Efficacy of RG1-VLP Vaccination against Infections with Genital and Cutaneous Human Papillomaviruses

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Licensed human papillomavirus (HPV) vaccines, based on virus-like particles (VLPs) self-assembled from major capsid protein L1, afford type-restricted protection against HPV types 16/18/6/11 (or 16/18 for the bivalent vaccine), which cause 70% of cervical cancers (CxCas) and 90% of genital warts. However, they do not protect against less prevalent high-risk (HR) types causing 30% of CxCas, or cutaneous HPV. In contrast, vaccination with the minor capsid protein L2 induces low-level immunity to type-common epitopes. Chimeric RG1-VLP presenting HPV16 L2 amino acids 17–36 (RG1 epitope) within the DE-surface loop of HPV16 L1 induced cross-neutralizing antisera. We hypothesized that RG1-VLP vaccination protects against a large spectrum of mucosal and cutaneous HPV infections in vivo. Immunization with RG1-VLP adjuvanted with human-applicable alum-MPL (aluminum hydroxide plus 3-O-desacyl-4′-monophosphoryl lipid A) induced robust L2 antibodies (ELISA titers 2,500–12,500), which (cross-)neutralized mucosal HR HPV16/18/45/37/33/52/58/35/39/51/59/68/73/26/69/34/70, low-risk HPV6/11/32/40, and cutaneous HPV2/27/3/76 (titers 25–1,000) using native virion- or pseudovirion (PsV)-based assays, and a vigorous cytotoxic T lymphocyte response by enzyme-linked immunospot. In vivo, mice were efficiently protected against experimental vaginal challenge with mucosal HR PsV types HPV16/18/45/33/52/58/35/39/51/59/68/73/26/69/34/70, low-risk HPV6/11/32/40, and cutaneous HPV2/27/3/76 (titers 25–1,000) using native virion- or pseudovirion (PsV)-based assays, and a vigorous cytotoxic T lymphocyte response by enzyme-linked immunospot. In vivo, mice were efficiently protected against experimental vaginal challenge with mucosal HR PsV types HPV16/18/45/33/52/58/35/39/51/59/68/73/26/69/34/70, low-risk HPV6/11/32/40, and cutaneous HPV2/27/3/76 (titers 25–1,000) using native virion- or pseudovirion (PsV)-based assays, and a vigorous cytotoxic T lymphocyte response by enzyme-linked immunospot. In vivo, mice were efficiently protected against experimental vaginal challenge with mucosal HR PsV types HPV16/18/45/33/52/58/35/39/51/59/68/73/26/69/34/70, low-risk HPV6/11/32/40, and cutaneous HPV2/27/3/76 (titers 25–1,000) using native virion- or pseudovirion (PsV)-based assays, and a vigorous cytotoxic T lymphocyte response by enzyme-linked immunospot.

INTRODUCTION

Human papillomaviruses (HPVs) are species-specific, epitheliotropic DNA viruses with over 120 types completely characterized today (Bernard et al., 2010). Infections are widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and inducing lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing widespread and induce lesions from benign papilloma to intraepithelial neoplasia to carcinoma, the latter representing.

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vaccine is unlikely to reduce the already very high costs of current HPV vaccines, which may impede delivery to the developing world with the highest CxCa burden. Furthermore, a vaccination strategy against the many types causing skin papillomas (warts) has not been established (Handisurya et al., 2009; Senger et al., 2009). Papillomavirus minor capsid protein L2–based immunogens represent an alternative strategy to multivalent L1-VLP vaccines. The amino (N) terminus of L2 contains highly conserved motifs that are buried in native virions and become exposed only shortly during the infectious process. However, immunization with L2 peptides alone can induce low-titer antibodies, which mediate cross-neutralization (Christensen and Kreider, 1991; Kawana et al., 1999; Roden et al., 2000; Pastrana et al., 2005) in vitro and cross-protection in animal models in vivo (Chandrachud et al., 1995; Gambhira et al., 2007a). An HPV16 L2 peptide comprising amino acids (aa) 17–36 is a broadly cross-neutralization B-cell epitope recognized by mAb RG1 (Gambhira et al., 2007b). Owing to its essential role for viral infectivity and its high conservation within many types, the RG1 epitope may represent an attractive target to develop a broad-spectrum HPV vaccine. We have previously introduced chimeric RG1-VLP as a possible strategy to improve immunogenicity of the RG1 epitope by its genetic insertion into the immunogenic DE-surface loop of HPV16 L1 (Slupetzky et al., 2007; Kondo et al., 2008; Schellenbacher et al., 2009; Caldeira JdO et al., 2010). Upon expression as a recombinant fusion protein, assembly into capsids repetitively displaying RG1 epitopes on the capsid surface (RG1-VLP) is highly efficient. Vaccination induced high-titer neutralizing antibodies against HPV16 and improved L2-specific antibodies. Then, available limited in vitro assays demonstrated cross-neutralization of mucosal HR HPV18/31/45/52/58, LR HPV HPV6/11, and a single genus β-type (HPV5) (Schellenbacher et al., 2009). This study comprehensively examines RG1-VLP vaccine efficacy to cross-protect against all relevant mucosal HR HPV5s in vivo and in vitro, endurance of protection (an important issue for L2-based vaccine development), and induction of cell-mediated immunity. Vaccine efficacy against natural infection was validated using authentic virion-based neutralization assays.

RESULTS

We have shown previously that vaccination of rabbits and mice with recombinant RG1-VLP plus alum-MPL (aluminum hydroxide plus 3-O-desacyl-4′-monophosphoryl lipid A) adjuvant (Schellenbacher et al., 2009) elicited high-titer neutralizing antibodies to HPV16 and cross-neutralizing antibodies to pseudovirion (PsV) of the limited number of then available mucosal HR HPV18/31/45/52/58, LR HPV6/11, and cutaneous β HPV5.

RG1-VLP vaccination induces a robust antibody response against the L2 epitope

To assess the robustness of the humoral immune responses to RG1-VLP, eight additional rabbits were vaccinated either 4 or 3 times (New Zealand White (NZW) nos. 1–6: 4 × 50 μg and nos. 7–10: 3 × 20 μg) and sera drawn 2 weeks after the last boost. Robust antibody responses to L2 (titers of 2,500–12,500) were detected for both vaccination protocols using the 16L2 N-terminal peptide (aa 11–200) as ELISA antigen (Supplementary Material online). Conversely, reactivity was absent for rabbit antisera to HPV16 wild-type L1-VLP as expected.

Antisera to RG1-VLP neutralize distantly related mucosal HPV types in vitro

The spectrum of cross-neutralization induced by RG1-VLP vaccination was further explored by analyzing rabbit antisera (n = 10) in neutralization assays for a large panel of genus-α HPV using additional PsV types (Table 1). Similar to findings reported previously for rabbits 1/2 (*), the additional eight rabbits’ immune sera contained high-titer neutralizing antibodies against HPV16 (titers of 10,000–100,000). Broad-spectrum cross-neutralization was found for species α9 HPV31, 52, 58, 33, 35 (in 2, 5, 9, 6, and 9 out of 10 sera), α7 HPV18, 45, 39, 59, 68, 70 (9, 6, 5, 1, 2, 7/10), α5 HPV51, 26, 69 (3, 10, 2/10), α1 HPV73 and 34 (10 and 8/10) (titers from 25 to 10,000), but not for αβ HPV56, 53, and 66. Cross-neutralization beyond mucosal HR types was analyzed for the most potent sera nos. 1/2 (Table 2). Apart from LR HPV6/11, α1 HPV32 (causing Heck’s disease) was neutralized (titers of 50/100), whereas HPV44 (α10) sporadically found in genital warts was not neutralized. To narrow the gap between PsV-based in vitro assays and natural HPV infection, RG1-VLP-induced neutralizing antibodies were also detected using infectious native virions (Handisurya et al., 2007). As shown in Figure 1a, HaCaT cells infected with HPV26 virions (lane 2) revealed a specific band corresponding to spliced viral mRNA in nested reverse transcriptase–PCR, in contrast to uninfected control cells (lane 1). Preincubation of virions with RG1-VLP antisera (1:400) completely abolished mRNA detection, indicating viral neutralization (lane 4), whereas preimmune serum (lane 3), mAb RG1 (Gambhira et al., 2007b) (lane 5), or serum from a Gardasil-vaccinated individual (lane 6) had no effect. RG1-VLP antisera also cross-neutralized LR HPV40 (Figure 1b; lanes 5 and 6) and HPV6 virions (see Supplementary Material online) at dilutions of 1:100–400. Taken together, the in vitro cross-neutralization spectrum of RG1-VLP vaccination includes almost all HR HPVs causing CxCa, as well as LR mucosal types in PsV and native virion–based assays.

Antisera to RG1-VLP cross-neutralize prevalent common cutaneous and HR β HPV5s

In immunocompetent individuals with palmoplantar and plane warts, the most frequently detected types are genus α HPV2/27/57 (Rubben et al., 1997), γ HPV4, μ HPV1, and β HPV3/10. Genus β HPV5/8 have been first identified in patients suffering from Epidermodysplasia verruciformis, whereas a possible role of β papillomavirus in non-melanoma skin cancer pathogenesis is still controversial. Thus, rabbit antisera nos. 1/2 were screened for cross-neutralization against available cutaneous HPVs in pseudovirion-based neutralization assay (PBNA; Table 2), showing previously reported cross-neutralization of HPV5(*), and additionally of...
HPV3 (α2) and HPV76 (β4) (titers of 1,000/100), but not of HPV4 (γ1) or HPV38 (β2). Furthermore, antisera nos. 1 and 2 cross-neutralized native virions of HPV2 with titers of 800 and 1,600 (Figure 1c, lanes 5 and 11), and HPV27 at dilution 1:400 (Supplementary Material online). Interestingly, antiserum to HPV2 L1-VLP neutralized HPV27, as did antiserum to HPV27 L1-VLP (1:400), indicating that closely related genotypes HPV2 and HPV27 (α4) may represent common serotypes (antisera to HPV2/27 L1-VLP were a kind gift from L Gissmann, DKFZ, Heidelberg, Germany). Neither HPV1 nor HPV4 virions (Supplementary Material online) were cross-neutralized by RG1-L1-VLP antiserum. Sera of 7/8 prepupertal girls were neutralized against highly prevalent cutaneous HPV1 (Supplementary Material online), indicating robust seroconversion after natural infection, whereas serum from a Gardasil-vaccinated individual did not cross-neutralize cutaneous HPV4. Moreover, the cross-neutralization spectrum of RG1-L1-VLP vaccine even extends to bovine papillomavirus type 1 in PBNA (Table 2).

### RG1-L1-VLP Vaccination Induces Cellular Immune Responses

The enzyme-linked immunospot analysis of splenocytes from mice vaccinated with RG1-L1-VLP or similarly HPV16 L1-VLP showed IFN-γ-producing cells when stimulated with a previously described HPV16 L1 cytotoxic T lymphocyte epitope (Supplementary Material online), indicating the induction of a strong cellular immune response (Ohlschläger et al., 2003). In contrast, stimulation of cells with the RG1 peptide did not result in significant IFN-γ production.

### RG1-L1-VLP Antisera Efficiently Protect Mice Against Experimental Vaginal Challenge with Mucosal HPV in Vivo

Given the broad spectrum of HR HPVs cross-neutralized in vitro, vaccine efficacy in vivo was determined using an experimental mouse model (adapted from Roberts et al., 2007). As expected, passive transfer of RG1-L1-VLP antiserum reduced vaginal infection in mice challenged with HPV16 PsV to background levels, similar to HPV16 L1 VLP antiserum (Figure 2a). Importantly, mice were also cross-protected from infection with phylogenetically divergent types HPV31/33/35/52/58 (α9), HPV18/45/39/56/68 (α7), HPV34/73 (α11), HPV53/55/66 (α6), and HPV26/51 (α5), and LR HPV6 (α10) and HPV43 (α8) (Figure 2b and c). Indicative, yet statistically insignificant, results were obtained for LR HPV44 (α10). The level of cross-protection by RG1-L1-VLP antiserum was

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### Table 1. Cross-neutralization of mucosal HR HPV by RG1-VLP antisera in PBNA in vitro

| HPV Type | Neutralizing Titers (Antisera Nos. 1 and 2) |
|----------|-------------------------------------------|
|          | 1:500                                      |
|          | 1:250                                      |
|          | 1:125                                      |
|          | 1:62.5                                     |
|          | 1:31.25                                    |
|          | 1:15.625                                   |
|          | 1:7.8125                                   |
|          | 1:3.90625                                  |
|          | 1:1.953125                                 |
|          | 1:0.97653125                              |

Abbreviations: alum-MPL, aluminum hydroxide plus 3-O-desacyl-4-monophosphoryl lipid A; HPV, human papillomavirus; HR, high risk; NZW, New Zealand White; PBNA, pseudovirion-based neutralization assay; VLP, virus-like particle.

Antisera of 10 NZW rabbits raised against RG1-L1-VLP were analyzed for cross-neutralization of 20 mucosal HR HPV pseudovirions in duplicates using end point serial dilutions of 1:25–1:100,000. Neutralization titers were determined as described earlier (Schellenbacher et al., 2009). Data previously published are indicated by “*” (Schellenbacher et al., 2009). Boxed titers indicate sera also tested for cross-neutralization in vivo (Figure 2a–c).
Induction of long-lasting B-cell memory and cross-protection

To examine long-term B-cell memory after RG1-VLP vaccination, rabbits 1/2 were housed for a further 10 months after the fourth immunization and boosted at week 52 with 50 μg of RG1-VLP (Supplementary Material online). When compared with sera drawn at week 10, cross-neutralization titers had declined 1–2 logs (HPV18/31) or beneath the level of detection (HPV45/52/58/6/5) in week 52. A similar 2-log decline was observed for neutralizing antibodies against HPV16, which are predominantly induced by the L1 scaffold of RG1-VLP. Importantly, boosting with RG1-VLP raised antibody titers to former levels or beyond. To determine whether cross-protection in vivo is also long lasting, antiserum drawn at week 52 was analyzed for cross-protection against in vivo challenge with HPV58. Although neutralization of HPV58 was no longer detectable in vitro at week 52, cross-protection was still conferred in vivo (Figure 2d), yet to a slightly lesser extent as compared with sera drawn at week 10 (Figure 2b).

DISCUSSION

The search for second-generation HPV vaccines is driven by the need to protect against the plurality of carcinogenic genital HPV by safe and affordable formulations. Because licensed vaccines do not target HR HPVs other than HPV16/18, causing 30% of CxCas, cytological screening programs cannot be superseded. This major limitation in particular affects developing countries, which bear >85% of the global CxCa burden. Moreover, precancerous cervical neoplasia with a substantial disease prevalence and morbidity in younger women is even more strongly associated with types other

| Pseudovirions | Neutralizing titer | Neutralizing titer |
|---------------|-------------------|-------------------|
|               | 1                 | 2                 |
| **Low-risk mucosal** |                   |                   |
| HPV6 *        | 100               | 50                |
| HPV11 *       | 100               | <25               |
| HPV32         | 50                | 100               |
| HPV44         | <25               | <25               |
| **Genus β**   |                   |                   |
| HPV5 *        | 100               | 50                |
| HPV38         | <25               | <25               |
| **Common cutaneous** |               |                   |
| HPV3          | 1,000             | 1,000             |
| HPV4          | <25               | <25               |
| HPV76         | 100               | 100               |
| **Nonhuman**  |                   |                   |
| BPV1          | 100               | 100               |

Abbreviations: BPV1, bovine papillomavirus type 1; HPV, human papillomavirus; LR, low risk; VLP, virus-like particle.

Antisera of two rabbits (nos. 1 and 2) raised against RG1-VLP were tested for cross-neutralization of 4 LR mucosal, 2 genus β cutaneous, 3 common cutaneous HPVs, and nonhuman BPV1 as indicated. Neutralizing titers were determined as described in Table 1.

| Pseudovirions | Neutralizing titer |
|---------------|--------------------|
|               |                    |
| **Low-risk mucosal** |                   |
| HPV6 *        | 100                |
| HPV11 *       | 100                |
| HPV32         | 50                 |
| HPV44         | <25                |
| **Genus β**   |                    |
| HPV5 *        | 100                |
| HPV38         | <25                |
| **Common cutaneous** |               |
| HPV3          | 1,000              |
| HPV4          | <25                |
| HPV76         | 100                |
| **Nonhuman**  |                    |
| BPV1          | 100                |
Chimeric L2-L1 VLP as a Pan-HPV Vaccine

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...in vitro chimeric RG1-VLP has been analyzed by a limited number of types. The spectrum of L2-mediated cross-neutralization by targeting even more mucosal and, additionally, nongenital L2-based vaccines are attractive as single-antigen formulations expected to significantly increase the breadth of coverage, trials. Although such a highly multivalent L1 vaccine is vaccine (comprising seven HR and two LR types) is in clinical...which cause more than 95% of CxCas worldwide, and also partially protects against the remaining four types tested (de Sanjose et al., 2010). Mice were protected from vaginal challenge with large doses of mucosal PsV despite considerable antisem dilution (~1:50) into the mouse circulation. Interestingly, undetectable cross-neutralization in vitro did not stringently imply incomplete protection in vivo. In line with the recently reported insufficient sensitivity of current in vitro assays to detect anti-L2 antibodies, in vivo testing has become the gold standard in detecting neutralizing HPV antibodies (Longet et al., 2011) and documenting vaccine efficacy. The results imply that the actual immunogenicity is substantially greater than that reflected by the standard in vitro neutralization assay, and suggest that this vaccine should have a significant impact not only on reducing the risk of CxCa but also on the overall incidence of genital HPV infection, thus providing a major advantage to HPV-based screening programs when compared...
with the current vaccines. Complementary in vitro assays were used to more extensively characterize the spectrum of cross-neutralization by RG1-VLP vaccination, which was not predicted by L1-based classification of papillomaviruses, the site of infection, or host species. In PBNA, variable (cross)-neutralization by RG1-VLP antiserum was detected for 17 mucosal HR types, whereas cross-protection in vivo was intact for the serum with least potency in vitro. Although technically demanding and limited to those few types available from productive lesions, neutralization assays using native virions may reflect viral infection more authentically than PBNA (McLaughlin-Dubin et al., 2004). Reassuringly, the RG1-VLP antiserum neutralized lesion-derived virions as well. The RG1-VLP antisera also cross-neutralized LR types HPV6/11 (a10), HPV32 (a1), HPV40 (a8), and cutaneous HPV2/27 (a4), HPV3 (a1), and HPV76 (b3), but not HPV1 (a), HPV4 (aγ), and HPV38 (b). On comparing the RG1 motifs of all types cross-neutralized, sequence homology to HPV16 RG1 was at least 60% (see Supplementary Material online). Whether this lower threshold of homology necessary for inducing cross-neutralization holds out in more sensitive in vivo assays needs to be demonstrated. Therefore, RG1-VLP vaccination is expected to protect against a substantial proportion of benign papillomas, including both sexually transmitted genital and nongenital skin warts. The latter is not only of tremendous clinical importance for the growing group of immunocompromised patients (e.g., organ-transplant recipients, HIV patients), but may have expanded relevance if these types contribute to the genesis of nonmelanoma skin cancers. Because the incidence of common skin warts peaks in early childhood, this would provide a rationale for implementing the vaccine into existing childhood programs. Various approaches to enhance cross-neutralizing L2 antibodies have been presented over the past years, including conjugation of L2 peptides to a T-helper epitope and a Toll-like receptor 2 ligand (Alphs et al., 2008), concatenated multimeric L2 peptide vaccination (Iagl et al., 2009), or display on bacteriophage (Tumban et al., 2012), adeno-associated virus (Nieto et al., 2012), or HPV L1-VLP (Zamora et al., 2006; Slupetzky et al., 2007; Kondo et al., 2008; Schellenbacher et al., 2009; Caldeira Jdo et al., 2010). Favorably, RG1-VLP vaccination plus alum-MPL (as in Cervarix) not only elicits robust antibody responses against RG1 in ELISA but also retains high-level protection against the most important HPV16. Therefore, RG1-VLP vaccination should provide strong protection not just against CxCas but also against the even higher percentage of noncervical cancers attributable to HPV16. In contrast, in a comprehensive direct comparison, HPV16 L1-VLP vaccination protected against HPV16 and 31 and only to a lower extent against 35, 39, 56, and 6. Licensed vaccines provide long-term protection of at least 8.5 years, and the antibody levels required are still unknown. Importantly, sera drawn 12 months after RG1-VLP vaccination completely protected mice against vaginal challenge with HR HPV58 in vivo. RG1-VLP holds promise as next-generation vaccine with broad efficacy against the vast majority of relevant mucosal and additional cutaneous HPVs, provides a rationale for childhood vaccination, and adds economic advantage of a single antigen formulation compared with multivalent L1-VLP formulations. As post-licensure data confirm excellent safety profiles for HPV L1-VLP vaccines, we infer that RG1-VLP vaccination may prove similarly safe, offering the possibility to evaluate vaccine immunogenicity in early-phase human trials.

**MATERIALS AND METHODS**

**Chimeric RG1-VLP**

Chimeric HPV16 L1–HPV16 L2 [aa 17–36] VLP (RG1-VLP) have been generated (Schellenbacher et al., 2009) by the insertion of HPV16 L2 peptide aa 17–36 (RG1) (Gambhir et al., 2007b) into the DE-surface loop of HPV16 L1 and expressed in insect cells (Kimbauer et al., 1992).

**Immunization**

NZW rabbits were immunized with 50 μg of RG1-VLP subcutaneously (weeks 0, 4, 6, and 8, n = 6; NZW nos. 1–6) or 20 μg of RG1-VLP (weeks 0, 3, and 6, n = 4; NZW nos. 7–10) (Charles River, Kisslegg, Germany) adjuvanted with alum-MPL (1:10) (Sigma Aldrich, St Louis, MO). Sera were drawn at weeks 10 (nos. 1–6) or 8 (nos. 7–10). Long-term antibody responses were determined for NZW nos. 1/2 kept at 10 months. Sera were drawn before and 14 days after final boost (week 52) and were stored at −20°C. For type-specific L1 antiserum, a single rabbit each was immunized with 20 μg PsV of HPV6/26/33/35/39/43/44/51/52/53/56/58/59/66/68/69, or 70, respectively (weeks 0, 3, and 6), in complete incomplete Freund’s adjuvant.

**Native virion–based neutralization assays (reverse transcriptase–PCR)**

Native virions HPV2/27/1/4 were extracted from plantar warts; HPV6 from genital warts; HPV26 from a highly differentiated carcinoma (Handisurya et al., 2007); and HPV40 virions were provided by Neil Christensen (Hershey, PA) (Jenkins et al., 2003). After mechanical disruption of wart tissue by high-speed homogenizer (Fastprep-24, MP Biomedicals, Eschwege, Germany) and centrifugation (14,000 r.p.m./4°C/5 minutes), supernatants containing virions were used for neutralization assays (Smith et al., 1995; Slupetzky et al., 2007). In brief, 3 × 10⁵ HaCaT keratinocytes were infected with virions that were either untreated or preincubated with rabbit antiserum and incubated overnight. Cellular RNA was reverse transcribed, and spliced viral E1^E4 mRNA was identified by 30-cycle nested PCR (95°C/1 minute, 60°C/1 minute, and 72°C/3 minutes; for primer pairs see Supplementary Material online).

**Murine vaginal challenge**

The intravaginal PsV challenge model (Roberts et al., 2007) was performed with slight modification (Karanam et al., 2010). Female Balb/C mice (groups of 5 or 10) were pretreated with 3 mg of progesterone subcutaneously medroxyprogesterone (Depocou, Pfizer, Vienna, Austria), and on day 3 they were passively transfected with 20μl rabbit antiserum: RG1-VLP (pre)immune serum (rabbits 1, 6, and 10), or sera to L1-VLP of HPV16/31/33/45/6, to L1/L2-VLP of HPV18 (J Schiller, NIH, Bethesda, MD), to L1-DNA of HPV59/73, or to L1/L2-PSV of HPV26/35/39/43/51/52/53/56/58/66/68/69 or 70, respectively. After 24 hours, vaginal microtrauma was induced by cervical cytobrush, and mice were challenged with luciferase-encoding PsV in 3% carbocymethylcellulose. After 3 days,
20μl of luciferin (Caliper, Waltham, MA; 7.5 mg ml⁻¹) was applied into the vagina, and infection was analyzed by bioluminescence imaging (IVIS 50, Caliper). Data are given with background signal subtracted (mice challenged with carboxymethylcellulose).

**Statistical methods**

Statistical analysis was performed using Microsoft Excel (heteroscedastic two-tailed unpaired t-test to evaluate P-values).

Standard techniques such as ELISA, PBNAs, and enzyme-linked immunospot assays are described in Supplementary Material online.

**Consent and approval**

Human sera and tissue samples were collected after written informed consent of the patient or the patient’s guardian in accordance with the Ethics Committee of the Medical University Vienna (ECS 1327:2012). The study was conducted according to the Declaration of Helsinki Principles.

**Animal welfare**

Animal studies have been approved (BMWF-66.009/0173-11/3b/2011) and animal care was in accordance with the guidelines of the Austrian Federal Ministry for Science and Research.

**CONFLICT OF INTEREST**

RK is a co-inventor on L1 patents licensed to GlaxoSmithKline (GSK) and Merck (MSD). RBSR has received an unrestricted educational grant from GSK. RBSR is a co-inventor on L2 patents licensed to PoxVax, Shantha Biotechnics/Sanofi, and GSK, and on PoxV technology licensed to GSK and MSD, and is a founder of Papivax LLC. The terms of these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies. The RG1-VLP vaccine is subject to a pending patent application (RK, CS, RBSR). The other authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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