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Nicht weil es schwer ist, wagen wir es nicht, sondern weil wir es nicht wagen, ist es schwer.

Lucius Annaeus Seneca

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Introduction

1 Human papillomaviruses

1.1. Phylogeny

Papillomaviruses (PV) are a large family of DNA viruses that are widespread in nature and infect mammals including humans, birds and even reptiles. At least 120 human PV (HPV) and 69 animal PV types have been completely characterized and grouped into 29 genera (Zhou, Sun et al. 1994; Bernard, Burk et al. 2010). Papillomaviruses have historically been grouped within the *Papovaviridae* family, which is nowadays split into *Papillomaviridae* and *Polyomaviridae*.

Papillomaviruses are characterized by means of nucleotide sequence homologies, biological and medical properties. A PV type refers to a full-length genome with an L1 sequence (encoding the major structural protein) that differs from another type by at least 10%. Subtypes' L1 gene nucleotides differ by 2-10%, and variants by less than 2%.

Papillomaviruses are species-specific and strictly epitheliotropic, infecting mucosal or cutaneous epithelia. Mucosal HPVs are further classified into high- and low- risk types, according to their potential to transform cells, the former causing pre-cancerous epithelial lesions that may progress into invasive cancer. The HPV are grouped into five genera: genus Alpha, Beta, Gamma, Mu and Nu (Figure 1). Genera are further grouped into species, and the majority of high-risk mucosal types are classified within species Alpha 7 and Alpha 9. The most important high-risk types HPV16 and HPV18, which together cause 70% of cervical cancers worldwide, belong to genus Alpha 9 and Alpha 7 respectively.

1.2. Virus genome

The HPV genome is a double stranded circular DNA of about 8kb condensed around cellular histones, and contains an early (E) region, a late (L) region, and a non-coding long control region (LCR) (or upstream regulatory region, URR) with the viral origin of replication (ORI). The genome contains open reading frames (ORF) that encode six early (E1, E2, E4, E5, E6 and E7, see Figure 2) and two late (L1 major and L2 minor capsid) proteins that all reside on one strand (Doorbar 2005;

Doorbar 2006). E1 is a helicase that binds weakly to the consensus sequence (AACNAT), occurring six times in the viral ORI, and to cellular factors like RPA (replication protein A) and the cellular DNA polymerase. The DNA-binding protein E2 recognizes the palindromic sequence [AACCg(N₄)cGGTT] in the non-coding LCR. For example, in HPV16 there are four such E2 binding sites near the ORI, and the DNA is bound by an E2-dimer with the Carboxy (C)-terminus. E2 binding to the viral DNA is necessary to recruit the E1 helicase by its amino (N)-terminus. After dissociation of E2, E1 builds up a double bi-hexameric ring essential for replication. E2 additionally anchors the viral episomes to mitotic chromosomes crucial for correct segregation into daughter cells, and can act as a transcriptional factor regulating the early promoter p97, which controls expression of the two viral oncogenes E6 and E7. It has been shown that at low levels E2 acts as a transcriptional activator, whereas at high levels E2 inhibits E6 and E7 expression by dispelling the activator SP1 from its site near the promoter. These opposed abilities are thought to result from E2's differential affinity to the various binding sites. Further, E1 and E2 together are necessary for viral genome maintenance.

E4 and E5 seem to contribute to viral genome amplification. The role of E4 in the amplification process is yet not fully known, however, it is able to bind cyclinB/Cdk2 relocating the complex to the cytoplasm, which prevents its nuclear accumulation and therefore inhibits mitotic progression antagonizing E7-mediated proliferation. The E4 protein also disrupts the cytokeleton network affecting the integrity of the superficial cornified envelope, which facilitates viral escape from the cornified layer. Further, E4 has been shown to bind E2 indicating an additional yet unknown role of E4. E5 is a transmembrane endoplasmatic reticulum (ER) protein, which can bind the vacuolar proton ATPase, delaying the endosomal acidification process. This modulates cell signaling by affecting the recycling of growth factor receptors, which increases epidermal growth factor (EGF)-mediated receptor signaling and maintains a replication-favorable environment.

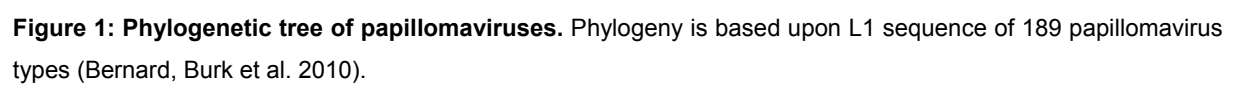
E6 and E7 are both expressed from a polycistronic mRNA and are the two oncogenes responsible for the extensive suprabasal epithelial cell proliferation necessary to expand the few infected cells that will later produce infectious virions. E7 binds to proteins important for cell proliferation, like histone deacetylases and

associates with members of the pocket protein family like the pRb (retinoblastoma protein) disrupting its links to the family of E2F transcription factors. pRB in uninfected cells inhibits E2F transcription activity for the expression of proteins necessary for S-phase entry. Interaction with E7 degrades pRb and facilitates E2F-mediated expression, resulting in proliferation. This unscheduled cell cycle entry, however, increases the level of p53, which normally leads to cell cycle arrest, DNA repair mechanisms or apoptosis. Consequently, the virus has found a way to counteract these p53-mediated effects in the anti-apoptotic E6 protein. The E6 protein of high-risk HPV types (and to a lesser extent of low risk types) is able to interact with E6AP ubiquitin ligase, which enhances the degradation of p53 and complements the function of E7.

The E proteins are expressed by the p97 or p670 promoter during different stages of infection and cell differentiation. The L1 and L2 proteins, as well, are expressed from the p670 promoter after a splicing site change and a shift in polyadenylation site usage.

1.3. Structural proteins and the viral capsid

The PV virion has a spherical structure with a T=7 icosahedral symmetry and a diameter of 50-60nm. The “naked” capsid lacks a lipoprotein envelope and consists of 360 copies of L1 and to a much lesser extent of the L2 minor capsid proteins. Five L1 monomers build a pentamer (or capsomer) and the capsid is composed of 72 pentameres. Previously it has been estimated that one virion contains 12 L2 molecules (a 1:30 ratio of L2:L1). However, more recent studies have shown that up to 72 L2 molecules can be incorporated into PsV in a 5:1 stoichiometry (Buck, Cheng et al. 2008). Additionally, it is also said that neighboring L2 molecules might interact with each other and that L2 in general can build up a network of contacts. A crystal structure of a small T=1 (12-pentamer) L1/L2 virus-like particle (VLP) of HPV16 is available, but L2 is less ordered than L1 and therefore can only be seen as an area of additional density at the base of a capsomer and no additional difference can be seen between L1-only and L1/L2 VLP (Chen, Garcea et al. 2000; Buck, Cheng et al. 2008).



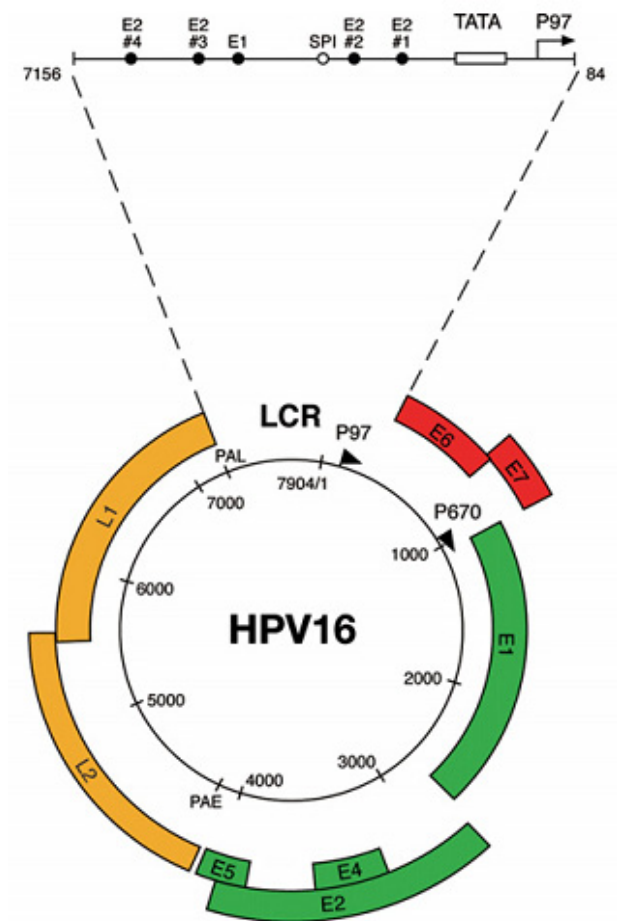


Figure 2: HPV16 genome. There are eight open reading frames: the early genes E1, E2, E4, E5, E6 and E7 and the late structural genes L1 and L2. The early proteins are expressed by the p97 or p670 promoter during different stages of infection and cell differentiation. The two late proteins L1 and L2, as well, are expressed from the p670 promoter after a splicing site change and a shift in polyadenylation site usage. All genes are encoded on one strand of the double-stranded viral genome. The long control region (LCR) is illustrated enlarged to show the four E2 binding sites of HPV16, recruiting E1 and the cellular replication machinery (Doorbar 2006).

1.4. Life Cycle

Papillomaviruses are thought to infect basal stem cells or transient amplifying cells of mucosal or cutaneous epithelia (Doorbar 2005). The initial infection requires a micro-trauma allowing the virus to reach the basal cell layer. Cell division is a requirement for PV infection *in vivo* and *in vitro*, which is consistent with the idea that wound healing is tied to infection.

Virus uptake *in vitro* is thought to be reliant on the cell surface receptor heparin sulphate, alpha integrin or laminin 5. The entry process appears very slow with disassembly occurring 4-6 hours after infection. Entry is dependent on a proteolytic event prior to viral endocytosis, which seems to be a requirement for establishment of infection both *in vivo* and *in vitro*. This includes the cleavage of the minor protein L2 by furin/proprotein convertase 5/6 (Day and Schiller 2009). Prior heparin sulfate

proteoglycan binding results in conformational change of the capsid and exposure of the furin cleavage site.

Furin is a type 1 membrane protein found in the trans-Golgi network; however, its active form is also localized throughout suprabasal epithelial layers and at a higher level at sites of trauma. This endonuclease removes aa residues 9-12 of the N-terminus of L2 and the cleavage site appears conserved among many different PV types. Cleavage of the papillomavirus minor capsid protein L2 at a furin consensus site is necessary for infection (Richards, Lowy et al. 2006). Further, it seems that only in mature virions the L2 N-terminus is accessible for cleavage, and that binding to the primary surface receptor facilitates furin cleavage, which again leads to the exposure of a second (unknown) receptor binding site on the major protein L1. Furin cleavage additionally exposes L2-specific neutralization epitopes (e.g. RG1, see below) until the capsid is internalized. Recently it has been found out that cyclophilin isomerase as well may play an important role in PV infection after furin cleavage.

Internalization of HPV16 occurs in a clathrin-dependent manner (Conway and Meyers 2009), though other mechanisms, like an aberrant pathway via the heparin sulfate receptor binding, might occur as well. For the virus to escape the endosome a C-terminal hydrophobic L2 sequence appears to destabilize and permeabilize the limiting membrane with its activity linked to the acidification of the endosome (Kamper, Day et al. 2006). Further, it seems that the reducing environment within the cell helps dissolve the disulphide bonds leading to the uncoating of the viral DNA. Furin cleavage of L2 might be essential for the release of the L2-bound genome from the endosome into the cytosol, while L1 does not leave this compartment (Richards, Lowy et al. 2006). Further, the conserved characteristic of L2's N-terminus may indicate possible interactions of L2 with intracellular receptors or chaperons helping endosomal escape.

L2 contains two nuclear localization sites (NLS) at the C- and N-terminal end, and it is thought that only the C-terminus is involved in the nuclear entry (Bordeaux, Forte et al. 2006). Additionally, L2 is able to bind DNA, whereas L1 is not (Zhou, Sun et al. 1994).

The viral genome is maintained as episomes in the basal cell layer in low copy number of around 10-200 per cell nucleus. In this state, the early proteins E6, E7, E1 and E2 are only expressed at low levels from the early promoter (p97), which is active independently from the cell's differentiation status. The progeny cells of the infected basal cell undergo the process of terminal differentiation as they move upwards into the suprabasal cell layers. In contrast to normal suprabasal cells, virus infected cells do not exit the cell cycle, but keep the cells in a proliferation state due to E6 and E7 activities (see above). Viral genome amplification begins in a subpopulation within the proliferating layers and is thought to be initiated as the late promoter (p670) increases the expression of all early proteins as a consequence of changes in the cellular environment as the cells move upwards the layers. The late promoter, in contrast to the early promoter, is only active when the cells differentiate.

The two late structural proteins L1 and L2 are only expressed in the upper layers of the epithelium. Although L1 alone is able to assemble into virus particles, L2 increases virus packaging and infectivity and cellular proteins like chaperons might assist with virus assembly. Virion assembly and productive infection only take place in terminally differentiated keratinocytes. Since HPV is non-cytolytic, infectious virions are shed with the cornified squame, possibly with the help of E4, which is able to disrupt the keratin network.

1.4.1. Cancer progression

If the immune system fails to resolve a persistently active high-risk HPV infection, progression to high-grade squamous intraepithelial lesion (hSIL) may occur (Doorbar 2005; Doorbar 2006). These dedifferentiated cells do not support productive HPV infection (i.e. release of infectious virions). The viral E7 oncoprotein retains cells continuously in S-phase stimulating cell proliferation, and E6-mediated loss of DNA repair by its interaction with and degradation of p53, allowing accumulation of secondary mutations in the host's genome, promoting oncogenic progression.

Integration of high-risk HPV DNA into the cellular genome is a frequent event in cancer progression, occurring randomly but more likely at common fragile sites of the host's chromosome, and is most often associated with deletion of E2. Loss of E2 de-represses E6 and E7 promoting unregulated growth of epithelial cells.

1.5. Medical importance

Papillomaviruses of the genus Beta have initially been found in skin cancers of Epidermodysplasia verruciformis (EV) patients (formerly known as EV-HPV types). EV is an autosomal recessive rare genetic disease and patients show generalized cutaneous warts caused by a large number of different Beta-HPV types. Strikingly, EV-patients develop non-melanoma skin cancer (NMSC) at sun exposed areas early in life. These cancers contain Beta-HPV DNA (most often types 5 and 8) at large copy numbers that is actively transcribed. Using sensitive nested PCR, DNA of Beta types has also been found in hair bulbs, a likely reservoir for these types, both in immunocompetent and immunosuppressed (non-EV) individuals.

Ultraviolet (UV) radiation is the main carcinogenic factor for development of non-melanoma skin cancer (NMSC), which is the most common skin cancer in Caucasian populations. Although unproven, there is accumulating experimental and epidemiologic evidence that Beta-HPV may be indirectly involved as adjunct carcinogenic factor in NMSC carcinogenesis. Prevalence of HPV DNA was higher in squamous cell carcinomas (SCC) than in basal cell carcinomas (BCC) (Patel, Karagas et al. 2008). Among the 25 human Beta types, especially HPV5, 8, 15, 20, 24, 36 and 38 might play a role in NMSC. This might be due to the ability of the viral oncogene E6 to inhibit UV-B-induced apoptosis and altering DNA repair, resulting in the accumulation of somatic mutations in infected keratinocytes. *In vitro* and transgenic animal studies have shown that UV-B irradiation has an effect on the viral promoter activity, promoting viral replication and transcription (Akgul, Lemme et al. 2005). E6 of HPV8 and 38 are capable of inducing S-phase entry and tumor invasion, although to a lesser extent than HPV16 (Karagas, Nelson et al. 2006; Pfister 2008). Chances of the development of NMSC increase with the duration of the PV infection and multiple infections are frequent, but HPV5 and 8 have been identified in 90% of the NMSC cases and not all types use the same biologic mechanisms. Studies showed that cutaneous Beta-HPV infections persist for more than 6 years (Harwood, Suretheran et al. 2000; de Koning, Struijk et al. 2007; Hazard, Karlsson et al. 2007). Genetic predisposition, immune status, persistence, and multiple infections are perceived risk factors.

Infection with ano-genital (mucosal) HPV is the most common viral sexually transmitted infection (STI). The virus is passed commonly by direct genital contact during intercourse (<http://www.cdc.gov/hpv/>). Infection with low-risk types causes ano-genital warts or condylomata acuminata, which may also develop at the oropharynx, larynx or conjunctive. The rare occurrence at the larynx is called recurrent respiratory papillomatosis (RRP) and is characterized by the decreased ability to breath and a hoarse voice, often requiring repeated surgeries. Genital warts may be surgically removed, destructed or treated by local application.

Persistence infection with high-risk HPV is known to be the causative agent for the development of cervical cancer (zur Hausen 1989; Durst, Glitz et al. 1992; zur Hausen 1994; Bosch, Lorincz et al. 2002). Although the prevalence of genital HPV especially in young adults, and life-time risk of acquiring an infection are quite high, progression to cervical cancer is very rare (about 0.03% incidence), because 90% of infections are resolved by the immune system within two years. Nevertheless, per year, more than 500.000 women worldwide develop cervical cancer and 250.000 die. Therefore, regular screening is an important issue, which can be achieved by the Papanikolaou test (Pap test or cervical smear) (Cronje 2011; Nishino, Tambouret et al. 2011) to identify abnormal or (pre-)malignant cervical epithelial cells. In developed countries Pap screening, eventually with adjunctive HPV DNA testing, effectively prevents most cervical cancers, but there is lack of resources in developing countries where 80% of all cervical cancer cases occur. Further, the Pap test has quite a low sensitivity (about 50%) and alternative screening methods, like HPV DNA testing or visual inspection of the cervix and colposcopy may help improve medical care in these settings.

In the absence of symptoms infections will often go unnoted. Further, multiple infections with different HPV types can take place. Precursor lesions appear about 5-10 years prior to progression to invasive cancer, and cervical cancer mostly occurs at age 40-50 (Banik, Bhattacharjee et al. 2011).

1.6. Vaccines

More than 120 different HPV have been completely characterized and at least 15 high-risk mucosal types are associated with the development of cervical

carcinomas (Munoz, Bosch et al. 2004). HPV DNA can be found in 99% of cases, with HPV16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 39, 51, 73, 68 and 66 being the most common types. The two licensed HPV vaccines, which will be discussed below, are expected to prevent 70% of the cervical cancer cases, induced by infection with the two most common types HPV16 and HPV18, but they do not prevent infection with the other 13 high risk types that account for the remaining 30% of cervical cancer cases. Analysis of the prevalence of different HPV types has also revealed significant geographic variation. Whereas HPV16 accounts for 50% of cervical cancers worldwide, HPV18 is prevalent in South Asia; HPV45 in Africa, South Asia, Europe and North America; and HPV31 is observed more frequently in Central and South America. Nevertheless, the set of the most frequent HPV types around the world is quite homogenous, and a vaccine effective against all 15 high-risk types would find worldwide use.

The virus has evolved many mechanisms to avoid host immunity (Mariani and Venuti 2010). For example, there is no viremia, no necrosis, no cell lysis or inflammation, the virus stays intraepithelial thus only small amounts of virus are exposed and the shed viruses do not usually reach vascular or lymph vessels. The natural primary immune response induced by HPV is said to be weak and slow, although ultimately a cellular immune response will induce regression of lesions and/or clearance of infection. As infected basal cells do not produce the structural proteins, they are no target by natural or vaccine-induced immune responses to L1 or L2 capsid proteins.

The anti-capsid antibodies induced by natural HPV infection or L1+L2 VLP vaccination are mainly L1 targeted, because L2 is largely internal in the virions and co-assembled VLP (Stanley 2008). The mechanisms of virus neutralization by antibodies (Ab) may include binding the receptor necessary to enter the cell and inhibiting conformational changes of the capsid required to initiate productive infection.

1.6.1. 1st generation HPV vaccines

Because of HPV's worldwide distribution, common incidence, and its causal role in the development of anogenital and particularly cervical cancer, two prophylactic

vaccines are already available. Gardasil® (MSD-Sanofi) and Cervarix® (GSK) both take advantage of L1's feature to spontaneously assemble into VLP when overexpressed recombinantly in yeast or insect cells (Kirnbauer, Booy et al. 1992; Kirnbauer, Taub et al. 1993). VLP are immunologically similar to wild-type (wt) virions and systemic immunization induces a strong immune response against L1. Subunit vaccines based on VLP are considered safe because VLP do not have potentially oncogenic viral DNA incorporated, they are not infectious and cannot replicate.

Following VLP vaccination the protective immune response is mainly due to high-titer and neutralizing antibodies. The structures of the VLP internal parts of L1 are highly conserved, whereas the external surface loops show hypervariability between different HPV genotypes. These correspond to different serotypes that probably evolved due to selection to evade any neutralizing antibodies (Roden, Monie et al. 2006; Roden and Wu 2006; Kwak, Yemelyanova et al. 2011). VLP vaccination induces type-restricted neutralizing antibodies directed against conformational-dependent epitopes. Studies have shown peak serum antibody titers after the final immunization that decline over the next years and then titers remain at a plateau several fold higher than titers following natural infection. These levels are upheld and are still able to maintain protection at least for 9 years. Another feature that increases the effectiveness of the VLP vaccines is that immunogenic epitopes are closely packed and therefore can crosslink B cells because of increased avidity. Additionally, L1 VLP activate immature human myeloid and plasmacytoid dendritic cells (DC) and are easily taken up by antigen-presenting cells (APC) to trigger a robust T-helper immune response. However, VLP do not or only inefficiently induce a cytotoxic T cell response and that may be another reason why this vaccine does not have a therapeutic effect on already existing infection.

Gardasil® (Merck) is a quadrivalent vaccine, containing L1 VLP from the two high-risk mucosal HPV16 and HPV18 and therefore may prevent 70% of cervical cancer. Additionally, it includes L1 VLP from the mucosal low-risk types HPV6 and HPV11, which cause 90% of genital warts. The vaccine has been approved by the Food and Drug Administration (FDA) in 2006 and is administered intramuscularly on a three-dose schedule at months 0, 2 and 6 (Pomfret, Gagnon et al. 2011). Reported side effects are injection site swelling, pain and bruising, fever, nausea and

dizziness. Other more severe side effects have been reported (e.g. blood clots, allergic shock and nervous system damage), but have not been causally associated with the vaccine. Studies have shown that Gardasil in women is up to 100% effective in preventing cervical intraepithelial neoplasia (CIN) and adenocarcinoma in situ (AIS; the incidence of CIN) mediated by the four HPV types, can reduce 90% of genital lesions at other sites and AIN (anal intraepithelial neoplasia), and can prevent 89% of genital warts. Further, it has been shown that the vaccine is able to induce low levels of cross-neutralizing antibodies against HPV31, a closely related type to HPV16.

Cervarix® (GSK) is a bivalent vaccine containing L1 VLP from HPV16 and 18. It shows similar side effects as Gardasil and it is administrated intramuscularly at months 0, 3 and 6 (Monie, Hung et al. 2008; Kemp, Hildesheim et al. 2011). The FDA has approved the product in 2009, when it has already been on the pharm market in other countries, like Australia or Europe. In addition to protection against HPV16 and HPV18, Cervarix induces partial cross-protection against closely related types not included in the vaccine, consistent with measurable levels of cross-neutralizing antibodies against HPV31, 33 and 45. HPV16 is closely related to HPV31 and HPV33, and HPV18 to HPV45, but cross-neutralization of further closely related types to HPV16, like HPV52 or 58, has not been identified. The cross-neutralizing titers of these antibodies were about 100-fold lower compared to levels for HPV16 and 18. Therefore, it is uncertain how long protection against these types may last.

Although L1-VLPs are immunogenic by themselves, both vaccines contain adjuvants to increase immunogenicity. Aluminum salt (alum) in Gardasil acts as a depot for vaccine antigen, increases antigen uptake by APC, and promotes a T_h2 response effective mainly against extracellular pathogens. Together with a TLR ligand (e.g. MPL) it induces transcription of IL1 β and IL18.

Cervarix combines alum with the Toll-Like receptor 4 (TLR4) agonist MPL (3-O-desacyl-4'-monophosphoryl lipid A), a proprietary adjuvant called ASO4 (Giannini, Hanon et al. 2006; Didierlaurent, Morel et al. 2009). MPL is the non-toxic derivative of lipopolysaccharide (LPS) from the Gram-negative bacterium *Salmonella minnesota* R595 strain. MPL, which is absorbed onto the hydroxide salt of aluminum, promotes induction of a primary innate response. It signals through TLR4 and induces NF- κ B

activity and cytokine production (e.g. TNF- α , IL12 and IL6) by DCs and monocytes, which leads to a higher level of antigen-loaded DC's in the lymph draining system near the injection site and therefore to increased activation of T cells. Additionally, MPL is reported to trigger IFN- γ production by CD4⁺ helper T cells, which leads to a T_h1 response required for protection against intracellular pathogens, and increases the level of L1 VLP memory B cells by cytokines like IFN- γ , TNF- α and IL10 produced by CD4 T cells. Altogether, ASO4 is said to induce a higher and longer lasting antibody response, resulting from T cell activation through APCs and B cell activation, when compared with aluminum salt alone.

Although vaccination is highly effective when given prophylactically, there is no therapeutic effect on established infections or dysplastic diseases. In addition, the best antibody titers have been found in pre-teen/adolescence girls and boys, therefore, the recommended age for vaccination with both products starts with 10 years, before initiation of sexual activity. Additionally, catch-up vaccination up to 26 years of age is recommendable, although cost-benefit ratio declines rapidly at increasing age. Protection lasts for at least 9 years, but may even be life-long, and studies about the broadness of protection induced by the vaccines are still ongoing.

1.6.2. 2nd generation HPV vaccines

Both licensed HPV vaccines induce high-level protection against persistent incident infection and pre-neoplastic anogenital disease associated with HPV16 and HPV18, and against HPV6 and HPV11 associated genital warts for the Merck vaccine. However, only low level cross-protection against the most closely related types (HPV31, 33 and 45) has been observed, thus both vaccines will not protect against the other 13 additional high-risk HPV types causing 30% of cervical carcinomas. Thus PAP screening is still necessary even in vaccinated women, increasing the need for new approaches to generate more broad-spectrum and cheaper HPV vaccines.

Merck has currently a nonavalent L1-VLP vaccine in clinical trials, adding five additional VLP of mucosal high-risk types to its current quadrivalent formulation. However such a multi-type L1 VLP vaccine will neither reduce complexity nor costs of the vaccine.

1.6.2.1. L2-based vaccines

An alternative approach to broaden the type-spectrum of HPV vaccines is to induce an immune response to the minor capsid protein L2, which is overall less conserved than L1, but harbors many cross-protective epitopes at the N-terminus, most of which map to amino acids (aa) 11-200 (Gaukroger, Chandrachud et al. 1996; Kawana, Matsumoto et al. 1998; Kawana, Yoshikawa et al. 1999; Roden, Yutzy et al. 2000). Native virions or L1+L2 VLP induce a type-restricted neutralizing antibody response to L1, because L2 in these contexts is immunologically subdominant to L1. However, when injected as an isolated protein, N-terminal L2 peptides induce low-titer antibodies that cross-neutralize many PV types (Karanam, Jagu et al. 2009). Notably, low-titer antibodies induced by the bovine papillomavirus 1 (BPV1) L2 aa 1-88 are able to cross-neutralize even HPV types 6, 11, 16, 18 and 31, indicating shared (cross-neutralization) epitopes between divergent species (Pastrana, Gambhira et al. 2005).

Several studies have attempted to enhance L2's feature to trigger broad-spectrum immunity. For example, multi-type L2 fusion protein with i) aa 11-88 from L2 from five different HPV types, ii) antigens with the aa 11-200 from three or iii) aa 17-36 from altogether 22 types have been generated for vaccination alone, combined with an L1-VLP vaccine, or in the context of L1/L2 co-assembled VLP (Jagu, Karanam et al. 2009; Jagu, Kwak et al. 2010). Antibody titers induced by L2 multi-type peptide alone were high and robust and cross-neutralized some related HPV types. When injected in combination with L1 VLP however, antibodies were mainly directed against the L1 protein. Comparison of an L1- and L2-response against the same type showed that the L2 antibody titers were much lower, raising concerns about the longevity of the L2 response. Anti-L2 antibodies that cross-neutralized heterologous HPV were weak or even non-detectable. If detected, they were 2-3 orders of magnitude lower compared to antibodies against homologous types used to generate this vaccine, thus neutralizing homologous types more efficiently than heterologous types. Another approach is the multimeric presentation of specific cross-neutralization sequences of L2 using PV-VLP as scaffold. For example, genetic insertion of aa 69-81 or 108-120 from HPV16 L2 into an L1 immunodominant surface loop of BPV1 (bovine papillomavirus 1) generated chimeric VLP, and vaccination

induced L2 antibody titers 1-2 orders of magnitude higher than those against synthetic L2 peptides alone (Slupetzky, Gambhira et al. 2007). Others have modified chimeric VLP by insertion of HPV16 L2 aa 56-75, protecting against five closely related HPV types (Kondo, Ochi et al. 2008; Kanda and Kondo 2009).

1.6.2.2. RG1 VLP

In another study, a monoclonal antibody (mAb) named RG1 was generated against HPV16 L2 protein that proved to (cross-)neutralize HPV16 and HPV18. The mAb recognizes aa 17-36, a common B-cell epitope located at the N-terminus of L2 (Gambhira, Karanam et al. 2007). The epitope is only exposed and accessible for antibodies after virion binding to the basement membrane (BM) and furin cleavage of the N-terminal residues aa 9-12 of L2 (Gambhira, Jagu et al. 2009). Sequence alignment of the 20 aa RG1 epitope has shown that it is highly conserved among phylogenetically divergent HPV types. For example, it exhibits aa sequence identity of 75% with the corresponding sequence of HPV45 (high-risk mucosal type) and HPV5 (high-risk Beta type), 80% identity with that of HPV18 (high-risk mucosal type), HPV6 and 11 (benign mucosal types), and 85% with the L2 sequence of HPV2 (cutaneous wart type; table 1). This might indicate an important unknown role of the RG1 peptide for the virus, like allowing interactions with cellular factors during early events of the viral life cycle. Immunization with the RG1 peptide has shown protection against divergent mucosal (HPV16, 18, 6, 11, 45, 31, 52 and 58) and cutaneous (HPV5) HPV types (Gambhira, Karanam et al. 2007; Alphas, Gambhira et al. 2008).

	RG1 aa sequence	homology to HPV16
HPV16	QLYKTCKQAGTCPPDI IPKV	
HPV2	DLYRTCKQAGTCPPDI IPRV	85%
HPV5	HIYQTCKQAGTCPPDV INKV	75%
HPV6	QLYQTCKLTGTCTPPDV IPKV	80%
HPV11	QLYQTCKATGTCTPPDV IPKV	80%
HPV18	DLYKTCKQSGTCPPDVVPKV	80%
HPV45	DLYRTCKQSGTCPPDV INKV	75%

Table 1: Alignment of RG1 aa sequence (aa17-36) of HPV16 with other medically important HPV types.

To take advantage of the type-restricted and high-titer anti-L1 immune response to HPV16 VLP and the broadly cross-neutralization feature of the RG1 epitope, the HPV16 RG1 epitope has been genetically engineered into the DE surface loop of HPV16L1 (Schellenbacher, Roden et al. 2009). The chimeric protein was expressed in Sf9 (*Spodoptera frugiperda*) insect cells by recombinant baculovirus. Analysis by transmission electron microscope (TEM) confirmed that insertion into the hypervariable DE loop did not impair the ability of L1 to self-assemble into VLP, as the recombinant protein assembled into particles (RG1 VLP) with high efficiency similar to the wt HPV16 L1 protein. Consequently, RG1 VLP showed improved immunogenicity for the now 360-fold on the VLP surface displayed RG1 epitope; immunization of rabbits using MLP-alum adjuvant induced readily detectable L2-specific immune response. Robust neutralizing antibodies were induced against the mucosal high-risk HPV types 16, 18, 31, 45, 52 and 58, the low-risk HPV6 and 11, and against the Beta type HPV5, when analyzed in PsV assays.

1.7. The aim of the study

The aim of this study (and to achieve the degree of *magistra rerum naturalium*) was to produce RG1 VLPs, analyze them using SDS-PAGE, Western blot, ELISA and TEM. To further analyze the robustness of RG1 VLP vaccination and broadness of cross-neutralization of heterologous types, four rabbits were immunized using alum-MPL adjuvant. Immunization took place at Charles River Laboratory (Germany) at weeks 0, 3 and 6, and serum was obtained before prime (pre-immune) and two weeks after the final boost (immune). Sera were evaluated by *in vitro* (cross-) neutralization assays (see below) and further for (cross-) protective efficacy in an *in vivo* mouse vaginal challenge model (Roberts, Buck et al. 2007). Groups (n=5) of progesterone treated mice were passively transferred (immunized) with rabbit pre-immune or immune sera. Progesterone treatment synchronizes the oestrus and thus makes the genital tract more susceptible for infection. One day later, mice were vaginally challenged with each of a large panel of mucosal HPV PsV types (see below) that enclosed luciferase as reporter gene. Three days later, anesthetized mice were evaluated for genital pseudo-infection as readout to evaluate RG1 VLP vaccine efficacy for (cross-) protection, using bioluminescence imaging (IVIS).

The PsV were produced according a slightly modified version of the protocol by Buck et al. (<http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm>). Modifications attempted to optimize the amount of PsV obtained; to this end, a “large-scale production” procedure was established. Two different versions of PsV, each containing a specific reporter gene (encoding either secreted alkaline phosphatase/SEAP or luciferase) were produced. Those containing SEAP were used to evaluate neutralization by immune serum *in vitro*; those with luciferase were used in the mouse challenge model. Although luciferase-based *in vitro* neutralization assays were established as well, *in vitro* neutralization assays mainly were done using SEAP PsV, for economic reasons and because these newly obtained SEAP results can be better compared to already existing SEAP results.

Newly produced PsV were further used as antigens to immunize one rabbit per HPV type to obtain type-specific high-titer neutralizing antisera as control for the *in vitro* neutralization and *in vivo* experiments. Altogether, antisera to 15 new HPV types were generated in a week 0-3-6 three-dose immunization scheme with 20µg PsV antigen each dose, using incomplete Freund’s adjuvant (IFA) for the prime injection and complete Freund’s adjuvant (CFA) for the boosts.

As mentioned before, RG1 VLP vaccination induces both neutralizing Ab to homologous HPV16 and cross-neutralizing Ab against many heterologous HPV types. However, they do not cross-neutralize (distantly related) types that show <60% aa identity in the RG1-related sequences, like the cutaneous HPV1, 4 and 38 (unpublished). Therefore, three new RG1 VLP constructs were generated that are expected to protect against medically relevant HPV types, whose “RG1” sequence is less conserved to the HPV16 RG1 sequence. For example, the “RG1” sequences of HPV1 and HPV4 show only a sequence identity of 50% compared to the HPV16 RG1 and the sequences of HPV17 75% (a Beta type), respectively. Therefore, RG1 VLP that specifically target Beta and cutaneous HPV might induce protection against such groups of HPV16-unrelated types. A new HPV1L1-4RG1, with HPV1 L1 as the carrier to display the HPV4 RG1 sequence, aims to protect against prevalent cutaneous wart types. In addition, a HPV5L1-17RG1 chimeric protein has been designed to target Beta HPV types such as HPV5, 14, 15, 17, 20, 23 or 24. Because the HPV45 RG1 sequence shows aa homology of only 75% to HPV16 RG1, a third construct

HPV18L1-45RG1 has been designed. HPV18 and 45 are particularly prevalent in adenocarcinomas of the cervical canal, which may escape PAP detection more often than squamous cell carcinoma and thus account for a progressively larger proportion of cervical cancer in industrialized countries. All newly designed VLP vaccines were expressed by and purified from Sf9 cells, analyzed by Western blot, for correct assembly into VLP by TEM, and by mAb in ELISA for the presence of neutralization epitopes.

2. Material and methods

2.1. Pseudovirions (PsV)

2.1.1. 293TT

The 293TT cell line was kept at 37°C in culture flasks in DMEM media + 10% FCS + 1% NEAA (non-essential amino acids) + 400 μ g/ml hygromycin B in a 5% CO₂ humidified incubator. The 293TT cell line originates from human embryonic kidney cells, which stably express the SV40 large T antigen, and thus enhancing expression of SV40 ORI-containing plasmids, as are used for HPV L1+L2 expression vectors (Pyeon, Lambert et al. 2005).

2.1.2. Transformation

Pseudovirion expression vectors were kindly sent by Richard Roden's lab¹ on filter paper. Altogether, DNA plasmids to generate 21 PsV types were provided: HPV1, 6, 8, 11, 13, 18, 26, 31, 34, 38, 40, 42, 43, 44, 51, 52, 53, 66, 69, 70, 82 (unpublished data, [15]), and the pCDNA^{Luc} reporter plasmid (firefly luciferase (Luc)). The parts of the filter containing DNA were punched out with a single-use sterile blade and eluted into 100 μ l double distilled (dd) H₂O. For transformation of highly competent E.coli (AG1, Agilent Technologies), cells were slowly thawed on wet ice, and 2.8 μ l β -mercaptoethanol followed by one or 10 μ l HPV DNA sample were gently added. A non-related kanamycin resistant plasmid and PUC18 were used as positive and negative controls, respectively. Bacteria were placed on ice for 30 minutes, heat-shocked in a 39°C water bath for exactly 45 seconds and cooled on ice for two minutes. S.O.C medium (250 μ l) (Invitrogen) was added, samples were incubated for 30 minutes at 37°C on a shaker, plated on LB-Agar plates +30 μ g/ml kanamycin (Dilco Luria Agar Basis, Invitrogen) and incubated at 37°C overnight.

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2.1.3. Bacterial culture

A single transformed bacterial colony was inoculated into 3ml of LB medium containing the appropriate antibiotic (e.g. 30^{µg}/ml kanamycin). These preparatory cultures were incubated overnight on a 37°C shaker, 100-150µl were added to 100-150ml main cultures and incubated the same way. In another approach to amplify DNA from bacteria that seem to loose the plasmid early while culturing, one single colony was used to directly inoculate the 100ml main cultures (e.g. HPV4, 53 and 70).

Storage of bacterial stocks: 0.5ml of preparatory cultures and 0.5ml glycerol were transferred into a Cryo-tube and stored at -20°C.

2.1.4. DNA isolation (Maxi plasmid DNA preparation)

DNA isolation was carried out according peqlab's or Qiagen's DNA preparation protocol.

In brief, the pellets of 100ml main cultures were put into resuspension buffer, incubated with lysis buffer for 5 minutes at room temperature (RT), and mixed with neutralization buffer for 20 minutes. After centrifugation at 14,000 rpm (Beckman JA20) for half an hour, supernatants were applied onto equilibrated tips, washed twice and eluted. The DNA was precipitated with isopropanol, washed with ethanol (centrifugation at 15,000 rpm (Beckman ultracentrifuge SW28) at 4°C for 30 and ten minutes respectively, and dried DNA pellets were resuspended in 100µl ddH₂O.

2.1.5. DNA concentration measurement

The DNA concentration was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

2.1.6. Restriction enzyme digestion

A single restriction enzyme (RE) digestion was performed using *Bam*HI (Boehringer Mannheim). For all reactions 1µl of enzyme, 1µl of 10x buffers

(Boehringer Mannheim) and 1µg DNA were added and the volume adjusted with distilled water to 10µl. Digestions were carried out at 37°C for 60 minutes and stored at 4°C.

The digestion products were visualized on agarose 1% gel (Invitrogen or Sigma). The gel-electrophoresis was operated at 100V / 80mA using 1x TBE as running buffer.

2.1.7. Pseudovirion production

PsV were produced in 293TT cells and purified by Optiprep (Sigma) gradient centrifugation according the protocol of Buck et al. in a slightly modified version (<http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm> (Buck, Pastrana et al. 2004)).

In brief, $7-10 \times 10^6$ 293TT cells were incubated for one day in 20ml transfection media (DMEM + 10% FCS, 1% NEAA and 1% Hepes) at 37°C in a 75cm² tissue culture flask. Importantly, for PsV production polystyrene tubes, siliconized pipette tips and eppendorf tubes were used, since PsV easily attach to polypropylene. On the second day, cells were transfected with 20µg L1-L2 double expression vector, e.g. HPV16L1-16L2-pVITRO-neo-mcs, or 13µg L1 + 13µg L2 single expression vectors (eg. HPV18) combined with an equal amount of reporter gene (SEAP or luc). The plasmids used for PsV production were based on pVITRO-neo-mcs' for expression of L1+L2 of HPV1, 2, 4, 8, 11, 13, 18, 26, 27, 31, 33, 35, 38, 39, 40, 41, 42, 43, 44, 51, 52, 53, 56, 57, 59, 66, 68, 69, 70, 73, 82, or HPV18 L1 (pEL1fβ), HPV18 L2 (pEL2bhb), HPV6 L1/L2 (p6shell), HPV11 (p11L1hpUF3 and p11L2hpUF3²), HPV45 (p45shell) and secreted alkaline phosphatase (SEAP³; pYSEAP). The DNAs were diluted into 2ml Optimem (Gibco) and for each sample 85µl Lipofectamine 2000 (Invitrogen) in 2ml Optimem was prepared. Lipofectamine and DNA were incubated at RT for 20 minutes, combined, and after further 30 minutes transferred onto 293TT cells. Transfection took place for 4-6 hours at 37°C, before the medium was exchanged. On the third day, cells were split 1:2 into two new

² kindly obtained from Martin Müller, DKFZ Heidelberg

³ kindly obtained from John Schiller, NIH, Bethesda

75cm² tissue culture flasks. On the following day, the cells were trypsinized, centrifuged (5 minutes, RT at 1,000 rpm), the pellet resuspended in 1ml sterile PBS and harvested by centrifugation (5 minutes, at 4°C, 1,500 rpm). The cell pellet was lysed by adding 1ml of PBS +9,5mM MgCl₂ + 0,2% benzonase, 0,25% brij 58, and incubated for at least 16 hours at 37°C with inverting the samples every 30 minutes for the first two hours. On day five, Optiprep (Sigma Aldrich) step gradients were prepared (27%, 33% and 39%) in 4ml ultracentrifuge tubes (11x60mm, Beckman) and equilibrated for one hour. In the meantime, cell lysates were chilled on ice for 5 minutes, mixed with 17% (volume) 5M NaCl and incubated on ice for 20 minutes. The supernatant after centrifugation (10 minutes, 4°C, and 2,500 rpm, Centrifuge 5402, Eppendorf) was saved, the pellet resuspended in an equal volume of 0,8M NaCl in PBS and the supernatant (10 minutes, 4°C, 2,500 rpm) from both centrifugation steps loaded carefully on top of the Optiprep gradient. Following centrifugation for 3.5 hours at 16°C and 50K rpm using a SW60 rotor (Beckmann) in an ultracentrifuge, 6 fractions (600-800µl each) were taken from top, stored at 4°C, quantified for L1/L2 content by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, see below)/ Coomassie staining and evaluated in a PsV neutralization assay.

2.1.7.1. “high-yield” PsV production

In order to produce a large batch of PsV to provide consistency especially among Luc-containing pseudovirions for later in vivo experiments, a high-yield production method was established. Briefly, 20x10⁶ 293TT cells were incubated in 25ml incubation media in a 175cm² culture flask. Per type, five such flasks were used to produce Luc-containing PsV, whereas one flask was used for SEAP-PsV. The cells were then handled according to the original protocol, but PsV-Luc samples were loaded onto larger Optiprep gradients (8ml thick wall 25x89mm centrifuge tubes; SW28 rotor, Beckman ultracentrifuge).

2.1.8. Evaluation of PsV production

2.1.8.1. SEAP-PsV

For analysis of PsV-SEAP fractions, 3×10^4 293TT cells (in assay medium DMEM + 10% FCS, 1% NEAA, 1% Hepes, 1% Penicillin/Streptomycin) were seeded into each well of a 96-well plate (flat bottom tissue culture; Costar; Corning) and incubated at 37°C until cells were settled. In the meantime, PsV dilutions were prepared in duplicates (U-bottom 96-well plate; Costar; Corning). Aliquots of six PsV fractions were diluted serially 1:2 (1:100, 1:200 and 1:400); an additional 1:100 PsV dilution was incubated with type-specific mAb or antiserum (at dilution 1:4,000) for one hour on ice before transferred onto cells. The assay was evaluated after three days of incubation at 37°C.

20µl of 0.05% CHAPS was added to each well to lyse the cells. After centrifugation of the plates (10 minutes, RT, 200g) 40µl supernatant was transferred into a new 96-well ELISA plate (Nunc) and incubated at 65°C for half an hour. The plate was then put on ice for 3 minutes, incubated at RT for additional 5 minutes, 200µl of SEAP solution (one tablet of 4-Nitrophenyl phosphate disodium salt hexahydrate substrat (Sigma) dissolved in 20ml diethanolamine) was transferred into each well, and the plate was wrapped with aluminum foil for light protection until a change of color occurred. PsV yield was analyzed colorimetrically at 405nm (OpsysMR from Dynex Technologies). Generally, two to three gradient fractions with highest OD values were combined and aliquots stored at -20°C.

2.1.8.2. PsV-Luc

PsV containing the LUC reporter plasmid were analyzed by SDS PAGE/Coomassie staining and PsV neutralization assay. After three-day incubation, plates were frozen at -20°C and finally evaluated with an IVIS 50 bioluminescence imager (Caliper-Perkin Elmer, Mainz, Germany; installed at the Veterinary University of Vienna) for 30 seconds, by addition of Caliper Vivo Glow™ Luciferin (Promega) in a final $150 \mu\text{g}/\mu\text{l}$ concentration without prior lysis of the cells.

Neutralizing rabbit-antisera against VLP of HPV6, 11, 16, 18, 31, 33 and 45 have been generated previously (R. Kirnbauer, unpublished; 1993 and 1995; John Schiller, NCI, NIH, Bethesda, USA). Additional 15 immune sera have been obtained

by immunization of rabbits (Charles River, Sulzfeld, Germany) with the respective PsV types as mentioned before.

2.1.9. *In vitro* PsV-neutralization assay

Antisera from four New Zealand White (NZW) rabbits immunized with RG1 VLP + alum MPL were analyzed for (cross-)neutralization *in vitro* using SEAP-PsV assays as described above. RG1 VLP pre-immune and immune sera were serially diluted from 1:25 to 1:100 for pre-immune and 1:25 to 1:100,000 for immune sera. If available, HPV type-specific neutralizing antisera were used as positive control. Additionally, immune sera after HPV16 L1 VLP vaccination of two NZW rabbits were pooled and used to compare with RG1 VLP immune sera.

2.2. Virus-like particles (VLP)

2.2.1. Sf9 insect cells

Sf9 insect cells were kept in suspension cultures in Grace's medium supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco) and 0.5% Pluronics (a surfactant to reduce shearing of cells; Gibco) at 27°C on a magnetic stirrer using tissue culture flasks (Bellco).

2.2.2. Generation of new RG1-L1 constructs

Three novel recombinant RG1-L1 constructs were designed using CLC DNA workbench software (CLC bio A/S) (Figure 3). HPV18, HPV5, or HPV1 L1 ORF's were chosen to serve as scaffold to genetically insert coding sequences for L2 RG1-homology peptides of HPV45, HPV17, HPV4 (Table 2), respectively, into the DE surface loop, thus encoding the following three fusion-proteins:

- HPV18 L1-45 RG1
- HPV5 L1-17 RG1
- HPV1 L1-4 RG1

HPV16 RG1:	QLYKTCKQAGTCPPD I IPKV	(L2 aa 17-36)
HPV45 RG1:	DLYRTCKQSGTCPPDV INKV	(L2 aa 16-35)
HPV17 RG1:	DIYRGCKQAGTCPPDV INKV	(L2 aa 14-33)
HPV4 RG1:	NLYAKCQLSGNCLPDVKNKV	(L2 aa 14-33)

Table 2: RG1 peptide-alignment of HPV16, HPV45, HPV17, and HPV4. Indicated L2 aa sequences homologous to the HPV16 RG1 epitope (L2 aa 17-36) are aligned.



Figure 3: Design of the new chimeric L1-RG1 constructs. The RG1-homology sequences of HPV4, HPV45 and HPV17 L2 were inserted into L1 of HPV1, HPV18 or HPV17, respectively. Insertion sites were chosen analogous to the original HPV16L1-16RG1 construct, resulting in the repetitive (360 times) exposure of the RG1 sequence by each DE surface loop of a fully assembled VLP. HPV4 RG1 (L2 aa 14-33) was inserted between HPV1 L1 aa 140/141; HPV45 RG1 (L2 aa 16-35) between HPV18 L1 aa 134/135; and HPV17 RG1 (L2 aa 14-33) between HPV5 L1 aa 137/138. Designing steps were done using the CLC DNA workbench software (CLC bio A/S).

Selection of insertion sites in L1:

For HPV1 L1-4RG1 between aa 140/141 of HPV1; for HPV18 L1-45RG1, the L2 peptide was inserted between aa 134/135 of HPV18 L1; for HPV5 L1-17RG1 between aa 137/138 of HPV5 L1 (Figure 3).

2.2.2.1. Transformation, DNA enrichment and Maxi Kit preparation

DNA constructs were synthesized, expressed and codon optimized for *Spodoptera frugiperda* by GeneArt® (Invitrogen). Lyophilized DNA was resolved in ddH₂O to a concentration of 0.1^{µg}/µl., *E.coli* AG1 competent cells (Agilent) were transformed, a single bacterial colony was expanded into 100ml medium and DNA isolated using Quiagen's Maxi Plasmid DNA Kit.

2.2.2.2. Cloning

2.2.2.2.1. Restriction enzyme (RE) digestion

Synthetic fusion genes were provided in Geneart's pMK-RQ vectors with RE sites 5' *KpnI* and 3' *BglII* flanking the fused L1-RG1 sequences. Therefore, double RE digestion with *KpnI* and *BglII* (Roche) was done to release the L1-RG1 fragments (Table 3) and analyzed by 1% agarose gel electrophoresis (80mA).

ddH ₂ O	29 µl	
Buffer L	3.5 µl	
KpnI	1.5 µl	
DNA		1.5µg/µl
→ 45 minutes, 37°C		
Buffer M	3.5 µl	
BglII	1.5 µl	
→ 45 minutes, 37°C		
Table 3: Double restriction enzyme digestion using <i>KpnI</i> and <i>BglII</i>		

2.2.2.2.2. QIAquick Gel extraction kit

QIAquick Gel extraction kit (Qiagen) was used to purify the released L1-RG1 DNA fragments from the agarose gel. Briefly, the band of the right size (compared to DNA standard) was visualized under long-wave UV light, cut out of the gel, weighted and mixed with 3 volumes of buffer QG, incubated for ten minutes at 50°C and periodically vortexed to dissolve the agarose. One volume of isopropanol was added, the sample was applied onto the QIAquick column, and centrifuged for one minute. The supernatant was discarded and additional 0.5ml buffer QG was transferred onto

the column, which was again centrifuged for one minute, washed with 0.75ml buffer, and DNA finally eluted with 30µl buffer EB.

2.2.2.2.3. Ligation into the baculovirus transfer vector (pSynwtVI-)

Purified L1-RG1 DNA was ligated into the pSynwtVI- baculovirus transfer vector (digested by BglII and KpnI and gel purified) by T4 ligase (Roche) at 14°C overnight, using either equal molar ratio of vector and insert, or excess of L1-RG1 DNA (Table 4).

	1)	2)
ddH ₂ O	6 µl	-
Buffer	1 µl	1 µl
T4 Ligase	1 µl	1 µl
pSYNwtVI-	1 µl	2 µl
L1-RG1 fragment	1 µl	6 µl
Table 4: Recipes of ligation reaction for subcloning of L1-RG1 sequences into the baculovirus expression vector. 1) with equal volume of L1-RG1 and vector. 2) with excess of L1-RG1 DNA		

2.2.2.3. Transformation and Maxi Plasmid DNA Preparation

AG1 competent E.coli (Agilent) were transformed with ligation reactions of 18L1-45RG1, 5L1-17RG1 or 1L1-4RG1 DNA and pSynwtVI-, selected by ampicillin resistance (as mentioned above) and plasmid DNA isolated with Qiagen's Maxi Prep Plasmid kit (see above).

2.2.2.4. Post ligation PCR screening, RE double-digestion and sequencing

Bacterial colonies growing on selective agar plates indicated successful transformation. PCR (2720 Thermal cycler; Applied Biosystems) of picked colonies was performed to directly screen for insertion of the full-length L1-RG1 fragment into the transfer vector, using primers flanking the multiple cloning site (synthesized at VBC Biotech Vienna). 40µl of PCR mastermix were aliquoted before 10µl polymerase mix was added (Table 5). Sterile single pipette tips were used to pick the colonies, which were directly put into the aliquoted PCR-mix. As a positive control, 0.5µl

BPVL1-31RG1 pSynwtVI⁻ DNA was used (Table 6). Amplimers were separated by 1% agarose gel and visualized by ethidium bromide staining under UV-light.

Additionally, a control double RE digestion using *KpnI* and *BglII* was done (see above).

	1x mastermix	1x polymerase mix
ddH ₂ O	30µl	8.5µl
Taq buffer	4µl	1µl
dNTP (FinEnzyme)	1.5µl	-
Forward primer	1.5µl	-
Reverse Primer	1.5µl	-
Taq polymerase (MP Q-Biogene)	-	0.5µl
Table 5: Recipe for the baculovirus DNA colony screen by PCR		

	95°C	5 min
30 cycles	95°C	25 sec
	47°C	30 sec
	72°C	1 min
	72°C	10 min
	4°	∞
Table 6: PCR cycles		

To rule out mutations/deletions introduced by sub-cloning, constructs were verified by DNA sequencing (VBC Biotech).

2.2.2.5. Baculovirus transfection

Two ml Grace's insect medium (unsupplemented) + 5µl baculovirus genomic DNA (BaculoGold, BD Bioscience) + 35µl Lipofectin Reagent (Invitrogen) + 2µg transfer vector DNA + 13µg carrier DNA (PUC19) were combined and incubated for ten minutes at RT prior to co-transfection of 2.5×10^6 Sf9 cells for one hour at RT with periodical tilting of culture plates. The supernatant was replaced by 4ml complete Grace's medium (+5% FCS), plates were kept at 27°C for five days to allow for homologous recombination to occur, and the resulting supernatant containing recombinant baculoviruses saved at 4°C.

2.2.2.6. Plaque Assay

To isolate single clones of recombinant virus, 5×10^6 Sf9 cells were transferred into 10cm culture dishes and infected one hour with 10-fold serial dilutions of transfection supernatant (ranging from 10^{-2} to 10^{-8} in Grace's medium without FCS or Pluronics). The supernatant was discarded and 5ml 2x Grace media + 20% FCS + 1% Penicillin/Streptomycin were mixed with 5ml sterile baculo-agarose (1.25% in distilled water; Invitrogen; brought to 47°C in a water bath) and the agarose overlay transferred onto the cells. The samples were then incubated for 4-7 days at 27°C (David O'Reilly, Lois Miller and Verne Luckow: Baculovirus expression vectors, 1992, Freeman).

For each baculovirus expression construct, five (or in the case of 18L1-45RG1 eleven) plaques visualized under a microscope were picked and directly used to infect 2.5×10^6 Sf9 cells for one hour, 4ml complete Grace's media were added and cells incubated at 27°C for seven days. Further passaging of the infectious recombinant baculovirus supernatants (P1, seed stocks) was done to produce high-titer working stocks (P2, P3, P4; (O'Reilly, Miller et al. 1994).

2.2.2.7. PCR screen for recombinant baculovirus

2.2.2.7.1. Proteinase K digestion

Ten or 120µl of baculovirus supernatant from each newly designed construct was mixed with 90µl or 1.08ml of lysis buffer (10mM Tris (pH 8.3), 50mM KCl, $1^{mg}/_{100ml}$ gelatin, 0.45% NP-40 (=Triton X100; Sigma), 0.45% Tween 20 (BioRad) and $400^{ng}/_{mg}$ proteinase K (Boehringer Mannheim)) and incubated for one hour at 55°C before the proteinase K was inactivated by boiling for ten minutes. 25µl of each sample were used for direct PCR or DNA isolation using TriReagent (MCR Gene).

2.2.2.7.2. TriReagent DNA Isolation

DNA was isolated according to protocol either from proteinase K digested samples, Sf9 cell pellets after three-day infection, or directly from baculovirus supernatants.

Briefly, 0.3ml proteinase K-digested samples were homogenized with 1ml TriReagent, stored for 5 minutes at RT and mixed with 0.2ml chloroform. After a 2-5 minutes incubation at RT, samples were centrifuged at 12,000g at 4°C for 15 minutes. The aqueous phase was removed and DNA precipitated from the inter- and organic phase by addition of 0.3ml 100% ethanol, centrifuged at 2,000g for five minutes and DNA pellets washed twice in 1ml 0.1M sodium citrate in 10% ethanol for 30 minutes with periodic mixing and centrifugation at 2,000g in between the washing steps, and DNA pellet suspended in 1.5ml 75% ethanol for 10 minutes to remove the pinkish color. The pellets were then air- and resuspended in 50µl ddH₂O or 8mM NaOH.

2.2.3. Production of high-titer baculovirus supernatant

2.5×10^6 cells in 4ml Grace's medium w/o FCS/pluronic were transferred into 60mm tissue culture-dishes and left for 15 minutes for the cells to adhere. The medium was removed and 100µl of recombinant baculovirus supernatant in 1ml Grace's medium w/o FCS was added. The cells were then incubated for 1 hour with gentle tilting the culture plates every 15 minutes. Subsequently, 3ml Grace's medium + 5% FCS were added, the plates were incubated at 27°C for about 7 days until cells were completely lysed, and virus-containing supernatants were saved at 4°C. Similarly, virus stocks (P2, P3, P4,) were further amplified by successive rounds of infection of 1×10^7 cells in 10cm tissue culture-dishes using 1ml supernatant and ten milliliter Grace's, or 3×10^7 cells in 175cm² flasks using 1ml supernatant and 20ml Grace's.

Virus supernatants were harvested in a 15 or 50ml falcon tube by low-speed centrifugation at 300g at RT for 3 minutes to remove cell debris and stored at 4°C or -20°C.

2.2.4. VLP production and purification

For large scale production of VLP, 500ml suspension culture at 3×10^6 Sf9 cells per ml were collected by low-speed centrifugation and infected with 10ml wt HPV16L1 or HPV16L1-RG1 baculovirus supernatant, or 20ml of 18L1-45RG1, 5L1-17RG1 or 1L1-4RG1 baculovirus at high multiplicity of infection (MOI) in 50ml

Grace's w/o FCS for 1 h with periodic gentle agitation. Ten 245x245mm tissue culture dishes (Nunc) with 90ml Grace's + 5% FCS and 5ml of cell/virus mix were plated and incubated for 3 days at 27°C. Cells were harvested into 250ml conical tubes using a household window wiper and a funnel by centrifugation at 2,500 rpm (Hettich Rotanta rotor 4410). The pellets were pooled into a 50ml falcon tube using 40ml PBS, centrifuged at 3,000 rpm at 4°C for five minutes, and pellets shock frozen in liquid N₂.

After thawing on ice in equal volume of breaking buffer (PBS + 0.8M NaCl + 2mM CaCl₂ + 1mM PMSF) pellets were homogenized by sonication for three minutes (UW 2070; Bandelin Sonopuls, Bandelin electronic; 30% Power). Brij58 was added to a final concentration of 0.5% and samples were rotated overnight at 4°C, centrifuged (Beckman JA20) at 9,500 rpm for 40 minutes at 4°C, supernatants loaded onto 35% (wt/vol) sucrose cushions in PBS + 0.5% NaCl + 0.1% Brij58 into 40ml tubes and ultracentrifuged at 25 krpm (110,000g) for 2.5 hours at 4°C using a Beckmann SW28 swinging bucket rotor. The supernatants were discarded, pellets were resuspended by short sonication in 2ml 29% (wt/wt) CsCl/PBS + 0.05% Brij58 and centrifuged to equilibration (Beckman TI70 rotor) in 25x89mm Quick seal ultracentrifuge tubes at 55,000 rpm for 24 h at 4°C.

Visible bands containing particulate structures were collected using needle and syringe, pooled into two 16x76mm Quick seal tubes, filled with 29% (wt/wt) CsCl/PBS + 0.05% Brij58, and centrifuged (Beckman TI75 rotor) at 55,000 rpm for 48 h at 4°C. The bands were again collected and stored in a polystyrene falcon tube at 4°C (left in the CsCl gradient solution to stabilize VLP integrity).

2.2.5. Dialysis of VLP

To remove CsCl VLP were dialyzed against PBS + 0.5M NaCl + 1mM CaCl₂ and 0.01% Tween 80 (Pierce) over night at 4°C using Slide-A-Lyzer Dialysis Cassettes (Pierce, cutoff 10kD).

2.2.6. SDS Page

The expression of wt HPV16 L1, chimeric HPV16L1-RG1, 18L1-45RG1, 5L1-17RG1, or 1L1-4RG1 proteins (approximate molecular weight (MW) of 55kD) was

verified by SDS-Page of Sf9 cell lysates or purified VLP. The concentration of L1 was estimated by comparison to known concentrations of bovine serum albumin (BSA; MW of 66,5kD).

Routinely four 10% SDS-gels were poured simultaneously in a Mighty Small II device (Hofer Scientific Instruments; Table 7). A 10x running buffer with 30.3g Tris, 144.1g glycine and 50ml 10% SDS, filled up to 1 liter with ddH₂O, was prepared and diluted to a 1x buffer prior usage.

	Separation gel	Stacking gel
dd H ₂ O	20ml	11.76ml
30% acrylamide/bisacrylamide	20ml	2.08ml
Tris/HCl pH 8.8	15ml	-
Tris/HCl pH 6.8		2ml
APS	420µl	160µl
TEMED	42µl	8µl
Table 7: Recipe for four 10% SDS protein gels		

A commercial protein marker (MW 26,6-180kD) SDS7 or SDS7-B2 (Sigma Aldrich), and BSA of 0.125 ^{mg}/_{ml}, 0.2 ^{mg}/_{ml}, 0.5 ^{mg}/_{ml} and 1 ^{mg}/_{ml} concentrations were used as standards. A non-infected Sf9 cell lysate was used as a negative control. Prior to loading, an equal volume of Lämmli buffer + 2% freshly added 5-mercaptoethanol (ME) was transferred to the samples, which were then boiled for 5 minutes in a water bath, 20µl of each sample were loaded onto the gel and electrophoresis was performed at 30mA per gel. Afterwards the gel was fixed and stained for one hour in Coomassie dye (5g Coomassie Brilliant-Blue R (Sigma) + 100ml acetic acid + 500ml methanol and 400ml H₂O) and destained in tap water overnight.

2.2.7. Western blot

After electrophoresis, wet Western blotting was performed in a Mini Blot cell (Biorad) device at 45V for one hour using blotting buffer composed of 200ml methanol and 100ml 10x SDS running buffer filled up to one liter with ddH₂O. The blotted membrane (Immobilon transfer membrane; Millipore) was blocked with 0.5% milk/PBS for 15-30 minutes. Incubation with the first Ab in a 1:5,000 or 1:10,000 dilution in 0.5% milk/PBS were performed for 1 hour at RT or at 4°C ON on a rotary shaker.

HPV16 wt L1 sample was detected using mAb Camvir-1, and Camvir-1 and anti-RG1 were used to identify the chimeric RG1-L1 protein. Since the Camvir-1 epitope in HPV16 L1 is 100% identical to the respective epitope in HPV1 L1, Camvir-1 was used as well to detect the 1L1-4RG1 protein. The anti-BPV1 L1 mouse mAb AU1 was used to detect the 5L1-17RG1 protein, and Camvir-1, AU1, polyclonal rabbit and mAb H18.E20 anti-HPV18 L1 were used to identify the chimeric 18L1-45RG1 fusion protein. Threefold washing with 0.5% milk/PBS was done prior incubation with the second antibody goat anti-mouse IgG (H+L) (or rabbit) coupled to HRP (horse raddish peroxidase, BioRad), in a 1:20,000 or 1:40,000 dilution for one hour.

After a threefold washing step, ECL Western blotting substrate (Pierce, Thermo Scientific) was applied for one minute onto the membrane according the manual's description for HRP detection, and the membrane exposed to an X-ray film (Hyperfilm ECL, Amersham-Pharmacia).

2.2.8. Transmission electron microscopy (TEM)

Assembly of HPV16 wt L1, chimeric HPV16L1-16RG1 (RG1 VLP), 18L1-45RG1, 5L1-17RG1, or 1L1-4RG1 proteins into particulate structures was visualized by TEM.

Two copper grids per sample were discharged using Salzers CTA 010. Immediately after discharging the grids were put on a drop of gradient-purified protein sample for 10 minutes, fixed for 20 minutes on 2.5% glutaraldehyde, rinsed with

ddH₂O, and stained with 1% uranylacetate for 3 minutes. A JEOL 1010 electron microscope at 80 kV was used for visualization and micrographs were taken at 30,000x magnification.

2.2.9. ELISA

Antigenicity of purified VLP was analyzed by Enzyme-linked Immunosorbent Assay (ELISA) under native or denatured conditions. For native ELISA, 0.1 or 0.6µg VLP in 100µl cold PBS per well were plated into a 96-well plate (Nunc, Maxisorp) and incubated over night at 4°C. For denatured ELISA, 0,1^{µg}/100µl PBS VLP were dried onto the ELISA plate in 0.2M NaHCO₃ (pH 10.6) + 0.01M Dithiothreitol (DTT) denaturation buffer by incubation at 37°C overnight with air-circulation.

On the second day plates were washed three times with PBS and wells blocked with 0.5% milk/PBS for 1 hour at 4°C. Serial 1:4 antibody dilutions in 0.5% milk/PBS ranging from 1:200 to 1:204,800 were applied in triplicates and the ELISA plate was gently rocked on a rotary shaker for 1 hour at RT. The RG1, Camvir-1, H16.E70 and H16.V5 mAb were used for probing both HPV16 wt L1 and HPV16-RG1 proteins. The 1L1-4RG1 protein was analyzed with mAb to HPV1 L1 or HPV1 L2⁴. Fusion protein 5L1-17RG1 was contacted with mAb HPV5 2.1.1. and HPV5 52, recognizing L1 of the HPV5 variant a and c, or variant b, respectively⁵.

After a threefold washing step, goat anti-mouse IgG (H+L)-HRP was applied in a 1:10,000 dilution (100^{µl}/well) for 45 minutes at RT on the ELISA shaker and again washed 4 times. As substrate, one tablet ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; Roche) was dissolved in 5ml 10x ABTS buffer and 45ml ddH₂O, 200µl per well transferred, the plate incubated in the dark for 20 minutes at RT, and the OD at 405 nm determined in an ELISA reader (Opsys MR, Dynex Technologies).

⁴ kindly obtained by J.Carter, Fred Hutchinson, University of Washington, Seattle

⁵ kindly obtained by Michel Favre, Institut Pasteur, Paris

2.3. Immunization of NZW rabbits

2.3.1. PsV immunizations

Prior to immunization, Optiprep PsV fractions with a visible 55kD protein band on SDS/Coomassie gel were further analyzed by Western blot using the antibody Camvir-1. Immunizations were performed for a total of 15 PsV types: HPV26, 35, 39, 42, 44, 51, 52, 53, 56, 58, 59, 66, 68, 70 and 73.

One NZW rabbit per HPV type was immunized 3 times (week 0-3-6) with 20µg PsV each dose, using CFA for the prime and IFA for the boost applications. Sera were obtained before priming or at week 8 (Charles River, Germany).

2.3.2. HPV16-RG1 and wild-type HPV16L1 VLP immunizations

Immunizations were performed at Charles River (Germany). Two or four NZW rabbits were immunized in a 3-dose regimen, with 20µg wt HPV16L1 VLP or HPV16L1-RG1 VLP, respectively, at week 0, 3 and 6 using alum-MPL adjuvant. VLP were dialyzed into PBS and diluted buffer to a final volume of 500µl. Per injection, one vial of MPL adjuvant (Sigma, S6322) was resuspended in 500µl saline and vortexed for a few minutes. The adjuvant was mixed with antigen, vortexed for 1 minute, left at RT for 5 minutes, and 400µl Alum (Sigma) was added. Three times 700µl of this adjuvant/antigen solution was used to immunize one rabbit, and blood drawn before prime and two weeks after the final boost (week 8).

2.4. *In vivo* mouse vaginal PsV challenge

2.4.1. Mice

Six to 8 weeks old Balb/c mice (purchased from Charles River, Germany) were kept under SPF (specific pathogen-free) conditions at the Veterinary University of Vienna (Institute of Laboratory Animal Science, Prof. Rülcke, chief).

2.4.2. Procedure

At first, pilot experiments were performed to establishing optimal conditions for HPV PsV infection. Setting a minor trauma by Cyto-brush 15x intra-vaginal rotation exposes the basement membrane, which is a critical step for efficient epithelial infection. First naïve mice were inoculated with PsV to ensure infection in non-immunized animals. For experimental immunization and PsV challenge the following established protocol was used (Figure 4, <http://www.nature.com/protocolexchange/protocols/249>):

Day 0: Subcutaneous (s.c.) injection with 100µl Depocon (3mg; Pfizer) to synchronize the oestrus.

Day 3: Intravenous (i.v.) immunization (passive transfer) with 20µl of pre-immune or immune serum of HPV16 L1- or RG1 VLP-vaccinated rabbits.

Day 4: Mice were anesthetized by intraperitoneal (i.p.) injection with Ketazol/Rompun (each 2,5ml (6.25%) + 35ml ddH₂O; Graeb and Bayer), and HPV16 PsV from different preparations⁶ were diluted 1:1 in 3% carboxymethylcellulose (CMC; Sigma Aldrich). The vaginal challenge was preceded by mechanical disruption of the vaginal mucosa with a cyto-brush (Cooper Surgical). 15µl PsV-CMC were deposited intra-vaginally using a positive displacement pipette (Gilson), the cyto-brush rotated 20 times clock- and anticlockwise and again 15µl PsV-CMC transferred intra-vaginally.

Day 7: Mice were anesthetized by isoflurane inhalation (ca. 2 Vol%; Isoba, Essex Tierarznei), and 40µl D-luciferin (Promega Caliper Vivo Glow™; 15mg/ml stock) were transferred intra-vaginally. Infection was evaluated by analyzing luciferase expression using an IVIS50 bioluminescence imager (Caliper) and Igor Pro 4.09A software. The pictures were recorded after 10 minutes exposure counting the $\text{photons}/\text{second}$ emitted. A luciferase signal that significantly exceeded the background signal emitted by non-challenged mice indicated successful infection.

⁶ one preparation kindly provided by Kihyuck Kwak, Pathology, Johns Hopkins University, Baltimore, USA

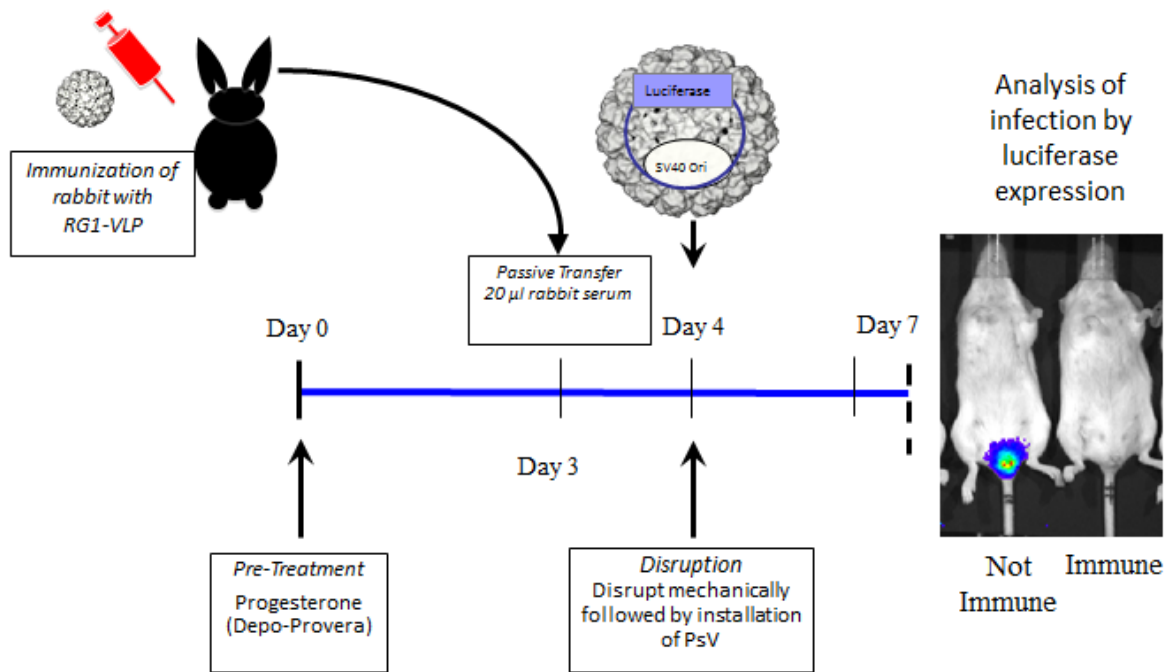


Figure 4: HPV PsV mouse vaginal challenge model. Illustration from Schellenbacher (adapted from Roberts, Buck et al. 2007)

3. Results

3.1. Pseudovirions

3.1.1. Plasmid DNA isolation

In total 21 DNA vectors for capsid protein expression of different HPV types were received, used to transform competent E.coli, and amplified DNA was isolated by maxi plasmid DNA preparation of 100ml cultures. Typically, a DNA concentration higher than 1µg/µl was obtained for transfection to produce PsV.

Restriction enzyme digests with *BamHI* were performed to verify DNA constructs. L1 open reading frames (ORF) have uniformly been cloned into multi cloning site (MCS) 2, and L2' ORF into MCS 1 of pVITRO-neo-mcs (for plasmid map see http://www.invivogen.com/PDF/pVITRO1-neo-mcs_TDS.pdf). Although the plasmid map of parental pVITRO-neo-mcs indicated two *BamHI* sites, digestion with this RE of plasmids with HPV structural genes inserted resulted in only one band in all of the samples. This was due to loss of the second *BamHI* site at MCS2 by insertion of *BglII*-restricted HPV L1 genes, resulting in a non-cleavable site combined from both sequences of *BglII* and *BamHI*. With this information *BamHI* digestion was predicted to linearize the vectors.

As examples, L1+L2 double expression vectors for HPV34, 44, and 52 (Figure 5) migrated as single bands after *BamHI* digestion at approximately 9,400bp (see marker), which size equates to the sum of the parental vector (6,300bp) plus L1 (~1500bp) and L2 (~1500bp) (total plasmid size of 9,300bp), indicating successful transformation and isolation (an identical restriction pattern was observed for the remaining 18 PsV types, data not shown). The parental pVITRO-neo-mcs vector was used as control (with or without *BamHI* restriction) (Figure 5, lanes 4 and 5) and showed the expected linearized band near 6,500bp.

3.1.2. Pseudovirion production

Papillomaviruses are species-specific and productive infection is restricted to highly differentiated epithelia. Thus HPV cannot easily be propagated in tissue

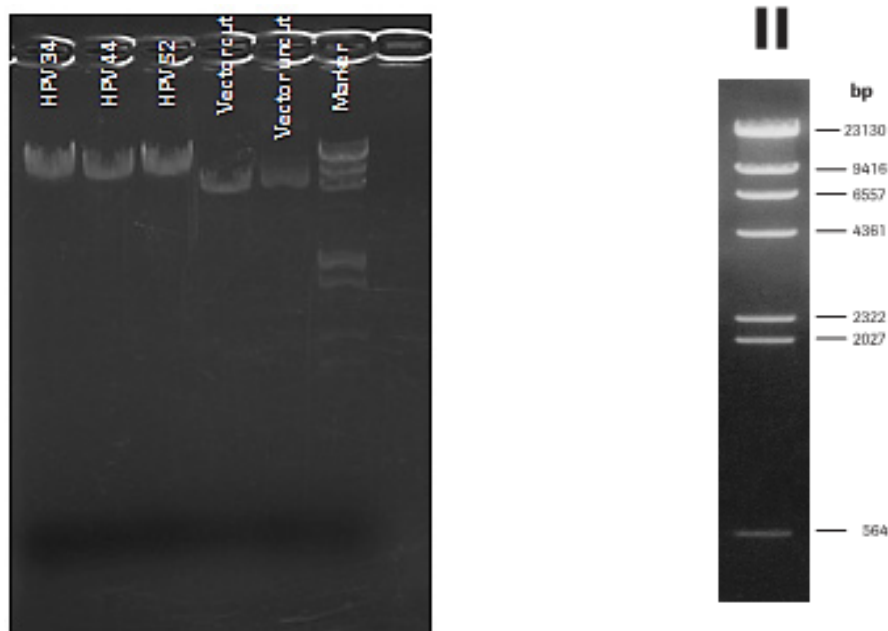


Figure 5: a) *Bam*HI digestion of pVistro-neo-mcs HPV34, 44 and 52 L1+L2 double expression vectors ($1\mu\text{g}/\mu\text{l}$; Lane 1-3), Lane 4: **pVistro-neo-mcs parental vector**, Lane 5: **pVistro-neo-mcs uncut** and Lane 6: DNA size markers II and VI ($1\mu\text{g}/\mu\text{l}$). The three linearized HPV vectors migrate at 9,500bp. **b) DNA marker II** (Roche) with the indicated bp range of fragment size.

culture or laboratory animals. Important progress has been achieved by the recent generation of PsV as proxies to study early events of papillomavirus infection.

Production of PsV is a lengthy and complex process. For some HPV types (e.g. HPV16, HPV6) PsV production at high concentration is very robust, whereas for other types (e.g. HPV11, HPV1) production proved far more difficult. Although the reason(s) for this different behavior is largely unknown, this may relate at least in part due to differential codon-usage requirements by mammalian cells. Accordingly, improved PsV production has been achieved by expressing capsid proteins from different subtypes of HPV (R. Roden, personal communication).

For immunization of NZW rabbits with a total of 60 μg of PsV to generate type-specific immune sera, and for the *in vivo* PsV challenge, it was necessary to establish a “high-yield” PsV production protocol. PsV-SEAP from high-yield productions were compared to conventional productions, generally demonstrating slightly increased PsV concentrations at similar total volumes. As a result, it was possible to generate PsV from HPV types that normally did not successfully produce useful PsV (e.g. HPV4 and HPV11, see figure 6A and B, respectively).

3.1.2.1. Evaluation of HPV18 PsV

Figure 7 shows the evaluation of HPV18 PsV produced by the original protocol. Fractions were harvested from an OptiPrep gradient (see methods) and analyzed for the ability to infect 293TT cells. After 3 days, detection of SEAP activity in the supernatant served as readout. A clear signal was derived from infections with gradient fractions 5 at dilutions 1:100, 1:200 and 1:400. Importantly, the signal was reduced to background (cells only) by pre-incubation of PsV with a neutralizing antiserum, indicating correct HPV18 PsV assembly. Consequently, fraction 5 was further used at a 1:400 dilution.

To avoid inconsistency between batches of PsV, a “high-yield” production protocol was established. Figure 8 shows the evaluation of “high-yield” HPV18 PsV preparation following gradient purification. Again, 600-800µl gradient fractions were harvested, 293TT with small aliquots infected and supernatants analyzed as before. Robust signals were generated by fractions 4 and 5, which were both neutralized to background by the type-specific antiserum. These two fractions were pooled and further used at a 1:400 dilution. From repeated experiments it appears that this “high-yield” production can result in several fold higher total yields of PsV compared to the standard protocol, since at least two fractions, for some HPV types even up to four fractions, contained useable PsV. Typically, at least twice the volume of PsV, useable at high dilution, were obtained. For some types (e.g. HPV31, HPV33) we even obtained 4 fractions containing PsV that could be used in a 1:400 dilution (data not shown). For other types (e.g. HPV6) though, PsV yield was very comparable to ‘normal’ production protocol.

PsV containing the LUC reporter gene were analyzed similarly in a PsV-neutralization assay, but evaluated by bioluminescence imaging (IVIS50). Cells infected with PsV were mixed with luciferin, exposed for 30 seconds and the flux (photons per second) measured. Preparations of efficient PsV showed flux values of 10^7 - 10^8 (e.g. HPV45, HPV16), whereas preparations of more difficult-to-produce HPV types typically showed values of 10^5 - 10^6 photons/sec (e.g. HPV11; data not shown).

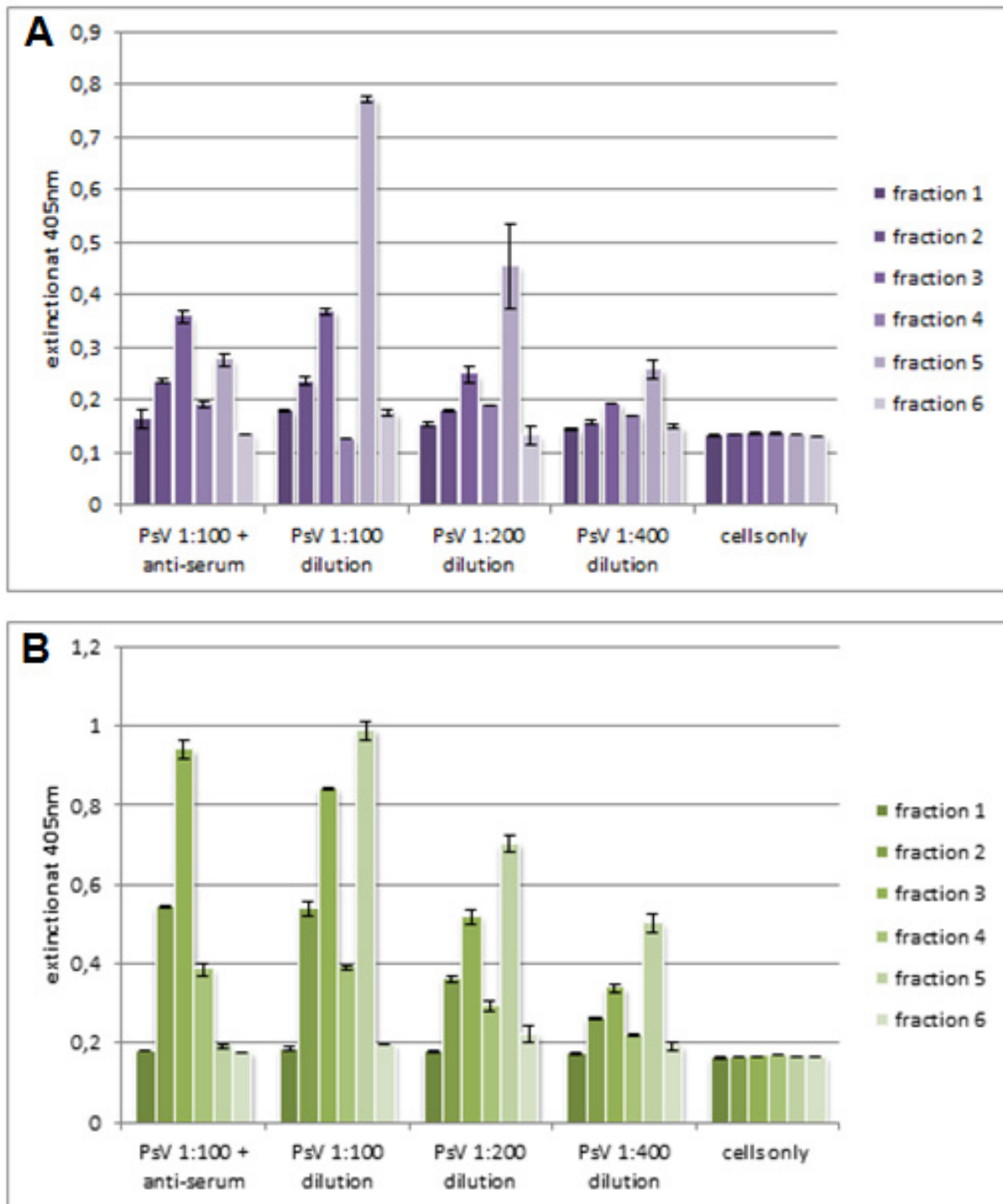


Figure 6: Analysis of HPV4 PsV and HPV11 PsV obtained by “high-yield” procedure: Altogether, six fractions 600-800µl each were harvested from OptiPrep gradient, serially diluted and used to infect 293TT cells for 3 days, and supernatants were analyzed calorimetrically by SEAP signals at 405nm. In order to verify the presence of correctly assembled pseudovirions, PsV fractions in a 1:100 dilution were pre-incubated with a HPV type-specific neutralizing anti-serum (1:1,000) before infecting 293TT cells. For HPV4 (A) and HPV11 (B) PsV productions the bars show a high signal at fractions 5 at all PsV dilutions, which, is reduced to background-level for PsV + anti-serum, indicating specific neutralization. These fractions were stored at -20°C and further used at a 1:100 dilution. As background, signals from cell cultures only were measured. The error bars indicate the standard deviation from the mean.

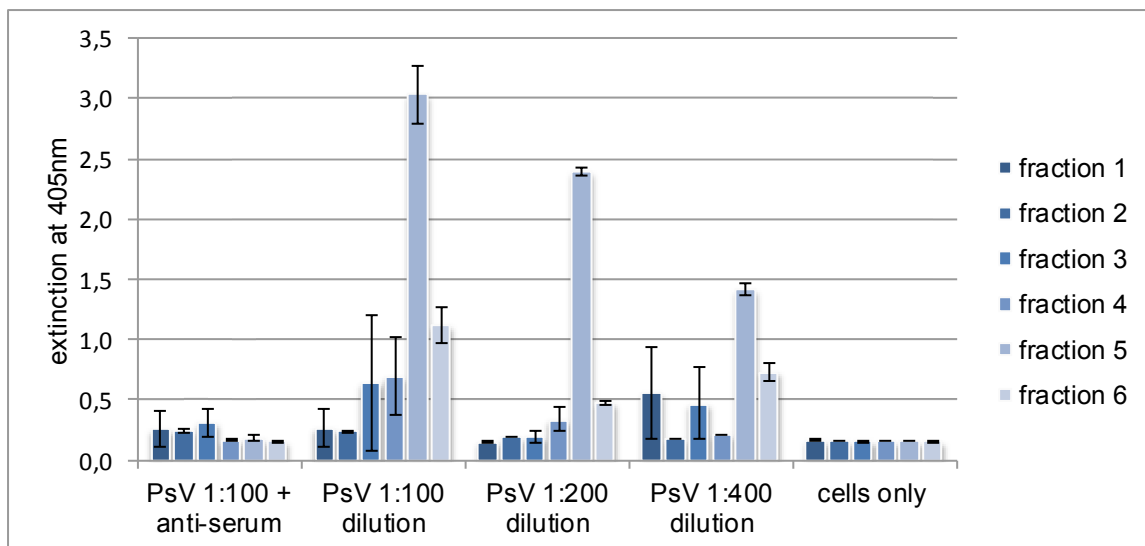


Figure 7: Analysis of HPV18 PsV generated by the original protocol: Six 600-800µl fractions each were harvested from OptiPrep gradient, diluted and used to infect 293TT cells for 3 days, and supernatants analyzed calorimetrically for SEAP signals at 405nm. In order to verify the presence of correctly assembled pseudovirions, PsV in a 1:100 dilution were neutralized using a HPV type-specific anti-serum before infecting 293TT cells. The bars show a high extinction at fraction 5 at all dilutions and to a lesser extent at fraction 6; however, there is only background-level signal for PsV + anti-serum, indicating successful neutralization of pseudovirions. Fraction 5 was stored at -20°C and further used at a 1:400 dilution. As background, supernatants from uninfected 293TT (cells only) were measured. The error bars show the standard deviation from the mean.

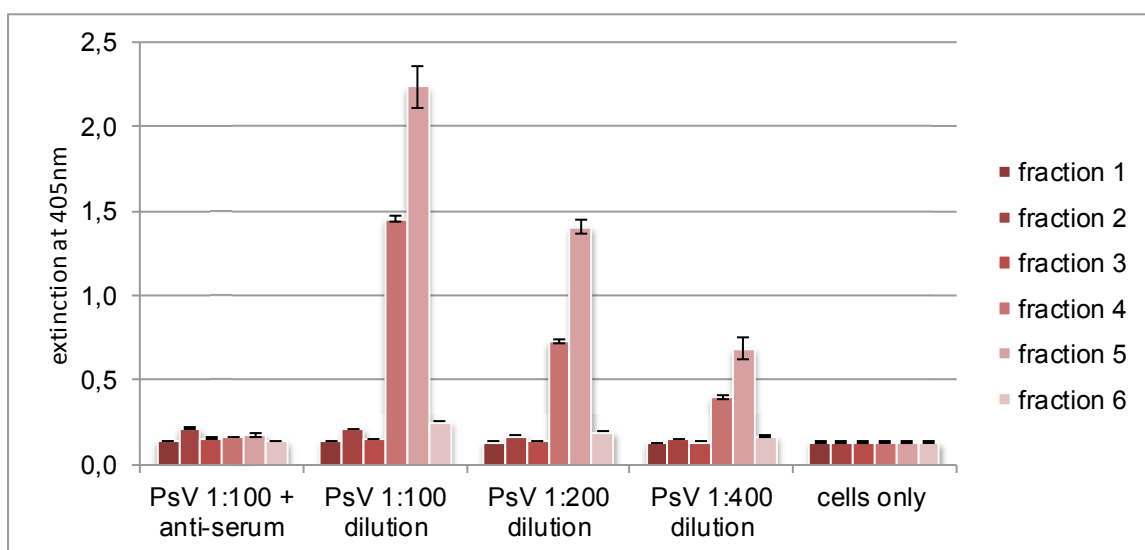


Figure 8: Analysis of HPV18 PsV obtained by "high-yield" procedure: Six 600-800µl fractions each were harvested from OptiPrep gradient, diluted and used to infect 293TT cells for 3 days and supernatants analyzed calorimetrically by SEAP signals at 405nm. In order to verify the presence of correctly assembled pseudovirions, PsV in a 1:100 dilution were neutralized using a HPV type-specific anti-serum before infecting 293TT cells. The bars show a high signal at fraction 4 and 5 at all dilutions; however, there is only background-level signal for PsV + anti-serum, indicating successful neutralization of pseudovirions. These two fractions were pooled and stored at -20°C and further used at a 1:400 dilution and. As background, cell supernatant from uninfected 293TT (cells only) were measured. The error bars show the standard deviation from the mean.

We and others have observed difficulties to produce PsV for types such as HPV4 or HPV1 (our unpublished observation; Richard Roden, personal communication), and the production of infectious PsV for types HPV1, HPV82 and HPV73 has failed despite our multiple attempts (data not shown). The reason for these failures is yet unclear as both L1 and L2 proteins are expressed (not shown); generating constructs with modified codon usage and/or using capsid gene sequences derived from other subtypes may overcome these limitations.

3.1.2.2. Evaluation of PsV used for immunization

Prior to immunization freshly produced PsV were analyzed by Western blot for the expression of the structural protein L1. Figure 9 shows an example of 7 PsV types (HPV26, HPV35, HPV39, HPV42, HPV44, HPV51 and HPV52) that express the 55-60kDa L1 capsid protein, using mAb Camvir-1 that detects a type-common linear epitope. Additional 8 PsV types (HPV53, 66, 58, 59, 66, 68, 70 and 73) were analyzed similarly, confirming respective L1 expression (data not shown). HPV16 VLP were used as a positive and 293TT cells as a negative control.

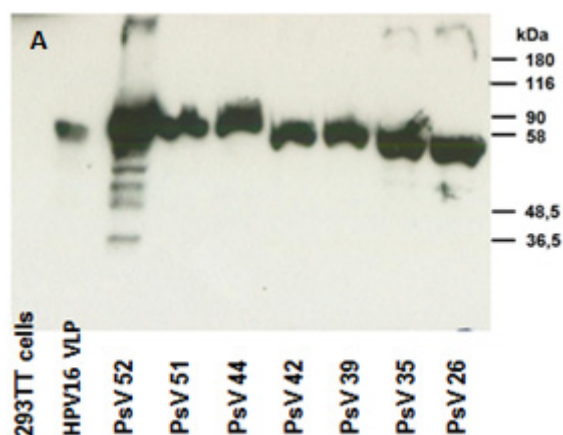


Figure 9: Western blot of HPV PsV types 26, 35, 39, 42, 44, 51 and 52. The figure shows seven of the 15 PsV types used to immunize NZW rabbits. The L1 proteins are recognized by mAb Camvir-1 and migrate in the range of 55 to 60 kd. HPV16 wt L1 VLP were used as positive control and a 293TT cell lysate as negative control.

3.2. Evaluation of antisera raised by RG1 VLP vaccination

Four NZW rabbits were immunized 3 times at weeks 0, 2, 6, each with 20 µg RG1 VLP and alum-MPL adjuvant. This immunization scheme is more in line with immunization protocols of licensed HPV vaccines (3 injections of 20-40 µg of each VLP over six months), yet less intense than the vaccination protocol used in our

previous study (Schellenbacher 2009). Sera were drawn before first injection and 2 weeks after the final boost and evaluated by PsV neutralization assays. Since previously generated RG1 VLP sera (unpublished data, Schellenbacher) have been evaluated with PsV containing the SEAP reporter plasmid, for comparison neutralization assays were performed with SEAP-PsV as well.

Table 8 shows results of the PsV assays indicating neutralization titers of RG1 VLP immune sera against 18 mucosal and 2 cutaneous HPV types. For 18 mucosal alpha PsV types, all four sera showed neutralization titer of 100 up to 10,000 to the most important high-risk type HPV16, and titers from 25 to 100 against HPV18. Further, all sera neutralized HPV35, which is most closely related to HPV16 with titers up to 1,000, and HPV26 with titers up to 100. All 4 sera were non-neutralizing for cutaneous Beta HPV38 and Gamma-type HPV4. At least one immune serum showed titers against HPV6, 11, 31, 33, 40, 44, 52, 53, 66 and 69, respectively. Two sera did neutralize HPV45, and three sera HPV34, 39 and 70. Two pooled immune sera raised by HPV16 wt L1 VLP immunization showed titers ranging from 50 – 1,000

Nr of serum PsV Type	Serum 1	Serum 2	Serum 3	Serum 4	Anti-HPV16 Serum	
HPV6	0	0	100	0	100	mucosal alpha-HPV
HPV11	0	0	50	0	0	
HPV16	100	100	10 000	10 000	1000	
HPV18	100	50	100	25	0	
HPV26	<25	100	50-100	50	0	
HPV31	0	0	25	0	100	
HPV33	0	0	100	0	50	
HPV34	<25	0	50	<25	0	
HPV35	100	100	1000	50	100	
HPV39	<25	<25	50	0	0	
HPV40	0	<25	0	0	0	
HPV44	0	0	0	100	0	
HPV45	0	25	100	0	0	
HPV52	0	0	25	0	0	
HPV53	0	0	0	<<25	0	
HPV66	0	0	<25	0	0	
HPV69	0	0	<25	0	0	
HPV70	25	50	100	0	0	
HPV4	0	0	0	0	0	Gamma-PV
HPV38	0	0	0	0	0	Beta-PV

Table 8: Results of PsV-neutralization assays analyzing RG1 VLP sera. Four RG1 VLP antisera raised in NZW rabbits were evaluated in neutralization assays using indicated mucosal genus Alpha or cutaneous Beta or Gamma HPV PsV types containing the SEAP reporter gene. The table shows the (cross-)neutralizing antibody titers against indicated types. Neutralization titers refer to reciprocals of the highest serum dilution causing 50% reduction of SEAP activity when compared to pre-immune sera.

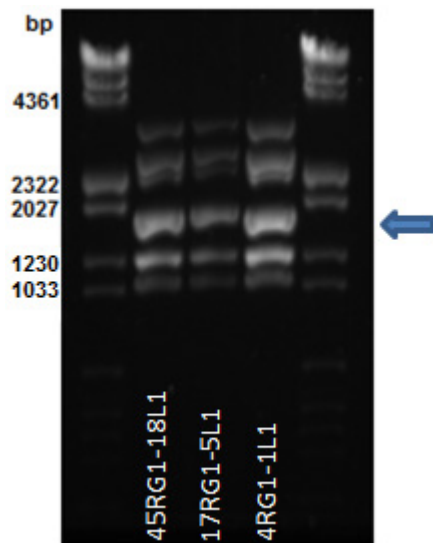


Figure 10: Double restriction digestion with *KpnI* and *BglII* to release L1-RG1 sequences from the pMK-RQ backbone. Band 1 and 5 show the DNA molecular marker (Roche). Since the commercial backbone vector showed additional *KpnI* and *BglII* sites, double digestion with these enzymes resulted in six DNA fragments. The blue arrow indicates full length L1-RG1 sequences at approximately 1600bp. Gel slices were cut out of the gel and DNAs isolated.

restricted to HPV6, 16, 31, 33 and 35. Pre-immune sera used as negative controls were consistently non-neutralizing (data not shown).

3.3. VLP

3.3.1. Generation of additional 'RG1'-L1 fusion proteins

Our unpublished data indicate that immunization with RG1 VLP does not induce cross-neutralizing antibodies against important cutaneous types. Presumably this is due to lower sequence homology (generally $\leq 70\%$) of the HPV16 RG1 aa sequence to corresponding L2 sequence of many cutaneous types (contrary to most mucosal types). Therefore, chimeric L1-L2 fusion proteins that incorporate analogous L2 peptides ('RG1') of HPV45, HPV4 and HPV17 have been designed similarly, which target additional clades of mucosal high-risk, cutaneous, and beta HPV types, respectively. We hypothesize that immunization with 'RG1' VLPs that specifically target Beta- and other cutaneous HPV might result in cross-neutralizing antisera against these genetically less related types. In addition, the HPV18L1-45RG1 construct is expected to target the (after HPV16) most important mucosal HPV18 and HPV45 that are frequently found in adenocarcinomas of the cervical canal.

The designing steps included the designation of RG1-homologous aa sequences of the respective type by visual inspection (or using BLAST), the determination of possible insertion sites into the DE-loop, codon optimization, and verification of chimeric fusion sequences (using CLC data).

Designed chimeric L1-RG1 DNA constructs with flanking RE sites were synthesized by GeneArt (Invitrogen) and inserted into backbone vector pMK-RQ. For expression in Sf9 insect cells, L1-RG1 sequences were released by *Bgl*III and *Kpn*I RE digestion (Figure 10), the fragments of approximately 1,600bp were separated by agarose gel, isolated and subcloned into pSynwtVI- baculovirus transfer vector (Kirnbauer, Taub et al. 1993). Following transformation of competent E.coli, colonies grown on selective media plates were screened by PCR.

Figure 11 shows faint amplimers at the expected size of 1,800-1,900bp for several colonies, using primers RK100 and RK101 that recognize baculovirus vector-specific sequences just outside the cloning site thus amplifying the whole inserted sequence. These results indicate that both ligation reactions, either with similar molar ratio of vector pSynwtVI- and L1-RG1 insert, or with excess of L1-RG1 DNA, were successful. Two clones for each construct (marked red in Fig.11) were selected and further characterized.

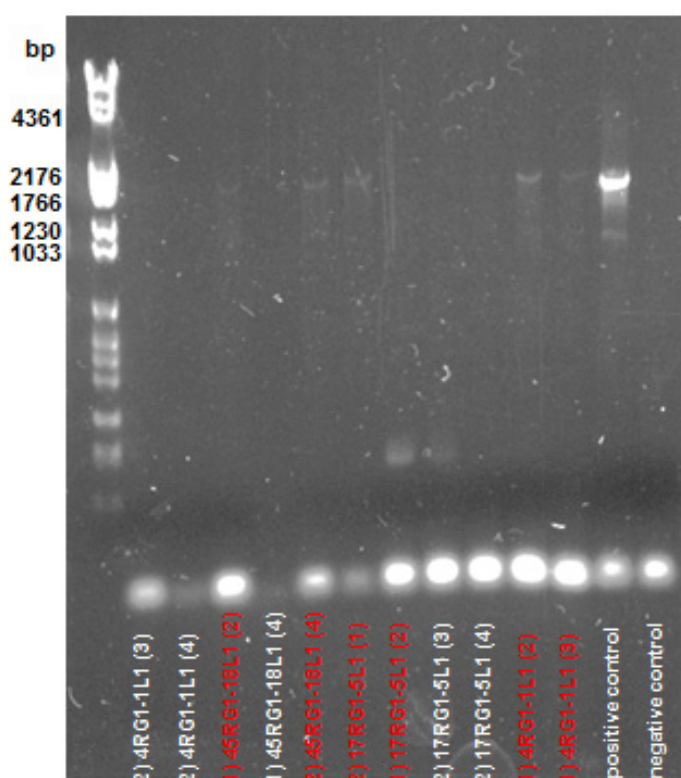


Figure 11: PCR screening for L1-RG1 subcloned into pSYNwtVI-. L1-RG1 of HPV18L1-45RG1, HPV5L1-17RG1 and HPV1L1-4RG1 were subcloned into pSynwtV- and obtained bacterial colonies screened by PCR using vector-specific primers. Faint amplimers at approximately 1,800-1,900bp can be seen. Two samples of each L1-RG1 construct were chosen for further use (in red). As seen, ligation according both protocols [either with 1) equal molar ratio of baculo expression vector and L1-RG1 DNA; or with 2) three times more L1-RG1 DNA as expression vector, indicated by the number before the sample's name; see methods] was successful. The numbering after the sample's name indicates the number of the picked bacteria colony. A BPV1L1-31RG1 was used as positive control, showing a HPV31RG1 band at approximately 1,800-1,900bp

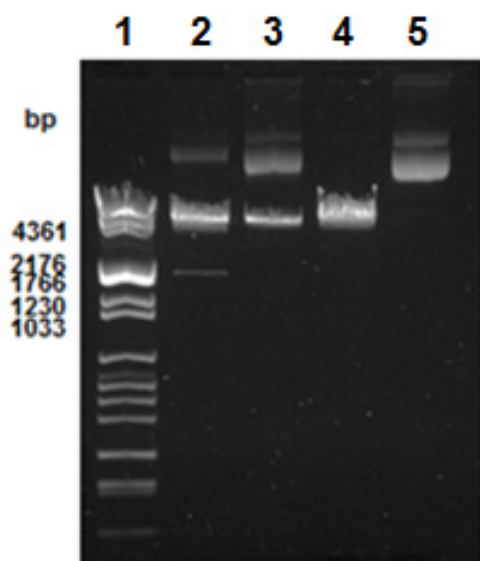


Figure 12: Verification of a 5L1-17RG1 pSynwtVI- DNA clone by single and double restriction digestion with *KpnI* and *BglII*. These RE sites flank the L1-RG1 sequence and double digestions of successfully ligated samples (partially) releases a band of approximately 1,800-1,900bp as expected (lane 2). Single digestions with *KpnI* (lane 3) or *BglII* (lane 4) linearizes the DNA with the expected size of around 6.500bp. The undigested sample (Lane 5) acts as control, also indicating the presence of undigested DNA (migrating similarly) in lanes 2 and 3.

Colonies were grown in 100 ml cultures, plasmid DNA was isolated and double digested by RE *BglII* and *KpnI*. Figure 12 shows digestion of HPV5L1-17RG1, a similar outcome was seen with HPV1L1-4RG1 and HPV18L1-45RG1. Single digestions with either enzyme or undigested DNA served as controls. Lane 2 shows a faint band of approximately 1,800bp corresponding to the excised L1-RG1 sequence. The other two bands show the undigested plasmid (uppermost band) or incomplected digest (linearized plasmid, second band). Equal results were obtained for all constructs' samples, thus one clone each was chosen for further work.

The absence of point mutations or deletions in the L1-RG1 flanking regions, that may result from subcloning, and integrity of the ORFs were verified by DNA sequencing of the junction sequences using vector-specific primers flanking the insert (not shown; VBC Biotech, Vienna).

3.3.2. Plaque assay and production of high-titer recombinant baculoviruses

Production of infectious high-titer baculovirus supernatants for expression of HPV16 wt L1 VLP and chimeric RG1 VLP required passaging from originally frozen seed-stocks, as we observed loss of infectivity for viral supernatants that had been stored at 4°C for more than a year. Following 10 to 12 passages high titer and large volume baculovirus working stocks were obtained for large scale VLP production.

New recombinant baculoviruses were generated by co-transfection of transfer vector and baculovirus genomic DNA into insect cells (see materials and methods), followed by plaque purification using established protocols. After an infection time of seven days, for all new L1-RG1 constructs well-defined single plaques were identified visually by naked eye and under the microscope, picked with a sterile pipette and transferred into insect medium to infect new Sf9 cells. The resulting clonal baculovirus was further passaged and amplified by several rounds of infection. High-titer working stocks were generated to sufficient volumes (ca. 100 ml) that are required for large-scale infection of Sf9 cells at high MOI to efficiently express recombinant proteins and produce preparative amounts of VLP.

3.3.3. Protein expression and VLP purification

3×10^6 Sf9 insect cells/ml (from 500ml suspension culture) were harvested by centrifugation, pooled into 250 ml and infected at high MOI with 10ml baculovirus supernatant expressing HPV16 wt L1 or RG1-L1, or 20ml for HPV18L1-45RG1, 5L1-17RG1 and 1L1-4RG1, and plated using 10 245x245mm culture dishes. Three days later cells were harvested, lysed and particulate structures purified by several rounds of density gradient ultracentrifugation (see materials). Typically, ca. 6 - 7ml VLP/CsCl fractions (concentration ranging from 0.1-0.5^{mg}/ml L1 protein, see below) were obtained and aliquots used for overnight dialysis, SDS-PAGE, and TEM analysis.

3.3.4. SDS Page

In order to estimate concentration and purity of recombinant proteins, samples of 20 μ l (plus same volume Lämmli-buffer added) were separated by SDS-PAGE, stained with Coomassie dye, and protein bands compared to those of known concentrations of BSA (MW of ca. 66kD) run in parallel.

The HPV16 L1 VLP sample (Figure 13A, Lane 6) showed a prominent band at the expected size of approximately 55kDa at a concentration of ca 0.1 ^{mg}/ml when compared to BSA standards (0,125 ^{mg}/ml; 0,25 ^{mg}/ml; 0,5 ^{mg}/ml and 1 ^{mg}/ml; lanes 2-5). A second prominent band at 36kDa likely corresponds to a proteolytic degradation product of L1. The HPV16 L1 VLP are kept in CsCl, which stabilizes VLP conformation, when routinely stored at 4°C.

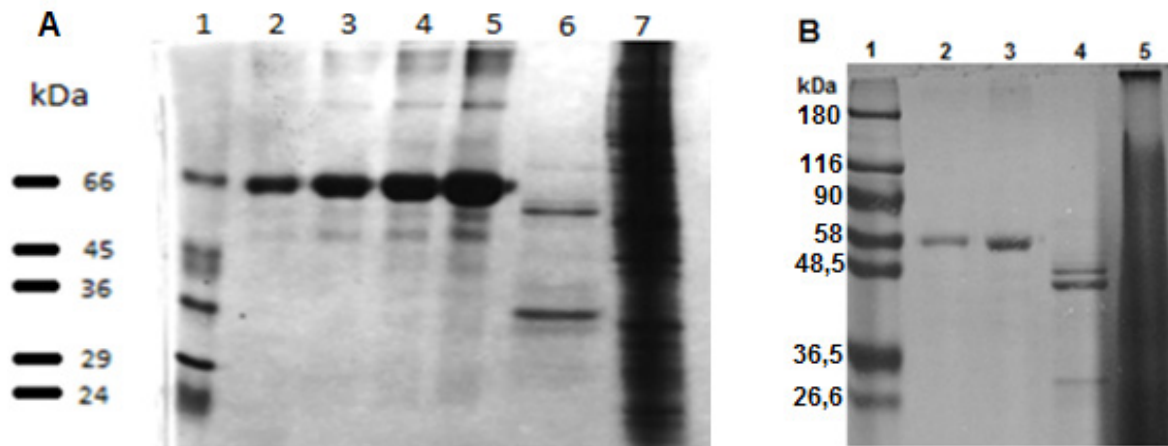
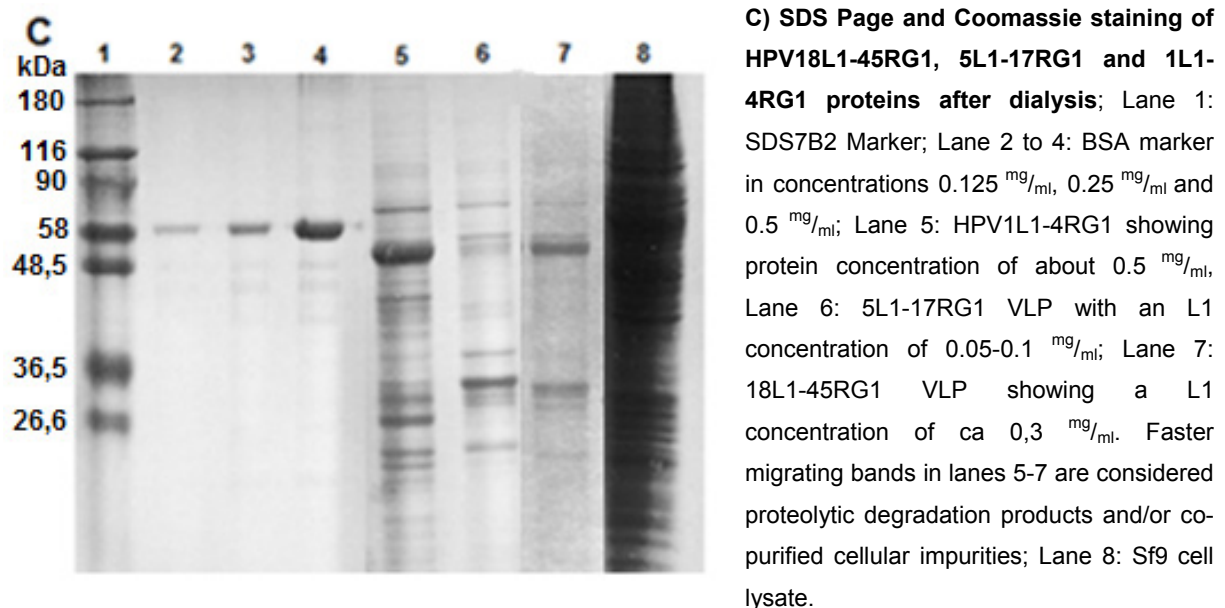


Figure 13: A) SDS Page and Coomassie staining of HPV16 wt L1 VLPs in CsCl; Lane 1: SDS7 Marker; Lane 2-5: BSA marker in concentrations 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml; Lane 6: HPV16 L1 VLP showing L1 proteins at 55kDa (first band) of about 0.1 mg/ml, the faster migrating band is considered a proteolytic degradation product of L1; Lane 7: Sf9 cell lysate. **B) SDS Page and Coomassie staining of RG1 VLPs after dialysis;** Lane 1: SDS7B2 Marker; Lane 2 and 3: BSA marker in concentrations 0.125 mg/ml and 0.25 mg/ml; Lane 4: RG1 VLP showing L1 proteins at 55-60kDa (double band) of about 0.2 mg/ml; Lane 5: Sf9 cell lysate.



C) SDS Page and Coomassie staining of HPV18L1-45RG1, 5L1-17RG1 and 1L1-4RG1 proteins after dialysis; Lane 1: SDS7B2 Marker; Lane 2 to 4: BSA marker in concentrations 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml; Lane 5: HPV1L1-4RG1 showing protein concentration of about 0.5 mg/ml, Lane 6: 5L1-17RG1 VLP with an L1 concentration of 0.05-0.1 mg/ml; Lane 7: 18L1-45RG1 VLP showing a L1 concentration of ca 0.3 mg/ml. Faster migrating bands in lanes 5-7 are considered proteolytic degradation products and/or co-purified cellular impurities; Lane 8: Sf9 cell lysate.

However, before used for immunizations, VLP need to be dialyzed and re-evaluated by SDS-PAGE.

The dialyzed RG1 VLP sample (Figure 13B, lane 4) shows a double band at approximately 58kDa and 55kDa. For unknown reason two closely migrating L1 species are often resolved in SDS-PAGE of purified VLP.

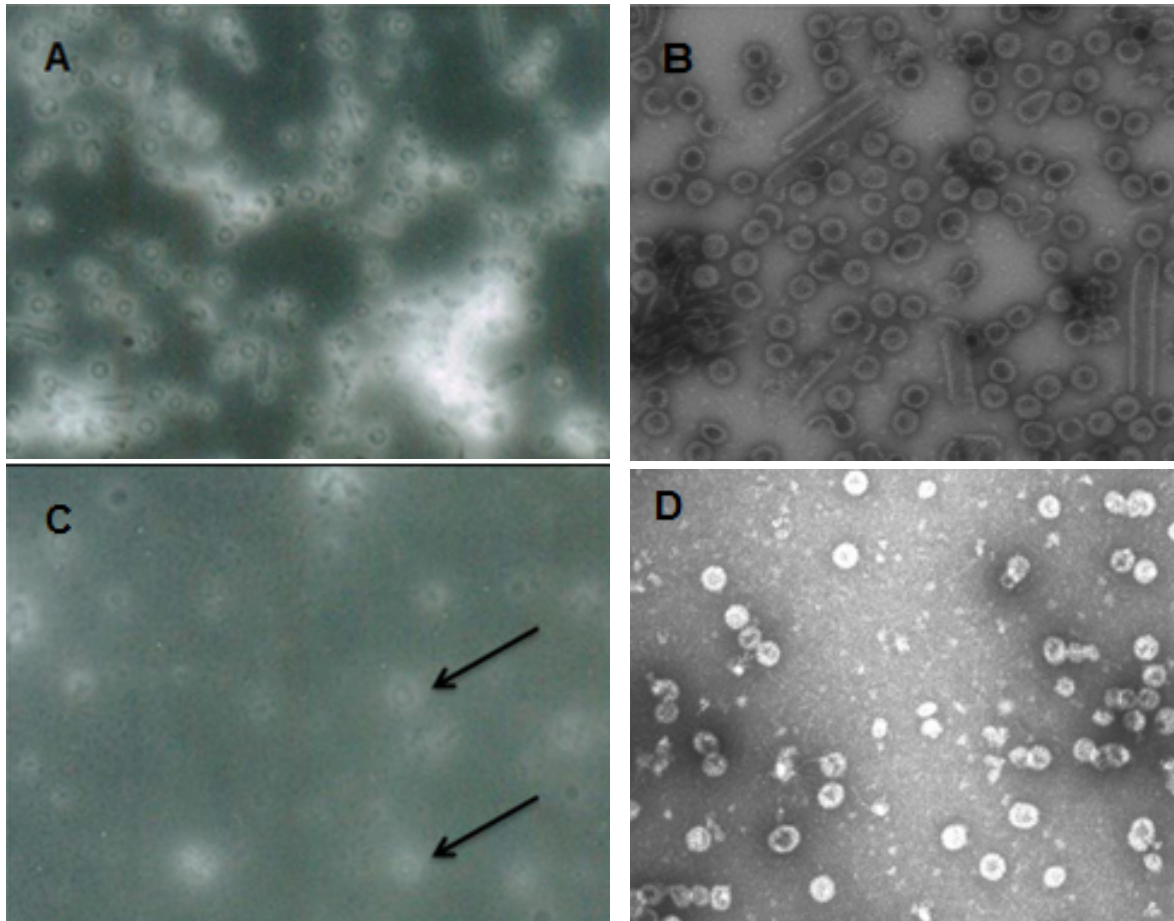


Figure 14: TEM following negative staining of RG1 VLP, HPV16 L1 VLP, 5L1-17RG1 and 18L1-45RG1 VLP. Negatively stained protein preparations were visualized at 30,000-fold magnification using a JEOL 1010 electron microscope. **A) RG1 VLP:** Spherical bright capsids with darker (empty) center and rod-like structures are visible. **B) HPV16L1 VLP:** Spherical VLP and rod-like structures are visible. **C) 5L1-17RG1:** Only a few small heterogeneous particles are detectable (arrows). **D) HPV18L1-45RG1 VLP:** Multiple spherical VLP of homogenous size, as well as smaller and incomplete assemblies are visible.

This heterogeneity is not due to post-translational modification, and both species appear to assemble into VLP (our unpublished observations). The faster migrating band around 40kD most likely corresponds to a proteolytic degradation product. The RG1 VLP protein concentration was estimated to be about 0.2 mg/ml by comparison to BSA standards.

As shown in Fig. 13C, 18L1-45RG1, 5L1-17RG1 and 1L1-4RG1 proteins migrate at approximately 60kDa (Lane 5-7). A higher concentration of 1L1-4RG1 around 0.5 mg/ml is detectable, whereas the 5L1-17RG1 and 18L1-45RG1 proteins are far less concentrated (around 0.05 and 0.2 mg/ml respectively). For all three

samples faster migrating degradation products and/or co-purified cellular proteins are seen.

3.3.5. Transmission electron microscopy (TEM)

Gradient-purified protein preparations were negatively stained with uranyl acetate and analyzed for assembly into particles using TEM at 30,000-fold magnification. VLP are characterized by spherical structure with a darker center indicating empty capsids, as can be seen for RG1 VLP, HPV16L1 VLP, and HPV18L1-45RG1 (Figure 14A, B, D). In addition, rod-like structures aberrantly assembled from pentameric L1 (pentamers or capsomers) were observed.

In contrast, micrographs of HPV1L1-4RG1 did not reveal particulate structures (not shown) indicating that this chimeric protein is not able to correctly assembly into VLP. This negative result was unexpected in light of the distinct protein band observed by SDS PAGE after gradient purification (Figure 13C, lane 5), yet confirmed with a second protein preparation expressed and purified independently (not shown). For the 5L1-17RG1 construct (Figure 14C) only sporadically small and heterogeneously assembled particles, but not full size VLP were seen (see arrows). In contrast, micrographs of HPV18L1-45RG1 revealed efficient assembly into complete VLP as well as smaller and incomplete assemblies (Figure 14D).

3.3.6. Characterization of chimeric L1-RG1 proteins by Western blot

Western blot was used to probe antigenicity of recombinant wt and chimeric fusion proteins.

In figure 15 samples of HPV16 wt L1 and a further RG1 VLP preparation were contacted by mAb Camvir-1 that recognizes a seven aa epitope (GFGAMDF) present in both wt and chimeric HPV16 L1. As expected, chimeric RG1 protein migrated slightly slower (ca. 57kD) than the HPV16 wt L1 protein (50kDa; Figure 15A, Lane 5 and 6). Both proteins were additionally probed with mAb RG1, which recognizes RG1 VLP (Fig 15B, Lane 2) but not wt HPV16 L1 (Fig 15B, lanes 3).

Camvir-1 recognizes the linear HPV16 L1 epitope GFGAMDF that is highly conserved among the L1's of many papillomavirus types. Sequence comparison

revealed a homologous peptide in HPV18 L1 (GYGAMDF), consistent with Camvir-1's ability to recognize chimeric 18L1-45RG1 as well as HPV18 wt L1 (Fig 16A). Following plaque purification of co-transfection supernatants and virus amplification (see material and methods), recombinant baculovirus plaques #1, 7, 10 and 11 readily expressed 18L1-45RG1 that co-migrated as ca. 60kDa proteins, and plaque #1 was further amplified and used to produce VLP in quantitative amounts.

Recombinant baculovirus plaques expressing HPV5 L1-17RG1 were identified using mAb AU1 that has been raised against bovine papillomavirus (BPV) L1 and recognizes the 6 aa linear epitope DTYRYI (Shafti-Keramat, Schellenbacher et al. 2009). Homology search revealed that HPV5 L1 ORF encodes the identical epitope. When contacted in immunoblot AU1 identified bands at 58-60kDa which represent the chimeric HPV5 L1-17RG1 protein, and plaque #2 was chosen arbitrarily for amplification and VLP production (Figure 16B). Faster migrating bands likely present degraded peptides.

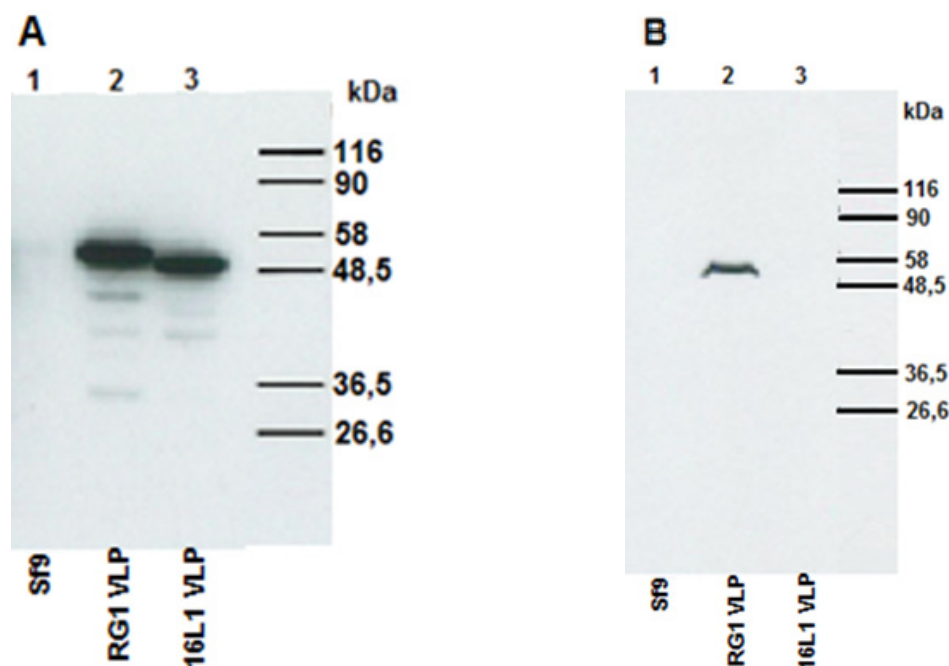


Figure 15: Western Blot of RG1-L1 and 16L1 VLP preparations, using A) mAb Camvir-1 against the HPV16 L1 epitope GFGAMDF. HPV16 L1 migrates as 50kDa protein (lane 3) and the chimeric RG1-L1 protein (lane 2) at approximately 57kDa. Sf9 cells only were used as a negative control lane 1). **B) mAb RG1.** The RG1 antibody only recognizes the RG1-L1 protein of about 57kDa. Sf9 cells only were used as a negative control (lane 1).

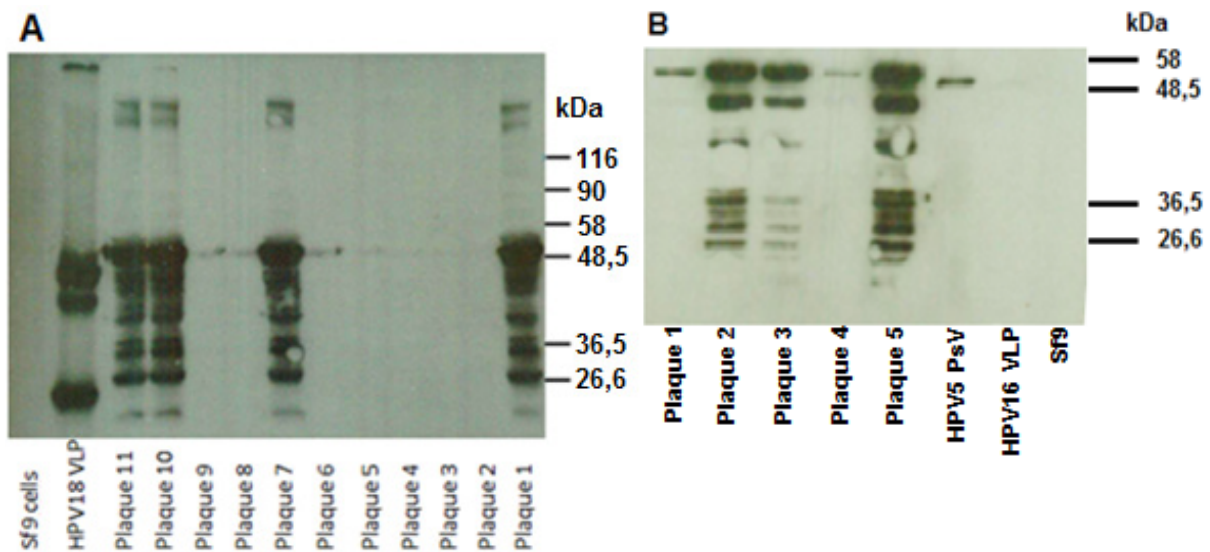
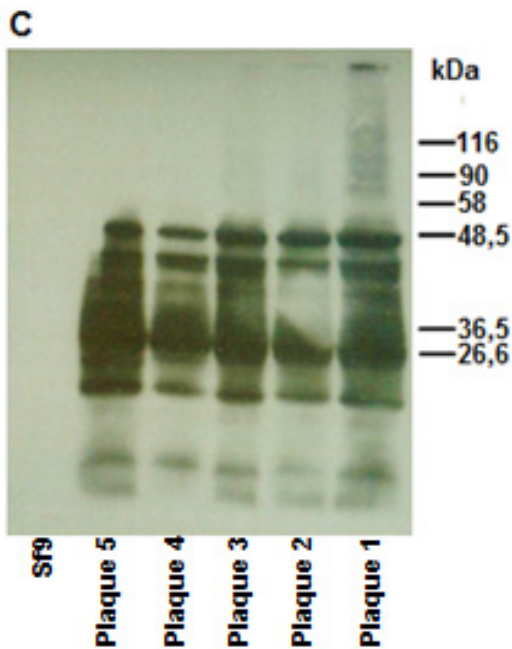


Figure 16: Identification by Western blot of HPV18L1-45RG1, HPV5L1-17RG1 and HPV1L1-4RG1 recombinant baculovirus plaques using the mAb Camvir-1 and AU-1. Infection of Sf9 cells with the respective recombinant virus, obtained by co-transfection of genomic DNA and baculovirus pSynwtVI- transfer vector, resulted in plaque formation, several of which were further amplified and analyzed by Western blot. **A) HPV18L1-45RG1:** Camvir-1 recognizes aa GFGAMDF at position 204-210 of HPV16 L1, the corresponding site in HPV18 L1 is to 85% identical (GYGAMDF). HPV18 VLP were used as a positive and Sf9 cells only as a negative control. Plaque #1, 7, 10 and 11 readily express the recombinant protein. **B) HPV5L1-17RG1:** MA b AU1 recognizes a 6 aa epitope (DTYRYI) of BPV-L1, which is 100% identical in HPV5 L1. HPV16 wt L1 VLP and Sf9 cell lysate were used as controls.



C) HPV1L1-4RG1: Infected cell cultures were lysed and samples probed with mAb Camvir-1 at 1:10,000 dilution. The Camvir-1 epitope GFGAMDF is 100% identical in HPV1 L1. Uninfected Sf9 cell lysate were used as controls.

Expression of the HPV1L1-4RG1 protein was similarly verified with mAb Camvir-1, the recognition site of which is 100% identical in HPV1 L1. All five baculovirus plaques analyzed showed a full-length band at 58kDa, as well as smaller degradation products, and plaque 1 was further amplified for VLP production (Figure 16C).

3.3.7. ELISA

ELISA was performed to analyze conformational neutralization epitopes present on assembled wt or chimeric VLP (or at least pentamers), but absent on non-assembled (denatured/linearized) monomers, and to verify antigenicity of the inserted RG1 epitope. HPV16 wt L1 VLP and RG1 VLP were analyzed using mAb Camvir-1, H16.V5, H16.E70 and RG1. Camvir-1 recognizes the linear 7 aa epitope GFGAMDF (residues 204-210) of HPV16 L1 accessible on denatured L1 (McLean, Churcher et al. 1990; Christensen, Dillner et al. 1996; Roden, Armstrong et al. 1997; White, Wilson et al. 1999). The non-continuous conformational epitopes of H16.V5 and

H16.E70 have been partially characterized and localized to the FG loop of the L1 protein (Roden, Armstrong et al. 1997). The H16.V5 epitope appears particularly immunogenic, as it is recognized by most human immune sera following natural HPV16 infection or Gardasil vaccination. Both mAb H16.V5 and H16.E70 neutralize HPV16 PsV.

Figure 17A shows titration curves of these 4 mAb by ELISA using native or denatured HPV16 wt L1 as antigen. H16.V5 and H16.E70 are conformational-dependent and neutralizing mAb and hence bind to VLP only. Although Camvir-1 recognizes a linear epitope and gives high binding signals to the denatured form, it also modestly binds to the native antigen, because of epitope exposure by partially disassembled or monomeric L1 contaminating native VLP preparations. The RG1 epitope is absent in HPV16 wt L1 VLP, thus the RG1 mAb does not bind both native and denatured wt L1 proteins. ELISA using native RG1 VLP or denatured protein as antigen demonstrates mAb RG1 binding to native VLP, but not denatured ones (Figure 17B). Similar to HPV16 wt L1 VLP, Camvir-1 binds robustly to denatured RG1 VLP and to a minor extent to native protein. Interestingly, the conformational mAb H16.V5 and H16.70 do not bind RG1 VLP, indicating that RG1 epitope insertion

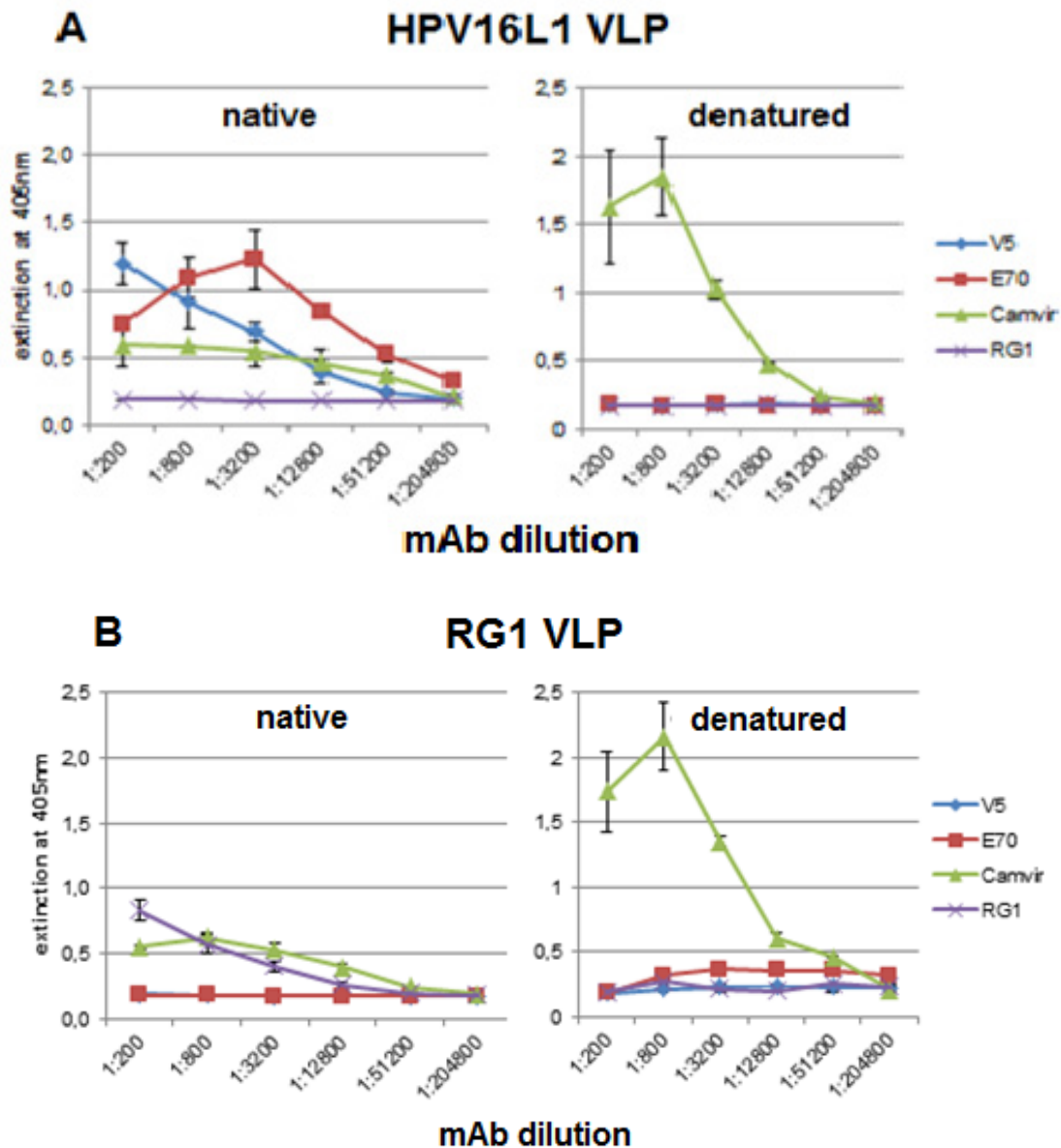


Figure 17: A) ELISA of native and denatured HPV16 L1 VLP. The mAb Camvir-1, H16.V5, H16.E70 and RG1 were used in ELISA to contact native or denatured protein antigen. Camvir-1 binds both native and denatured HPV16 wt L1 VLP. H16.V5 and H16.E70 recognize conformational epitopes and thus only bind native VLP. As expected, mAb RG1 does not bind HPV16 wt L1 VLP. The error bars show the standard deviation from the mean. **B) ELISA of native and denatured RG1 VLP.** The same 4 mAb were used to analyze chimeric native or denatured RG1 VLP. Camvir-1 recognizes a linear epitope present in denatured monomeric (linearized) L1, but also contaminating native VLP preparations in variable amounts. Therefore, both native and denatured VLP show a Camvir-1 signal. H16.V5 and H16.E70 both recognize L1-dependent conformational epitopes present on native HPV16 wt L1 VLP, which appear absent in assembled native RG1 VLP. The mAb RG1 is directed against the conformational RG1 epitope present in assembled RG1 VLP, but absent in denatured protein. The error bars show the standard deviation from the mean.

into the DE loop prevents epitope recognition. Although this might indicate the potential for decreased L1 immunogenicity, HPV16-neutralization titers following RG1 VLP vaccination are similar to HPV16 wt L1 VLP vaccination (Schellenbacher, Roden et al. 2009). Additional RG1 VLP batches were evaluated for H16.V5 binding with similar (negative) results (data not shown).

Next, we attempted to evaluate HPV5L1-17RG1 and HPV5 L1 wt proteins by ELISA using the only two known mAb raised against L1 proteins from the HPV5b and HPV5c variant⁷ (HPV5 2.1.1. and HPV5 52)(Favre, Orth et al. 1998). Unfortunately, in our hands both mAb did neither bind to wt HPV5 L1 VLP nor chimeric 5L1-17RG1 VLP, indicating they were non-functional (data not shown).

We next evaluated HPV1L1-4RG1 protein by ELISA. The mAb HPV1 L1 binds both native and denatured HPV1L1-4RG1, indicating recognition of a linear epitope. This result was not unexpected given TEM has shown that the construct does not assemble into VLP. As expected, mAb to HPV1 L2 does not bind native antigen (Figure 18).

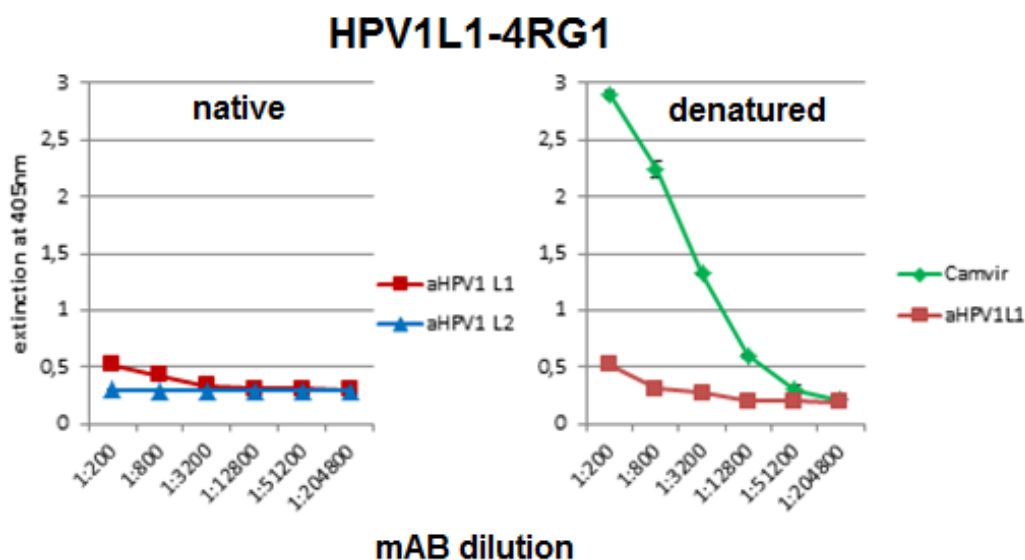


Figure 18: ELISA of native and denatured HPV1L1-4RG1 protein. ELISA was performed similar to figure 17. MAb HPV1 L1 is able to bind both native and denatured VLP with the same strength indicating binding to a linear L1 epitope. As expected, the antibody HPV1 L2 does not react in both ELISA, whereas Camvir-1 is able to bind denatured HPV1L1-4RG1 proteins. The error bars show the standard deviation from the mean.

⁷ a kind gift of Michel Favre, Pasteure Institute, Paris

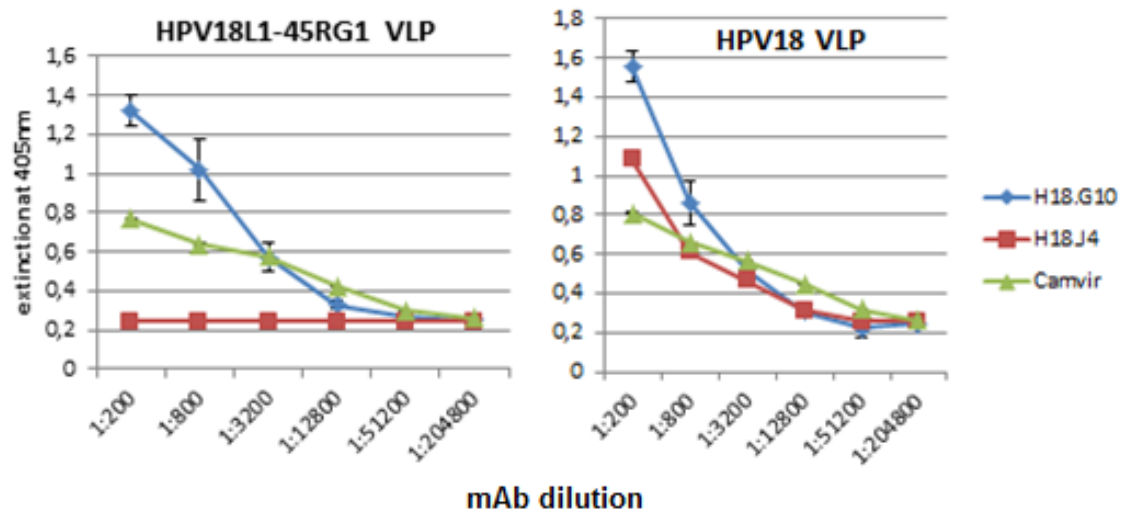


Figure 19: ELISA of HPV18 wt L1 VLP and HPV18L1-45RG1 VLP. H18.G10 and H18.J4 are conformational-dependent mAb that specifically neutralize HPV18. MAb H18.G10 binds to both chimeric and wt L1 VLP, whereas mAb H18.J4 binds wt HPV18 VLP only. This indicates that HPV45 RG1 sequence insertion prevents recognition of the latter neutralization epitope. The HPV16 L1 specific mAb Camvir-1 is directed against a linear HPV16 L1 epitope that is shared among many mucosal and cutaneous HPV L1 proteins and therefore acts as a control. The error bars show the standard deviation from the mean.

Furthermore, HPV18 wt L1 and HPV18L1-45RG1 VLP were analyzed by ELISA using two conformational-dependent neutralizing mAb against HPV18 L1, H18.G10 and H18.J4, and the non-neutralizing Camvir-1 recognizing a linear HPV16 L1 epitope also present in HPV18 L1. As shown in figure 19 H18.G10 binds to both wild type and chimeric HPV18 VLP; H18.J4 however is able to recognize wt VLP only. This indicates that insertion of the HPV45 RG1 sequence has disrupted the H18.J4 neutralization epitope. Camvir-1, as expected, binds both HPV18 wt L1 and 18L1-45RG1 VLP.

3.4. *In vivo* murine vaginal PsV challenge

3.4.1. Pilot experiments

In order to evaluate RG1 VLP vaccination efficacy *in vivo*, a mouse vaginal challenge model is currently being established. Briefly, progesterone synchronized mice are vaginally traumatized by a cytobrush and challenged with PsV. Passive immunization with RG1 VLP immune (or pre-immune) serum will determine if serum antibodies are protective against infection with the respective PsV types. So far, pilot

experiments have been performed to improve handling and ensure minimal variance within and between experiments.

The first pilot experiments using a cyto-brush alone to set a minor vaginal trauma in anesthetized mice did not result in any detectable bioluminescence signal indicating unsuccessful HPV16 PsV infection. The trauma was gradually increased by fully inserting the brush and rotating it 15 times instead of 10 times as per protocol. Further, we obtained a HPV16 PsV preparation, which had already been successfully used for *in vivo* experiments⁸.

Infection using the latter PsV occurred when the trauma was increased; whereas our own PsV preparation did not result in detectable infection (not shown). We therefore compared both batches by *in vitro* PsV-neutralization assay and determined that our preparation resulted in about 10-fold less PsV-luc signal ($\frac{\text{photons}}{\text{sec}}$) than PsV from Johns Hopkins (data not shown). In concordance, SDS-PAGE confirmed that PsV preparation from Johns Hopkins were about 4-fold higher concentrated compared to our own PsV (Figure 20; compare lanes 4 and 5).

Figure 21 shows the result of an HPV16 PsV challenge after mechanically disrupting the vaginal mucosa by cyto-brush. All 3 mice showed signs of infection, with the weakest signal emitted from the mouse on the left side, and the strongest

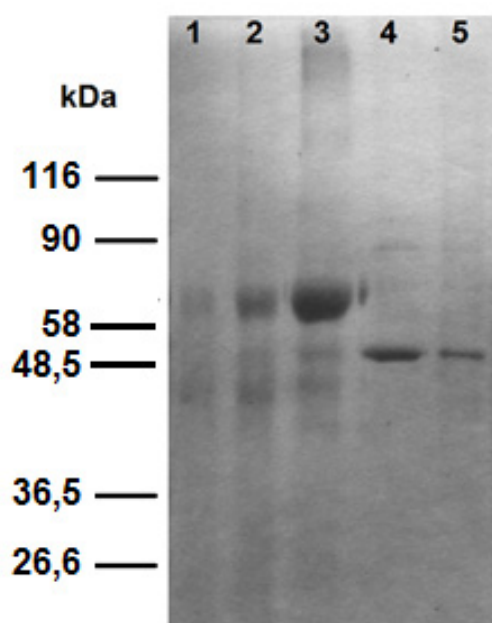


Figure 20: SDS-PAGE and Coomassie staining of two HPV16 PsV samples. Concentration of PsV from Johns Hopkins (lane 4) is about 4-fold higher compared to our own PsV (lane 5). Lane 1-3 show BSA standard markers in the concentrations 0.125 $\frac{\text{mg}}{\text{ml}}$, 0.25 $\frac{\text{mg}}{\text{ml}}$ and 0.5 $\frac{\text{mg}}{\text{ml}}$.

⁸ kindly provided by Kihyuck Kwak, Johns Hopkins University, Baltimore

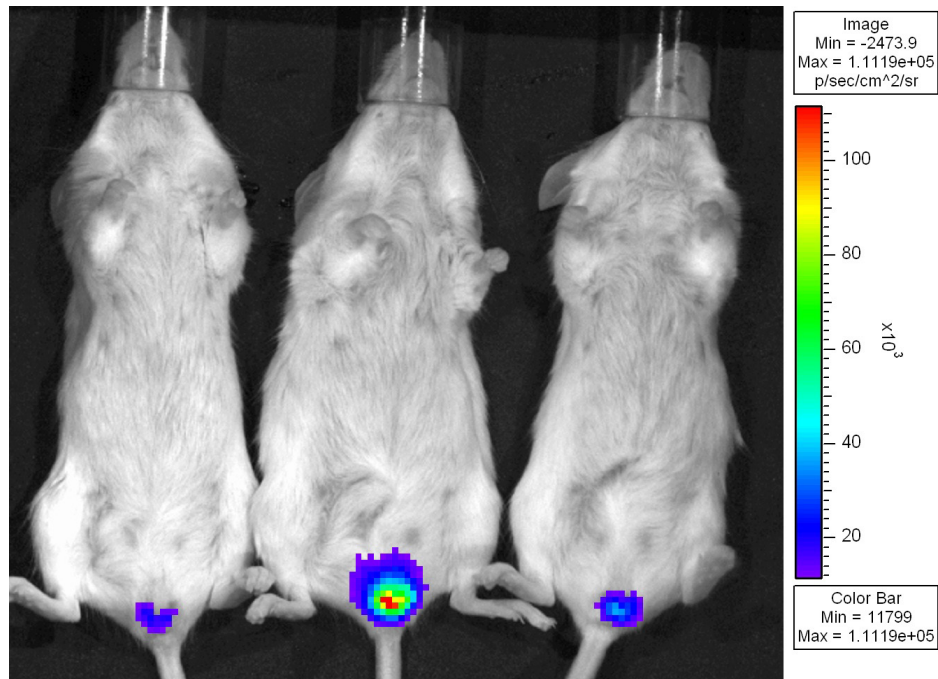


Figure 21: Mouse vaginal challenge pilot experiment using HPV16 PsV (obtained from Kihyuck Kwak, Johns Hopkins). The vaginal epithelium of anesthetized mice was mechanically disrupted by rotating 15x with a cervical cyto-brush and 1:4 diluted HPV16 PsV in carboxymethyl-cellulose (CMC) were installed into the vagina (total volume 30 μ l). Three days later, 40 μ l luciferin were applied intra-vaginally and the flux (photons per second) was measured for 30 seconds (IVIS50, Caliper). It can be seen that all 3 mice have been infected as indicated by bioluminescence signal from the genital tract, with the highest signal intensity emitted from the mouse in the center and the weakest on the left.

signal emitted from the mouse in the center. These up to 10-fold differences may occur because of inadvertent differences in the strength of applied trauma.

4. Discussion

4.1. Large scale PsV production

The protocol by Buck et al. describes how to produce HPV L1/L2 PsV containing a reporter gene (SEAP). However, the process of PsV production is not very robust and takes eight days to accomplish. Important variables that may affect PsV production include the quality/purity and concentration of DNA used for co-transfection and the status of the 293TT producer cell. Experience has taught us that DNA at higher concentration (obtained by Maxi Prep) is preferable to DNA at lower concentration (e.g. by Mini Prep). The plasmids used to express L1+L2 capsid genes play an essential role in PsV production as well. For example, it appears that pViro-neo-mcs does not work that well for some types (e.g. HPV6) when compared to psheLL, which might mostly be due to codon optimization differences. However, results could be different if for example the more sensitive LUC was used rather than the SEAP reporter system. Another strategy to increase robustness of type-specific PsV production might be to alter the codon usage, or to express capsid genes from a different HPV subtype. Additionally, it is important to use healthy, rapidly growing and subconfluent 293TT cells.

A “high-yield” PsV production protocol was established in order to avoid possible variation between PsV preparations that might make it difficult to compare results between experiments, both *in vivo* and *in vitro*. Another aim is to increase PsV yield for less effective HPV types. This strategy was successful for example, for HPV11 PsV that were not easily produced in a standard scale, but rather at the larger scale protocol. For LUC-containing PsV an additional aim was to increase the volume of PsV-containing fractions. These PsV as well as other newly designed L1-RG1 constructs will find application in the *in vivo* genital mouse model analyzing RG1 VLP vaccination efficacy.

4.2. VLP

The baculovirus system is an established method to produce preparative amounts of correctly folded and modified protein of interest by eukaryotic expression in Sf9 insect cells. It was possible for all wt and functional recombinant proteins to produce about 4-7ml VLP-containing solution at a concentration of at least 0.1-0.2

mg/ml (for HPV16 L1 wt and RG1-L1) and up to 0.4 mg/ml (for HPV1L1-4RG1). Thus, using the above mentioned protocol offered an effective way to express and purify VLP at sufficient amounts required for described further use.

Following gradient purification, VLPs are very stable when kept at 4°C in CsCl, or frozen at -20 to -70°C . However, CsCl is considered a potentially hazardous compound, the LD_{50} (rat) is 2004mg/kg , thus prior to injection into animals samples were dialyzed against PBS + 0.5M NaCl + 1mM CaCl_2 + 0.01% Tween 80. High salt concentrations ($>0.5\text{M NaCl}$) protect VLP from aggregation and non-ionic surfactant like Tween-80 keep VLP stable against surface absorbance as well as aggregation (Shi, Sanyal et al. 2005). Of note, analyzing VLP kept in CsCl may underestimate protein concentration by SDS-PAGE (our unpublished observations), and thus we routinely verify sample concentration by SDS-Coomassie gel after dialysis.

4.2.1. RG1 VLP and HPV16 L1 VLP

Visualization of VLPs by electron microscopy is a convenient way to estimate efficacy of VLP assembly by estimating concentration and morphology of full sized VLP ($50\text{-}60\text{nm}$ spheres) or incorrectly folded aggregates (Schellenbacher, Roden et al. 2009). Correctly assembled VLP display conformation-dependent epitopes required for induction of neutralizing antibodies, a prerequisite for HPV vaccine efficacy. By TEM HPV16 wt L1 and RG1 VLP showed a similar size and morphology suggesting correct assembly of the chimeric protein. RG1 VLP were therefore used to immunize 4 NZW rabbits to further evaluate robustness of vaccination efficacy, and immune sera were drawn and evaluated by *in vitro* PsV neutralization assays. Further, purified VLP were analyzed by ELISA for the presence of important immunogenic epitopes that induce effective anti-L1 immune responses. Regardless of type, HPV L1 VLP induce type-specific neutralizing antibodies. Type-restriction arises from diversity of epitope conformation of the hypervariable surface loops, whereas overall L1 sequence homology among different HPV types, forming a jellyroll β -sandwich in the assembled VLP, is very high. Thus, most PV genotypes represent distinct serotypes (Bernard, Burk et al. 2010). In HPV L1 VLP there are five surface-exposed loops (BC, DE, EF, FG and HI), showing flexible structures with conformational differences across different types and L1 assemblies (Joshi, Cheluvaraja et al. 2011). L1 assembled into pentamers or VLP show structural

similarities in their loop regions, yet pentamers are 20-40 times less immunogenic indicating that fluctuations and temporal variability in the loop conformations and neutralization epitopes might critically influence immunogenicity (Thones, Herreiner et al. 2008).

MAB H16.V5 and H16.E70 raised against HPV16 L1 VLP recognize different yet overlapping epitopes. H16.E70 binds leucine at position 50 and serine at position 282 from the FG loop, and inhibits binding of PsV to the cell surface (Roden, Armstrong et al. 1997; Bishop, Dasgupta et al. 2007). The exact recognition site of H16.V5 is not known, but the discontinuous epitope may locate to the FG and possibly HI loops and is bound by most human sera past HPV16 infection. Negativity for H16.V5 by ELISA indicates the absence of this major neutralization epitope on native RG1 VLP (figure 17), whereas HPV16 wt VLP score positive for H16.V5 as expected. Although this might indicate the possibility for reduced immunogenicity, neutralizing titers against HPV16 raised by RG1 VLP immunization are similar to those raised by HPV16 wt L1 VLP. Changes in the neighboring DE loop because of the RG1 insertion might induce slight changes in the conformation of the H16.V5 epitope that prevent mAb recognition without diminishing the overall polyclonal neutralizing immune response to HPV16.

In this study, immunizations of two NZW rabbits with RG1 VLP have used a different protocol, which might have contributed to the overall attenuated antibody responses observed compared to previous results (Schellenbacher, Roden et al. 2009). In this study (Schellenbacher et al 2009) using a four-dose protocol, rabbits received 50µg VLP per injection at week 0, 4, 6 and 8. PsV neutralization assays have revealed that RG1 VLP are able to elicit a neutralizing antibodies against HPV16, 18, 31, 45, 52 and 58. Importantly, it has been shown that RG1 VLP vaccination induced titers against HPV16 of 100,000 and against HPV31 of 1,000-10,000. In contrast, in the study herein rabbits were given 20µg in a three-dose scheme at week 0, 3 and 6. PsV assays indicated immune titers against HPV16 of 1,000-10,000, and only 1 of 4 NZW sera revealed a titer of 25 against HPV31 (table 8). The latter result was surprising since titers of 50-1,000 against another closely related type, HPV35, were induced. Although neutralization titers against 18 mucosal high- and low-risk types were observed, overall immune responses varied between the four NZW (table 8). These overall attenuated responses might be due to a

different genetic background of the outbred rabbits or, perhaps more likely, due to the less intense immunization protocol used. Nevertheless, passive transfer of immune sera will allow us to evaluate if *in vivo* challenge is a better estimate for protective efficacy than inferred from PsV neutralization titers (Longet, Schiller et al. 2011). In addition, we are currently immunizing additional four NZW rabbits according to the initial four-dose scheme (Schellenbacher, Roden et al. 2009).

4.2.2. Newly designed constructs

To achieve even broader protection against medically important cutaneous and high-risk mucosal types, we have generated recombinant baculoviruses for expression of chimeric HPV18L1-45RG1, HPV1L1-4RG1 and HPV5L1-17RG1 proteins.

HPV18 and HPV45 are frequently found in adenocarcinomas of the cervical canal which are more often overlooked by PAP screens (Bulk, Berkhof et al. 2006). Thus the HPV45 RG1 sequence has been chosen for insertion into the HPV18 L1 protein, to more efficiently induce a (cross-)neutralizing antibody response to these more distantly related mucosal types. Importantly, HPV18 and HPV45 are more closely related to HPV39 (accounting for 2% of the cancer cases), 51, 59 (responsible for 1% of the cancer cases), 68, 70, 26, 69, 51, 82, 53, 56 and 66 (accountable for less than 1% of the cancer cases) (de Sanjose, Quint et al. 2010), as compared to HPV16. RG1 VLP vaccination induces cross-neutralizing antibodies against some of these types, but only at low titers. The new HPV18L1-45RG1 fusion protein assembles very efficiently into VLP (figure 14C) and might improve this response, and we have already started to immunize NZW rabbits to determine broadness and efficacy of this new vaccine candidate.

By TEM, the chimeric HPV18L1-45RG1 protein efficiently assembled into correctly folded VLP (see figure 14D). By ELISA, the conformational-dependent mAb H18.J4 binds to wt HPV18 VLP, but not chimeric HPV18L1-45RG1 VLP, indicating that HPV45 RG1 sequence insertion has altered the H18.J4 neutralization epitope (figure 19). This might be due to direct interruption of the epitope, or indirectly by negatively affecting the loop formation by the neighboring structures. The possible impact of this epitope loss on immunogenicity will be analyzed using immune sera

against HPV18L1-45RG1 VLP by HPV18 PsV neutralization assay. However, it has been shown in a subset of Gardasil-vaccinated patients that despite absence of anti-HPV18 antibodies (analyzed with H18.J4 in competitive luminex immunoassays) 4 years after immunization, effective protection against HPV18 remains (Olsson, Villa et al. 2007; Brown, Garland et al. 2011). Therefore, HPV18L1-45RG1 VLP vaccine may elicit effective L1-mediated protection against HPV18 despite missing the H18.J4 epitope and in addition, induce anti L1-mediated cross-protection to HPV45 and other related types (e.g. HPV33, 51, 59, 68, 69)

HPV1 and HPV4 frequently cause common and palmo-plantar warts; HPV5 and HPV17 are cutaneous Beta prototypes found in EV-cancers, which have also been implicated to play an indirect role in the development of NMSC in immunocompetent patients. The chimeric HPV5L1-17RG1 protein was designed to trigger a humoral immune response against genus beta-PV that cause, in combination with UV radiation, NMSC in EV patients, and possibly also contribute to SCC in immunosuppressed patients. In addition, there is accumulating evidence that Beta-PV may play an indirect role in NMSC of immunocompetent patients, which are the most common cancers in Caucasians. HPV5 and 8 are the most important oncogenic types of genus beta, thus L1 of HPV5 ($\beta 1$ species, figure 22) was chosen as scaffold to present the RG1 sequence of HPV17 ($\beta 2$ species), another representative of six beta-HPV types commonly found in NMSC (Orth 2006). We hypothesize that induced anti-L1 antibodies will cross-neutralize HPV 8 and especially 14, 20 and 47 (showing a L1 sequence homology of 87%, 77%, 75%

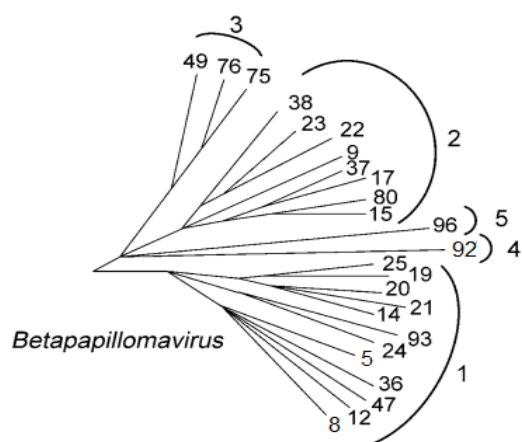


Figure 22: Phylogenetic tree of genus Beta PV. Beta types are grouped within 5 species (Bernard, Burk et al. 2010).

	RG1 sequence	Homology
HPV17	DIYRGCKQAGTCPPDVINKV	100%
HPV5	HIYQTCKQAGTCPPDVINKV	85%
HPV14	NIYRTCKQAGTCPPDVINKV	90%
HPV20	NIYRTCKQAGTCPPDVINKV	90%
HPV47	HIYQTCKQAGTCPSDVVINKV	75%
HPV76	HIYQSCKAAGTCPPDVLNKV	75%
HPV96	NIYRGCKAAGTCPPDVINKV	90%

Table 9: Alignment of RG1 aa sequence of HPV17 to those other medically important Beta-PV types.

HPV17's RG1 aa sequence shows homology of 75-90% even to beta types of other species (e.g. HPV76, 96).

and 82%), which are closely related to HPV5 (Handisurya, Gambhira et al. 2009). In addition, L2 peptide HPV17RG1 might trigger cross-neutralization antibodies even against more diverse cutaneous types, because RG1 aa sequence shows homologies at 75-100% to medically important further Beta types (see table 9 and figure 22).

The HPV1L1-4RG1 construct was designed to target the distantly related HPV1 belonging to genus Mu (species 1), and HPV4 representing a Gamma-PV (species 1). Although biological benign, both cause common, cutaneous and palmo-plantar warts, which are frequent nuisance especially in school children. RG1 VLP sera do not cross-neutralize HPV1 and HPV4 PsV *in vitro* (our unpublished results), consistent with the fact that the respective RG1 epitopes shows 50% dissimilarity to the HPV16RG1 sequence.

Although both HPV1L1-4RG1 and HPV5L1-17RG1 proteins were expressed to high levels by baculoviruses, only HPV1L1-4RG1 was purified to high concentration of 0,5^{mg}/ml on density gradient, whereas HPV5L1-17RG1 preparation reached a concentration of ca. 0,1^{mg}/ml only (Fig 13C). Furthermore, TEM revealed no correctly assembled VLP for HPV1L1-4RG1 and HPV5L1-17RG1 constructs, respectively. This indicates that insertion of the RG1 epitopes into the respective L1 proteins interferes with the ability to assemble into VLP, although insertion sites have been chosen according to the respective insertion site used for RG1 VLP that assemble with high efficacy. ELISA has shown that non-denatured HPV1L1-4RG1 protein is bound by anti HPV1L1 mAb⁹, but the mAb binds denatured proteins as well indicating recognition of a linear epitope. Since a conformation-dependent anti-HPV1L1 mAb is not available we cannot distinguish if this protein adopts a conformation of a pentamer, which is the least assembly recognized for induction of a neutralizing immune response. ELISA with anti-HPV5L1 Ab¹⁰ has not shown meaningful results, probably because of Ab deterioration (data not shown). Unfortunately, we were unable to obtain a new batch of mAb.

Taken together, it appears unlikely that current HPV1L1-4RG1 and HPV5L1-17RG1 proteins will be useful as prophylactic vaccines. New projects have been

⁹ kindly obtained by J. Carter

¹⁰ kindly obtained by Michel Fauvre, Institut Pasteur, Paris, France

designed to attain chimeric VLP for the intended use. These include generation of a HPV16L1-17RG1 fusion protein, and a modified HPV5L1-17RG1 construct in which the complete DE-loop of HPV5 L1 is replaced by the HPV17 RG1 sequence. In addition, a multimeric concatenated fusion protein consisting of five 17RG1 sequences will be generated and immunogenicity of peptide vaccination compared to chimeric VLP vaccination.

4.3. *In vivo* murine vaginal challenge model

Murine vaginal challenge with PsV has proven a valuable *in vivo* model for experimental genital HPV transmission (Roberts, Buck et al. 2007; Alphs, Gambhira et al. 2008). To take advantage of this model to determine vaccine efficacy, we have performed pilot experiments. Challenge of naïve mice with HPV16 PsV was used to develop a sense of PsV concentrations required to obtain meaningful signals, and to learn appropriate animal handling to minimize variance between experiments. Initially, the “high-yield” PsV production using 8 ml of each Optiprep gradient solution yielded PsV samples that were less concentrated and less infectious, as even by challenge with higher PsV/CMC volumes only poorly measurable infections were detected. Consequently, drawing smaller volumes of from Optiprep gradients resulted in higher concentrated PsV samples that recently resulted in robust and useful infections for several high-risk PsV types including HPV16, 31 and 35 (our unpublished results, not shown).

4.4. Outlook

Based on results presented in this thesis, our medium-term goal is generating a vaccine with efficacy against a broad spectrum of low-risk and high-risk mucosal and cutaneous HPV types, including those that cause common warts as well as Beta-HPV causally implicated in NMSC. Appropriate L1-RG1 VLP vaccines have been designed as outlined above and PsV of the most important mucosal and cutaneous HPV types will be generated to reveal the full spectrum of vaccine-induced cross-neutralization in pre-clinical studies. Finally, the mouse model will be adapted from vaginal to cutaneous challenge to evaluate vaccine efficacy also at the relevant (cutaneous) site, with the long-term goal to initiate human clinical trials.

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5. Appendix

5.1. HPV protein sequences

HPV1 L1

MYNVFQMAVWLPAQNKFYLPQPITRILSTDEYVTRTNLFYHATSERLLLVGHPLEISSNQT
VTIPKVSPNAFRVFRVRFADPNRFAFGDKAIFNPETERLVWGLRGIEIGRGQPLGIGITGHPL
LNKLDDAENPTNYINTHANGDSRQNTAFDAKQTQMFLVGCTPASGEHWTSSRCPGEQVKL
GDCPRVQMIESVIEDGDMMDIGFGAMDFAALQQDKSDVPLDVVQATCKYPDYIRMNHEAY
GNSMFFFARREQMYTRHFFTRGGSVGDKEAVPQSLYLTADAEPRTTLATTNYVGTPSGSM
VSSDVQLFNRSYWLQRCQGQNNNGICWRNQLFITVGDNTRGTSLSISMKNNASTTYSNANF
NDFLRHTEEFDLSEFIVQLCKVKLTPELAYIHTMDPNILEDWQLSVSQPPTNPLEDQYRFLGS
SLAAKCEQAPPEPQTDPPYSQYKFWEVDLTERMSEQLDQFPLGRKFLYQSGMTQRTATSS
TTKRKTVRVSTSAKRRRKA

HPV4RG1

NLYAKCQLSGNCLPDVKNKV

HPV5L1

MAVWHSANGKVYLPPSTPVARVQSTDEYIQRRTNIYYHAFSDRLLTVGHPYFNVYNINGDKLE
VPKVSGNQHRVFRKLKLPDPNRFALPDMSVYNPDKERLVWACRGLEIGRGQPLGVRSTGHP
YFNKVKDTENSAYITFSKDDRQDTSFDPKQIQMFIVGCTPCIGEHWDKAVPCAENDQQTG
LCPPIELKNTYIQDGMADIGFGNMNFKALQDSRSDVSLDIVNETCKYPDFLKMQNDIYGDA
CFFYARREQCYARHFFVRGGKTGDDIPRAQIDNGTYKNQFYIPGADGQAQKTIGNSMYFPT
VSGSLVSSDAQLFNRPFWLQRAQGHNNGILWANQMFIITVDNTRNTNFSISVYNQAGALKD
VADYNADQFREYQRHVEEYEISLILQLCKVPLKAQVLAQINAMNSSLLEDWQLGFVPTPDNP
IQDTYRYIDSLATRCPDKNPPKEKEDPYKGLHFWDVDLTERLSLDLDQYSLGRKFLFQAGLQ
QTTVNGTKAVSYKGSNRGTKRKRKN

HPV17 RG1

DIYRGCKQAGTCPPDVINKV

HPV18 L1

MALWRPSDNTVYLPSPSVARVVNTDDYVTRTSIFYHAGSSRLLTVGNPYFRVPAGGGNKQ
DIPKVSAYQYRVFRVQLPDPNKFGLPDTSIYNPETQRLVWACAGVEIGRGQPLGVGLSGHP
FYNKLDDTESSHAATSNVSEDVRDNVSVDYKQTQLCILGCAPAIGEHWAKGTACKSRPLSQ
GDCPPLELKNTVLEDGDMVDTGYGAMDFSTLQDTKCEVPLDICQSICKYPDYLQMSADPYG
DSMFFCLRREQLFARHFWNRAGTMGDTVPSLYIKGTGMRASPGSCVYSPSPSGSIVTSD
SQLFNKPYWLHKAQGHNNGVCWHNQLFVTVDTRSTNL TICASTQSPVPGQYDATKFKQ
YSRHVEEYDLQFIFQLCTITLTADVMSYIHSMNSSILEDWNFGVPPPPTTSLVDTYRFVQSV
ITCQKDAAPAENKDPYDKLFWNVDLKEKFSLDLDQYPLGRKFLVQAGLRRKPTIGPRKRS
PSATTSSKPAKRVVRARK

HPV45RG1

DLYRTCKQSGTCPPDVINKV

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5.3. Abbreviations

aa	amino acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AIN	anal intraepithelial neoplasia
AIS	adenocarcinoma in situ
Alum	aluminum salt
APC	antigen presenting cell
APS	ammoniumpersulfate
AS04	adjuvant system 04
BCC	basal cell carcinomas
BM	basal/basement membrane
BPV	bovine papillomavirus
BSA	bovine serum albumin
C-terminal	carboxy-terminal
CIN	cervical intraepithelial neoplasia
DC	dendritic cell
ER	endoplasmatic reticulum
EV	Epidermodysplasia verruciformis
FDA	Food and Drug Administration
HPV	human papillomavirus
i.p.	intra-peritoneal
i.v.	intra-venous
LCR	long control region
LUC	luciferase
mAb	monoclonal antibody
MCS	multiple cloning site
5-ME	5-mercaptoethanol
MPL	monophosphoryl lipid A
MW	molecular weight
N-terminal	amino-terminal
NLS	nuclear localization sequence
NMSC	non-melanoma skin cancers
NZW	New Zealand White
ON	overnight
ORF	open reading frame
ORI	origin of replication
PAP	Papanicolaou test/screening
RPA	replication protein A
PsV	pseudovirion
RT	room temperature
PV	papillomavirus
RE	restriction enzyme
s.c.	sub-cutaneous
SCC	squamous cell carcinomas
SDS	Sodium dodecyl sulfate
SEAP	secreted alkaline phosphatase
(h)SIL	(high grade) squamous intraepithelial neoplasia
STI	sexually transmitted infections
TEMED	Tetramethylethylenediamine
URR	upstream regulatory region
VLP	virus-like particle
wt	wild-type

5.4. Summary

Human papillomaviruses are a medically important large family of DNA viruses. The circular, double-stranded genome is surrounded by a “naked” protein capsid of 55-60nm in a T=7 symmetry. More than 120 different HPV genotypes are already characterized based on the viruses’ epitheliotropism into mucosal and cutaneous types. Additionally, mucosal HPV are further divided into high- and low-risk types based upon their oncogenic potential. Long term persistent infection with high-risk mucosal types has been associated with the development of anogenital and oropharynx cancer, while low-risk types (e.g. HPV6) cause genital warts. The most important types HPV16 and 18 are responsible for 70% of all cervical cancer cases, the additional 14 high-risk types for the remaining 30%. Furthermore, some Beta-HPV types have recently been associated, in connection with UV exposure, in the development of non-melanoma skin cancer; other cutaneous types induce the development of benign common cutaneous warts.

Although two vaccines against HPV16 and 18, and one additionally against HPV6 and 11, are available, they do not protect against any Beta-types or the other 14 high risk mucosal HPV. Current vaccines are based upon L1’s feature to assemble into “virus-like particles” (VLP), that induce a type-restricted neutralizing and protective antibody response. A multivalent vaccine directed against all medical relevant HPV types would be technically most demanding and expensive.

An approach to broaden immunity is a L2-based vaccine, which contains cross-neutralizing epitopes. Immunizations with purified L2 proteins (the minor capsid protein) induce low-titer neutralizing antibodies protecting against even heterologous, farther related types. However, L2 in native virions or L1+L2 co-assembled VLP is only subdominant to L1, presumably because it is partly hidden from the immune system and present in a low 1:30 ratio (L2:L1) only.

Our group has recently established a chimeric L1-L2 vaccine that repetitively presents on the VLP surface a conserved N-terminal L2 epitope (“RG1”) inserted into the HPV16 L1 DE loop (RG1 VLP). Immunization of NZW rabbits induces broadly cross-neutralizing antibodies against further mucosal high-risk and low-risk types, as well as against the cutaneous high-risk HPV5.

The aim of this work is to further evaluate the RG1 VLP vaccine. At first, four NZW rabbits were immunized in week 0-3-6, together with the alum-MPL adjuvant, and the immune sera analyzed in the PsV-neutralization assay. Result have shown that induced

antibody titers against 20 tested PsV were diverse between the sera, because only one serum showed cross-neutralizing antibody titers against HPV6, 11, 31, 33, 40, 44, 52, 53, 66 und 69, two sera against HPV45, three sera against HPV34, 39 und 70 and all immune sera showed protection against HPV16, 18, 26 und 35. All four sera were non-protective against the two tested cutaneous HPV4 and 38. ELISA has shown that the important L1-antibody epitope V5 is missing in the RG1 VLP, which might explain lower antibody titers against HPV16 and closely related types. Further, low titers might also result from the less intense immunization protocol that was used. Thus, establishing a robust vaccination protocol and recreation of the initial data is important before proceeding with *in vivo* challenge experiments.

Similar to HPV16L1/L2 based RG1 VLP, new chimeric RG1-L1 constructs have been designed. To emphasize the most important mucosal HPV types HPV18 and 45 that are less related to HPV16, i) HPV18 L1 was used as carrier to display the RG1 peptide from HPV45. Further, to generate a vaccine against the most cutaneous HPV types, ii) the HPV1 L1 was used as scaffold for the HPV4 RG1 and the iii) HPV5 L1 protein as carrier for the HPV17 RG1 peptide. Transmission electron microscopy revealed that the HPV5L1-17RG1 construct is able to sporadically assemble into VLP, while the HPV1L1-4RG1 construct is only present as artifacts or smaller components. In contrast, the HPV18L1-45RG1 construct was shown to effectively assemble into correct VLP and comparison of HPV18 wt VLP to these chimeric VLP by ELISA using mAb revealed that one analyzed conformational-dependent mAb did not bind the chimeric VLP but only wt VLP, whereas binding of the second neutralizing mAb was unaffected. To evaluate whether loss of one neutralization epitope resulting from RG1 insertion is relevant, cross-neutralization ability of this vaccine candidate will be evaluated using immune sera assayed by the PsV-neutralization assay. Additionally, by ELISA we were unable to confirm the presence of neutralizing epitopes of HPV5L1-17RG1 and HPV1L1-4RG1 using mAb raised against the respective L1 scaffold proteins. Given these results and the fact that both do not assemble into full VLP, it was planned to re-design both cutaneous constructs.

In vivo efficacy of the RG1 VLP vaccine against the majority of mucosal HPV types will be analyzed by a mouse vaginal challenge model. Pilot experiments have already been performed and revealed that the settling of the micro trauma and the used concentration of PsV is very important. For the future we aim to establish a cutaneous *in vivo* challenge model to analyze vaccine efficacy against important cutaneous HPV types at its relevant natural infection site.

5.5. Zusammenfassung

Papillomviren sind DNA Tumoviren mit einem Durchmesser von 55-60nm und der Symmetrie eines T=7 Ikosaeders. Virionen bestehen aus einem Proteinkapsid ohne Lipoproteinhülle, welches die zirkuläre, doppelsträngige virale DNA umschließt. Mehr als 120 verschiedene HPV Typen sind bereits komplett charakterisiert und werden in Haut- oder Schleimhaut-infizierende Typen unterteilt. Eine persistente Infektion mit mukosalen hoch-Risiko Typen spielt eine ursächliche Rolle in der Entwicklung des Gebärmutterhalskrebses (Zervixkarzinoms), des Analkarzinoms, eines Teils anderer Genitalkarzinome sowie Oropharynxkarzinome, während sogenannte niedrig-Risiko Schleimhauttypen gutartige Genitalwarzen verursachen. Vor allem HPV16 und HPV18 sind für 70% der Zervixkarzinome weltweit verantwortlich, etwa 14 weitere hoch-Risiko Typen für die weiteren 30%. In den letzten Jahren wurden Papillomviren der Spezies beta (zusätzlich zum Hauptkarzinogen UV-Strahlung) mit der Entwicklung von „nicht-melanozytären“-Hautkarzinome (weißer Hautkrebs) in Verbindung gebracht. Andere kutane Typen verursachen die gewöhnlichen Hautwarzen.

Beide zugelassene HPV Impfstoffe schützen gegen hoch-Risiko HPV16 und HPV18, jedoch nicht gegen 14 weitere hoch-Risiko Schleimhauttypen, und nicht gegen kutane bzw. beta-Typen. Ein Impfstoff schützt auch gegen niedrig-Risiko HPV6 und HPV11. Diese Spaltimpfstoffe bestehen aus dem Hauptkapsidprotein L1, welches spontan „Virus-ähnlichen Partikel“ (VLP) bildet und rufen eine Typen-restringierte protektive Antikörperantwort hervor. Ein multivalenter Impfstoff gegen die Vielzahl medizinisch relevanter Typen wäre technisch aufwändig und entsprechend teuer.

Eine Möglichkeit, das Spektrum der protektiven Immunantwort zu erweitern, basiert auf dem Nebenkapsidprotein L2, welches hochkonservierte Regionen enthält. Immunisierung mit isoliertem L2 kann niedrigtitrige neutralisierende Antikörper induzieren, welche auch heterologe, entfernt verwandte HPV Typen kreuz-neutralisieren. Im Kontext mit L1+L2 oder natürlichen Virionen ist L2 jedoch immunologisch subdominant, da es in einer geringeren molaren Ratio als L1 vorliegt (Verhältnis L1:L2 etwa 1:30), sowie teilweise im Inneren der Virionen vor dem Immunsystem verborgen ist.

Unsere Arbeitsgruppe hat vor kurzem einen experimentellen chimären L1-L2 VLP Impfstoff konzipiert, welcher ein hoch konserviertes N-terminales L2 Epitop („RG1“) in die DE-Oberflächenschlinge (loop) des HPV16 L1 Proteins inseriert hat (RG1 VLP). RG1 VLP immunisierte Kaninchen kreuz-neutralisierende Antikörper gegen weitere mukosale hoch- und niedrig-Risiko und den kutanen hoch-Risiko Typ 5 aufweisen. Das Ziel dieser Arbeit ist es nun, den RG1 VLP Impfstoff weiter zu charakterisieren. Zunächst wurden erneut RG1 VLP produziert, gereinigt und 4 NZW Kaninchen in einem 0-3-6 Wochen Schema, zusammen mit Alum-MPL Adjuvans, immunisiert. Die Immunseren wurden *in vitro* in

Pseudovirionen (PsV) Neutralisations-Assays evaluiert. Insgesamt wurden 20 verschiedene PsV Typen hergestellt. Die Assays zeigten Unterschiede zwischen den Immunseren auf; jeweils ein Immunserum zeigte kreuzneutralisierende Titer gegen HPV6, 11, 31, 33, 40, 44, 52, 53, 66 und 69, jeweils zwei Seren zeigten Titer gegen HPV45, drei Seren gegen HPV34, 39 und 70, und alle Immunseren neutralisierten hoch-Risiko HPV16, 18, 26 und 35. Allen Seren waren nicht-neutralisierend für kutane HPV4 und 38 PsV. ELISA Resultate zeigten, dass RG1 VLP das wichtige HPV16 L1 Epitop V5 nicht aufweisen, was die schwächeren Antikörpertiter gegen HPV16 und nahe verwandte Typen, im Vergleich zur HPV16 L1 wild-Typ Vakzine, erklären könnte. Es bleibt herauszufinden, ob eine Immunisierung nach dem (intensiveren) Originalschema die humorale Immunantwort verbessert.

Nach dem Prinzip der RG1 VLP wurden weitere chimäre VLP Konstrukte generiert. i) HPV18 L1 wurde als Carrier für die HPV45 ‚RG1‘ Sequenz verwendet, da HPV18 und HPV45 nahe verwandt und nach HPV16 wichtige hoch-Risiko Schleimhauttypen darstellen. Zwei ‚kutane Vakzinen‘ bestehen aus ii) HPV1 L1 welches das HPV4 ‚RG1‘ Epitop tragen, und iii) HPV5 L1 welches das HPV17 ‚RG1‘ Peptid trägt, um (kreuz-)neutralisierende Antikörper gegen die Mehrzahl an Hauttypen hervorrufen. Transmissions-Elektronenmikroskopie zeigte, dass das HPV5L1-17RG1 Konstrukt in der Lage ist, vereinzelt wenige partikuläre Strukturen zu bilden, während HPV1L1-4RG1 keine VLP Bildung zeigte. Im Gegensatz dazu konnten für das HPV18L1-45RG1 Fusionsprotein viele effizient und korrekt assemblierte VLP nachgewiesen werden. Die Untersuchung mit mAb zeigte im ELISA, dass einer von 2 untersuchten konformationellen Antikörpern nicht in der Lage ist, die chimären VLP (im Vergleich mit Wildtyp HPV18 L1 VLP) zu binden. Der Verlust des L1 Epitops in den chimären VLP ist offensichtlich durch die RG1 Insertion verursacht. Wir werden die Immunogenität der HPV18L1-45RG1 VLP durch Analyse von Kaninchen-Immunseren in PsV-Neutralisierungstests auf ihre (Kreuz-)Neutralisierung weiter untersuchen. ELISA zeigte auch, dass die HPV1L1-4RG1 und HPV5L1-17RG1 Proteine keine konformationellen Epitope aufweisen, welche für die Induktion einer neutralisierenden (protektiven) Immunantwort unerlässlich sind.

Die RG1 VLP-Immunseren werden in einem murinen vaginalen PsV-Challenge Modell evaluiert. Erste Pilotversuche zeigten, dass das Setzen eines epithelialen vaginalen Mikrotraumas und die Konzentration der eingesetzten PsV für die Robustheit des Modells von großer Bedeutung sind. Nach Etablierung wird die RG1-Vakzine Effizienz gegen eine Vielzahl an mukosalen HPV Typen getestet werden. Im weiteren Verlauf wird ein kutanes *in vivo* Challenge Model etabliert werden, um die Effizienz der Vakzine gegen kutane HPV am natürlichen Infektionsort zu testen.

5.6. Curriculum Vitae

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