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Antibody to the gp120 V1/V2 Loops and CD4+ and CD8+ T Cell Responses in Protection from SIVmac251 Vaginal Acquisition and Persistent Viremia

Shari N. Gordon,* Melvin N. Doster,* Rhonda C. Kines,† Brandon F. Keele,‡ Egidio Brocca-Cofano,§ Yongjun Guan,¶ Poonam Pegu,* Namal P. M. Liyanage,* Monica Vaccari,* Nicolas Cuburu,† Christopher B. Buck,† Guido Ferrari,‖ David Montefiori,¶ Michael Piatak, Jr.,‡ Jeffrey D. Lifson,‡ Anastasia M. Xenophontos,* David Venzon,† Marjorie Robert-Guroff,§ Barney S. Graham,** Douglas R. Lowy,† John T. Schiller,‡ and Genoveffa Franchini*

The human papillomavirus pseudovirions (HPV-PsVs) approach is an effective gene-delivery system that can prime or boost an immune response in the vaginal tract of nonhuman primates and mice. Intravaginal vaccination with HPV-PsVs expressing SIV genes, combined with an i.m. gp120 protein injection, induced humoral and cellular SIV-specific responses in macaques. Priming systemic immune responses with i.m. immunization with ALVAC-SIV vaccines, followed by intravaginal HPV-PsV–SIV/gp120 boosting, expanded and/or recruited T cells in the female genital tract. Using a stringent repeated low-dose intravaginal challenge with the highly pathogenic SIVmac251, we show that although these regimens did not demonstrate significant protection from virus acquisition, they provided control of viremia in a number of animals. High-avidity Ab responses to the envelope gp120 V1/V2 region correlated with delayed SIVmac251 acquisition, whereas virus levels in mucosal tissues were inversely correlated with antienvelope CD4+ T cell responses. CD8+ T cell depletion in animals with controlled viremia caused an increase in tissue virus load in some animals, suggesting a role for CD8+ T cells in virus control. This study highlights the importance of CD8+ cells and antienvelope CD4+ T cells in curtailing virus replication and antienvelope V1/V2 Abs in preventing SIVmac251 acquisition. The Journal of Immunology, 2014, 193: 000–000.

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The sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers KF646830–KF647217.

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; HPV, human papillomavirus; MPL, monophosphoryl lipid A; N9, nonoxynol 9; PsV, pseudovirion.
Repeated low-dose mucosal challenges with simian HIV or SIV viruses in macaques are reasonable models of HIV sexual transmission (11). The SIVmac251 challenge used in this study is a pathogenic CCR5 user that is resistant to neutralization, similar to most HIV primary isolates. To date, HIV vaccine candidates tested in this macaque model, using mucosal repeated low doses of SIV, have recapitulated the results of HIV clinical trials in humans (12–14).

Preventing HIV transmission remains the primary goal of HIV vaccines; however, once infection has occurred, the reduction of chronic-phase viremia and the slowing or halting of disease progression are also important objectives. Increasing evidence suggests that although a vaccine-induced humoral response is important for protection from virus acquisition (15–17), CD8+ T-cell responses contribute to virus control after lentiviral transmission (16, 18–20).

In the RV144 Thai trial, the ALVAC-HIV/gp120 regimen induced negligible CD8+ T-cell responses, and vaccinees that became infected had virus levels and CD4+ T-cell counts similar to those of the placebo group, requiring the initiation of antiretroviral therapy (21). Multiple lines of evidence implicate CD8+ T cells in the control of HIV/SIV replication; for example, CD8+ T-cell depletion of macaques during SIV infection causes a rapid increase in viral burden (22, 23). In addition, during primary HIV infection, the postpeak decline in viremia is temporally associated with the induction of CD8+ T-cell responses (24, 25). The immunologic pressure imposed by CD8+ T cells on HIV is evidenced by the emergence of MHC I–restricted escape mutations (26–28). Intriguingly, recent studies have demonstrated potent control of SIV infection by broadly distributed T-cell responses, induced by rhesus CMV vaccine vectors that generate unusual MHC class II–restricted CD8+ T cells targeting promiscuous SIV epitopes (29, 30).

Our goal was to develop a novel vaccine regimen that induces mucosal CD8+ T cells together with binding functional Abs and to ask whether this vaccine regimen alone could protect, or whether prior priming with systemic immunization could further improve protection. Human papillomaviruses (HPVs) are small non-enveloped DNA viruses that naturally infect epithelial cells within the genital tract; we used HPV-based vectors as a delivery system to specifically target Ag expression to the vaginal epithelium. We created HPV-pseudovirions (PsVs) that express SIV genes and delivered them to basal epithelial cells in the female genital tract. Infection in the vaginal tract is facilitated by macrotransduction that allows access to the basal epithelial layers (31). HPV-PsV–mediated gene expression in the female genital tract has been shown to be transient, lasting for ~5 d, during which priming of T cells in the genital draining lymph nodes and Ag recall in the genital mucosa have been reported in murine models (32, 33). Initiation of an adaptive response is likely enhanced by the adjuvant-like potential of the HPV capsid, with its ordered protein arrangement (34). HPV-like particles have been shown to induce maturation of dendritic cells, resulting in the production of IL-6, IL-12, and TNF-α, and may be recognized by TLRs on mucosal cells engaging pathogen–associated molecular patterns on the HPV capsid (33, 35, 36). In previous studies, we demonstrated the feasibility of this vaccine approach using model Ags and the ability of HPV-PsVs to express foreign genes in the vaginal tract and induce HPV capsid–specific Abs in serum to each HPV serotype (37).

In this study, we evaluated whether the local mucosal immune responses, induced by HPV-PsV vaccines paired with a gp120 protein boost, might prevent SIVmac251 intravaginal transmission. In addition, because ALVAC/gp120 regimens demonstrate limited but significant protection from infection in humans as well as in nonhuman primates (8, 13, 20, 38, 39), we examined whether an ALVAC-SIV systemic prime, paired with an HPV-PsV-SIV/gp120 boost, by inducing also higher systemic response, could increase vaccine efficacy.

Materials and Methods

Animals, immunization, and SIV challenge

This study used 36 female rhesus macaques of Indian origin, aged 3.5–7 y. All animals were housed and cared for under the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care, and the study was conducted with the approval of the Institutional Animal Care and Use Committee at Advanced Biosciences Laboratories in Rockville, MD. The animals were divided into MD, aged 3.5–7 y. All animals were housed and cared for under the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care, and the study was conducted with the approval of the Institutional Animal Care and Use Committee at Advanced Biosciences Laboratories in Rockville, MD. The animals were divided into 3 groups of 12 animals, each based on their MHC alleles. In one group, the 12 animals were vaccinated with 10^9 PFU of ALVAC-SIV encoding SIVmac251 gag, pol, and env (gp160) by i.m. injection in the thigh at weeks 0 and 4. The ALVAC-SIV vector was made as previously described (40). The 12 ALVAC-SIV–vaccinated animals and 12 additional macaques were vaccinated intravaginally with HPV-PsVs expressing SIVmac251 genes (HPV-PsV-SIV) at weeks 10, 14, and 24. HPV-PsVs were produced in 293T cells, and the adjuvants alum and pertussis toxin were cotransfected into 293T cells. The resulting PsVs were purified, propagated, and titrated. At 28 wk after each HPV-PsV-SIV vaccination, macaques were given 30 mg/kg Depo-Provera i.m. to thin the vaginal epithelium, and 1 wk prior to vaccination, macaques were treated with antibiotics to prevent vaginosis. At 6 and 24 h before vaccination, a vaginal application of nonoxynol 9 (N9), a nonionic detergent, was administered as a 10% gel mixed with 4% carbamoyl cellulse (Sigma-Aldrich, St. Louis, MO). N9 induces microtrauma in the epithelia, which facilitates HPV-PsV vaccination. At 6 h after the last N9 treatment, a standard 500-μl inoculum, consisting of 10^5 IU (infectious units) of HPV-PsVs and carbamoyl cellulose, was instilled into the vaginal vault, using a positive displacement pipette. In addition, all 24 vaccinated macaques were given 2 i.m. injections with 200 μg gp120 protein, as done previously (39). The protein was mixed with the adjuvants alum and monophosphoryl lipid A (MPL) and administered in the thigh muscle at weeks 10 and 24. A total of 12 macaques were used as controls. Control animals were given the ALVAC vector that did not express SIV genes, HPV-PsVs that expressed luciferase, and the adjuvants alum and MPL at similar doses and times as the vaccinated animals.

At week 28, 4 wk after the last HPV-PsV vaccination, all 36 rhesus macaques were challenged intravaginally with 250 tissue culture–infective doses, 50%, of SIVmac251. The virus was kindly provided by Nancy Miller in the Division of AIDS, National Institutes of Health. Blood was collected, and SIV RNA was quantified in plasma 7 d after challenge; animals with virus loads <50 copies per milliliter were challenged. Animals with two successive viral determinations >10^4 were considered persistently SIV infected; repeated SIV challenges were stopped, and virus loads were monitored weekly in the acute phase and monthly in the chronic phase. Animals with virus loads between 50 and 10^4 copies were restated at day 10. If the virus load increased at day 10 to >10^4, the animal was considered persistently SIV infected; the challenge phase was stopped, and virus load in plasma was monitored thereafter. If, however, the virus load at day 10 was <50 copies per milliliter, the animal was considered transiently infected, and repeated low-dose challenges were resumed. A maximum of nine repeated low doses of SIVmac251 were administered at 10-d intervals.

Mucosal Abs

Vaginal secretions were collected using absorbent cotton sponges. To elute secretions, the sponges were incubated for 10 min in elution buffer, on ice; transferred into a Salivette column (Sarstedt); and centrifuged at 3000 rpm for 30 min at 4°C. For SIV-specific IgA and IgG, serially diluted 1:2. Inoculum, consisting of 10^6 IU (infectious units) of HPV-PsVs and carbamoyl cellulose, was instilled into the vaginal vault, using a positive displacement pipette. In addition, all 24 vaccinated macaques were given 2 i.m. injections with 200 μg gp120 protein, as done previously (39). The protein was mixed with the adjuvants alum and monophosphoryl lipid A (MPL) and administered in the thigh muscle at weeks 10 and 24. A total of 12 macaques were used as controls. Control animals were given the ALVAC vector that did not express SIV genes, HPV-PsVs that expressed luciferase, and the adjuvants alum and MPL at similar doses and times as the vaccinated animals.
Statistical analysis

Comparisons between groups were performed using the Mann–Whitney–Wilcoxon test for continuous factors, and paired comparisons between two times were assessed by the Wilcoxon signed rank test. Correlations were performed using the Spearman rank correlation method. The difference between groups in the binding of each of the overlapping gp120 peptides was tested using the exact Mann–Whitney–Wilcoxon test, and the p values were corrected for multiple comparisons by the Hochberg method. Graphical analysis was performed using GraphPad Prism, and error bars on graphs represent the SEMs.

IFN-γ ELISPOT

SIV-specific T cells were assessed using an IFN-γ ELISPOT kit from Mabtech, as previously described (39). Cryopreserved PBMCs were thawed, resuspended in PBS, and stained with either SIVmac251, Gag or gp120 (Env) overlapping 15-mer peptides, Con A, or were left unstimulated. PBMCs and stimulants were added to IFN-γ–coated plates and incubated for 24 h. The plates were developed, and the frequency of IFN-γ–positive spot-forming cells per 10^6 PBMCs was determined after background subtraction.

Pentamer staining and intracellular cytokine assays

Ten-color flow cytometric analysis was performed on mononuclear cells from blood and from mucosal biopsy specimens. Pinch biopsy specimens obtained from the cervix, vaginal tract, or rectum were washed and incubated for 1 h with collagenase D at a concentration of 2 mg/ml in Iscove’s medium with antibiotics and amphotericin. Following incubation, the remaining tissue was mechanically disrupted to obtain a mononuclear cell suspension. Filtered single-cell suspensions of mononuclear cells were used in an intracellular cytokine assay performed as previously described (37). Cells were stimulated with either Env peptides at a concentration of 2 μg/ml or with PMA and ionomycin, or were left unstimulated in the presence of Golgi transport inhibitors, CD107a, clone H4A3; anti-CD28-EC, clone CD28.2 (eBioscience); and CD49D, clone 9F10 (BD Biosciences) for 6 h. Cells were then surface stained with CD3 (clone SP34-2), CD4 (clone L200), CD8 (clone RPA-T8), CD95 (clone DX2), and the LIVE/DEAD yellow fixable amine dye (Invitrogen). Stained, permeabilized, and pseudopatinized samples were acquired on an LSR II flow cytometer, and the frequency of CD3+, CD4+, CD8+, and CD95+ T cells and the proportion of mono- and polyclonal (simultaneous production of multiple cytokines) responses were determined. For Gag CM9 pentamer detection (obtained from Proliumne), cells were stained for 15 min with the Gag CM9 PE pentamer, washed, and then stained with the amine dye and CD3, CD4, CD8, CD28, and CD95, using the same clones as above. Samples were washed, permeabilized with Cytofix/Cytoperm, and stained intracellularly with K67 (clone B56, BD Biosciences). All cells were fixed with 1% paraformaldehyde and acquired on an LSR II (BD Biosciences). Data analysis was performed with FlowJo (TreeStar) and with SPICE (National Institute of Allergy and Infectious Diseases) (42).

CFSE proliferation assay

The lymphoproliferation assay was performed as previously described (39). Cells were briefly incubated with 5 μM CFSE (Invitrogen), washed, enumerated, and stimulated with 5 μg/ml SIV Env or Con A, or were left unstimulated for 5 d. Cells were then harvested and stained with CD3, CD4, CD8, CD28, and CD95, and the amine dye, as described above. Samples were fixed, and a LSRII flow cytometer, and the frequency of CD3+, CD4+, CD8+, or CD3+CD8+CD95+ T cells with diminished expression of CD95 were enumerated, and stimulated with 5 μg/ml SIV Env or Con A, or were left unstimulated in the presence of Golgi transport inhibitors, CD107a, clone H4A3; anti-CD28-EC, clone CD28.2 (eBioscience); and CD49D, clone 9F10 (BD Biosciences) for 6 h. Cells were then surface stained with CD3 (clone SP34-2), CD4 (clone L200), CD8 (clone RPA-T8), CD95 (clone DX2), and the LIVE/DEAD yellow fixable amine dye (Invitrogen). Stained, permeabilized, and pseudopatinized samples were acquired on an LSR II flow cytometer, and the frequency of CD3+, CD4+, CD8+, and CD95+ T cells and the proportion of mono- and polyclonal (simultaneous production of multiple cytokines) responses were determined. For Gag CM9 pentamer detection (obtained from Proliumne), cells were stained for 15 min with the Gag CM9 PE pentamer, washed, and then stained with the amine dye and CD3, CD4, CD8, CD28, and CD95, using the same clones as above. Samples were washed, permeabilized with Cytofix/Cytoperm, and stained intracellularly with K67 (clone B56, BD Biosciences). All cells were fixed with 1% paraformaldehyde and acquired on an LSR II (BD Biosciences). Data analysis was performed with FlowJo (TreeStar) and with SPICE (National Institute of Allergy and Infectious Diseases) (42).

Binding Abs and pepscans

An ELISA was used to detect SIVmac251-gp120 binding Abs in blood, as previously described (40), and to detect binding to overlapping peptides spanning gp120. A serial dilution of plasma was added to microtiter plates coated with native purified gp120 Env protein of SIVmac251 or individual peptides, and the Ab titer determined. The absorbance at OD 450 nm was reported for peptide mapping. For binding Abs to gp120, the endpoint titers were defined as 2× the OD 450 of the negative control serum.

B cell ELISPOT

SIV Env-specific or total IgG or IgA Ab-secreting cells were analyzed by a B cell ELISPOT, as described previously (39). Briefly, MultiScreen 96-well plates (Millipore MAIPS4510) were incubated with 70% ethanol, rinsed, and coated with SIVmac251 gp120 protein or goat anti-mouse IgG or IgA (KPL). Coated plates were incubated at 4°C overnight, washed, and blocked for 2 h at 37°C. PBMCs were stimulated with CpG (ODN-2006; Operon), CD40L, and IL-21 (PeproTech) for 3 d at 37°C in 24-well plates. Stimulated PBMCs were next harvested and washed, and 3×10^6 cells were plated and incubated overnight at 37°C. Plates were then washed and incubated with biotinylated goat anti-mouse IgG or IgA (Rockland), and HRP–biotin D conjugate (Vector Laboratories) was added. After several washes, plates were developed using 3-amin-9-ethyl-carbazole (Sigma-Aldrich). Spot quantification was performed with an ELISPOT reader.

ADCC

ADCC activity mediated by Abs in plasma samples was detected by the GranzoLux (GTL) procedure, as previously described (20, 43). Briefly, CEM.NKCCR5 target T cells were coated with recombinant SIVmac251 gp120 and labeled with a fluorescent target-cell marker and a viability marker. Labeled target cells were washed and plated. Cryopreserved human PBMCs from an HIV-seronegative donor served as effectors and were added to the assay wells at an E:T ratio of 30:1. Fluorogenic granyme B substrate (OncoImmulin) was added to each well. After incubation, serially diluted plasma samples were added to the assay wells. The plates were incubated for 15 min at room temperature, centrifuged, and incubated for 1 h at 37°C. The plates were then washed, cells were resuspended in PBS, and 2×10^5 events representing viable target T cells were acquired for each well, using an LSR II flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo 8.8.4 software (TreeStar). The final results are expressed as ADCC titer and maximum granyme B activity.

Neutralization assays

Neutralization was measured as a reduction in luciferase reporter gene expression after a single round of infection in TZM-bl cells, as described previously (20, 44). TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. Virus was incubated with serial 3-fold dilutions of samples in duplicate. Freshly trypsinized cells were added to each well. One set of control wells received cells and virus (virus control), and another set received cells only (background control). After a 48-h incubation, cells were transfected to 96-well black solid plates (Costar) for measurements of lucimencence. Neutralization titers are the dilution at which relative luminescence units were reduced by 50% compared with that in virus control wells after subtraction of background relative luminescence units. Assay stocks of molecularly cloned Env-pseudotyped viruses, SIVmac251, 14, SIVmac251, 30, were prepared by transfection in 293T cells and titrated in TZM-bl cells. The SIVmac251 challenge stock was obtained from Nancy Miller in the Division of AIDS, National Institutes of Health, expanded on rhesus PBMCs, titered, and used.

Ab Avidity

Three recombinant SIV envelope proteins—full-length gp120, gp120 deleted of the V1V2 region, and the V1V2 mini protein—were made from Env-cultured SIVmac251. The V1V2 mini protein was fused to the C-terminal tag of HIV-1 gp120. These proteins were used as an Ag for the capture ELISA to detect SIV Abs against conformational epitopes, as previously described (39). Parallel ELISAs were used to determine Ab avidity. Heat-inactivated plasma samples were serially diluted and applied to a 96-well plate capturing SIVmac251 gp120 proteins in parallel duplicates. After 1 h of incubation, the plate was washed, and half the samples were treated with TBS, whereas the paired samples were treated with 1.5 M sodium thiocyanate (Sigma-Aldrich) for 10 min at room temperature. The plate was washed, and a goat anti-mouse IgG detecting Ab (Fitzgerald) was used. The avidity index (%) was calculated by taking the ratio of the sodium thiocyanate–treated plasma dilution, giving an OD of 0.5 to the TBS-treated plasma dilution giving an OD of 0.5, and multiplying by 100. Plasma of uninfected normal macaques served as negative controls. A high-avidity monkey mAb of 3.11H was included on every plate as the standard.

Viral load and transmitted founder variants

Plasma SIV RNA was quantified by nucleic acid sequence–based amplification, as previously described (45, 46). SIV DNA was quantified in mucosal tissues 3 wk after SIV infection by a real-time quantitative PCR assay with sensitivity ≤10 copies per 10^7 cells, as previously described (47). Briefly, genomic DNA was extracted from nasal washes, treated with the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer’s protocol, except the DNA elution step. The quantity and quality of the DNA were assessed by OD 260 measurements using an ND-1000
CD8\(^*\) cell depletion

CD8\(^*\) lymphocyte depletion was performed in 11 macaques. Animals were infused with the αCD8-depleting rhesus recombinant Ab M-T807R1, ob-
Vaccination induced mainly monofunctional responses in the blood and vaginal tract. (A) Cytokine production following Env peptide stimulation of mononuclear cells from vaginal biopsies obtained at week 17. Shown is the sum of IFN-γ, TNF-α, IL-2, and CD107 production after background subtraction in CD95+CD4+ and CD95+CD8+ T cells. (B) The functional capacity of the SIV-specific response is represented by the pie charts; they show the proportion of cells that responded to stimulation by producing either IFN-γ, TNF-α, IL-2, or CD107, or a combination thereof. The fraction of cells that responded to stimulation by producing one cytokine is shown in gray; two cytokines, black; three cytokines, green; or four cytokines, orange. CD4 responses are in the top pie panel and CD8 responses are in the lower pie panel (C) Total Env-specific cytokine production in blood after the last vaccination, week 26, in CD95+CD4+ and CD95+CD8+ T cells. Shown is the sum of IFN-γ, TNF-α, IL-2, and CD107 production. (D) Pies show the fraction of cells that responded to stimulation by producing one cytokine (gray), two cytokines (black), three cytokines (green), or four cytokines (orange). CD4 responses are in the top pie panel and CD8 responses are in the lower pie panel.
In the ALVAC/HPV group, Gag-specific CD8+ T cells were not detected in the rectum of vaccinated animals at week 17 (data not shown). The increased T cell response, observed in the vaginal tract of the ALVAC/HPV group, and the absence of Gag-specific T cells in the rectum of ALVAC-SIV–primed animals suggest that systemic priming followed by intravaginal boosting recruits and/or expands cell-mediated responses in the female genital tract.

Pinch biopsies of cervicovaginal tissues yield a limited number of mononuclear cells. Mononuclear cells isolated from the Mamu-A*01–positive animals were used to measure the frequency of GagCM9+ CD8+ T cells, whereas cells from the remaining 27 Mamu-A*01–negative animals were used to measure functional mucosal responses to envelope peptides at week 17 (Fig. 2A). Intracellular cytokine staining for IFN-γ, TNF-α, IL-2, as well as the expression of CD107, was determined following 6-h stimulation with overlapping Env peptides. Vaccination induced mainly monofunctional CD4+ and CD8+ T cell responses that secreted IFN-γ, TNF-α, or CD107 (Fig. 2B). The frequency of Env-specific T cells was similar in the two vaccination regimens. At 2 wk before the first SIV challenge (week 26), a similar analysis of cytokine profile was performed in the blood of all vaccinated animals (Fig. 2C). ALVAC/HPV–vaccinated animals had a greater frequency of blood CD4+ T cell responses compared with the HPV group. Similar to the vaginal tract, primarily monofunctional memory responses were induced in blood (Fig. 2D); however, TNF-α was the dominating cytokine response in the ALVAC/HPV group, whereas either TNF-α, IFN-γ, or IL2 was produced in the HPV group.

**Systemic and mucosal gp120-specific Abs induced by vaccination**

ALVAC-SIV priming induced gp120-specific IgG in the blood, but by the end of the vaccination regimen, both groups had similar levels of high-titer binding Abs (Fig. 3A). To assess Abs in mucosal secretions, we collected vaginal swabs after the last vaccination. Equivalent levels of gp120-specific IgG were found in the ALVAC/HPV and HPV groups presented as titer per microgram of total IgG to normalize for the levels of total IgG isolated from each animal (Fig. 3B). Before normalization by total IgG, we directly compared the gp120-specific titers in each animal’s blood and vaginal mucosa and observed that, on average, Env-specific IgG was approximately one log lower in the vaginal mucosa than in blood. Low levels of gp120-specific IgA were detected in the vaginal secretions of both vaccinated groups (Fig. 3B). A similar frequency of Env-specific memory B cells was measured in both groups 1 wk prior to SIV challenge (Fig. 3C). Although both vaccine regimens induced measurable IgG+ gp120-specific B cells, no IgA+ gp120-specific B cells were detected in blood (data not shown).

The functional capacity of Abs induced by the two vaccine regimens was determined in the blood, owing to the limited quantity of protein extracted from vaginal swabs. The two vaccine regimens induced serum Abs that mediated similar levels of ADCC, measured as % maximum granzyme activity and ADCC titer (Fig. 3D). In contrast, the ALVAC/HPV group had significantly greater neutralization titers for the tier-1–like SIVmac251.6 virus compared with the HPV group \( p = 0.0023 \) (Fig. 3E). Neither vaccine regimen induced Abs that neutralized the tier-2–like SIVmac251.30 isolate (data not shown) or the SIVmac251 challenge stock (Fig. 3E).

Abs to the V1/V2 loop of gp120 were found to be a correlate of reduced HIV risk in the RV144 Thai trial, using ALVAC-HIV and gp120 immunogens (9). In another study involving ALVAC-SIV/gp120 vaccination, we found that animals that resisted SIVmac251 infection had high-avidity Abs directed to the V1/V2 region (39).

**FIGURE 3.** Vaccine-induced binding Abs and their functional capacity. (A) gp120–specific IgG titer in blood during the vaccination phase, with the ALVAC/HPV group represented as circles and with a large dashed line; the HPV group, squares with a small dashed line; and controls, triangles with a solid line. Vertical lines indicate the time when a vaccine was given: ALVAC, weeks 0 and 4; HPV-PvVs, weeks 6, 10, and 24; and gp120/adjuvant, weeks 10 and 24. (B) Gp120 binding Ab titers in mucosal secretions per microgram of total IgG/IgA measured after the last vaccination, week 25. IgG is shown on the left and IgA is on the right. The ALVAC/HPV group is represented by white bars and the HPV group by hatched bars. (C) Percent gp120–specific IgG memory B cells measured by B cell ELISPOT in PBMCs at week 27. (D) ADCC in the blood shown as % granzyme B activity (left) or ADCC titer (right) measured at week 26. Circles represent animals in the ALVAC/HPV group; squares, the HPV group; and triangles, the control group. (E) Neutralization of an easy-to-neutralize tier-1–like virus SIVmac251.6 (left) and the SIVmac251 challenge stock (right) measured at week 26 after the last vaccination. A significantly higher level of neutralization was observed in the ALVAC/HPV group, indicated by the asterisk, using the Mann–Whitney–Wilcoxon test, with \( p = 0.0023 \).
We therefore measured vaccine-induced Ab binding to overlapping linear peptides that spanned gp120, including the V1/V2 region, and Ab avidity. Both regimens had an overall similar recognition of overlapping peptides spanning the constant and variable regions of gp120 (Fig. 4A). We compared the average binding of each peptide in the two vaccination regimens, using the Z statistic of the Mann–Whitney–Wilcoxon test (Fig. 4B). After correction for multiple comparisons by the Hochberg method, two peptides, 24 and 29, in the V2 loops had significantly greater Ab recognition in the HPV group (Fig. 4B). The ALVAC/HPV group showed increased binding to peptides 16 and 17 within the C1/V1 region and to peptide 40 in the C2 regions of gp120, but the difference was not statistically significant. The avidity of Abs to the whole gp120 protein of SIVmac239 was evaluated after sodium thiocyanate treatment. On average, Abs from both vaccine regimens had a similar avidity index (Fig. 4C).

To determine the contribution of the V1/V2 region of gp120, the avidity index of vaccine-induced Abs was assessed using a gp120 protein in which the V1/V2 loop was deleted (D

\text{V1/V2}) and a conformational protein containing the entire V1/V2 stem loop of SIVmac239, linked to a tag from the C-terminal of HIV gp120. A significant reduction in the avidity index was observed when the V1/V2 region of gp120 was deleted ($p < 0.0001$) (Fig. 4C). The average avidity to the entire gp120 was 20.1, whereas the $\Delta$V1/V2 avidity index was 6.4. Furthermore, when the avidity index of Abs to the V1/V2 mini protein was assessed, an average avidity of 30.9 was observed, a significant increase when compared with gp120 protein ($p = 0.0011$) (Fig. 4C). In some animals, the V1/V2 avidity was greater than 40. Of interest, an avidity index of 35–44 has been observed in other vaccination regimens in macaques protected from SIVmac251 and SIVsmE660 infection (15, 39).

**Vaccination with ALVAC-SIV/HPV-PsV-SIV/gp120 influences persistent viremia**

The efficacy of each vaccination regimen was assessed by challenging animals with up to nine intravaginal low doses of SIVmac251 (250 tissue culture–infective doses, 50%), given every 10 d, beginning 4 wk after the last vaccination. The level of SIV RNA was determined in plasma by nucleic acid sequence–based amplification, 7 d after each challenge; animals that tested negative (<50 copies per milliliter) were rechallenged. All three groups acquired SIV at a similar rate (Fig. 5A), and at the end of the challenge phase five vaccinated animals, two in the ALVAC/HPV group and three in the HPV group, remained SIV negative in plasma.
whereas one control animal remained negative. Because most HIV infections are initiated with a single or few viral variants, we aimed to model this outcome in our mucosal challenge experiment in macaques. The number of transmitted viral variants is an independent analysis of a limiting dose challenge (47). Thus, we quantified the number of variants in all SIV-infected animals that had at least two viral load measurements >10^4 RNA copies per milliliter and created neighbor joining trees for each group (Supplemental Fig. 1). No difference in the number of transmitted variants was observed between the three groups. Each group had a median of one viral variant and a maximum of three (Figure 5B, Supplemental Fig. 1). This finding suggests that our intravaginal SIVmac251 was given at a dose that models HIV heterosexual transmission. Furthermore, neither the intravaginal vaccination nor the progesterone/N9 treatment caused a significant increase in the number of transmitted variants. A similar number of variants (median 1) were also observed in naive macaques that were given a low-dose challenge by the vaginal or rectal route (39) (N. Miller, unpublished observations). No significant associations were observed between vaccine-induced immune responses and the number of transmitted variants.

Most infected animals demonstrated high peak (10^6–10^8) and set point (10^5–10^7) plasma virus (Fig. 5C). However, of the vaccinated animals, 8 had no detectable plasma virus or transient plasma viremia that remained below the limit of assay detection: 50 SIV RNA copies per milliliter (Fig. 5C). In total, 16 of 24 vaccinated animals and 10 of 12 controls demonstrated persistent SIV viremia. Persistent viremia was defined as at least two successive plasma viral load measurements >10^4 SIV RNA copies per milliliter. We next compared the viremia over the 16 wk of follow-up in the persistently SIV-infected animals. No significant differences in either peak or set point viremia in vaccinated animals or controls were observed (Fig. 5D). In addition, a similar loss of CD4^+ T cells was observed in the blood of all persistently SIV-infected macaques (Fig. 5E). To assess virus levels in mucosal tissues, pinch biopsies were collected from the vagina and rectum during acute SIV infection, and the levels of SIV DNA were determined (Fig. 6A, 6B). Unlike our findings for plasma viremia, significantly less SIV DNA was measured in the vaginal and rectal mucosa during the acute phase in the ALVAC/HPV-vaccinated animals in comparison with controls (p = 0.014 and p = 0.022) (Fig. 6A, 6B). Reduced viral DNA in the mucosa

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Vaccine efficacy and plasma viral loads after SIV infection. (A) The rate of SIV infection is shown by the percentage of uninfected animals at each challenge in the control group (solid black line), the ALVAC/HPV group (large dashes), and the HPV group (small dashes). (B) The number of transmitted founder viral variants is shown for each vaccine group, with the ALVAC/HPV group represented by open bars, the HPV group by hatched bars, and the control group by black bars. The number of variants was determined during the first 2 wk of infection in animals that had two successive positive tests for SIV RNA in plasma, with >10^4 copies per milliliter. (C) Plasma viral load over time in the ALVAC/HPV group is represented by open circles, in the HPV group by squares, and in the control group by triangles. The animal codes of animals with transient plasma viremia, or those that tested negative for SIV RNA in plasma, are shown to the right of each graph. (D) Geometric mean of plasma viral load in animals that were persistently SIV infected. Persistent infection was defined as two successive positive tests for SIV RNA in plasma, with >10^4 copies per milliliter. (E) The average absolute number of CD4^+ T cells in the blood per cubic millimeter is shown for persistently SIV-infected animals in the ALVAC/HPV group (open circles), the HPV group (squares), and the control group (triangles).
during the acute phase of SIV infection was also temporally associated with the expansion of GagCM9+ CD8+ T cells measured 10 d after SIV infection (Fig. 6C), with the vaginal tract having the highest frequency of SIV-specific CD8+ T cells and the lowest virus DNA levels in the acute phase of infection.

**CD4+ T cells and Abs to V1/V2 correlate with protection from persistent viremia**

We investigated potential associations between mucosal SIV DNA levels in the acute phase and vaccine-induced responses. We observed an inverse correlation between the level of Env-specific CD4+ T cell proliferation in blood measured 2 wk after the last vaccination and SIV DNA in the vaginal and rectal tract after SIV infection (r = −0.5 and −0.47 and p = 0.014 and 0.027 for vaginal and rectal tissues, respectively) (Fig. 6D). We did not observe a correlation between vaccine-induced CD8 T cell responses and SIV viral loads. However, the temporal expansion of vaginal CD8 T cells during the acute phase and association of CD4 helper responses with reduced viremia may indicate that the increased CD4+ T cell responses induced by the ALVAC/HPV may have helped the development of a secondary CD8+ T cell response, which in turn affected virus replication in the mucosa.

Next, we investigated the four animals with transient viremia; these animals had virus loads ranging from 50 to 10^4 copies per milliliter and then controlled viremia during the remaining repeated low-dose challenges and for 14 wk of follow-up. We questioned whether their SIV-specific immune responses were boosted during the successive SIV challenges. We observed a reduction in gp120 binding Abs and IFN-γ ELISpot responses to Gag in the vaccinated animals, comparing responses before the first SIV challenge with samples collected after the fifth challenge (Supplemental Fig. 2A). In addition, we did not detect a Vif-specific response in the cervicovaginal tract at the end of the challenge phase (Supplemental Fig. 2B). Vif is not in any of the vaccines administered but is abundant in the challenge virus. Furthermore, the levels of gp120 binding Ab titers in the vaginal secretions had also declined following the ninth and final SIV challenge, when compared with pre-SIV levels, consistent with the findings in blood (Supplemental Fig. 2C).

The two distinct outcomes observed in this study, that is, persistent SIV infection versus protection from infection or high virus replication, gave us the opportunity to investigate any associations between the measured immune responses and outcome. A comparison of immune responses in protected animals and persistently SIVmac251-infected macaques yielded a significant difference in the levels of Abs with high avidity to the V1/V2 region (Fig. 7A). Furthermore, we found a significant correlation between the number of challenges to attain persistent infection and the avidity index of V1/V2 Abs in blood (Fig. 7B). In all, these data highlight the importance of Env-specific CD4+ T cells in the containment of virus replication at mucosal sites and of high-avidity Abs targeted to the V1/V2 region of gp120 in the prevention of SIVmac251 acquisition.

**CD8 T cells contribute to protection from persistent viremia**

At 3 wk after the ninth SIV challenge, SIV-negative animals and animals with transient viremia had <50 copies of SIV RNA in plasma and were tested for SIV DNA in the mucosa (Fig. 6A, 6B, arrows). With the exception of one of the control animals that had 24 copies of SIV DNA per 10^6 cells in the rectum, the remaining nine animals tested negative for SIV DNA in the vaginal and rectal tract, using an assay that detects >10 SIV DNA copies per 10^6 cells (45). We followed these 10 animals for 14 wk, testing for SIV RNA in blood, and all remained SIV negative.

To investigate in more detail the immune control of viremia, we performed CD8 depletion in nine of the ten animals (one animal

**FIGURE 6.** Reduced SIV DNA in mucosal tissues of ALVAC/HPV-vaccinated animals. (A) Vaginal SIV DNA levels per 10^6 cells. Circles represent the ALVAC/HPV group, squares the HPV group, and triangles the controls. Significantly less SIV DNA was present in the vaginal tissues of the ALVAC/HPV group compared with the control group, denoted by an asterisk using the Mann–Whitney–Wilcoxon test, with p = 0.014. Arrows indicate animals that had either transient plasma viremia or tested negative for SIV RNA in plasma. (B) Rectal SIV DNA per 10^6 cells in vaccinated macaques and controls. Significantly less SIV DNA was present in the rectal tissues of the ALVAC/HPV group compared with controls, denoted by an asterisk using the Mann–Whitney–Wilcoxon test, with p = 0.022. (C) Mononuclear cells from the blood and from vaginal and rectal biopsy specimens were obtained 10 d after SIV infection. The frequency of memory (CD95+ GagCM9-specific CD8+ T cells is shown in the vaccinated animals (white bars) and controls (black bars). (D) An inverse correlation was observed between the levels of SIV DNA in the vaginal tract (left) and the rectum (right), and the Env-specific proliferating memory (CD95+ CD4+ T cells presented as the square root of the data. The correlation was assessed using a nonparametric Spearman test with r values of −0.5 and −0.47 and p values of 0.014 and 0.027 for the vaginal and rectal tracts, respectively.
in macaques (49), similar to the bottleneck described during most HIV infections (48). Thus, in this study, we used a repeated intravaginal challenge with a SIVmac251 swarm to test the efficacy of a novel mucosal vaccination regimen HPV-PsV-SIV, with and without ALVAC-SIV priming. Neither vaccine regimen significantly altered the rate of SIV acquisition compared with controls, although several animals were either protected from SIV infection or had transient viremia. In all, 16 of 24 (∼67%) vaccinated animals, and 10 of 12 (∼83%) of controls, developed persistent infection. Vaccinated animals that became persistently viremic had similar peak and set-point plasma virus levels, which was not surprising, given the low levels of systemic CD8+ T cell responses induced by these vaccines. However, we observed a reduction in viral burden in mucosal tissues in vaccinated animals compared with controls, and this reduction was significant in the ALVAC/HPV group, which had the highest levels of vaginal Gag-specific CD8+ T cell responses. The limited number of mononuclear cells isolated from the vaginal tract precluded our assessment of vaginal Gag-specific responses in all animals and of ret, tat, and nef responses. In addition, we observed an inverse correlation between proliferating Env-specific CD4+ T cells in blood and the levels of mucosal SIV DNA. A number of vaccinated animals had a long-lasting control of viremia. Altogether, these data suggest that T cell responses induced by HPV-PsV vaccination exerted early mucosal virus control, but virus expansion likely outpaced T cell expansion, leading to systemic dissemination and uncontrolled viremia. Surprisingly, the mucosal T cell response induced by the HPV-PsVs/gp120 regimen did not curtail local virus levels or provide sustained virus control, whereas priming with ALVAC-SIV induced an overall higher Gag and Env proliferative T cell response (Fig. 1C) and better control of mucosal virus levels (Fig. 6A, 6B). This suggests a role for SIV-specific T cells in early protection from virus replication. In a murine model, intravaginal HPV-PsV vaccination induces long-lived CD103+ CD8+ T cells that home to the site of vaccination and intercalate throughout the epithelial layers of the vaginal tract (32). If HPV-PsV vaccination similarly induces tissue-resident CD8+ T cells in humans as it does in mice, these CD8+ T cells would be well positioned to combat HIV at the site of virus entry. The exposed columnar epithelium in the cervix of young women is potentially susceptible to HPV infection and a likely site of HIV transmission (51).

Progestosterone treatment was used to facilitate vaccine delivery and may have influenced the vaccine-induced immune response, as progesterone has been shown to reduce antiviral responses (52). Intravaginal delivery of vaccines and the disruption of the epithelium used to facilitate delivery are cumbersome, and they introduce several challenges for clinical applications. However, vaccination in the secretory phase may eliminate the need for

### Table 1. Virus in mucosal tissues before, during, or after CD8 depletion

<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>SIV Infected</th>
<th>Vaginal Biopsies</th>
<th>Rectal Biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>P448</td>
<td>HPV</td>
<td>No</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>P449</td>
<td>HPV</td>
<td>No</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P779</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P793</td>
<td>HPV</td>
<td>Yes</td>
<td>Pre-αCD8+ tx</td>
<td>–</td>
</tr>
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<td>P781</td>
<td>ALVAC/HPV</td>
<td>No</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>Yes</td>
<td>Pre-αCD8+ tx</td>
<td>–</td>
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<td>–</td>
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</tr>
<tr>
<td>P468</td>
<td>Control</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Bold text denotes SIV infected animals. pos, positive for SIV RNA or DNA or both; tx, treatment.
hormonal treatment, and the collection of a cytology specimen as routinely done during a Papanicolaou test causes sufficient microtrauma to facilitate HPV vaccination (53). Thus, intravaginal HPV vaccination could be easily incorporated into a routine gynecologic visit and would potentially confer protection against both HPV and HIV.

Both regimens (ALVAC/HPV and HPV) elicited high-titer gp120-specific IgG in the blood and vaginal secretions, but neither regimen induced appreciable levels of IgA in the vaginal secretions, nor detectable gp120-specific IgA* memory B cells in the blood. Thus, IgA was unlikely to have played a role in the outcome of these studies. We found similar levels of ADCC, and the blood. Thus, IgA was unlikely to have played a role in the data suggest the relevance of this animal model for the preclinical delayed virus acquisition, as in the case of HIV in RV144. These studies demonstrated that Abs targeting the V1/V2 region were associated with achievement only when an Ab-inducing protein boost was added to the regimen (54). Our results similarly support this concept, as delayed persistent SIV infection was associated with the avidity of Abs directed to the V1/V2 region of gp120 and not with T cells. The functional role of Abs to the V1/V2 region in the efficacy of HIV vaccines remains to be clearly defined, and recent data suggest that mAbs to V2 synergize with other envelope regions to neutralize the virus (55). The studies described underscore the importance of this immunologic target, as we demonstrated that Abs targeting the V1/V2 region were associated with delayed virus acquisition, as in the case of HIV in RV144. These data suggest the relevance of this animal model for the preclinical evaluation of HIV vaccine candidates.

Acknowledgments

We thank Dr. Nancy Miller and the Division of AIDS, National Institutes of Health, for the SIVmac251 Virus stock and for supporting the measurement of several Ab responses; Dr. Jean Charles Grivel for a processing protocol of-concept phase 2b study, Lancet Infect. Dis. 11: 507–515.


responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68: 4650–4655.


Supplemental Data 1

a ALVAC/HPV+gp120

b HPV+gp120

c Control

SUPPLEMENTAL DATA 1: Number of transmitted viral variants (a) Neighbor joining tree (radial) for ALVAC/HPV+gp120 vaccinated, SIV infected animals. Animals that are infected with a single variant are in black, while animals infected with multiple viral variants are colored. (b) Neighbor joining tree (radial) for HPV+gp120 vaccinated, SIV infected animals. (c) Neighbor joining tree (radial) for control animals that were SIV infected animals.
SUPPLEMENTAL DATA 2: Repeated low dose SIV challenges do not increase SIV-specific responses in animals with transient viremia.

(a) Gp120 specific antibody titers and Gag specific IFN-γ ELISPOT responses in blood. Samples were obtained 1-2 weeks before the first SIV challenge (Pre SIV) and during the challenge phase i.e. seven days after the fifth SIV challenge (Post 5th Exp). The ALVAC/HPV animals are shown in circles, HPV group squares, and control triangle. 

(b) After the 9th and final intra-vaginal SIV challenge, vaginal biopsies were obtained. Representative flow plots of mononuclear cells obtained from these biopsies that were either left unstimulated (left) or stimulated with overlapping peptides that span Vif (right). Shown is the frequency of IFN-γ and TNF-α cytokine production. 

(c) Gp120 specific binding antibodies in mucosal secretions collected 3 weeks before SIV challenge (Pre SIV) and after the 9th low dose SIV challenge (Post 9th Exp). 

(d) Three doses of CD8 depleting antibodies (αCD8) were administered and the absolute number of CD4 T-cells (small dashed line), CD20+ cells (dashed line), and CD8+ T-cells (solid line) is shown before and after antibody administration. 

(e) The frequency of CD8+ T-cells in the lymph nodes (LN) and rectal pinch biopsies (RB) before CD8 depletion (Baseline) and 9 days post the first antibody administration (Day 9) is shown. A significant reduction in the frequency was observed at Day 9 indicated by *. A Wilcoxon signed rank test was used to compare the differences, and a p value of 0.001 and 0.002 was obtained for the lymph nodes and rectal biopsies respectively. 

(f) Viral loads over time of the two persistently SIV infected animals that were CD8 depleted. Arrows indicate time of anti CD8+ administration.