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Developments in L2-based Human Papillomavirus (HPV) Vaccines

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Abstract

Infections with sexually transmitted high-risk Human Papillomavirus (hrHPV), of which there are at least 15 genotypes, are responsible for a tremendous disease burden by causing cervical, and subsets of other ano-genital and oro-pharyngeal carcinomas, together representing 5% of all cancer cases worldwide. HPV subunit vaccines consisting of virus-like particles (VLP) self-assembled from major capsid protein L1 plus adjuvant have been licensed. Prophylactic vaccinations with the 2-valent (HPV16/18), 4-valent (HPV6/11 /16/18), or 9-valent (HPV6/11/16/18/31/33/45/52/58) vaccine induce high-titer neutralizing antibodies restricted to the vaccine types that cause up to 90% of cervical carcinomas, a subset of other ano-genital and oro-pharyngeal cancers and 90% of benign ano-genital warts (condylomata). The complexity of manufacturing multivalent L1-VLP vaccines limits the number of included VLP types and thus the vaccines' spectrum of protection, leaving a panel of oncogenic mucosal HPV unaddressed. In addition, current vaccines do not protect against cutaneous HPV types causing benign skin warts, or against beta-papillomavirus (betaPV) types implicated in the development of non-melanoma skin cancer (NMSC) in immunosuppressed patients. In contrast with L1-VLP, the minor capsid protein L2 contains type-common epitopes that induce low-titer yet broadly cross-neutralizing antibodies to heterologous PV types and provide cross-protection in animal challenge models. Efforts to increase the low immunogenicity of L2 (poly)-peptides and thereby to develop broader-spectrum HPV vaccines are the focus of this review.

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Disclosures

Under a licensing agreement between Sanofi-Aventis and the Johns Hopkins University, RBSR is entitled to a share of royalty received by the university on sales of products. The value of these products could be impacted by the outcome of the study described in this article. Under a licensing agreement between PaxVax, the NIH, and the Johns Hopkins University, RBSR is entitled to a share of royalty received by the university on sales of the vaccine described in this article. RBSR is a co-founder of and has an equity ownership interest in Papivax LLC. Also, he owns Papivax Biotech, Inc. stock options and is a member of Papivax Biotech, Inc.'s Scientific Advisory Board. Under a licensing agreement between Papivax Biotech, Inc. and the Johns Hopkins University, the university is entitled to royalties on an invention described in this article. Under an option agreement between PathoVax LLC, the Johns Hopkins University, and University of Vienna Medical School, CS, RBSR and RK are entitled to distributions of payments associated with an invention described in this publication. CS, RBSR and RK also own equity in PathoVax LLC and are member of its scientific advisory board. These arrangements have been reviewed and approved by the Medical University of Vienna and the Johns Hopkins University in accordance with their conflict of interest policies.

Keywords

Human Papillomavirus (HPV) Vaccine; Minor Capsid Protein (L2); Cervical Cancer (CxCa); Squamous Cell Cancer (SCC); cutaneous and anogenital warts (condylomata); HPV infection and disease

1. Infections with Human Papillomaviruses (HPV)

Human Papillomaviruses (HPV) infect the epithelia of skin and mucosa in humans, and more than 200 genotypes have been completely sequenced (pave.niaid.nih.gov; International Human Papillomavirus Reference Center; http://www.hpvcenter.se)(Bernard et al., 2010). The diseases caused by HPV range from benign skin warts and mucosal papillomas (condylomas) to dysplasias and carcinomas of the ano-genital tract and oro-pharynx, although many infections remain subclinical (Bruni et al., 2010). Most often infections are cleared and regression of lesions is mediated by a cellular immune response (reviewed in (Stanley, 2001)). Persistent infection with high-risk HPV (hrHPV) types can give rise to high grade squamous intraepithelial lesions (HSIL) that can eventually progress to invasive cancers if left untreated. Classification of HPV by their dominant site of infection discriminates cutaneous and mucosal types, although site-specificity is not complete and interchange of anatomical sites occurs. Taxonomically, HPV types are classified into genera, species, subspecies and types by sequence similarities of the major viral capsid protein (L1) (De Villiers et al., 2004).

Mucosal HPV (all found in genus alpha) infect ano-genital and oro-pharyngeal mucosal surfaces almost exclusively by sexual contact. Genital HPV is the most frequent sexually transmitted infection (STI) with an estimated cumulative lifetime prevalence of ~ 70% in both sexes. A subset of low-risk HPV (lrHPV), mostly HPV 6 and 11, cause benign, yet incommodious and sometimes tediously manageable benign genital warts or condylomata acuminata (Hu and Goldie, 2008). However, infrequently these types cause recalcitrant laryngeal infections (recurrent respiratory papillomatosis) that can be life-threatening, or rarely, genital verrucous carcinoma Buschke-Löwenstein tumor.

Persisting infection with one of more than 15 types of mucosal hrHPV increases the risk for malignant transformation of the epithelial keratinocytes resulting in squamous intraepithelial neoplasias (SIL) that may progress into invasive cancers of the cervix, anus, vulva, vagina, and penis. Virtually all cervical cancers (CxCa) are caused by hrHPV, predominantly by HPV16 (~50%) and HPV18 (~20%) and less frequently at least a dozen other hrHPV types (e.g. HPV31, 33, 45, 52, 58, ….) (de Sanjose et al., 2010; Muñoz et al., 2004). An estimated number of 275.000 women die every year from CxCa worldwide, with an overwhelming proportion of victims in developing countries, whereas introduction of cytological (PAP) screening has considerably decreased CxCa in developed countries. Although rare overall, anal cancers are prevalent in high-risk populations (e.g. HIV-positive individuals) and ~90% are positive for hrHPV (mostly HPV16), while about 40% of cancers of the vulva, vagina and penis are attributable to hrHPV infections (Lowy and Schiller, 2012). Remarkably, the growing number of oro-pharyngeal (tonsils and base of tongue) cancers appear causally associated to HPV with ~85% positive for HPV16 (Kreimer et al., 2005).

The highly divergent group of common cutaneous HPV types within genus alpha, gamma, mu and nu types are transmitted via direct contact or contaminated objects (e.g. floors) and cause common warts of the skin (most often hand and feet), mainly affecting school children with self-limited disease (Van Haalen et al., 2009). Yet persisting warts are a common nuisance and can be a disfiguring affliction in immunodeficient individuals (e.g. organ transplant recipients) (Lally et al., 2011). Patients with the rare genodermatosis Epidermodysplasia Verruciformis (EV) develop generalized flat warts and macular lesions due to genetic susceptibility to infection with the distinct large group of genus beta HPV types (betaPV). EV patients develop early in life non-melanoma skin cancers (NMSC), i.e. basal cell cancers and squamous cell cancers, predominantly in sun-exposed areas indicating a causal role of chronic betaPV infection as adjunct to UV-light in skin carcinogenesis. Importantly, betaPV have also been implicated in NMSC development in the growing number of organ transplant recipients (OTR).

PV have co-evolved with their respective hosts and have acquired biological and immunological features required for their strict epitheliotropic life-cycle. PV are non-enveloped double-stranded DNA tumor viruses with ~8 kb genome encoding for early (E) and late (L) proteins. Following initial infection of basal epithelial cells exposed by microtrauma, the virus genome is maintained in low copy number in basal cells. The productive viral life cycle is tightly linked to the differentiation of the stratified epithelial layers. Production of capsid proteins L1 and L2 and encapsidation of the genome occurs only in the uppermost layers of the epithelium, and assembled infectious virions are shed off with detached keratinocytes. The icosahedral virus capsid is mainly constructed from 72 pentamers of the major capsid protein L1 (360 copies of L1). The minor capsid protein L2 is believed located mostly within the center of the pentamers in an estimated L2:L1 ratio of 1:5–1:10. Persistence of HPV infections and induced lesions are facilitated by the immunoprivileged site of local infection within the external layers of the epithelium providing insufficient access of antigen presenting cells, the non-lytic virus replication cycle and the lack of systemic viremia.

2. Licensed HPV Vaccines (L1-VLP Vaccines)

The finding that recombinantly expressed L1 protein alone can self-assemble into virus-like-particles (VLP) launched the development of prophylactic HPV vaccines (Hagensee et al., 1993; Kirnbauer et al., 1992, 1993; Rose et al., 1993). These VLP are morphologically and immunologically similar to native virions with type-specific conformation-dependent epitopes repetitively presented on the VLP’s surface. The dense arrangement of identical epitopes effectively triggers B-cell immunological responses by pattern recognition receptors (e.g. toll-like receptor TLR-4) when the vaccine is administered systemically (e.g. intramuscularly) (Einstein et al., 2009). High-titer and long lasting neutralizing antibodies against conformational epitopes are induced when administered with and even without adjuvant. Early animal models (CRPV, BPV1) have demonstrated VLP vaccine efficacy irrespective of the presence of L2 (Breitburd et al., 1995; Ghim et al., 1996). Although the mechanism of protection is not fully understood, protection against experimental challenge is conferred by passive transfer of serum, which underlines the crucial role of neutralizing antibodies to confer sterilizing immunity thereby preventing virus binding to the cell surface

and productive virion uptake. In 2006 two L1-VLP vaccines were licensed; Cervarix and Gardasil. The bivalent Cervarix contains VLP of hrHPV16 and 18 produced in insect cells and ASO4 adjuvant (aluminum hydroxide plus monophosphoryl lipid A). The quadrivalent Gardasil contains VLP of hrHPV16 and 18, and additionally of lrHPV6 and 11, produced in yeast and formulated with aluminum hydroxyphosphate sulfate adjuvant. Extensive clinical and post-licensure studies have reported high-efficacy (up to 100%) against cervical, vulvar, vaginal intraepithelial neoplasias for both vaccines when given to virus-naïve individuals. Protective efficacy against anal intraepithelial neoplasias and genital warts was demonstrated for Gardasil (Lehtinen et al., 2012; Muñoz et al., 2010; Palefsky et al., 2011). Highest efficacy is achieved by prophylactic vaccination of sexually naïve adolescents with a rapid decline in impact following sexual debut, since the vaccine has no therapeutic effect on prevalent infections or existing genital lesions.

The induced systemic antibody titers are dramatically higher than those after natural infection, and following initial decline seem to plateau to several fold higher for at least 10 years as observed in clinical studies. No severe side effects have been causally related to the vaccine in post-licensure surveillance, while minor side effects include mostly local erythema and pain (Einstein et al., 2014). The introduction of HPV vaccines in Europe, North and South America, Japan and Australia has achieved varying coverage rates, resulting from differences in public awareness, financial reimbursement and parental acceptance. Four years after high-coverage vaccination of young girls in Australia with the 4-valent vaccine the prevalence of genital warts has dramatically declined in vaccinated women, but also in young men due to herd effect (Fairley et al., 2009).

For all the exceptional success of the first generation 2- and 4-valent HPV vaccines several limitations remain to be addressed. Most importantly, efficacy of L1-VLP vaccines is restricted to the included types, although limited cross-neutralization has been reported for closely related hr types HPV31, 45 (Toft et al., 2014). Thus persistent infections with other than vaccine types cannot be significantly reduced, which leaves hr types causing ~30% of all CxCa unaddressed and renders the vaccines even less satisfactory for developing countries without existing cervical screening programs. This inadequate protection has led to the recent licensure of a 9-valent L1-VLP vaccine (Gardasil-9), which includes VLP of mucosal hrHPV16, 18, 31, 33, 45, 52, 58 and lrHPV6, 11 with the potential to protect against 90% of both CxCa and genital warts (Joura et al., 2015, 2014). A crucial impediment for introducing HPV vaccination programs in many low-resource countries is the high costs of L1-VLP vaccines. This is temporarily relieved by agreements of the GAVI Alliance with vaccine manufacturers, which has negotiated vaccine prices as low as 5$(US) for Gardasil-4 and has started vaccination support in eligible developing countries (Hanson et al., 2015; Herrero et al., 2015). Additional impeding factors for global vaccine programs are the required cold chain and the need for repeat vaccinations. Recently, a 2-dose vaccination protocol has been established in adolescents. If extended to young adults, such simplified programs could be expected to significantly reduce costs and raise acceptance of vaccination programs (Kreimer et al., 2015; Sankaranarayanan et al., 2016).

Despite this considerable progress, it is as yet unclear whether manufacturing costs can be reduced to make highly multivalent L1-VLP vaccines widely attractive to populations most

in need. Due to technical complexity, the development of highly multivalent VLP formulations, that protect against all relevant mucosal hrHPV, and moreover against so far unaddressed cutaneous HPV types, is unlikely. Such a vaccine against cutaneous genus alpha HPV could target a considerable proportion of skin and palmo-plantar warts, a common nuisance in children and immunocompromised adults. In addition, if an adjunct causal role of betaPV in the development of NMSC in immunosuppressed patients can be proven, targeting the large spectrum of betaPV would be highly desirable for patients before organ transplantation to reduce their high risk for skin cancer.

3. Immunogenicity of the minor capsid protein L2

The minor capsid protein L2 is involved in papillomavirus entry of the cells (Y. Kawana et al., 2001; Yang et al., 2003a), the escape from endosomal vesicles and the subsequent transport of the viral genome into the nucleus (Day et al., 1998; Yang et al., 2003b). L2 is nonessential for capsid formation (Chen et al., 2000) and is mostly inaccessible to antibody and thus presumably located below the surface of the native virion (Kines et al., 2009; Richards et al., 2006). Following contact of the PV virion with the epithelial basal membrane at a minor abrasion, a conformational change of the capsid occurs. This shift exposes the first few positively charged amino acids (aa) which are then cleaved off at a specific site by the cell surface proprotein convertase furin. Furin cleavage subsequently uncovers L2 cross-neutralization epitopes (Kines et al., 2009). The minor representation of L2 compared to the dense and highly repetitive display of L1 epitopes (and its likely location below the capsid surface) renders L2 immunologically subdominant to L1 in natural infections or vaccinations with co-assembled L1+L2 VLP. Due to immune evasion mechanisms of HPV in natural infection L1 specific antibody responses are induced in about 50% of infected individuals (Carter et al., 2000), with titers several magnitudes lower than achieved by systemic VLP administration. In contrast, L2 specific antibodies following natural infections are exceptionally rare (Wang et al., 2015a). However, several studies have demonstrated the induction of low-titer and protective L2 specific antibodies in animals vaccinated with bacterially expressed L2 polypeptides (Campo et al., 1997; Chandrachud et al., 1995; Christensen et al., 1991). Antibodies were cross-reactive (Kawana et al., 1998; Roden et al., 2000) and in particular cross-protective against phylogenetically highly divergent papillomavirus types in vivo. The responsible linear neutralization epitopes were found at the N-terminus between L2 aa 11–200, which is highly conserved between different types. For L2 based vaccines, a widely used single L2 epitope designated ‘RG1’ of HPV16 L2 comprises aa 17–36. The RG1 epitope was initially identified by the neutralizing mouse monoclonal antibody ‘RG1’ and L2 17–36 peptide immunizations provide broad cross-neutralizing activity against heterologous papillomavirus types (Gambhira et al., 2007a, 2007b). In addition, cross-neutralization was observed by vaccination with several polypeptides, including aa 1–88 or 11–200 of BPV1 L2 (Gaukroger et al., 1996; Pastrana et al., 2005), or L2 peptides of HPV16 L2 aa 69–81, aa 108–120 or aa 58–64 (Kawana et al., 1999; Wang et al., 2015b). Since antibody levels induced by L2 peptides are generally several orders of magnitude lower that those induced by L1 VLP vaccines, the successful employment of L2 peptides as vaccines is dependent on the augmentation of their immunogenicity to induce robust antibody responses. Additionally the selection of the

epitope(s) is largely empirical, as L1 based taxonomical relationships and sequence homologies of L2 are either non-predictive or of inconsistent relevance for expected induction of cross-protective antibodies. Several vaccine approaches have employed equivalent L2 epitopes of different HPV types to widen the spectrum of protection. The objective of developing an L2 based vaccine is to generate a single- or oligo-valent antigen with a distinctively larger or ideally holistic spectrum of protection against genital hrHPv and lrHPV and potentially cutaneous HPV. The latter types might be targeted alone or in combination with mucosal HPV. Although speculative due to the limited amount of data available, development of a cutaneous broad-spectrum HPV vaccine based on type-common L2 epitopes against the multitude of relevant cutaneous types might represent an alternative to a complex and highly multivalent L1-VLP vaccine. The latter approach may not be feasible in particular since dominant high-risk cutaneous types involved in skin cancer development have not been clearly identified.

As for many other vaccines including HPV L1-VLP, the main mechanism of protection by L2 based HPV vaccines is the induction of sterilizing immunity by systemic neutralizing antibodies. Various methods have been used to improve both antibody titers and the spectrum of protection, which include L2 epitope display on virus capsids, composition of multimeric L2 peptides (concatenated peptides of multiple types), or fusion with TLR ligands and shall be discussed in this review.

3.1. Viral capsid based L2 Vaccines

A favorable immunogenic characteristic of a VLP is that it can act as natural adjuvant when presenting foreign epitopes within the densely spaced repetitive array of surface loops thus inducing robust B-cell activation and consequential durable high-titer neutralizing antibodies. Carrier scaffolds that have been employed to develop L2 vaccines include Papillomaviruses, Bacteriophages, Adeno-associated Viruses, Adenoviruses and Tobacco Mosaic Virus.

3.1.1. Use of the papillomavirus capsid as a scaffold for L2—Previous work has demonstrated the capability to generate chimeric fusion proteins, by inserting foreign epitopes into papillomavirus L1 surface loops, that retain the ability to self-assembly into VLP, although the process is difficult to predict and likely influenced by the epitope inserted. Slupetzky et al demonstrated the feasibility of inserting a foreign epitope into various bovine papillomavirus type 1 (BPV1) L1 or HPV16 L1 capsid surface loops without compromising the antigenicity of the carrier VLP (Slupetzky et al., 2001). Varsani et al generated chimeric HPV16 L1 that assembled into VLP following insertion of HPV16 L2 aa 108–120 into different surface loops, and L2 specific antibodies were induced following immunization in mice (Varsani et al., 2003). Importantly, VLP assembly and maintenance of the neutralizing immune responses against conformational epitopes of the L1 scaffold was dependent on the site of insertion. Kondo et al then succeeded in inducing cross-neutralizing antibodies against HPV18/31/52/58 following vaccination of animals with chimeric HPV16 L1-VLP, using C-terminal insertion between L1 aa 430–433 to present HPV16 L2 peptides aa 18–38, 56–75, or 96–115 (Kondo et al., 2008). Chimeric L1-VLP (BPV1, HPV16) displaying overlapping N-terminal HPV16 L2 peptides from aa 1-200 (length 14 to 95 aa) within the

DE-surface loop were generated by Schellenbacher et al. The DE-loop of BPV1 L1 tolerated insertions up to 40 aa while retaining the capacity to assemble into VLP. Immunization with chimeric VLP plus Freund's or human applicable aluminum hydroxide plus monophosphoryl lipid A (alum + MPL) adjuvants into rabbits and mice revealed that the broadest spectrum of cross-neutralizing antibodies was induced by vaccination with peptide RG1 displayed by BPV1 or HPV16 L1-VLP. Antisera were cross-neutralizing in vitro against heterologous mucosal hrHPV and lrHPV, betaPV, and common cutaneous HPV types, although neutralization was not universal. High-titer neutralization of homologous HPV16 indicated the majority of conformational neutralization epitopes were retained by the modified HPV16 L1 VLP carrier (Schellenbacher et al., 2009). A comprehensive spectrum of protection has been achieved by RG1-VLP vaccination against mucosal hrHPV, lrHPV and even cutaneous HPV. Importantly, passive transfer of rabbit immune sera into mice cross-protected animals against vaginal challenge with pseudovirions (PsV) of all relevant mucosal hrHPV (HPV16/18/45/31/33/52/58/35/39/51/59/68/56/73/26/53/66/34) and lrHPV (HPV6/43/44) in vivo and protective titers lasted over one year (Schellenbacher et al., 2013). Based on these encouraging preclinical data, RG1-VLP are currently being produced under cGMP, sponsored by the US NCI/NIH PREVENT program, for IND-enabling and subsequent clinical phase I studies planned to start in 2017.

A potential advantage of using HPV VLP for display of L2 peptides is that chimeric VLP are based on licensed HPV vaccines with the relatively minor modification of adding a single epitope. However, there are limitations for designing L2 based chimeric HPV L1-VLP vaccines. Both toleration of epitope insertion without loss of functional VLP assembly and immunologically favorable epitope presentation can only be empirically determined. Consequently, insertion of peptides of L2 from both HPV33 and HPV58 into two different sites of HPV18 L1 was recently examined as an approach for further increasing the breadth of protection (Boxus et al., 2016), and HPV18 L1-VLP with inserted HPV45 L2 peptide have been tailored to more specifically target the group of alpha 7 mucosal hrHPV (Huber et al., 2015). A combination of different chimeric L1-L2 VLP with complementary spectra of cross-neutralization can be envisaged to develop a pan-spectrum HPV vaccine.

3.1.2. Bacteriophage display—As alternative approach for L2 based vaccines, bacteriophage has been used as scaffold for L2 epitope display. Potential advantages are the ability to display different epitopes and the high-output bacterial production of bacteriophage. Also the encapsidated ssRNA activates toll-like receptors (TLR) 7, 8 and thus serves as natural adjuvant, although regulatory rules may limit the amount of viral RNA allowed in human vaccine formulations. Spray-dried bacteriophage formulations retain immunogenicity, potentially eliminating the requirement for a cold-chain for transport and storage.

Following early work using RNA bacteriophage MS2 to display foreign (HIV) epitopes (Peabody et al., 2008), VLPs of PP7, a bacteriophage of Pseudomonas aeruginosa, expressing HPV16 L2 epitope aa 17–31 were introduced. These VLP induced (cross-) protection against HPV16 and HPV45 challenge in immunized mice (Caldeira et al., 2010). An extension to this work showed that PP7 VLP presenting L2 aa 17–31 of HPV16 or homologs of different HPV types induced different spectra of cross-reactivity and superior

neutralization by a combination of different PP7 VLP (Tumban et al., 2011). Display of the HPV16 L2 epitope aa 17–31 within the N-terminus of the MS2 coat protein showed favorable anti L2 responses over presentation via the AB-loop of PP7. Cross-protection was demonstrated for intravaginal challenge with mucosal hrHPV16/31/33/35/39/45/51/53/58, lrHPV6, or intradermal challenge with beta-HPV5 (Tumban et al., 2012) and immune responses were long-lasting. Importantly, immunogenicity was maintained by a single shot, and thermostability was achieved by spray-drying of VLP (Tumban et al., 2015). Agilvax's HPV vaccine AX03 based on this technology is being developed with DMID/NIAID/NIH for further clinical development (http://www.agilvax.com/nAdvancingClinicalProductionOfItsLeadVaccineAgainstHumanPapillomaVirus.html).

3.1.3. Adeno-associated Virus (AAV) display—Adeno-associated virus 2 (AAV2) based VLP vaccines make use of a small ssDNA virus, which does not cause any specific disease. Following double insertions of HPV16 L2 aa 17–36 and HPV31 L2 aa 17–36 into the capsid subunit V3, the recombinant antigen self-assembled into VLP (AAVLP-HPV16/31L2). Vaccinations induced (cross-) neutralizing antibodies against HPV16/18/31/45/52/58 and BPV1 in mice (Nieto et al., 2012). Immunogenicity was also maintained with lyophilized and reconstituted recombinant AAV VLP. Unexpectedly, a booster effect of pre-existing antibodies against AAVP was described. Effective antibody induction was dependent on the inclusion of adjuvant montanide ISA51, which is not approved for human use, or MPL which proved less effective. Further investigation of the AAVLP-HPV16/31L2 vaccine without or with different adjuvants (alum +/− MPL or RIBI) showed sufficient antibody response with alum alone and superior responses with additional MPL or RIBI. Importantly, rabbits were protected against cutaneous challenge with hrHPV16/31/35/39/45/58/59 quasi-virions enclosing the cottontail rabbit papillomavirus (CRPV) genome at 6 or 12 months post-immunization with or without adjuvant (Jagu et al., 2015).

3.1.4. Adenovirus display—Recombinant adenovirus type 5 (Ad5) has been generated by insertion of HPV16 L2 aa 12–41 within hypervariable regions HRV1 or HRV5 of the hexon capsid protein, which form immunodominant neutralization epitopes. Vaccinations of mice with these L2-recombinant Ad5 and alum plus MPL adjuvant produced weak antibody titers to L2 and protected mice from vaginal challenge with HPV16 PsV, but not HPV56 PsV. This may reflect in part the inability of adenoviruses to replicate in mice. These constructs may be more immunogenic if used in a permissive host. Indeed, Ad5 infects humans causing non-fatal gastro-intestinal and respiratory disease. Such a live recombinant vector might have the advantage of inducing antibody responses by a single administration. In addition oral uptake of lyophilized virus could circumvent the need for parenteral administration and a cold-chain and thus improve vaccine acceptance (Wu et al., 2015).

3.1.5. Tobacco mosaic virus display—Palmer et al generated inactivated recombinant tobacco mosaic virus (rTMV) particles that display on their surface L2 aa 94–122 of CRPV or non-related rabbit oral papillomavirus (ROPV). Vaccinations of rabbits with CRPV L2, ROPV L2, or CRPV L2 + ROPV L2 rTMV induced neutralizing antibodies against CRPV

and induced protection against papilloma growth by CRPV infection (Palmer et al., 2006), although (cross-)protection against ROPV challenge was not determined.

3.2. *Lactobacillus casei* based L2 vaccine

An HPV vaccine candidate for mucosal delivery was developed using *L. casei* that displays the N-terminus of HPV16 L2 protein (aa 1–224) on the bacterial surface. Oral immunization of mice with inactivated recombinant *L. casei-L2* induced L2-specific vaginal IgG and IgA and also serum IgG that conferred (cross-) neutralization in vitro and (cross-) protection against genital challenge with HPV16/18/45/58 and BPV1 PsV in vivo (Yoon et al., 2012). The induction of systemic and intravaginal L2 antibodies by intranasal recombinant *L. casei* administration may offer the advantage of needle-free vaccination.

3.3. Synthetic peptides and multimers

Immunizations with L2 polypeptides or proteins made in *E. coli* were the initial approaches tested (Campo et al., 1997; Kawana et al., 1999; Roden et al., 2000). These antigens can be easily mass-produced and the breadth of protection further enhanced by including L2 peptides of different HPV types. To increase the generally low immunogenicity of polypeptides and to optimize the range of cross-protection, multimeric, concatenated peptides have been composed and formulated with strong adjuvant.

Kawana et al demonstrated induction of cross-neutralizing antibodies in serum and vaginal washes of mice, following intranasal immunization with HPV16 L2 aa 108–120 (K. Kawana et al., 2001). Ensuing work in humans confirmed that intranasal application of synthetic peptide HPV16 L2 aa 108–120 was well-tolerated in healthy adults (n=13) (Kawana et al., 2003). (Cross-) neutralizing antisera against HPV16 and HPV52 were induced by nasal inoculation with 0,5mg dose of synthetic peptide, but not by 0,1mg dose or placebo. These data were the first to show the potential of L2 peptides to induce (cross-) neutralizing antibodies by nasal application in humans, but also demonstrated insufficient immunogenicity of mucosal peptide application without potent adjuvants.

Vaccination of rabbits and mice with multimeric polypeptides of HPV16 L2 (aa 1–88; 11–200), or L2 multimers of 3, 5, or 22 different HPV types (aa 11–200 × 3 (HPV 6, 16, 18), aa 11–88 × 5 (HPV 1, 5, 6, 16, 18), or aa 17–36 × 22 (five cutaneous, two mucosal low-risk, and 15 oncogenic types)) with or without different adjuvants resulted in robust HPV16-neutralizing antibody responses. Vaccination with either of the multitype L2 fusion proteins 11–200 × 3 and 11–88 × 5 induced high serum neutralizing antibody titers against HPV6/16/18, but also cross-neutralized HPV45 and 58, although sequences of the latter two were not contained in the fusion proteins. Detection of HPV16 neutralization 4 months after immunization benefited from formulation with a potent adjuvant (Jagu et al., 2009). In subsequent work using phylogenetically divergent HPV L2 aa 11–88 × 8 (HPV 6, 16, 18, 31, 39, 51, 56, 73) or 11–88 × 5 (HPV 1, 5, 6, 16, 18) plus alum adjuvant, immunizations with either of the two antigens induced similar robust protection, indicating that multitype L2 epitope presentation can be tailored to selected types according to phylogenetic relationships to achieve broad protection against medically important HPV types (Jagu et al., 2013).

3.4. L2 polypeptide linked to T helper epitope and the Toll-like receptor ligand

As alternative strategy to increase L2 immunogenicity without additional adjuvant, Alphs et al have coupled the HPV16 L2 peptide aa 17–36 (RG-1) to a broadly recognized T-helper epitope (P25) and the Toll-like receptor-2 (TLR2) ligand dipalmitoyl-S-glyceryl cysteine (P2C) generating completely synthetic vaccines (P25-P2C-HPV). A potent L2-specific antibody response was generated to the P25-P2C-HPV lipopeptide when delivered either subcutaneously or intranasally that neutralized hrHPV16/18/45, beta HPV5 and BPV1 and conferred (cross-) protection against challenge with HPV16 and 45 (Alphs et al., 2008).

3.5. L2 peptide – bacterial thioredoxin (Trx) fusion protein

Incorporation of HPV16 L2 peptides into bacterial thioredoxin (Trx), which potently stimulates murine T-cell proliferation, greatly enhanced L2-specific immune responses when compared to immunizations with KLH-coupled peptide, and (cross-) neutralization against HPV16/18/31/45 and 58 was induced by vaccination with HPV16 L2 aa 20–38 – Trx fusion protein (Rubio et al., 2009). A trivalent Trx-L2 vaccine based on *Pyrococcus furiosus* Trx (which has low cross-reactivity to human Trx), incorporating L2 (aa 20-38) epitopes from HPV16, HPV31 and HPV51 and formulated in human-compatible alum + MPL adjuvant, induced broadly cross-neutralizing and cross-protective antibody responses in mice and guinea pigs (Seitz et al., 2015). Of note, *P. furiosus* Trx-L2 is a thermostable formulation that would be particularly suited for low-resource countries without functional cold chain and in addition allows for one step purification from expressed bacteria.

3.6. L2 peptide – bacterial flagellin (Fla) fusion protein

Genetically modified bacterial flagellin (Fla), a TLR5 ligand, has been fused to HPV16 L2 aa 11–200 or monomeric and multimeric forms of aa 11–88 and produced in *E. coli*. Following Fla-L2 immunization of mice and rabbits without adjuvant, mice were protected from vaginal challenge with HPV16/33/35/56 PsV (56 not significant for Fla 11–88 × 5), and rabbits were protected against warts development following cutaneous challenge by homologous and heterologous HPV quasivirions (Kalnin et al., 2014). In addition, passive serum transfer experiments underlined the protective role of antibodies as the major mechanism for vaccine efficacy.

3.7. L2 based vaccines with both therapeutic and prophylactic potential

Vaccines based on L2 antigens have been designed to confer not only prophylactic but also therapeutic potential, including chimeric VLP and polypeptide preparations. In addition to L2 peptides, these vaccines include papillomavirus early antigens E6 and E7. The latter are expressed in transformed epithelial cells where no productive infection (and expression of late proteins) can be detected. However, no therapeutic HPV vaccine has proven effective in phase III clinical trials nor gained licensure for clinical use. The development of purely therapeutic HPV vaccines is beyond the scope of this review.

3.7.1. HPV L2 plus E6 and E7 fusion peptides (TA-GW and TA-CIN)—A recombinant HPV6 L2-E7 fusion protein (TA-GW) vaccine was tested in a small dose escalation trial in humans and proved safe and immunogenic, inducing antibody and T-cell

proliferative responses (Lacey et al., 1999; Thompson et al., 1999). Three vaccinations over 4 weeks were associated with partial remission in patients with genital warts, although efficacy was not demonstrated in this uncontrolled study. A favorable effect in lesion clearance rate was suggested when the vaccine was co-administered with conventional therapies and long-lived T- and B-cell responses were elicited (Vandepapeliere et al., 2005).

TA-CIN is a recombinant HPV16 L2-E6E7 fusion protein vaccine designed to target genital intraepithelial neoplasia and cancer that express the HPV16 oncogenes. Induction of HPV16-specific CTL, T-helper cells, antibodies, and prevention of HPV16 containing tumor outgrowth were demonstrated in a preclinical vaccine mouse model. Safety and immunogenicity of TA-CIN was demonstrated in a dose-escalating, double blind, placebo-controlled phase I study conducted in 40 healthy volunteers. Both IgG antibodies and proliferative responses against TA-CIN were elicited at all three doses (De Jong et al., 2002). Further, a nonrandomized phase II prime-boost vaccine trial was conducted in patients with high-grade anogenital intraepithelial neoplasia (AGIN), with three doses TA-CIN prime followed by boosting with a recombinant vaccinia virus expressing HPV16 and HPV18 E6/E7 (TA-HPV). No serious adverse effects were recorded and a minority of women demonstrated clinical responses. However, the prime-boost approach offered no significant advantages over single TA-HPV vaccination (Fiander et al., 2006).

Another trial conducted in vulvar intraepithelial neoplasia (VIN) patients applied locally the topical immunomodulater imiqimod (a TLR 7 antagonist) for 8 weeks, followed by immunization with three doses of TA-CIN. This study achieved both lesion regression and HPV16 clearance in a fraction of women, with increased local infiltration of CD8 and CD4 T-cells in lesion responders, whereas T regulatory cells were increased in non-responders (Daayana et al., 2010). However, due to the lack of an included placebo-control both the role of TA-CIN and any specific effect of L2 remain unclear. Following studies have specifically looked into L2-specific antibody responses in TA-CIN vaccinees. Vaccination with relatively high-doses of TA-CIN induced HPV16 L2 specific antibody responses in healthy volunteers that were (cross-) neutralizing to HPV16 and HPV18, whereas serum responses in patients with anogenital dysplasia were infrequent. The authors speculated that the significantly lower antibodies in AGIN patients might point to immune tolerance (Gambhira et al., 2006). Interestingly, by passive transfer of serum from TA-CIN vaccinated patients, almost half of the sera (8/17) cross-protected mice against a vaginal challenge with HPV58 PsV (Wang et al., 2014b).

3.7.2. Chimeric HPV16 L1/L2-16E7 VLP—Recombinant HPV16 L1 self-assembled into VLP that incorporate the minor capsid protein L2 when co-expressed in the same cell (Kirnbauer et al., 1993). Chimeric HPV16 VLP that incorporated the entire HPV16 E7 protein fused to HPV16 L2 also assembled into VLP and induced L1 specific neutralizing antibody responses. Moreover immunizations with HPV16 L1/L2-16E7 VLP, but not parental HPV16 L1/L2 VLP, protected mice against challenge with HPV16 E7 expressing TC1 tumor cells via class-I restricted cytotoxic T-cell response (Greenstone et al., 1998). This study raised the possibility of generating VLP vaccines containing non-structural early (E) proteins to increase the therapeutic potential of prophylactic HPV vaccination.

3.7.3. Calreticulin-based HPV E6/E7/L2 DNA Vaccine (hCRTE6E7L2)—To generate both prophylactic and therapeutic immune responses, a DNA vaccine has been designed that consists of HPV16 L2 plus early proteins E6 and E7 fused to heat shock protein calreticulin (hCRTE6E7L2). Vaccination induced both E6/E7 specific CD8 T-cell responses with anti-tumorigenic effects against E6/E7 expressing tumors in mice. In addition L2 specific neutralizing antibodies to HPV16 were induced in mice that protected against PsV infection, demonstrating both therapeutic and prophylactic potential of the vaccine approach (Kim et al., 2008). Cellular immune responses were maintained in CD4 T-cell depleted mice, a possible therapeutic vaccine model for the high-risk population of HIV positive patients with reduced CD4 cell counts (Peng et al., 2014; Wang et al., 2015b). The hCRTE6E7L2 DNA vaccine is currently produced under cGMP for clinical trials by the NCI PREVENT program (unpublished data).

4. Assay development for evaluation of monovalent L2 vaccines to cross-protect against heterologous HPV infections

Since papillomavirus infections are species-specific there is no easy animal model for HPV infection, and authentic virions can be propagated in vitro only in limited amounts using differentiating raft culture systems, or can be isolated from patients' lesions. Papillomavirus L1+L2 capsid proteins are able to self-assemble in vitro with the ability to package foreign DNA of a specific size (about 6–8kbp). Co-transfection of HPV L1+L2 genes under a strong promotor plus a reporter encoding gene resulted in the generation of PsV (Buck et al., 2004), which simplifies modeling aspects of infection and detection of inhibition of infection by neutralizing sera or compounds (for a detailed protocol see http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm). An initial protocol of a PsV-based neutralization assay (PBNA) proved suitable in detecting neutralizing antibody responses in vitro (for a detailed protocol, see http://home.ccr.cancer.gov/lco/neutralizationassay.htm). However, when L2 specific antisera were tested for protection following passive transfer in mice and subsequent intra-vaginal challenge with chemoluminescent PsV, the challenge model was dramatically more sensitive to detect L2 antibody-mediated protection than the orignal PBNA (now designated 'L1-based PBNA'). However, more recently gained knowledge on the specific kinetics of HPV infection, including the secondary conformational change with disclosure of L2 epitopes following basal membrane binding, resulted in the development of in vitro furin-dependent neutralization assays, that more closely resemble the in vivo infectivity situation (Day et al., 2012; Wang et al., 2014a). These 'L2-based PBNA' are more sensitive to detect L2-specific neutralizing antibody responses with no expense at the L1-specific responses. These assays are more likely to give a more accurate estimate of L2 antibody mediated neutralization and protection, closer to in vivo assays which are not feasible for high-throughput testing.

5. Discussion

Licensed L1-VLP vaccines induce high-titer and long lasting neutralizing antibodies and have an excellent safety profile. When applied prophylactically, high protective efficacy against anogenital warts and intraepithelial neoplasias is achieved. An important limitation is

that vaccine efficacy and protection are largely restricted to the vaccine types, thus necessitating complex (multivalent) formulations with substantial costs of production to achieve broad protection.

For a next-generation HPV vaccine with improved efficacy, the desired spectrum of protection includes all relevant mucosal hrHPV, which could particularly benefit low-income countries where cytological screening programs are missing. Papillomavirus vaccines based on highly conserved L2 peptides induce cross-neutralizing and cross-protective antibody responses in experimental animal vaccine trials. As proof of principle, vaccinations with a single L2 epitope ‘RG1’ presented by a particulate structure conferred protection against PsV challenge with virtually all relevant hrHPV in animals (Schellenbacher et al., 2013).

Although differences in the spectrum and robustness of L2 specific antibodies in varying animal species and depending on the antigen formulation have been reported (our personal observation;(Kondo et al., 2008; Seitz et al., 2015)), the phenomenon of broad protection induced by L2 vaccines is consistent. However, there is only very limited data in humans that demonstrate weak L2 specific antibody responses and these were in response to vaccination with full-length HPV L2 protein or distinct HPV16 L2 peptides (Kawana et al., 2003; Wang et al., 2014b). Different strategies have been used to overcome the generally limited immunogenicity of (poly)peptide vaccination. Multimeric presentation on VLP, fusions as concatenated multimers, or direct coupling to T-cell stimulatory (lipo)peptides considerably increased L2 specific antibody titers, when compared to peptide vaccination alone, and provided cross-neutralization and cross-protection in animal studies. Stable plateau antibody levels (for at least 1 year) were achieved in animal vaccinations (Schellenbacher et al., 2013; Tumban et al., 2015). Importantly, L2 vaccine approaches differ in antigen dosage (high doses in proof-of-principle studies), adjuvant formulations (high-potent experimental versus human applicable adjuvants) and assays used to determine neutralizing antibody titers. However, head to head studies comparing efficacy between different experimental vaccine approaches are not available. Future clinical trials need to examine safety of L2 based vaccines and whether L2 specific immune responses, despite being lower titer, provide similar robust protection against infection as those induced by L1 VLP vaccines. L2-specific ELISA detect antibodies of which only a subgroup appears to be neutralizing and thus it needs to be shown if these are a useful correlate of protection. Pseudovirion-based neutralization assays have been initially developed to detect L1-VLP induced neutralization. For L2-based vaccinations, more sensitive L2 based PBNA have been introduced that better mimic the spacio-temporal kinetics of papillomavirus infection in vivo. In addition, even more sensitive murine challenge models can detect protection conferred by vaccination or passive transfer, and thus are currently regarded as the gold standard to predict vaccine efficacy. As with L1 VLP vaccines, the minimum level of antibodies induced by L2 based vaccines that still confers protection against challenge and disease remains unknown.

At least three VLP based prophylactic L2 vaccines are currently (or will shortly be) produced under cGMP for early phase clinical trials in humans. Chimeric 16L1-16L2 aa17-36 VLP (RG1-VLP), that have been designed to induce protection against all relevant mucosal hrHPV, are expected to resemble L1-VLP vaccines in safety profile, and cGMP

production is being sponsored by the US NCI PREVENT program. A bacteriophage that also displays HPV16 aa L2 17–31 is expected to confer protection against a broad spectrum of hrHPV. Of note the phage vaccine has been shown to retain immunogenicity following spray-drying and rehydration, a potential advantage for eventual vaccine distribution in the absence of a cold-chain in underdeveloped countries (Tumban et al., 2015). In addition, a thermostable Thioredoxin-L2 vaccine that has been shown to induce robust neutralization against multiple hrHPV types is close to cGMP production for a planned human trial ((Seitz et al., 2015); Martin Müller, personal communication).

A common characteristic of L2 based vaccines is a single antigen formulation with expected lower cost of production compared to complex multivalent VLP vaccines. High efficacy, a robust safety record and long duration of protection (at least 10 years) achieved by HPV L1-VLP vaccines provides a very high bar against which L2 vaccines will be judged by regulatory agencies. Low-level antibodies to L1 or L2 are sufficient for protection in animal models, and minimum protective levels have not been defined in humans, further complicating clinical development. However, immune-bridging has been used in younger populations in approvals of the L1 VLP vaccines, and for Gardasil-9, but it is less clear how this would be applied to L2-based vaccines. Various strategies for immunogenic preparations of L2 vaccines have been employed including multimeric assemblies, linkage to TLR or T-helper epitopes, and densely repetitive L2 presentation on diverse particulate carriers to induce robust and long-lived serological responses. Attractive alternative routes of antigen presentation via mucosal surfaces are adenoviruses or lactobacillus vehicles to preferentially induce mucosal immune protection (Wu et al., 2015; Yoon et al., 2012).

A range of phylogenetically diverse HPV types of genus alpha, gamma, mu, nu induce common and palmo-plantar warts, and the large number of genus beta types has been implicated in the development of NMSC in immunosuppressed patients. The vast diversity of types precludes the development of multivalent VLP vaccines against the plethora of potentially relevant HPV. However, cross-neutralizing immune responses against cutaneous HPV have been observed with different L2 based vaccines including RG1-VLP (our own observation). As L2 antigens are now being more systematically investigated for protective efficacy against cutaneous HPV, selected cross-neutralization epitopes of L2 may emerge as potential vaccine antigens to more directly target cutaneous HPV.

The spectacular success of licensed L1-VLP vaccines indicates that L2 based HPV vaccines are promising next generation candidate vaccines to further broaden the protective spectrum including the mucosal hrHPV not addressed by current vaccines, additional mucosal lrHPV and even common cutaneous and betaPV types. Several of the more advanced experimental mono- or oligo-valent vaccines are promising with regard to ease of production, potential breadth of protection and probably reduced costs as compared to licensed vaccines and are now close to or have entered the path for cGMP production, IND enabling and early clinical studies. If safety and robust protection can be demonstrated comparable to licensed HPV vaccines, L2 based vaccines may be developed specifically for immunosuppressed patients prone to infection with much more diverse HPV types, for cutaneous infections, and for developing countries which often lack the financial resources, adequate infrastructure to distribution including cold-chain, and cytological cervical screening programs.

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