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Targeting the Vaginal Mucosa with Human Papillomavirus Pseudovirion Vaccines Delivering Simian Immunodeficiency Virus DNA

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The majority of HIV infections occur via mucosal transmission. Vaccines that induce memory T and B cells in the female genital tract may prevent the establishment and systemic dissemination of HIV. We tested the immunogenicity of a vaccine that uses human papillomavirus (HPV)-based gene transfer vectors, also called pseudovirions (PsVs), to deliver SIV genes to the vaginal epithelium. Our findings demonstrate that this vaccine platform induces gene expression in the genital tract in both cynomolgus and rhesus macaques. Intravaginal vaccination with HPV16, HPV45, and HPV58 PsVs delivering SIV Gag DNA induced Gag-specific Abs in serum and the vaginal tract, and T cell responses in blood, vaginal mucosa, and draining lymph nodes that rapidly expanded following intravaginal exposure to SIVmac251. HPV PsV-based vehicles are immunogenic, which warrant further testing as vaccine candidates for HIV and may provide a useful model to evaluate the benefits and risks of inducing high levels of SIV-specific immune responses at mucosal sites prior to SIV infection. The Journal of Immunology, 2012, 188: 714–723.

The female genital tract is unique because of its hormonal responsiveness, commensal bacteria, biochemical processes, and immunological milieu (1, 2). These features may contribute to the increased rate of heterosexual male to female HIV transmission when compared with female to male transmission (3). Blocking vaginal transmission of HIV may require vaccines that target the female genital tract and induce local immunity. HIV vaccines based on viral vectors, proteins, or a combination thereof, tested in phase III vaccine efficacy trials in humans, induced mainly systemic immune responses using vaccines delivered by i.m. inoculation (4–6). Although these vaccine modalities induce variable levels of HIV-specific responses in the blood (4, 5), little is known about their ability to induce mucosal responses. A limited, but significant, protection from heterosexual transmission has been observed in individuals vaccinated with a combination of the recombinant poxvirus ALVAC-HIV and the gp120 envelope protein (5). This vaccine modality induces low CD8+ and CD4+ T cell responses and Abs to HIV that mediate Ab-dependent cellular cytotoxicity but have limited neutralizing activity (5). These findings suggest that a balance of T cell responses in conjunction with Abs to the envelope protein may be important. However, in animal models, vaccines that elicit predominantly effector memory CD8+ T cell responses can also control mucosal SIV infection (7, 8). Thus, defining the quantity, quality, and location of protective HIV/SIV vaccine-induced immune responses is necessary. We hypothesize that vaccine-induced cell-mediated and humoral memory responses, within the interstitial layers of the female genital tract, can curtail the local expansion of HIV/SIV and prevent its systemic dissemination. In the current study, we tested a vaccine delivery platform that specifically targets the vaginal mucosa. A subset of human papillomaviruses (HPVs) is sexually transmitted mucosal pathogens that naturally infect cervicovaginal keratinocytes (9). HPV–virus-like particle (VLP)–based vaccines are safe and very effective at preventing the HPV infections that cause cervical neoplasia in women (10). HPV capsid proteins, L1 and L2, can self-assemble into VLPs, and, when cotransfected with a plasmid containing a gene of interest, L1 and L2 will encapsidate the plasmid-forming pseudovirions (PsVs) (11, 12). HPV PsVs have been shown to effectively deliver reporter genes to the female genital tract in multiple animal models (13–15). HPV PsV infection is limited to keratinocytes and requires minor disruption of the epithelium (16). Thus, we treated macaques with progesterone to thin the vaginal epithelium and used mechanical and/or chemical disruption of the epithelium to facilitate efficient HPV PsV delivery to keratinocytes. Expression of the transgene is robust but transient, lasting ∼7 d in the mouse genital tract (13). Furthermore, HPV PsV may serve as adjuvants, engaging TLRs and facilitating the activation and maturation of APCs (17, 18).

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Abbreviations used in this article: Env, envelope; HPV, human papillomavirus; IU, infectious unit; N9, nonoxynol 9; PsV, pseudovirion; RFP, red fluorescent protein; VLP, virus-like particle.

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We have exploited the ability of HPV PsVs to target the female genital tract and used PsVs as vectors to deliver DNA encoding SIV genes to a site of SIV transmission in two nonhuman primate species. SIV Gag was chosen as our model Ag to initially test the immunogenicity of HPV PsVs in macaques, as Gag is easily cloned, expressed, and secreted. We demonstrate that this vaccination strategy induces local and systemic immune responses in both cynomolgus and rhesus macaques. In addition, HPV PsVs induced mucosal immune responses that rapidly expanded upon vaginal exposure to SIVmac251.

Materials and Methods

Animals, HPV vaccination, and SIV infection

Eight cynomolgus macaques and eight rhesus macaques were used in this study; all animals were housed and cared for under the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International and housed at Advanced BioSciences Laboratories in Rockville, MD.

HPV PsVs were produced as previously described (11, 12). Briefly, DNA constructs encoding the L1 and L2 capsid of HPV and a smaller SIV Gag, Gag-Pro, red fluorescent protein (RFP), or luciferase that could be efficiently packaged, were cotransfected into 293T T cells, and the resulting PsVs purified, propagated, and titrated. Twenty-eight days prior to vaccination, macaques were given 30 mg/kg Depo-Provera i.m. One week prior to vaccination, macaques were treated with antibiotics to prevent any occult vaginosis. At 6 and/or 18 h prior to vaccination, a vaginal application of nonoxynol 9 (N9), a nonionic surfactant used as the DHCP antigen, was administered either as a 10% gel mixed with 4% carboxymethyl cellulose (Sigma-Aldrich, St. Louis, MO) or as a 12.5% foam (VCF: Apothecus Pharmaceutical, Oyster Bay, NY). Six hours after the last N9 treatment, two of the cynomolgus macaques had the vaginal epithelium lightly abraded with a cytobrush. A standard inoculum of 500 μl that consisted of 10^5 to 10^10 infectious units (IU) HPV PsVs mixed with carboxymethyl cellulose was instilled using a positive displacement pipette into the cervix of each animal. Cynomolgus macaques received 2 × 10^5 IU HPV16- and 45-expressing SIV Gag and RFP given 30 d apart. Six rhesus macaques similarly received 2 × 10^10 IU HPV16 but 1 × 10^9 IU HPV45, both expressing SIV Gag-Pro after two N9 applications (three macaques received N9 gel and three N9 foam). Three months after the last vaccination, two of the cynomolgus macaques had the vaginal epithelium lightly abraded with a cytobrush. A standard inoculum of 500 μl that consisted of 10^5 to 10^10 infectious units (IU) HPV PsVs mixed with carboxymethyl cellulose was instilled using a positive displacement pipette into the cervix of each animal. Cynomolgus macaques received 2 × 10^5 IU HPV16 and 45-expressing SIV Gag and RFP given 30 d apart. Six rhesus macaques similarly received 2 × 10^10 IU HPV16 but 1 × 10^9 IU HPV45, both expressing SIV Gag-Pro after two N9 applications (three macaques received N9 gel and three N9 foam). Three months later, the rhesus macaques were boosted with a third vaccination of 2 × 10^9 HPV58, also expressing SIV Gag-Pro. Two rhesus macaques, used as unvaccinated controls for the study, were given HPV16, HPV45, and HPV58 expressing luciferase at similar doses and timing as animals vaccinated with HPV-Gag-Pro-PsVs. Two of the cynomolgus macaques had the vaginal epithelium lightly abraded with a cytobrush. A standard inoculum of 500 μl that consisted of 10^5 to 10^10 infectious units (IU) HPV PsVs mixed with carboxymethyl cellulose was instilled using a positive displacement pipette into the cervix of each animal. Cynomolgus macaques received 2 × 10^5 IU HPV16- and 45-expressing SIV Gag and RFP given 30 d apart. Six rhesus macaques similarly received 2 × 10^10 IU HPV16 but 1 × 10^9 IU HPV45, both expressing SIV Gag-Pro after two N9 applications (three macaques received N9 gel and three N9 foam). Three months later, the rhesus macaques were boosted with a third vaccination of 2 × 10^9 HPV58, also expressing SIV Gag-Pro. Two rhesus macaques, used as unvaccinated controls for the study, were given HPV16, HPV45, and HPV58 expressing luciferase at similar doses and timing as animals vaccinated with HPV-Gag-Pro-PsVs. Six weeks post-HPV vaccination, rhesus macaques were challenged intravaginally with (10^5) TCID50 of SIVmac251.

ELISPOT and immunohistochemistry assays

SIV-specific T cells were assessed using an ELISPOT kit from Mabtech (Mairemont, OH). Peripheral blood cells were stimulated with either SIV gag or envelope (Env) overlapping 15-mer peptides or Con A or left unstimulated and added to IFN-γ-coated plates for 24 h. The plates were developed, and the frequency of IFN-γ-positive spot-forming cells per 10^6 PBMC was determined after background subtraction.

All slides for immunohistochemistry were stained using the Dako Autostainer (DakoCytomation, Carpinteria, CA) as previously described (19). The primary Abs that were used included monoclonal anti-CD4 mouse serum (clone IF6; Vector Laboratories, Burlingame, CA), monoclonal anti-CD8 mouse serum (clone IAS5; Leica Microsystems, Bannockburn, IL), and polyclonal anti-Ki67 rabbit serum (Lab Vision, Fremont, CA). For all primary Abs, slices were subjected to an Ag retrieval step as previously described (19). Primary Abs were replaced by normal rabbit IgG, FITC-conjugated anti-rabbit IgG (Dako) or mouse IgG (DakoCytomation) and included with each staining series as the negative control. Binding of the CD4, CD8, and Ki-67 were detected simultaneously using Alexa Fluor 488-labeled polyclonal goat anti-rabbit IgG and Alexa Fluor 568-labeled polyclonal goat anti-mouse IgG (Molecular Probes, Eugene OR). Slides were visualized with epifluorescent illumination using a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood NY). Digital images were captured and analyzed using a Zeiss AxioCam System and OpenLab software (Improvision, Waltham, MA). The numbers of CD4/Ki67 positive are presented as cells per square millimeter of lamina propria.

SIV Abs in serum

Serum samples were tested for SIV-specific Ab responses by using an ELISA described elsewhere (23, 24). Briefly, samples for SIVgag testing were serially diluted and applied to a 96-well half-area plate (Greiner Bio-One), previously coated with 1 μg/ml SIVmac251-purified lysate (Advanced Biotechnologies) and blocked with 1% BSA block solution (KPL). After overnight incubation at 4˚C, the plate was washed with PBS-Tween, washed, and incubated for 1 h at room temperature. After washing, 3˚, 5˚, 5˚, 5˚-tetramethylbenzene peroxidase substrate solution was added to each well, followed by 20 min of incubation at room temperature until the color developed. The reaction was stopped by adding 2 M H2SO4, and the plate was read at 450 nm within 30 min. Ab titer was defined as the reciprocal of the dilution at which the OD of the test serum was two times greater than that of a naive control macaque serum diluted 1:50. SIV Abs were detected by ELISA specific for each of the different HPV types as previously described (13).

SIV Abs in vaginal secretions

Vaginal secretions were collected in triplicate using Weck-Cel sponges (Medtronic). To elute secretions, the sponges were incubated for 10 min in ice-cold elution buffer and then transferred into a Salivette column (Sarstedt, Germany) and centrifuged at 3000 rpm for 30 min at 4˚C. For SIV gag-specific IgA and IgG, serially diluted secretions were applied to a 96-well half-area ELISA plate (Greiner Bio-One), coated overnight at 4˚C with 1 μg/ml SIVmac251-purified lysate (Advanced Biotechnologies), and blocked. Serial dilutions of SIVgag-specific IgA or IgG standards of known concentration were prepared as previously described (24) and included on each plate. After overnight incubation at 4˚C, the plate was washed with peroxidase-conjugated anti-monkey IgA Ab (Alpha Diagnostic, San Antonio, TX), and incubated for another hour at room temperature. After washing, 3˚, 5˚, 5˚-tetramethylbenzene peroxidase substrate solution was added to each well, followed by 20 min of incubation at room temperature until the color developed. The reaction was stopped by adding 2 M H2SO4, and the plate was read at 450 nm within 30 min. Ab titer was defined as the reciprocal of the dilution at which the OD of the test serum was two times greater than that of a naive control macaque serum diluted 1:50. HPV Abs were detected by ELISA specific for each of the different HPV types as previously described (13).

Tissue collection, quantitation of viral RNA and DNA, and immunophenotyping

Mononuclear cells were isolated from blood, axillary, and obturator lymph nodes, rectum, endocervix, and vaginal tissue pre- or postvaccination as described (19, 20).

SIV RNA was quantified in the plasma by NASBA, as previously described (21), and SIV DNA was quantified in tissues by quantitative PCR (22).

Four- and nine-color flow cytometric analysis was performed on mononuclear cells from blood and tissues. Immunophenotype and intraacellular cytokine assays to detect SIV-specific cells were performed as previously described (19, 22). Cells were stained with the following Abs: CD3 (cloneSP34-2), CD4 (clone L200), CD8 (clone RPA-T8), CD95 (clone DX2), IFN-γ (clone B27), TNF-α (clone MAB11), IL-2 (clone MQI-17H12), and CD107 (clone H4A3), all obtained from BD Biosciences (San Diego, CA). In addition, the live/dead yellow fixable amine dye was obtained from Invitrogen (Carlsbad, CA), anti-CD28 (clone CD28.2) from eBioscience (San Diego, CA), and anti-CD45R (clone 30-F11) and CD107 (clone H4A3) from BioLegend (San Diego, CA). All cells were fixed with 1% paraformaldehyde and acquired on either a FACSCalibur or LSR II (BD Biosciences). Data analysis was performed with FlowJo (Tree Star).

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Statistical analysis

Comparisons between groups were assessed using either a Wilcoxon rank sum test or repeated-measures ANOVA. Graphical analysis was performed using GraphPad Prism (GraphPad).

Results

Efficient gene delivery and seroconversion to HPV Ags in macaques vaccinated with HPV PsVs

We produced HPV pseudovirions by cotransfecting 293T T cells, a human cell line, with a DNA plasmid containing the capsid (L1/L2) genes and a DNA plasmid encoding RFP, luciferase, or SIV Gag genes, as previously described (11, 12). Eight cynomolgus macaques were intravaginally exposed to $2 \times 10^{10}$ IU of HPV16, followed by HPV45 pseudovirions encapsidating RFP and SIV Gag (Fig. 1A). Efficient gene expression of intravaginally delivered HPV PsVs has been observed in progesterone-treated macaques, after microtrauma was induced in the vaginal tract (15). Thus, we treated macaques with 30 mg/kg of Depo-Provera and compared four methods of transient disruption of the cervicovaginal epithelium, all of which included N9. N9 is currently in use as an over-the-counter spermicide and known to disrupt genital epithelium (25, 26). N9 was prepared as a 10% gel formulation delivered either once (Gel 1X), twice (Gel 2X), or administered twice in combination with gentle abrasion with a cytobrush (Gel 2X+cytobrush). In addition, we tested a commercially available 12.5% foam application of N9 (foam) delivered twice. Forty-eight hours after vaccination, in vivo imaging detected RFP expression from the HPV-RFP constructs in the vaginal tract using an endoscope attached to a Maestro multispectral CCD camera (Fig. 1B). N9 delivered as a gel application resulted in punctate fluorescence, whereas N9 foam treatment facilitated diffuse fluorescence throughout the cervicovaginal tract (data not shown). HPV PsV vaccination induced seroconversion to HPV Ags, and we detected capsid-specific IgG Abs in the blood of all animals (Fig. 1C). The magnitude of the Abs was highest in animals treated with N9 gel in combination with a cytobrush; however, increased Abs to HPV are not related to immune responses to the encapsidated genes (J.T. Schiller, unpublished observations). Our findings confirm and extend the results from Roberts and colleagues (15), who demonstrated that, after transient disruption, macaque vagina epithelium is effectively transduced by HPV PsVs, leading to robust in vivo gene expression.

HPV PsV vaccination induces SIV Gag-specific cell-mediated and humoral responses in macaques

We next investigated the immunogenicity of intravaginally delivered HPV PsVs. Prior to vaccination, the expression of SIV-Gag was confirmed by Western blot on cell lysates from 293T T cells transduced by HPV Gag PsVs. We detected the SIV Gag protein precursor p55 by Abs that recognize the rSIV p27 Gag protein, used as a positive control (lane 1 of Fig. 2A). Following vaccination, T cell responses to SIV Gag were measured in PBMCs by ELISPOT using 15-mer peptides spanning the entire SIV Gag protein. Animals treated with N9 gel mounted T cell responses of greater magnitude than animals treated with N9 foam (Fig. 2B). Two treatments with N9 gel facilitated the development of high-titer Gag-specific serum IgG (Fig. 2C). Additionally, we measured Gag-specific IgA in the serum, and very low levels of IgA were detected in the two animals in which the epithelia was disrupted with the cytobrush (titers 50 and 100); all other animals were negative (data not shown). Interestingly, however, Gag-specific IgA in vaginal secretions was equivalent in all groups (Fig. 2D).

**FIGURE 1.** HPV transduces macaque epithelium and induces Abs to HPV. Cynomolgus macaques were vaccinated intravaginally with HPV PsVs expressing either RFP or SIV Gag. A, Schematic showing the design of the study. Eight cynomolgus macaques were vaccinated with HPV16 and HPV45, 30 d apart. Blood (black bars) and tissues (white diamonds) were sampled pre- and postvaccination. To obtain sufficient samples to analyze the female genital tract, animals were serially sacrificed at 37, 44, or 56 d postvaccination (stars). B, Forty-eight hours postvaccination, a camera fitted to an endoscope was used to visualize the cervix and vaginal tract. Successful HPV vaccination was confirmed by the detection of RFP in each animal. Shown is the level of in vivo fluorescence from HPV-RFP PsVs measured in a representative animal (original magnification ×10). C, Serum IgG to the HPV16 and -45 capsid L1 measured 14–56 d postvaccination. The vaginal epithelia were disrupted by four methods using N9. N9 was prepared as a 10% gel and delivered once (Gel 1X, white bars), twice, (Gel 2X, hatched bars), or twice in combination with abrasion using a cytobrush (Gel 2X+cytobrush, black bars). In addition, we used a 12.5% foam application of N9 also given twice (Foam, striped bars).
numbers of animals per group (n = 2) precluded statistical evaluation of the individual immune responses induced by these four immunization regimens; the data support the notion that two N9 gel applications may be superior to the foam in the ability to facilitate the induction of systemic serum IgG and T cell responses.

Cytokine production in the blood, lymph nodes, and female genital tract in cynomolgus macaques

To compare the frequency of SIV-specific cells at multiple sites, we serially sacrificed immunized macaques to obtain sufficient mononuclear cells from various tissues. Fig. 3 shows representative flow cytometric plots of the frequency of IL-2 produced in unstimulated and Gag-stimulated CD4+ T cells from the blood. SIVmac251-specific CD4+ T cell responses were detectable in the blood and the vagina as early as 14 d postvaccination (Fig. 3B, C, left panels). The responses in blood peaked 37 d postvaccination, whereas vaginal responses were highest 14 d postvaccination and boosted in some animals at day 44 (2 wk after HPV45 vaccination), but then waned over time. The magnitude of the CD4+ T cell response was lower in the obturator lymph nodes (Fig. 3D) and undetectable in the rectal mucosa (data not shown). A similar frequency and pattern were observed for SIVmac251 Gag-specific CD8+ T cell responses (Fig. 3B–D, right panels).

HPV PsVs recruit T cells to the vaginal mucosa and induce T cell responses

Next, we tested the immunogenicity of the HPV PsVs in Indian rhesus macaques; we chose to use a second model system to confirm and extend our results. The rhesus macaque is the most commonly used model for HIV vaccine development, and there are more reagents to phenotype the immune responses induced in these animals. Six MamuA01+ macaques were treated with Depo-Provera for 28 d and immunized sequentially with HPV16, -45, and -58 expressing the entire SIV gag and protease genes (Fig. 4A). Two additional animals (controls) were immunized with HPV16, -45, and -58 expressing the luciferase gene. We elected to disrupt the vaginal epithelium with either N9 delivered twice as a 10% gel (n = 4), because our data in cynomolgus macaques suggested a superior immunogenicity of this regimen, or with the 12.5% N9 foam (n = 4), because this preparation is widely available, easy to apply, and therefore a more practical approach. In this experiment, we used the Gag-pro SIVmac251/766 construct obtained from a virus variant cloned early in infection following mucosal exposure to SIVmac251. Expression of the uncleaved Gag precursor protein p55 and the cleaved p27 Gag protein from HPV16, HPV45, and HPV58 PsVs encoding SIVmac251/766 Gag-Pro was confirmed by Western blotting (Fig. 4B and data not shown for HPV58). Vaccination with HPV PsVs induced binding Abs to the HPV capsid in all macaques (data not shown). Because in mice, HPV intravaginal vaccination results in the recruitment of T cells to the site of vaccination (N. Cuburu and J.T. Schiller, unpublished observations), we assessed whether this is also the case in nonhuman primates. We obtained vaginal biopsies prior to vaccination and at 1 wk after HPV58 vaccination, but then waned over time. The magnitude of the CD4+ T cell response was lower in the obturator lymph nodes (Fig. 3D) and undetectable in the rectal mucosa (data not shown). A similar frequency and pattern were observed for SIVmac251 Gag-specific CD8+ T cell responses (Fig. 3B–D, right panels).
nation and was boosted by HPV58 at 131 d postvaccination (Fig. 4F). In contrast, SIV-specific IgG was not induced in foam-treated animals (Fig. 4G). In animals in which vaccination was facilitated by N9 gel, a modest increase was observed in IgA production after the third HPV vaccination at day 145, and SIV-specific IgG was also induced at this time point (Fig. 4F, 4G).

To characterize the quality and quantity of the cellular immune responses elicited by the HPV PsV-Gag-pro vaccines, we performed immunophenotyping and functional assays of SIV-specific T cells in the blood of the immunized rhesus macaques.

CD95+ (Ag-experienced) CD8+ T cells were gated and the frequency of the SIV-specific Gag CM9 tetramer-positive cells assessed. Detectable Gag-specific CM9 tetramer-positive cells were observed after the first HPV16 PsV vaccination; however, the frequency of tetramer-positive cells was only marginally increased following immunization with the HPV45 PsVs. A further boost with 2 × 10^{10} IU of HPV58 PsVs induced a marked in-

**FIGURE 3.** SIV Gag-specific cytokine production in systemic and mucosal tissues. A, Representative flow cytometric plots showing the frequency of IL-2 production in unstimulated or Gag-stimulated CD4+ T cells. B, PBMCs from HPV-SIV Gag-vaccinated macaques were stimulated with Gag peptides. The frequency of CD4+ T cells (left panel) and CD8+ T cells (right panel) producing IFN-γ and IL-2 (Cytokine+) is shown. Arrows above indicate the approximate time each HPV vaccine was administered. C, Mononuclear cells were isolated from the cervix and vaginal tract and stimulated with Gag peptides. However, in some animals, sufficient cervical mononuclear cells were not obtained to perform stimulations. The frequency of CD4+ T cells (left panel) and CD8+ T cells (right panel) producing cytokines is shown. D, At sacrifice, the genital draining obturator lymph nodes were collected from vaccinated macaques. Mononuclear cells were isolated and stimulated with SIV Gag peptides. The frequency of CD4+ T cells (left panel) or CD8+ T cells (right panel) producing cytokines is shown.
crease in tetramer-positive cells in animals that had been treated with either the N9 gel or the foam (Fig. 5A).

Ag-experienced T cells are characterized as central memory or effector memory cells, with central memory cells releasing IL-2 upon stimulation, whereas effector memory cells are more likely to produce markers of degranulation, such as CD107. Using these molecules, along with IFN-γ and TNF-α production, we characterized the cytokine profile of the SIV Gag-specific memory

FIGURE 4. HPV PsVs are immunogenic in rhesus macaques, recruit CD4+ and CD8+ T cells to the site of vaccination, and induce vaginal humoral responses. A, Schematic showing the vaccination and sampling schedule in rhesus macaques. Macaques were vaccinated on days 0, 30, and 121 with HPV16, -45, and -58, respectively. Blood (black squares) and tissues (white diamonds) were collected pre- and post-HPV vaccination. B, Western blot showing the expression of SIV Gag polyprotein p55 and processed p27 protein in 293T T cells transduced with HPV16 and HPV45 Gag-Pro constructs (lanes 2 and 3). Nontransduced 293T T lysates was used as a negative control (lane 1). C, Vaginal biopsies were obtained prior to and 1 wk post-HPV vaccination and paraffin-embedded. A representative example of immunohistochemical staining performed on embedded tissue, stained for CD4 (red), Ki67 (green), and nuclear material stained by DAPI (blue) prior to (left panel) and post-HPV vaccination (right panel). D, The absolute number of CD4+ and CD8+ T cells enumerated from vaginal biopsies pre- (white bars) and postvaccination (black bars). A repeated-measures ANOVA demonstrated that the difference is statistically significant (p = 0.0049 and p = 0.012). E, The absolute number of Ki67+CD4+ and Ki67+CD8+ cells in vaginal biopsies pre- and post-HPV vaccination. A repeated-measures ANOVA demonstrated that the difference is statistically significant (p = 0.040 and p = 0.034). F, SIV Gag-specific IgA as a function of total IgA in vaginal secretions. G, SIV Gag-specific IgG as a function of total IgG in vaginal secretions.
We found that the majority of memory circulating cells induced by this vaccine modality are monofunctional (i.e., secrete only one cytokine). Although limited cytokine production was observed after HPV16 PsV and -45 PsV vaccinations, HPV58 PsVs caused a robust increase in the frequency of SIVmac251-specific CD4+ cytokine-producing cells in both the gel- and foam-treated animals after background subtraction, and these CD4+ T cells produced either IL-2 or IFN-γ/TNF-α (Fig. 5B). Of interest, the phenotype of the cells differed between the groups; vaccination in the presence of the gel resulted in higher IL-2 production (Fig. 5B). Gag-specific CD8+ memory cells produced either CD107 or IFN-γ/TNF-α and negligible IL-2 (Fig. 5C). HPV58 PsV vaccination boosted CD8+ T cell cytokine production in the N9 foam-treated animals but not in the N9 gel-treated animals (Fig. 5C). The low frequency of cells obtained from vaginal biopsies in rhesus macaques precluded reliable longitudinal analysis of T cell phenotype and function at this site. Altogether, these data show that in rhesus macaques, this vaccine modality recruits and/or expands CD4+ and CD8+ T cells at the site of vaccination and induces effector memory CD8+ T cell responses and central and effector memory CD4+ immune responses in the blood.

HPV PsV vaccination does not exacerbate SIVmac251 replication but primes SIV-specific responses in the vaginal mucosa, lymph nodes, and the blood

The aim of mucosal HIV vaccines is to induce a population of virus-specific resident memory B and T cells at the portal of entry that are able to prevent the systemic spreading of the virus. However, a possible caveat in the case of HIV infection is that the recruitment and activation of CD4+ T cells may result in exacerbation of virus replication. To investigate the kinetics of the expansion of SIV-specific responses and a possible exacerbation of infection, the six vaccinated and two control rhesus macaques were exposed to a high dose (10^5 TCID50) of SIVmac251 by the vaginal route at 6 wk after the final HPV58 PsV vaccination and euthanized at 1 or 2 wk postinfection as indicated in Fig. 6A. We quantified plasma viral RNA and viral DNA levels in vaginal tissues collected at euthanasia. We did not observe differences in the levels of viral RNA in plasma 1 or 2 wk postinfection (Fig. 6B). Similarly, we observed no difference in the levels of SIV DNA in the vaginal tissues of vaccinated or unvaccinated animals collected 2 wk postinfection (Fig. 6C). Statistical analysis that included two additional naive controls challenged intravaginally with the same stock of SIVmac251 at the same dose, but at a dif-
ferent time, supported a lack of a significant difference in virus levels in plasma between vaccinated and unvaccinated animals. We next investigated the virus-specific immune responses in multiple tissues at 1 or 2 wk post-SIVmac251 infection. We measured SIV Gag CM9 tetramer CD8+ T cell responses in the blood, genital draining obturator lymph node, cervix, vagina, and rectum 2 wk postchallenge. Both intraepithelial and lamina propria CD8+ T cells were obtained from the female genital tract. The bottom panel shows the average frequency of SIV-specific Gag CM9 CD8+ T cells from vaccinated animals. E, Average IFN-γ-producing spot-forming mononuclear cells from axillary, obturator lymph nodes, and blood after stimulation with overlapping peptides spanning SIV Gag (left panel) or SIV Env (right panel) from vaccinated and unvaccinated controls, 2 wk postchallenge. A repeated-measures ANOVA demonstrated that the difference in IFN-γ production from the obturator lymph node between vaccinated and nonvaccinated animals is statistically significant (p = 0.034).
vaccinated macaques, we performed an IFN-γ ELISPOT in blood, obturator, and axillary lymph nodes using both the Gag peptides (included in the vaccine) and Env peptides (not included in the vaccine). We observed significantly higher Gag-specific IFN-γ responses in vaccinated animals when compared with unvaccinated controls in the genital draining obturator lymph node (p = 0.034; Fig. 6E, left panel). As expected, no Env responses were observed in all tissues tested (Fig. 6E, right panel). Furthermore, similar to what was observed by Gag CM9 tetramer staining, the blood and obturator lymph node had a greater magnitude of response compared with a distal site (i.e., the axillary node).

**Discussion**

Most HIV infections worldwide occur by mucosal routes, and recent studies demonstrate that mucosal exposure of humans to HIV, and of macaques to SIV, results in the transmission of a single or few viral variants (27–29). The founder viruses cross the mucosal epithelium, replicate in the lamina propria, and seed distal sites, producing a chronic viral infection. Mucosal immune responses may be needed to curb the initial local expansion of the founder virus and prevent systemic viral spread. This hypothesis would require a vaccine to induce durable protective memory immune responses at the relevant mucosal site. We tested the immunogenicity of a novel mucosal vaccine, HPV PsV, as this vaccine modality targets the cervicovaginal epithelium (14, 15). We demonstrated that HPV PsVs, used as vehicles for the delivery of SIV DNA, induced SIVmac251 specific cellular and humoral immune responses at the genital tract, in the genital draining lymph nodes, and in blood. The immunogenicity of HPV PsVs may be related to its ability to activate and mature dendritic cells, which, in turn, facilitates the induction of Th1 responses (17). In murine models, gene expression from HPV PsVs is observed in APCs in the lamina propria of the intestines after oral exposure to HPV PsVs (30). The adjutant effect of HPV PsVs has been demonstrated in studies where stronger T cell responses and Abs were elicited using PsVs as opposed to naked DNA in the respiratory, gastrointestinal, and female genital tract (13, 30, 31). Furthermore, intravaginal vaccination in mice (N. Caburu and J.T. Schiller, unpublished observations) and in macaques, as reported in this study, results in the recruitment of both CD4+ and CD8+ T cells to the site of vaccination, increasing the absolute number of T cells in the cervicovaginal mucosa. The HPV Gag PsV-induced mucosal immune response is likely underestimated, as vaccination may induce focal responses that may be missed by studying pinch biopsies and tissue sections. In addition, SIV-specific responses are determined as a percentage of isolated T cells does not account for the recruitment or increase in absolute number of cells or the distribution of vaccine-induced cells within the female genital tract. HIV/SIV-specific Abs in the female genital tract may represent a first line of defense against infection. In humans, IgG has been shown to be the dominant Ig isotype in the vaginal tract and is transported across epithelial cells via the neonatal FcR (32). In addition, HIV-specific IgG is predominant in vaginal secretions from infected women (33, 34). In our studies, we measured Gag-specific IgA and IgG in rhesus macaques post-HPV PsV-SIV vaccination and detected both subtypes in the vaginal tract. Surprisingly, we observed increased IgA as compared with IgG levels in vaginal secretions. HPV PsVs are potent inducers of IgA; however, further investigation is needed to determine if the predominance of Gag-specific IgA in our studies is related to the vector, route of administration, or hormonal environment.

We challenged immunized macaques with a high dose of SIVmac251, which precludes the assessment of relative vaccine efficacy, because our goal was to test the expansion of HPV-Gag-pro PsV-induced responses in tissues after vaginal exposure to SIV. Importantly, we have not observed exacerbation of SIV replication despite the recruitment or expansion of a significant number of activated CD4+ T cells in the female genital tract. Whether the extent and rate of expansion of SIV-specific immune responses will be sufficient to prevent SIV replication and spread to distal sites, or will favor virus immune escape, needs to be tested using repeated low doses of SIVmac251, whereby few virus variants are transmitted similar to heterosexual HIV transmission in women. Furthermore, the inflammatory profile, length of the eclipse phase (35), and initial innate and adaptive responses may vary in low-dose versus high-dose models. Indeed, we have observed diverse outcomes in macaques given the same vaccine regimen when half of the animals were challenged with a single high dose and the other half with lower repeated doses of SIVmac251 (M. Vaccari and G. Franchini, unpublished observations).

Although HPV PsV targeting of the vaginal mucosa makes this approach attractive, the intravaginal delivery of HPV PsVs and hormonal treatment is cumbersome; in addition, effective vaccination may require disruption of the epithelium with agents like N9 that can exacerbate HIV infection (36). Thus, this vaccination regimen, in its current form, may be somewhat impractical for clinical studies. However, vaccine administration in the early follicular phase of the cycle may eliminate the need for hormonal treatment. In addition, the N9 foam is easy to use, inexpensive, readily available, and could be self-administered prior to a gynecological visit. Thus, a clinical trial with HPV-PsV is feasible. Furthermore, adolescent girls have extensive cervical ectopy, and the exposed simple columnar epithelium may be susceptible to HPV PsV infection under less stringent conditions, eliminating the need for epithelia disruption. In addition, alternate mucosal routes or other alterations in the mode of delivery of HPV PsVs could be explored.

In this study, we have used HPV16, -45, and -58 PsVs; some of these serotypes, or closely related ones, are in the HPV vaccine. Thus, neutralizing Abs to these serotypes would preclude their efficacy in HPV VLP-vaccinated women. However, because HPV PsV-mediated genital transudation is not species restricted, animal papillomavirus serotypes, which are not cross-neutralized by VLPs in current HPV vaccines, could be selected for the development of a human HIV vaccine.

To date, there have been three large-phase II/III HIV clinical vaccine trials, and all have used systemically administered vaccine candidates (4–6). Among those, the recombinant canarypox ALVAC-HIV vaccine, administered together with HIV envelope proteins in the Thai RV144 trial, afforded 31% protection against mucosal heterosexual HIV infection (5). Similarly, nonhuman primate vaccine studies using ALVAC-SIV and SIVgp120 protein have protected some vaccinees from repeated low-dose SIVmac251 challenges (23) (P. Pegu and G. Franchini, unpublished observations). These vaccine regimens induce limited T cell responses and high levels of nonneutralizing Abs; however, the correlates of protection have not been defined, allowing only limited speculation regarding vaccine-elicted immune responses that may protect from HIV infection. An effective HIV vaccine may need to elicit mucosal high-titer Abs and high-frequency T cell responses, as opposed to only one of the aforementioned responses. In this study, HPV PsVs elicited both CD4+ and CD8+ T cell responses and low-level Gag Ab responses. Importantly, immune responses were induced locally at the mucosal site at risk for lentivirus transmission. Adding SIV Env Ags to induce Abs against the surface of the virus, as well as other accessory/regulatory genes.
will be a crucial next step in the evaluation of this vaccine concept trial.


6. Schiller, J. T., P. M. Day, and R. C. K. 2010. A limited number of simian immunodeficiency virus (SIV) env variants are transmitted to rhesus macaques vaginally inoculated with papillomavirus pseudoviruses encoding gag.


8. Schiller, J. T., P. M. Day, and R. C. K. 2010. A limited number of simian immunodeficiency virus (SIV) env variants are transmitted to rhesus macaques vaginally inoculated with papillomavirus pseudoviruses encoding gag.


10. Schiller, J. T., P. M. Day, and R. C. K. 2010. A limited number of simian immunodeficiency virus (SIV) env variants are transmitted to rhesus macaques vaginally inoculated with papillomavirus pseudoviruses encoding gag.