies detected are often very low or absent and it is unclear whether such cross-reactive immunity can be sustained in the long term [32,35,36]. Current formulations of both licensed HPV L1 VLP vaccines are made based on two oncogenic HPV genotypes, HPV 16 and 18 which account for ~70% of all cervical cancers cases. Gardasil® (Merck) also targets HPV 6 and HPV11 that cause benign genital warts. Vaccinated individuals however are either weakly or ineffectively protected from most of the remaining dozen oncogenic HPV genotypes that collectively cause ~30% of all cervical cancer cases [14,19,37,38]. Furthermore, the vaccines do not target the cutaneous genotypes such HPV1 and HPV2 that cause benign skin warts such as plantar and common warts. A solution towards the problem of type-restricted immunity would be to create more highly valent L1 VLP vaccines for comprehensive coverage [39]. Indeed, Merck currently has a nonavalent vaccine in advanced clinical trials which will likely broaden the efficacy to the other oncogenic HPV types. However, this is a complex and costly approach, and may therefore be difficult to produce at low cost [33]. Therefore, in addition to a therapeutic HPV vaccine, there remains a need to develop a second generation HPV vaccine that is both affordable and provides broad coverage protection against diverse oncogenic and benign HPV types.

Extending HPV vaccine coverage

HPV L2 as an alternative candidate

L2 is the other protein that makes up the HPV capsid and is necessary for PV infection [40,41]. Given its presence in the capsid, it was hypothesized that L2 could also be used as an antigen for HPV prophylactic vaccination. Initial studies using rabbit and bovine papillomavirus models showed that vaccination with the cognate L2 protein prepared by recombinant expression in bacteria confers protection against experimental PV challenge at both mucosal and cutaneous sites and induces low titers of neutralizing antibody[28,42-45]. Further studies showed that there are several cross-neutralizing epitopes on the N-terminus of L2 around amino acid regions 11-88 that offer the potential for broad protection from a single antigen [46-51]. One limitation of these seminal studies was that these animal PV models were unable to dissect the serologic relationship between HPV types [52]. However, this issue has been largely resolved with the development of HPV pseudovirion (PsV) technologies [53-55]. Briefly, HPV PsVs contain the outer capsid structures that mimic native HPV virions but instead of the viral genome they contain a reporter plasmid such as GFP or luciferase that is expressed upon successful “infection”. Importantly, these PsV can

be used to challenge mice intra-vaginally or cutaneously [55] and mouse vaccination studies using PsV are consistent with earlier animal PV studies. This can also be done with 'quasivirions' that contain bovine or cottontail rabbit papillomavirus genomes inside HPV capsid proteins to provide a measurable phenotype (e.g. focal transformation of mouse fibroblasts or warts in rabbits respectively)[56]. Studies using these HPV pseudovirions show that antisera to L2 can cross-neutralize a diverse array of medically significant HPV genotypes [57-60]. Cross-neutralization of a broad range of HPV genotypes by sera and protection against a diversity of HPV genotypes upon vaccination with a single L2 epitope is an important distinction from current L1 VLP vaccines because it suggests the possibility of a simple pan-HPV prophylactic vaccine derived from L2. L2 vaccines can be produced in bacteria, a simple, inexpensive and well characterized system [19] and provide broad immunity despite producing levels of neutralizing antibodies two orders of magnitude lower than L1 VLP. Thus bacterial production of a single broadly protective antigen in a low cost expression system represents an interesting alternative approach to highly multivalent L1 VLP vaccines. However, because of its weak immunogenicity compared to L1 VLP, a key challenge in L2-based vaccine development is effective display of these broadly cross-neutralizing L2 epitopes to the immune system to trigger high titer, high avidity and long lived neutralizing antibody responses.

Challenges in utilizing L2 in HPV vaccines

L2 is not required in HPV VLP formation but does co-assemble with L1 into VLPs. Thus, initial studies attempted to extend protection by purifying L1/L2-VLPs produced in insect cells for vaccination [61]. Disappointingly, this approach was unsuccessful as L2 antibody responses were either absent or very much sub-dominant to L1 in animals vaccinated with L1/L2-VLPs [61]. L1/L2 VLPs produced in insect cells contain approximately 12 copies of L2 per virion in contrast to the expected number of 72 copies [62]. This might explain in part L2's sub-dominance in the context of an L1/L2VLP produced in insect cells as the higher number and ordered arrangement of L1 epitopes would skew the immune response towards L1. However, natural infections trigger L1-specific but not significant L2 responses, suggesting that low L2 occupancy in L1/L2 VLPs is not the main reason for lack of response to L2. Rather *in vitro* data suggests that the virus capsid hides L2 until initiation of the infectious process [14,40] which occurs with the virion binding the extracellular matrix *in vitro*, or the basement membrane *in vivo*.

After binding, the virus undergoes a series of conformational changes which exposes the N-terminus of L2 on the capsid surface (Figure 1) [6,29,63-65]. L2 contains a furin cleavage site at its N-terminus and is thus susceptible to a single cut by the cellular protease furin to remove the conserved, positively charged amino terminus, and this cleavage of L2 by furin is essential to infection. These events display upon the capsid surface several broadly cross-neutralizing epitopes at the N-terminus of L2, including residues 17-36 recognized by the cross-neutralizing monoclonal antibody RG-1 [40,49,65,66]. At present, it is unclear how the altered conformation of L1 and/or cleaved L2 binds to secondary receptors on the basal keratinocyte to facilitate cell entry[61,63,67]. Nonetheless, this transient exposure of L2 epitopes prior to cell entry suggests a need to hide the broadly cross-protective epitopes from the immune system. The cloaking of L2 and subdominance to L1 in the context of the capsid may help explain the lack of evolutionary pressure for these epitopes to change, thereby resulting in the maintenance of this broadly cross-reactive neutralizing epitope. The conservation of the L2 neutralizing epitopes also suggests an evolutionary constraint that may reflect a role in binding to a cellular entry factor during infection. The challenge ahead for L2 vaccines thus, is to find methods to induce and maintain protective levels of antibodies to these L2 epitopes for many decades.

Methods to improve L2-based vaccines

HPV L1-VLPs vaccines displaying L2

To potentially overcome HPV L1 immune dominance by increasing the density of surface display of L2, several groups have produced chimeric HPV VLPs by inserting L2 neutralizing epitopes into the immunodominant L1 neutralizing epitopes of HPV L1-VLPs or BPV1 (Bovine Papillomavirus Type 1) L1-VLPs[68-70]. This concept is promising in that by displaying L2 epitopes on L1 the theoretical amount of L2 epitope exposure would increase approximately 5 fold from the estimated number of 72 to 360. Further, the L2 epitope would no longer be buried and the protective effect of L1 might also be retained. Vaccination studies utilizing this approach reported the presence of L2-specific antibodies via ELISA assays. However, in the context of HPV serology, ELISA assays have been criticized for overestimating the protective antibodies HPV L2 can produce because HPV L2 contains regions that can produce non-neutralizing antibodies. As a result, the HPV PsV *in vitro* neutralization assay is used for measuring HPV specific neutralizing antibodies. Most studies using this approach [68,69,71] showed a low but detectable amount of L2-specific cross-neutralizing antibodies in addition to the high titer L1-specific neutralizing antibody response. While these studies showed that placing L2 epitopes in an immunodominant loop of L1 epitopes did induce a cross-neutralizing L2-specific response, its impact on the antibody avidity and longevity remains to be assessed.

Display in L1 capsomers (made of 5 L1 proteins and the building block for VLPs) could also be another strategy. Garcea and colleagues[72] showed that by deleting the 9 and 26 amino acids from the N and C terminus of L1 respectively, L1 readily forms capsomers (made of 5 L1 proteins) instead of full VLPs. Interestingly, like L1-VLP, L1 capsomers (abbreviated as L1Δ onwards) retain the ability to induce a type-restricted neutralizing antibody, although at somewhat lower antibody titers [59,72]. It is possible that utilizing L1Δ as a platform for cross-presentation of L2 epitopes, one could overcome the problem of L2 epitopes being hidden in normal L1/L2-VLPs. In addition, high amounts of L1Δ can be made in bacteria readily. This vaccine display design and its subsequent production method would potentially solve both the cost of HPV L1-VLP manufacturing as well as the presentation of L2 epitopes.

Our group has also previously developed a broadly protective L2 peptide vaccine candidate ($L2_{11\text{-}88}X5$) by fusing 5 different HPV L2 regions 11-88 (HPV 1, 5, 6, 16, 18) and expressing this multimer in bacteria. While broadly protective against numerous HPV genotypes *in vivo*, our *in vitro* neutralization titer data of $L2_{11\text{-}88}X5$ are still lower than L1-VLP vaccines [58]. HPV VLPs can directly activate dendritic cells and therefore it is possible that they could act as an adjuvant. Thus a possible approach to augment the L2-antibody titers and broaden vaccine coverage at the same time would be to combine our $L2_{11\text{-}88}X5$ with current licensed HPV vaccines to extend protective coverage. To test this hypothesis, we examined mixing of $L2_{11\text{-}88}X5$ protein with either an L1-VLP (Cervarix®) or L1Δ. Neutralization assay data using HPV 16 revealed that $L2_{11\text{-}88}X5$-Cervarix® mixture was comparable to Cervarix® alone and no improvement in antibody titers was seen in the presence of L1Δ. In addition, there was no improvement in the L2-specific antibody titers against other genotypes. These two observations show that when mixing L1-VLPs or L1Δ with L2 peptide vaccines, the L1-specific and L2-specifc responses act essentially independently[59], and that the L2 epitopes likely need to be displayed directly on VLP rather than mixed with them to boost the response.

In another study [73], the L2 residues 13-47 from 3 different high risk HPV types (HPV 18, 31 and 45) were fused in tandem to the C-terminus of HPV16L1Δ, forming the vaccine candidate HPV16L1Δ-L2X3, this candidate was expressed in bacteria with a glutathione-S-

transferase (GST) tag for subsequent downstream protein purification. Vaccination with either HPV16L1Δ or HPV16L1Δ-L2X3 with alum and monophosphoryl lipid A (MPL) induced similar L1-specific protective responses, but the response to the $L2_{13\text{-}47}$x3 fusion peptide was weak suggesting that the epitopes need to be displayed in the immunodominant epitopes of the L1 capsomer structure.

Non-HPV VLPs displaying L2

Since display of L2 in the immunodominant epitopes of L1 VLP induced L2-specific antibody responses that were robust but still much lower than the L1-specific titers, others have opted to display L2-neutralizing epitopes using non-HPV VLPs (Table 2). The potential of non-HPV VLPs displaying L2 epitopes was first demonstrated by Palmer *et al.* [74] who created recombinant tobacco mosaic virus (rTMV) particles that either contained the L2 peptide region 94-122 of cottontail rabbit papillomavirus (CRPV) or rabbit oral papillomaviruses (ROPV). Vaccination studies show efficient protection against homologous challenges. More importantly, because CRPV and ROPV are distantly related, the authors highlighted that vaccination of rTMV-ROPV with an RIBI adjuvant conferred a weak but measurable cross-protective response against cutaneous CRPV challenge which further substantiates the cross-neutralizing ability of L2 in PVs. In a more recent study using HPV L2 instead of animal PVs, Cerovska*et al.* [75] utilized recombinant DNA technology to fuse the DNA of HPV L2 regions 108-120 onto the N or C terminus of a plant viral vector expressing the coat protein of potato virus X (PVX). Theoretically, expression of this viral vector in plants would yield Potato virus X coat-protein (PVX CP) virus-like particles with HPV L2 at the N or C-terminus of the coat protein. The study revealed that only the HPV $L2_{108\text{-}120}$ fused at the N-terminus of the PVX CP ($L2_{108\text{-}120}$-PVX CP) could be expressed. Subsequent vaccination in mice with $L2_{108\text{-}120}$-PVX CP via subcutaneous injection or via tattoo administration followed by ELISA of the vaccinated sera showed that this L2-VLP method was able to induce L2-specific antibodies. However the study did not test neutralization titers for comparison with L1 VLP. Nonetheless, both of these studies show the potential of using a non-HPV VLP for L2 display, although challenges remain in optimizing display. Another highlight of both studies was the use of the well-established plant protein expression system, *Nicotiana benthamiana* to generate the VLPs displaying L2. This has several advantages over current manufacturing methods of licensed VLP vaccines since plants are cheap and easy to grow for large biomass and most importantly, the proteins expressed are usually free from bacterial or animal pathogen toxins that might contaminate the vaccine[76].

Bacteriophage VLPs also have been utilized in generating a pan-HPV VLP vaccine by the Chackerian group whereby they utilized an RNA bacteriophage, PP7, to display the broadly neutralizing L2 epitope (regions 17-31) on the surface loop of the PP7 coat protein[77,78]. Initial studies were focused on creating two L2-PP7 VLPs derived from HPV16 and HPV45 sequences. The vaccination studies with incomplete Freund's adjuvant showed substantial cross-protection, for example, HPV 16 L2-PP7 vaccinated mice were significantly protected from HPV45 PsV vaginal challenge compared to PP7 VLP alone although not completely [78]. Subsequently in another study by the same group, a total of 8 different L2-PP7 VLPs were produced, each displaying the $L2_{17\text{-}31}$ of a different HPV type respectively (HPV 1,5,6,11,16,18,45 and 58)[77]. These high and low-risk HPV types were chosen presumably because they represent common members of medically relevant HPV types. Vaginal- and cutaneous- challenges were performed subsequently using these different PsV types and HPV31 whose L2 was not included in L2-PP7. Overall, the results showed the greatest protection was exhibited in mice that were vaccinated with a mixture containing all 8 types of L2-PP7 VLPs. These L2-PP7 mixture vaccinated mice also were protected from HPV31 PsV challenge highlighting substantial cross-protection. There are several useful features of

this system. Firstly, PP7 can be simply expressed at very high levels in bacteria. Secondly, two rounds of immunizations were sufficient in their studies to elicit protection. Thirdly, no adjuvant was requied for the L2-PP7 mixture vaccine as PP7 particles carry ssRNA which can potentially act as an endogenous adjuvant to activate TLR 7 or 8 signaling [77]. Taken together, the results suggest the potential of this bacteriophage method as a pan-HPV vaccine. It would be interesting to see future studies looking at the longevity and avidity of the L2 epitope-specific neutralizing responses to L2-PP7 vaccination compared to other approaches.

Non-HPV mammalian VLPs, such as Adeno-Associated virus-like particles (AAVLP), have also been explored as platforms to display L2. AAVLPs are a promising platform for heterologous HPV L2 display because the viral capsids are able to self-assemble efficiently despite insertion of foreign peptide epitopes into the sequence. In a recent pilot study to explore the potential of AAVLPs, Nieto *et al.* [79] generated prophylactic HPV vaccines made of AAVLPs displaying HPV16 and 31 regions 17-36 of L2. This method showed cross-neutralizing serum in both rabbits and mice. However, an adjuvant was required to elicit the high titers and the duration of the response is not known.

Therapeutic VLP-based HPV vaccines

Rationale of therapeutic HPV vaccination

Pap screening and intervention is a proven strategy in the control of cervical cancer[80] and can reduce the incidence by 60-90% within 3 years of its introduction to populations naïve to screening. It is possible that prophylactic HPV vaccines, when widely administered and proven as effective in reducing cervical cancer rates, will impact the need/interval for screening services or developing therapies such as therapeutic vaccination. However, at present, HPV infection and associated diseases are widespread, and the need for a therapeutic vaccine remains to help those currently afflicted with HPV disease and to accelerate the reduction of cancer rates. Furthermore, both vaccination and cytologic screening implementation is far from complete in many countries. This need is also compounded by the potential advent of first line HPV DNA testing for cervical screening, which will identify millions of infected individuals. The HPV infected patients must be continually screened until they clear the infection or develop high grade neoplasia, whereupon they undergo an ablative therapy. These ablative treatments of high grade neoplasia are associated with significant side effects and costs, and do not impact non-cervical disease. Therefore a therapeutic HPV vaccine could be helpful for these patients. Current licensed HPV L1-VLP vaccines (as well as the various L2 strategies discussed so far) are utilized for prophylaxis and offer little or no therapeutic value [25]. New, more effective treatments for existing non-cervical high grade neoplasia and HPV-associated cancer, which remain prevalent, are urgently needed [33].

During the viral life cycle, detectably expression of L1 and L2 capsid proteins occurs only upon terminal differentiation of the infected keratinocytes. Thus, neither cervical cancer cells, nor the infected basal cells attached to the basement membrane, express the capsid proteins. As a result, the early genes of HPV are more appropriate targets for therapeutic immunization. E1 and E2 are possible candidates but they are not typically expressed in cancer. E5 is very small and poorly immunogenic, and E4 is more of a late protein, being undetectable in basal cells. In contrast, E6 and E7 are expressed in all HPV infected cells, and are up-regulated in cancer cells, and thus are generally considered the logical targets for therapeutic vaccination. E6 and E7 have already been targeted in numerous HPV therapeutic vaccination strategies including peptide, protein, live vector, DNA and whole-cell based approaches. Due to space limitations, our discussion will be focused on the efforts using VLP-based therapeutic vaccine approaches. This is an attractive approach that can

potentially combine both prophylactic with therapeutic effects. For other therapeutic strategies, the reader is directed to [81,82].

Chimeric VLPs for therapeutic vaccination

Conventional therapies for cervical cancer are often invasive and toxic because they do not directly target the virus. The use of therapeutic vaccination against cervical cancer is thus attractive since it introduces the possibility of a non-invasive approach to directly target HPV infected cells via the induction of CD8 cytotoxic T-lymphocyte responses (CTLs), thereby reducing the potential for side effects. Indeed, most HPV infections are spontaneously cleared by the immune system, but this becomes a much less frequent outcome in immunosuppressed patients, or those with more advanced disease. HPV E6 and E7 peptide sequences contain several T cell epitopes which can elicit an anti-tumor CTL response [82-84]. However, typically the T-cell responses induced by natural infection are very low or undetectable, possibly reflecting the localized nature of the infection and viral immune evasion mechanisms, and indicating the potential for vaccination to improve E6/E7-specific immune responses. As mentioned, HPV VLPs are able to directly activate dendritic cells, and despite being an exogenous antigen, they can provide MHC Class I presentation by pseudo-infecting cells [12,85]. Thus, in addition to inducing high antibody titers, VLPs contain features that readily induce cell-mediated immunity.

Utilizing the advantages of VLPs, numerous groups have attempted to improve E6/E7 T-cell responses by forming chimeric HPV VLPs (cVLPs) in which the T-cell epitopes of HPV E6, E7 or both are fused to the C-terminus of either HPV L1-VLP or L1/L2-VLPs. Chimeric VLPs can thus act like a ‘delivery vehicle’ carrying the extra epitopes of the non-structural HPV proteins into the target cell for entry into the class I presentation pathway. Fusion of E7 protein to the C-terminus of L1 in VLPs provides a strong specific CTL response. However, there are significant constraints to the size of the E7 polypeptide fused to L1 before it results in impaired cVLP production. Conversely, fusing E7 to L2 in L1/L2E7-cVLPs is more tolerant to larger fusions without disrupting assembly, and we note that L2 is required for viral infection but not class I antigen presentation of the E7 epitopes included in the cVLPs [86]. Indeed, both approaches provide *in vivo* tumor protection in mouse models. The cVLPs have also been modified for inexpensive production in bacterial or plant expression systems [87-92].

The data discussed in this section on therapeutic cVLPs is largely pre-clinical and there is limited information about the safety and therapeutic efficacy of using cVLP in humans affected with cervical cancer. The first phase 1 clinical trial of cVLP was published in 2007. HPV16 L1E7 cVLPs were administered 4 times to patients with HPV16-positive high-grade cervical intraepithelial neoplasia (CIN2/3) [93]. The HPV16 L1E7 cVLPs were well tolerated and able to induce cellular immune responses in vaccinated patients. Unfortunately, in terms of clinical efficacy which was measured via 1) 50% lesion size reduction and 2) disappearance of HPV 16 DNA, there was no significant difference in either parameter between placebo and vaccinated groups. In addition, even though cellular immune responses were detected in patients, they did not clearly correlate with clinical response. This is in contrast to pre-clinical mouse data whereby the clearance of HPV induced tumors is clearly correlated with specific E6 or E7-CTL responses induced by the cVLPs.

One possible reason for the poor clinical efficacy of the HPV16 L1E7 cVLPs could be pre-existing neutralizing antibodies against HPV16 L1 (triggered by natural infection) that would limit the effectiveness of the cVLP vaccination, as shown in cVLP vaccinated mice by Da Silva and colleagues [94]. To circumvent this issue, Da Silva *et al*[95] successfully showed that a prime/boost strategy using heterologous cVLPs made from BPV and COPV

but still fused with HPV16 E7 epitope could potentially bypass the pre-existing neutralizing antibody effects. Since the safety profile of the vaccine is high and a strong T-cell response was observed, it would be interesting to see if this heterologous prime/boost vaccination method could improve correlative responses in future human trials with HPV cVLP vaccines. Another possible reason for the difference between the response to the cVLP vaccination against human high grade cervical disease and the mouse model is the locality of the disease. The mouse TC-1 challenge model is systemic, whereas CIN2/3 is restricted to the patient's cervical epithelium. Therefore, a vaccination strategy to target or 'pull' the induced E7-specific CTL to the site of infection in the cervical epithelium may be required.

In the case of cervical cancer, specific CTLs against the cervical lesions are 'stuck' at the lymphatic regions and are unable to interact with the tumor lesions. The factors hampering this have not been fully identified although it has been attributed to a combination of T-regulatory cells and myeloid-derived suppressor cells that impact the proper homing and killing function of tumor-specific CTLs [96-98]. Clearly, the local tumor microenvironment plays a huge role in hindering the success of immunotherapeutic approaches. Taken together, it would be pertinent to consider combining therapeutic cVLPs with either immunomodulatory agents or other therapeutic modalities to alter the tumor micro-environment to improve vaccine potency (e.g via CTL access to the lesion and overcoming the local immune suppressive environment). Such combinatorial studies have already been tested using HPV therapeutic DNA vaccines whereby the depletion of regulatory T-cells has helped to improve therapeutic potency [99]. In another interesting study, HPV therapeutic DNA vaccination combined with chemotherapeutic agents resulted in enhanced anti-tumor effects [100]. These approaches however have been tested in mouse models only and thus it remains to be seen if such methods can be used in clinical settings.

HPV VLPs as delivery platforms

HPV L1/L2-Pseudovirions as carriers for DNA vaccines

Naked DNA-based vaccines have great potential for the induction of therapeutic T cell responses. However, one major obstacle in the field of DNA vaccination is the delivery of the DNA vaccines into target cells, especially antigen presenting cells. The utilization of L1/L2 VLPs to encapsidate a DNA vaccine that creates infectious but non-replicative HPV pseudovirion (PsV) is a promising technology to overcome the challenge of plasmid delivery in vivo [53]. Several groups have tried previously using L1-VLPs to facilitate DNA vaccine delivery; however because L1-VLPs lack L2 that is necessary for HPV infection, this approach is likely inferior compared to using HPV PsV. HPV PsV have been tested as a vaccine delivery platform against non-HPV diseases such as respiratory syncytical (RSV) virus and in Human Immunodeficiency virus (HIV) vaccine research using an SIV (Simian immunodeficiency virus) model[101,102]. In both studies, the specific CD8+T cell responses towards the respective DNA antigen were far higher than naked DNA vaccination, and the mice were vaccinated intra-vaginally because HPV PsV do not infect intact normal epithelium. Intra-vaginal vaccination warrants particular consideration for mucosal immunization as many viruses such as HIV, Herpes simplex viruses, share this same site of infection; induction of immune responses at the mucosa (e.g vagina) locally could thus be advantageous in several disease systems. Peng *et al*[103] generated HPV 16 PsV encapsidating a DNA vaccine encoding the ovalbumin (OVA) antigen. The results showed that PsV delivery method was able to generate a stronger OVA-specific CD8+ response compared to other routes of administration such as gene gun delivery, providing further support for HPV PsV as a promising platform for DNA-based therapeutic vaccination.

Expert commentary and 5 year outlook

Although vaccine development has been successful, there are still numerous issues that need to be addressed. It is important to recognize that pre-cancer was the primary efficacy endpoint (high grade cervical intraepithelial neoplasia (CIN) or adenocarcinoma in situ (AIS)) of the vaccine trials. Thus the demonstration of the true impact of the vaccines on cervical cancer rates in the population will take time, reflecting the 10-30 year process of HPV carcinogenesis and the delays in vaccine delivery nationally. However, we feel that there is reason to be optimistic since efficacy has been demonstrated in preventing both genital warts and precursors of cervical cancer in a national vaccine program setting in Australia. In addition, there is also no information on the duration of vaccine efficacy beyond a decade given its recent development and we will have to wait for these phase 4 studies to mature. If immunity wanes, then a booster may be required. The sufficiency of only 2 doses instead of 3 (to increase cost effectiveness), or the need for an additional booster (4^{th} immunization) to achieve lifetime immunity remain open questions.

We also await the outcome of the efficacy studies of Merck's nonavalent HPV L1 VLP vaccine that are currently ongoing. This vaccine targets the two most common HPV types in genital warts, and most importantly, the seven most common oncogenic HPV types found in cervical cancer (HPV16, 18,31,33, 35, 52, 58). Should this vaccine prove safe and effective in providing the type of broad and durable coverage of oncogenic HPV types needed to eventually eradicate HPV-related anogenital malignancies, it would likely dramatically reduce or eliminate the need for cytologic screening in the appropriately vaccinated population.

From a public health perspective, the introduction of Merck's nonavalent HPV vaccine is unlikely to solve the problem of cost. Hence, in parallel with this are numerous efforts to develop generic HPV L1 VLP vaccines and L2-based approaches intended for local production at low cost to broaden access to this important opportunity for cancer prevention across the globe and to better reach under-served populations. Furthermore, efforts to simplify delivery of HPV vaccines must continue, including examining co-delivery of HPV VLP vaccines with vaccines for other agents, and whether HPV vaccination could be initiated with childhood vaccination. A careful consideration of the impact of HPV vaccination on screening also must be made with respect to cost benefit, and whether the consequent reduction in the prevalence of cervical neoplasia renders the Pap smear insufficiently predictive as a first line screening tool. Another critical issue to address is whether HPV VLP vaccines protect men and women from HPV infection in the oral cavity, and thereby from developing HPV-associated head and neck cancers. Currently there is early evidence to support this possibility[22] and rates of HPV-associated head and neck cancers should be followed in phase 4 vaccine studies in men and women.

For further improvements in the field of VLP vaccinology against HPV or other pathogens and diseases, it is important that the dissection of the B cell epitope display and T cell epitope deliver mechanisms continues. It is clear that VLPs do not all behave the same way; for example HPV VLP directly activates dendritic cells but human polyomavirus VLPs do not. The mechanisms and consequences of this for the immune response are not fully understood, and may impact the induction of cellular immune responses to heterologous T cell epitopes carried by chimeric VLP. The insertion of heterologous epitopes into VLP often reduces particle assembly warranting structural studies of the folding and assembly processes. The immunodominant VLP epitopes are typically conformational, i.e. formed from residues on multiple different loops, rather than linear like the heterologous epitopes being inserted (e.g. L2 17-36). This may explain why the inserted heterologous epitope is immunogenic, but not nearly to the level of the original immunodominant epitope site. More

research is needed to understand structurally how best to present these epitopes to B cells such that they are even more immunogenic to the level of HPV VLP. Perhaps a better approach is to use a monoclonal antibody with the appropriate properties to select the VLP structure from a random phage library, for example, especially if the monoclonal antibody recognizes a conformation epitope. We believe that this will be a fruitful area in VLP design in the near term for both xenogenic epitopes of infectious agents and even potentially self-epitopes such as those of tumor antigens. Indeed there is growing interest in prophylactic vaccination against tumor antigens in patients genetically at high risk for particular malignancies to generate protective humoral and cellular immune responses.

Conclusion

Since HPV is currently responsible for genital warts and 5% of all cancer cases worldwide, the licensure in numerous countries and implementation of many national immunization campaigns for the prophylactic L1 VLP vaccines is potentially of profound benefit to human health. Early indications of such benefits are already seen in Australia and we are optimistic that this vaccine or a more broadly protective 2nd generation successor will be a important complement to the demonstrated success of Pap screening in cervical cancer prevention, and the emerging implementation of HPV DNA screening. Nevertheless, much work remains to ensure global access to HPV vaccination and broadening coverage to all oncogenic HPV genotypes. How these HPV VLP vaccines are so effective should be studied in detail, and information about the key mechanisms can be utilized to drive rational development of VLP vaccines to targets other than HPV. Efforts to display broadly protective L2 epitopes in various VLP to develop a pan-HPV vaccine are ongoing, but much remains to be learned about the optimal approach to insert heterologous epitopes into conformation-specific immunodominant VLP epitopes. HPV VLP can also be utilized to display other epitopes and generate robust humoral responses, even to self-antigens, suggesting promise in many other applications such as combating arthritic disease, Alzheimer disease and other infectious agents. HPV VLPs are particularly interesting in their ability to directly active dendritic and other antigen-presenting cells, and to present heterologous epitopes, including viral and tumor antigens, via MHCI to generate therapeutic immune responses. With only a single trial using such chimeric HPV L1-E7 VLP, these studies remain in their infancy. However, it is clear that in addition to triggering robust E6 and/or E7 antigen-specific T cell responses, considerations of targeting cytotoxic T cell responses to disease sites, and overcoming local tolerogenic factors are likely to influence the success of therapeutic HPV vaccines based on chimeric VLP vaccines.

Acknowledgments

Funding: Public Health Service grants CA133749, CA118790, P50 CA098252 to RBSR

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Key Issues

- Cervical cancer and other HPV-associated malignancies are potentially preventable through HPV VLP vaccination, but continued follow up is required to demonstrate their impact on cancer incidence and mortality.
- Implementation of cytologic screening and intervention can reduce cervical cancer rates 60-90% in naïve populations and this must be considered with respect to the cost/benefit of vaccination.
- Careful consideration of how to globally implement of HPV vaccination is required to realize a dramatic reduction in rates of HPV-associated cancer, but there is an extended lead time to achieve this goal.
- Widespread HPV vaccination is likely to impact cytologic screening approaches (i.e. Pap screening versus HPV DNA testing first), timing (when to start and intervals). The costs and benefits of all these approaches requires careful consideration and proper implementation to achieve the best reduction rates in cervical cancer.
- Low cost vaccines and overcoming logistical barriers to delivery are critical as current vaccines are not practical for the developing world. Generic, local production of heat-stable formulations of L1 VLP in low cost expression systems that are effective as a single dose would be advantageous.
- To inform rationale design of VLP-based vaccines to other agents, further mechanistic study of the human immune response to HPV VLP, especially early innate events and the induction of longterm memory, is required to understand how they are so immunogenic and effective.
- Defining an immune correlate of protection is critical so that individual patients can know that they are effectively vaccinated (i.e. immune to HPV at that time)
- Robust and long lived protection must be extended to all oncogenic HPV types by developing highly multivalent L1 VLP formulations, or possibly by use of L2.
- VLP show promise for display of heterologous linear epitopes such as L2 to induce robust titers of high avidity and long-lived antibodies, but this potential has not been fully realized suggesting suboptimal presentation to the immune system. More study of immunodominant conformational epitopes in VLP is required to understand how best to generate similar responses to heterologous linear epitopes inserted into these structures.
- HPV infection and associated diseases are widespread, and the need for a therapeutic vaccine remains.
- Chimeric VLP can effectively deliver viral antigens to MHCI and generate a robust cellular immune response. However, to date, cVLP have not generated therapeutic responses in patients, suggesting consideration of targeting cellular immunity to the lesion site (e.g. route and site of immunization) and approaches to modulate the lesion microenvironment are warranted.

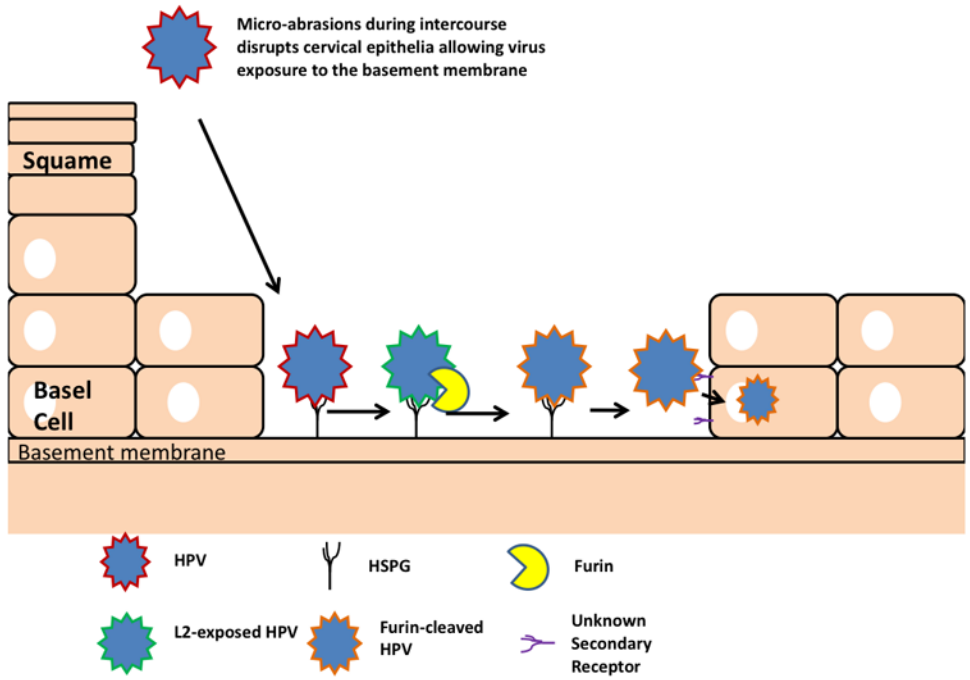

Figure 1.

Model of infection of the cervical epithelium by HPV (Developed by Day *et al.*). Upon access to the basement membrane, HPV binds to the basement membrane before undergoing a series of conformational changes in capsid structure that ultimately leads to the transient exposure of its L2 minor capsid protein (See text for full details). Upon its exposure, L2 is cleaved by furin, and the furin-cleaved virion is then taken up by the basal epithelial cells to initiate infection.

Table 1

Recognized limitations of current licensed HPV Vaccines- Cervarix® (GSK) and Gardasil® (Merck & Co.)

Limitation	Impact	Solution and Alternatives
Cost of manufacturing, trials and IP leads to high vaccine price	Developing countries that do not have the economic means to afford the vaccine continue to bear the burden of cervical cancer	Investigate manufacturing generic L1 VLP vaccines in cheaper production systems like plants or bacteria, and reduce number of doses or combining with childhood vaccines
Type restriction	Vaccine does not protect against all HPV types causing cervical cancer, thus screening must continue for HPV-vaccinated women.	More HPV VLP types to create a highly multivalent HPV vaccine Alternative vaccine targeting broadly protective antigen L2
No therapeutic value	No effect on individuals with pre-existing HPV infection	Alternative vaccine constructs displaying E6 and E7

Table 2

Summary of VLP-based prophylactic HPV vaccines displaying L2

2nd generation prophylactic HPV VLP candidate vaccines based on HPV L2	Potential or current advantages over licensed HPV L1-VLP vaccine	Potential or current disadvantages	References
L1-VLPs expressing L2 epitopes	Broad spectrum protection	L2-specific neutralizing antibody titers detected were low. Uses same expression system as L1-VLPs (Cervarix® or Gardasil®) which does not bring down cost of potential vaccine	[68-70]
L1-Capsomers	Bacterial expression system lowers the cost of manufacturing	Lower antibody avidity compared to L1-VLPs. Type restricted protection but can be overcome by creating a variety of capsomeres or combinatorial vaccination methods with L2	[59,72,88]
Plant-VLPs e.g Tobacco Mosaic Virus	Broad spectrum protection. Plant expression system lowers the cost of manufacturing. Reduced issues of bacterial endotoxin contamination	Studies using this method showed L2 antibody neutralizing titers that were low	[75,87]
Bacteriophage VLPs	Broad spectrum protection. Bacterial expression system lowers the cost of manufacturing. No adjuvant needed?	Endotoxin contamination	[77,78]
Non-HPV Mammalian virus VLPs e.g AAVLP	Broad spectrum protection	Requires adjuvant. Pre-existing neutralizing antibodies against the AAV or other carrier	[79]