

Chimeric Human Papilloma Virus–Simian/Human Immunodeficiency Virus Virus-like-Particle Vaccines: Immunogenicity and Protective Efficacy in Macaques

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Vaccines to efficiently block or limit sexual transmission of both HIV and human papilloma virus (HPV) are urgently needed. Chimeric virus-like-particle (VLP) vaccines consisting of both multimerized HPV L1 proteins and fragments of SIV gag p27, HIV-1 tat, and HIV-1 rev proteins (HPV–SHIV VLPs) were constructed and administered to macaques both systemically and mucosally. An additional group of macaques first received a priming vaccination with DNA vaccines expressing the same SIV and HIV-1 antigens prior to chimeric HPV–SHIV VLP boosting vaccinations. Although HPV L1 antibodies were induced in all immunized macaques, weak antibody or T cell responses to the chimeric SHIV antigens were detected only in animals receiving the DNA prime/HPV–SHIV VLP boost vaccine regimen. Significant but partial protection from a virulent mucosal SHIV challenge was also detected only in the prime/boosted macaques and not in animals receiving the HPV–SHIV VLP vaccines alone, with three of five prime/boosted animals retaining some CD4⁺ T cells following challenge. Thus, although some immunogenicity and partial protection was observed in non-human primates receiving both DNA and chimeric HPV–SHIV VLP vaccines, significant improvements in vaccine design are required before we can confidently proceed with this approach to clinical trials. © 2002 Elsevier Science (USA)

Key Words: HIV; vaccine; human papilloma virus; virus-like-particle; DNA vaccine; macaques.

INTRODUCTION

An HIV vaccine is urgently needed to prevent the millions of new HIV infections each year occurring primarily in underdeveloped countries (Piot *et al.*, 2001). Most HIV infections occur across the genital mucosa and vaccines that block or limit mucosal transmission are likely to be the most efficient vaccines. Vaccines delivered via mucosal surfaces appear most effective at the induction of mucosal immunity (Lehner *et al.*, 1999).

Although prevention of HIV transmission might most effectively occur by the induction of high-titer neutralizing antibodies, safe or viable HIV vaccine strategies have not to date induced antibodies capable of neutralizing diverse field strains of HIV-1 (Lacasse *et al.*, 1999). There is, however, a large body of evidence that HIV-1 or SIV specific T cells can dramatically curtail viral replication, in some cases sufficiently to render the virus nonpathogenic for considerable periods *in vivo* (Amara *et al.*, 2001; Koup *et al.*, 1994; Rosenberg *et al.*, 2000).

The induction of specific T cells by vaccines requires the presentation of specific peptides from within host cells. The entry of vaccine constructs into host cells greatly facilitates this process. Vaccine approaches ca-

pable of inducing HIV or SIV specific T cells include plasmid DNA vaccines, live vector vaccines, virus-like particle (VLP) vaccines, and live attenuated vaccines (Barouch *et al.*, 2000; Davis *et al.*, 2000; Kent *et al.*, 2001b, 1998). Live attenuated vaccines are generally considered too dangerous to enter human clinical trials at present (Baba *et al.*, 1995; Kent *et al.*, 2001b). DNA and live-vectored vaccines are by themselves relatively inefficient at induction of specific T cells; although when utilized together with cytokines or sequentially in prime/boost approaches DNA and live-vector vaccines induce high levels of HIV or SIV specific T cells and nonsterilizing protective immunity (Amara *et al.*, 2001; Barouch *et al.*, 2000). Nonreplicative VLP vaccines could be among the safest of these vaccine approaches and, if the vaccines efficiently enter host cells, could induce or boost HIV or SIV specific T cells.

Human papilloma virus (HPV) causes anogenital warts and cervical cancer and there is also an urgent requirement for the development of an HPV vaccine (Frazer, 1996; Galloway, 1998; Schiller and Lowy, 2001). HPV VLPs can be generated by expressing the HPV capsid protein L1 utilizing baculovirus or other expression systems where five L1 subunits multimerize into immunogenic pentamers and 72 L1 pentamers multimerize into a HPV VLP (Kirnbauer *et al.*, 1992; Peng *et al.*, 1998; Zhou *et al.*, 1991). HPV L1 VLPs are efficiently taken up by both

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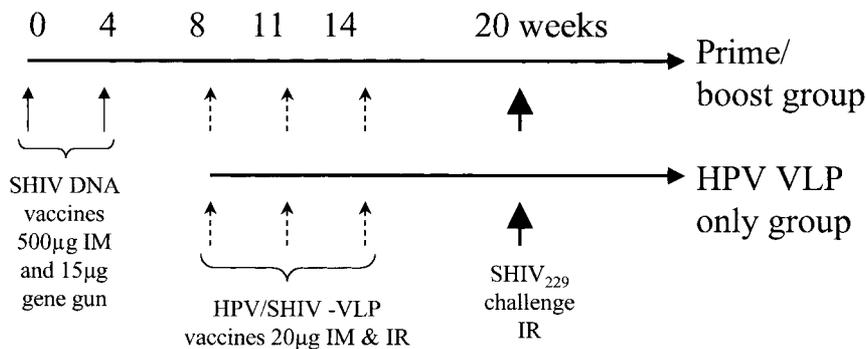


FIG. 1. Vaccination and challenge schedule. DNA and chimeric HPV-SHIV VLP vaccines were administered at the times noted.

mucosal epithelial cells and antigen-presenting cells (Liu *et al.*, 1998). HPV L1 VLPs have shown promise in mouse and rabbit papillomavirus models, where they are capable of inducing mucosal antibody and T cell responses when delivered mucosally, as well as providing protection from papillomavirus challenge (Liu *et al.*, 1998; Revaz *et al.*, 2001). HPV VLPs have been shown to be immunogenic in small human trials and have entered expanded human clinical trials for the prevention and treatment of cervical cancer and anogenital warts (Zhang *et al.*, 2000).

The L1 protein of HPV can be modified to express small (up to 60 aa) portions of additional proteins by deleting the coding sequence for the L1 C-terminal 25 aa and adding the foreign gene to generate a safe mucosal delivery vector. The resultant chimeric HPV VLPs can induce CTLs in murine systems, including to HIV-1 epitopes, and provide protection against tumor challenge models (Liu *et al.*, 1998; Peng *et al.*, 1998). The prospect emerges that an effective chimeric HIV-HPV VLP vaccine could be delivered mucosally to induce immunity against two important sexually transmitted pathogens, HPV and HIV.

A robust model in which to evaluate the efficacy of candidate mucosal HIV vaccine approaches is a pathogenic simian-human immunodeficiency virus (SHIV) infection administered to the rectal mucosa of outbred monkeys (Amara *et al.*, 2001; Barouch *et al.*, 2000; Li *et al.*, 1992). SHIV is a chimeric primate lentivirus virus utilizing an SIV backbone with the HIV-1 env, tat, and rev genes replacing the SIV env, tat, and rev genes. The SHIV genes we chose to express in the HPV-HIV chimeric VLP vaccines were: (a) HIV-1 tat and rev, since these are small genes more readily expressed in chimeric HPV VLPs, expressed early in the infection cycle, thereby potentially inducing CTLs which can clear infections prior to the formation of progeny virus, shown to be important targets for CTL responses (Addo *et al.*, 2001; Allen *et al.*, 2000a) and could be subsequently studied in human clinical trials if found effective and (b) SIV gag p27, since this portion of the virus is conserved, an important target for both CTLs and T-helper responses

(Gauduin *et al.*, 1999), and we had previous experience in evaluating SIV gag specific T cell responses in macaques (Kent *et al.*, 2001b, 1996).

We evaluated HPV-HIV VLPs for immunogenicity and protective immunity using a mucosal SHIV challenge model in macaques. Since we and others have previously shown that DNA priming followed by attenuated poxvirus boosting can induce high levels of HIV-1 or SIV specific T cells and protection from viral challenge in macaques (Allen *et al.*, 2000b; Amara *et al.*, 2001; Hanke *et al.*, 1999; Kent *et al.*, 1998), we also evaluated a DNA vaccine prime and HPV-HIV VLP boost approach to determine whether this approach could efficiently induce T cell mediated immunity in macaques.

RESULTS

Vaccinations

HPV-SHIV chimeric vaccines expressing all of HIV-1 tat (2 products, HPV6bL1/tat1, HPV6bL1/tat2), HIV-1 rev (3 products, HPV6bL1/rev1, HPV6bL1/rev2, HPV6bL1/rev3), and part of SIV p27 gag (HPV6bL1SIVp27c and HPV6bL1SIVp27d, spanning amino acids 91-188, 43% of the SIVp27 gag protein) were constructed and induced anti-HPV L1 antibodies in mice with 1 µg of the construct delivered twice IM without adjuvant (Liu *et al.*, manuscript in preparation). Five separate SIV p27 HPV VLPs were constructed, but only two (p27c and p27d constructs) induced anti-HPV L1 antibodies in mice and were included in this study (Liu *et al.*, manuscript in preparation). Of the seven constructs immunogenic in mice, three (tat1 and tat2 and rev3) were shown to form VLPs and the other four constructs formed chimeric pentamers by electron microscopy. The combination of seven immunogenic HPV-SHIV VLPs was administered to macaques three times by both IM and intrarectal routes (Fig. 1, VLP only).

The group of five macaques receiving the DNA prime and HPV-SHIV VLP boost vaccine regimen received three separate DNA vaccines expressing HIV-1 tat, rev, and SIV gag. The SIV gag vaccine was codon optimized for expression in mammalian cells (Egan *et al.*, 2000;

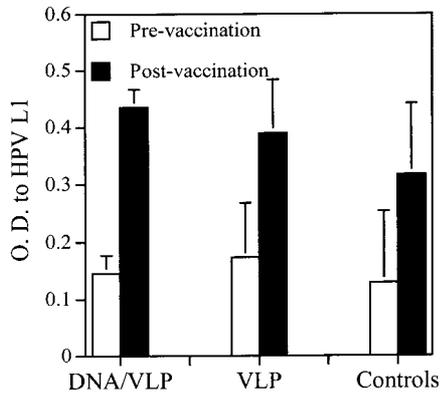


FIG. 2. Anti-HPV L1 antibodies pre- and postvaccination. HPV L1 antibodies in plasma were measured by EIA at week 0, prior to any vaccinations, and at week 15, after the animals had received all vaccines. Mean (\pm SD) anti-HPV L1 antibodies are shown for the three groups of five macaques receiving the DNA prime/HPV–SHIV VLP boost regimen (DNA/VLP), the HPV–SHIV VLP vaccines alone (VLP), or control HPV VLPs not expressing SHIV antigens (controls).

Shiver *et al.*, 2002). The expression of HIV-1 tat vaccine was driven from a CMV promoter and showed high levels of functional activity *in vitro* and in macaques (Kent *et al.*, 2001a). The HIV-1 rev DNA vaccine was expressed off a RSV promoter and showed high levels of functional activity *in vitro*, inducing a 44-fold increase in rev activity from the pDM128 CAT-RRE reporter relative to mock control. The DNA vaccines were administered both IM (500 μ g) in saline and epidermally via gene gun (15 μ g) twice (Fig. 1, prime boost). All vaccines were well tolerated by the macaques.

Antibody responses to HPV and SHIV antigens following vaccinations

Both anti-HPV and anti-SHIV antibody responses were assessed in plasma prior to and following vaccinations. All 15 macaques received HPV VLP vaccinations, with the control macaques receiving nonchimeric HPV VLPs. HPV L1 specific antibodies were increased in each individual macaque and across all groups following the HPV VLP vaccinations (Fig. 2). SIV gag, HIV-1 tat, and HIV-1 antibody responses were evaluated by EIA, using recombinant proteins as antigens and reference antibodies or SIV-positive sera as positive control responses. No significant anti-SHIV antibody responses were detected by EIA in sera from any of the animals, although responses were readily detected in reference HIV-1 tat, HIV-1 rev, and SIV antibodies (Fig. 3A). Anti-SIV gag responses were also assessed by Western blot using cross-reactive HIV-2 antigens. Weak anti-gag bands were detected in three of five animals receiving the DNA vaccine prime/HPV–SHIV VLP vaccine boost vaccine regimen, but in none of the HPV–SHIV VLP vaccinated animals nor in controls (Fig. 3B and data not shown). The anti-gag responses detected by Western blot in a subset of DNA

prime/HPV–SHIV VLP boost vaccinated animals recognized both p27 and p17 gag antigens (Fig. 3B), suggesting that the antibody response was primarily recognizing the entire gag protein expressed by the pSIVoptgag DNA vaccine rather than the fragment of p27 expressed by the chimeric HPV–SIVp27 VLPs.

Lymphoproliferative responses following vaccinations

To assess the CD4⁺ T cell immunogenicity of the vaccinations, lymphoproliferative assays were performed using freshly isolated PBMC stimulated by antigens expressed by the vaccines (Fig. 4A). Prior to vaccination there were no lymphoproliferative responses to SIV gag antigens and a mitogenic response was present in all animals. Following either HPV–SHIV VLP vaccines alone or the DNA prime/HPV–SHIV VLP boost vaccine regimen there were no significant lymphoproliferative responses to vaccine antigens. Using identical or similar SIV or HIV-1 antigens in recent or concurrent live attenuated vaccine studies or DNA prime/fowlpoxvirus boost vaccine studies, lymphoproliferative responses with stimulation indices of 5–20 have been observed in vaccinated pigtail macaques (Kent *et al.*, 1997, 1998, 2000, and Kent *et al.*, unpublished data).

IFN γ ELISPOT responses following vaccinations

Antigen specific production of IFN γ by T cells was quantified by ELISPOT assays in response to whole inactivated SIV and overlapping 15-mer peptides on batched frozen PBMC performed on the same day. IFN γ responses were weak or undetectable following either of the vaccine regimens and not significantly greater than responses in animals receiving only control vaccines (Fig. 4B). In concurrent or recent experiments studying either live attenuated or DNA prime/fowlpoxvirus boost vaccinations in *Macaca nemestrina*, antigen specific responses of up to 650 IFN γ spot-forming cells/million PBMC are observed using the same methods and similar or identical SIV or HIV-1 antigen preparations (Dale *et al.*, 2000; Kent *et al.*, 2001b, and Kent *et al.*, unpublished data).

T cell responses by intracellular cytokine staining

To further quantify and phenotype vaccine-induced T cell responses, antigen specific responses in whole blood were assessed by intracellular IFN γ staining and flow cytometry following a 6-h *in vitro* culture with either overlapping peptides or whole inactivated SIV. Weak SIV specific responses (mean \sim 0.1% of gated lymphocytes) were detectable only in macaques following the DNA prime/HPV–SHIV VLP boost vaccine regimen and not in controls or animals receiving the HPV–SHIV VLP vaccines alone (Fig. 5). Differences between DNA prime/HPV–SHIV VLP boost vaccinated animals and controls of borderline significance were present to the whole inac-

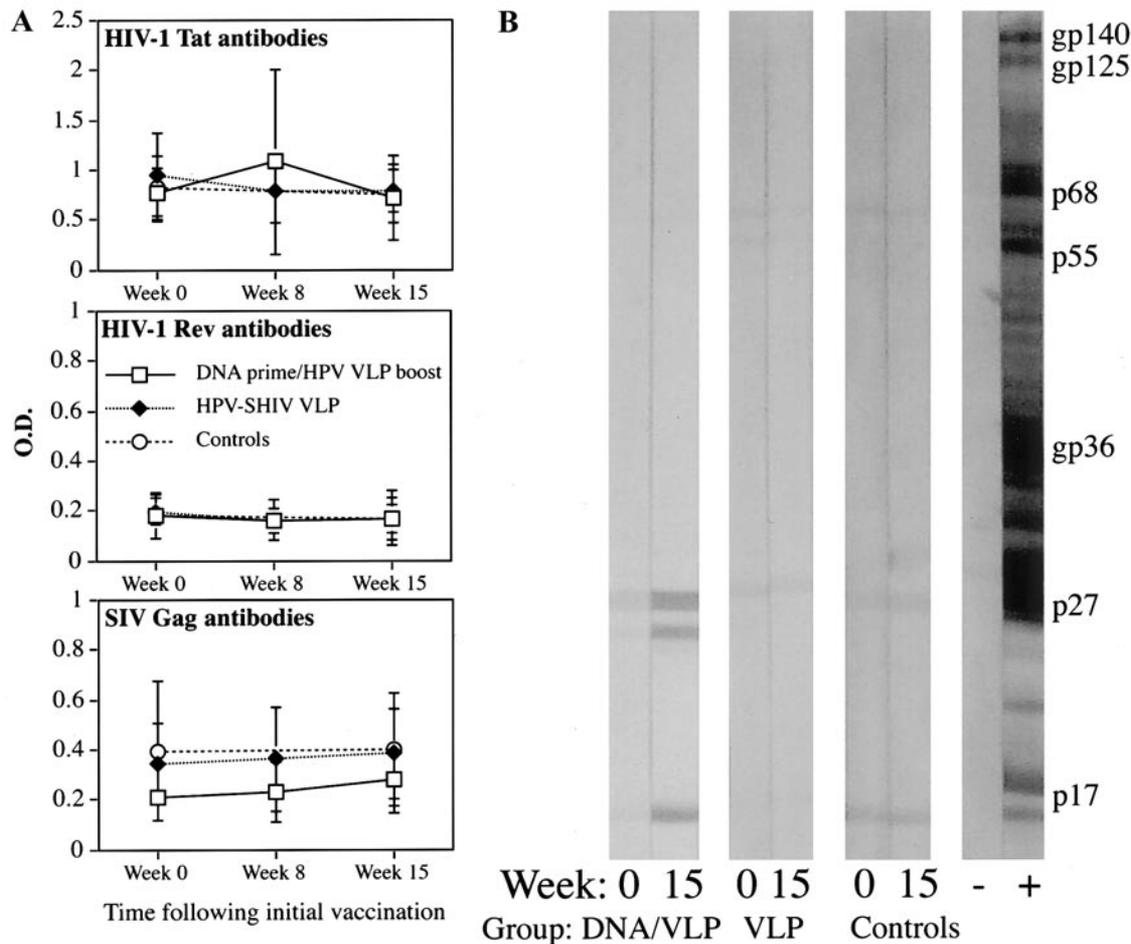


FIG. 3. Anti-SHIV antibody responses following vaccinations. (A) Anti HIV-1 tat, HIV-1 rev, and SIV gag were measured in plasma by EIA. Plasma was diluted 1 in 50 for the anti HIV-1 tat responses and 1 in 500 for the anti HIV-1 rev and SIV gag responses. Antibody responses prior to vaccination, after DNA vaccinations only (week 8), and after all vaccinations (week 15) are shown. Mean (\pm SD) responses for each vaccine group of five macaques are shown. Positive control responses to reference rabbit anti-HIV-1 tat (1 in 1600) and rev (1 in 1600) antibodies and plasma from a SIV-infected macaque (1 in 100) were OD_{450} (\pm SD) 2.8 ± 0.3 , 2.2 ± 0.1 , and 1.2 ± 0.1 , respectively. (B) Western blot to HIV-1 antigens pre- (week 0) and postvaccinations (week 15) in a macaque from each vaccine group. Control human sera negative (-) and positive (+) for HIV-2 is shown.

tivated SIV antigen ($P = 0.057$, Student's t test, Fig. 5A). Although some individual animals appear to be responding to SIVgag and HIV-1 tat peptides in the prime/boost group (Figs. 5B and 5C), as a group these responses were not statistically significant in comparison to the control group ($P = 0.08$ for SIVgag and $P = 0.18$ for HIV-1tat). No rev specific responses were detected (Fig. 5D). Responses were approximately equivalent in the CD3+ only and CD3+CD8+ populations, suggesting that the responses induced and detectable by this technique were primarily CD8+ T cell responses.

SHIV challenge stock

An HIV-1_{IIIB}-based SHIV strain, SHIV_{229(mn)}, previously shown to be pathogenic in *M. nemestrina* was kindly obtained from Dr. Mike Agy, University of Washington, Seattle, and amplified on *M. nemestrina* PBMC (Thompson *et al.*, 2000). Prior to the challenge of the vaccinated

animals, the SHIV_{229(mn)} stock was assessed for infectivity via intrarectal administration in pigtailed macaques at two doses (2×10^5 TCID₅₀ in two animals: M21 and M26, and 4×10^4 TCID₅₀ in two animals: M24 and M25) as assessed by serial analyses of peripheral CD4+ T cells by FACS and plasma SHIV RNA levels by real time PCR. All four inoculated animals had an abrupt loss of CD4+ T cells within 3 weeks (Fig. 6A) and had high levels of SHIV RNA detected, peaking 2 weeks postinoculation (Fig. 6B).

Outcome of SHIV challenge

The SHIV_{229(mn)} challenge stock was inoculated into all 15 macaques on the same day, 6 weeks after the last vaccination at a dose of 10^5 TCID₅₀ intrarectally. CD4+ T cells and plasma SHIV RNA were followed for 12 weeks. All 5 control macaques had high levels of SHIV RNA at peak levels at 2 weeks (mean $8.6 \log_{10}$ copies/ml) fol-

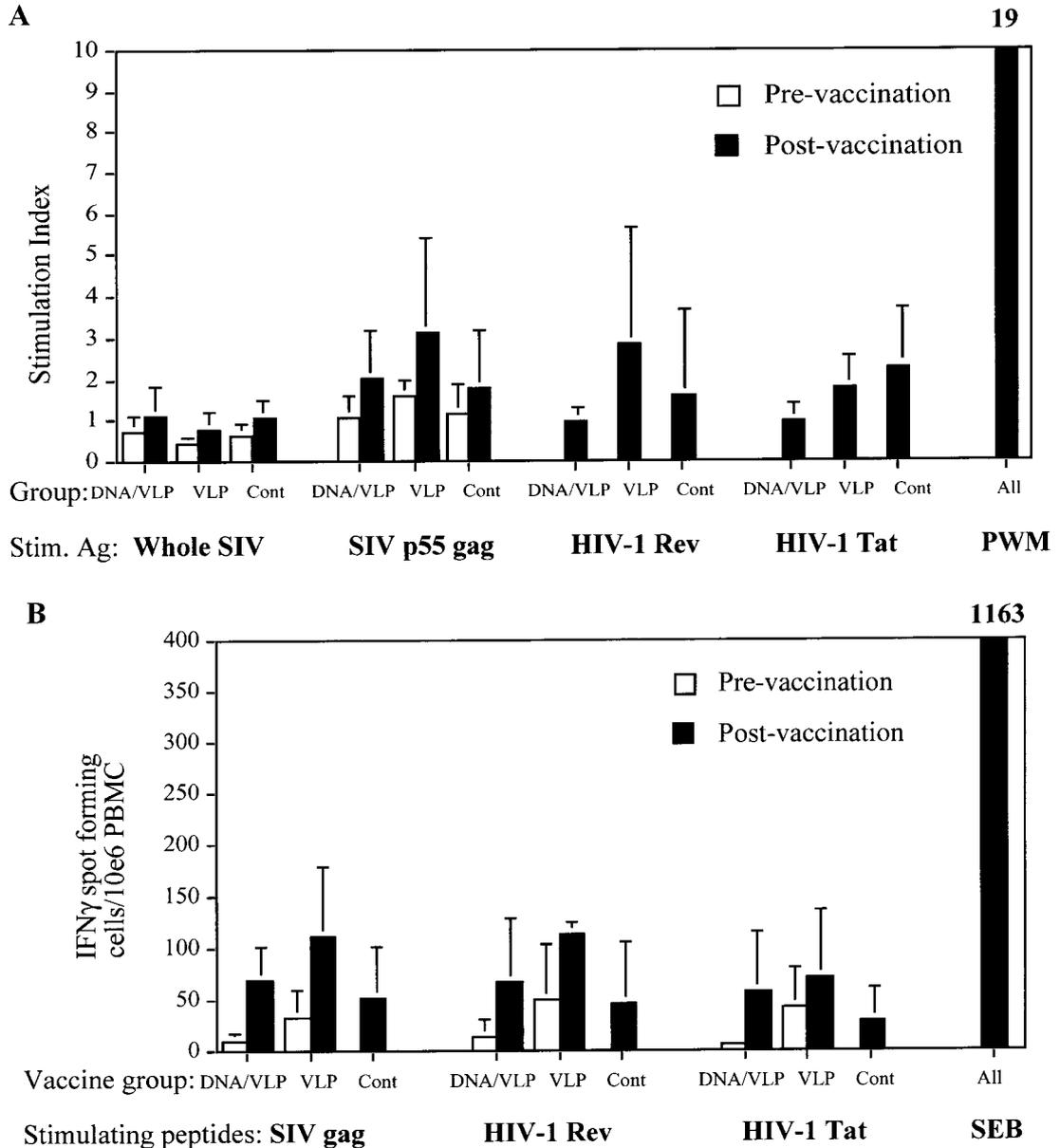


FIG. 4. Anti-SHIV T cell responses to vaccination. **A.** Lymphoproliferative responses to SHIV antigens. Proliferation of freshly isolated macaque PBMC to whole inactivated SIV (SIV), SIVp55 gag protein both prior to (week 0) and following all vaccinations (week 15), and overlapping 15-mer HIV-1 rev or tat peptides following vaccinations are shown. Proliferation was assessed by [³H]thymidine incorporation and expressed as stimulation index of mean counts from active antigen wells/counts from relevant control antigen wells. Mean (\pm SD) responses are shown for the three groups of five macaques receiving the DNA prime/HPV-SHIV VLP boost vaccine regimen (DNA/VLP), the HPV-SHIV VLP regimen alone (VLP), or control DNA and HPV VLP vaccinations (Cont). Mean positive control responses to pokeweed mitogen (PWM) in all groups are also shown. **B.** IFN γ ELISPOT responses to SHIV antigens. ELISPOT responses to pools of overlapping 15-mer peptides of SIV gag, HIV-1 tat, and HIV-1 rev were assessed prior to (week 0) and following (week 15) vaccinations. Macaque IFN γ ELISPOT responses were evaluated on batched frozen PBMC using a macaque-specific kit and enumerated using an ELISPOT counter. Mean (\pm SD) responses are shown for the three groups of five macaques receiving the DNA prime/HPV-SHIV VLP boost vaccine regimen (DNA/VLP), the HPV-SHIV VLP regimen alone (VLP), or control DNA and HPV VLP vaccinations (Cont). Mean positive control responses to the superantigen Staphylococcal enterotoxin B (SEB) in all groups are also shown.

lowing inoculation and at set point at 8 weeks (mean 6.7 log₁₀ copies/ml) (Fig. 7A). Similarly, all 5 control macaques lost CD4⁺ T cells precipitously, being 4.8% at week 2 and 0.7% of total lymphocytes by week 8 (Fig. 7C). The group of 5 macaques receiving the three doses of HPV-SHIV VLP vaccines alone was not significantly pro-

duced from the SHIV challenge. The mean SHIV RNA level in this group was 8.4 log₁₀ copies/ml at 2 weeks and 6.4 log₁₀ copies/ml at 8 weeks (Fig. 7B), and the mean CD4⁺ T cell level was 14.0% at 2 weeks and 1.3% at 8 weeks (Fig. 7D). Interestingly, one of the HPV-SHIV VLP vaccinated animals, M38, had a slightly lower SHIV RNA

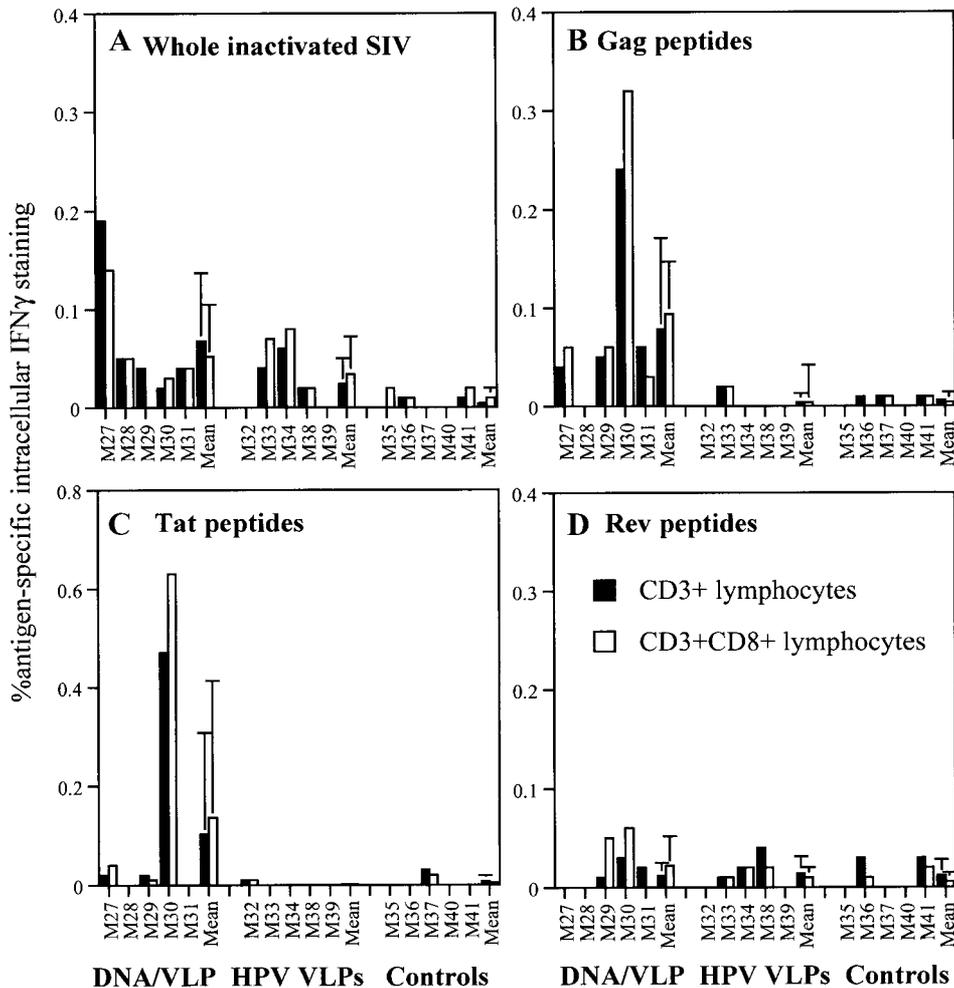


FIG. 5. Intracellular IFN γ expression following vaccinations. One week following all vaccinations, whole blood from the three groups of five macaques was stimulated with various antigen preparations: whole inactivated SIV (A), overlapping 15-mer SIV gag peptides (B), overlapping 15-mer HIV-1 tat peptides (C); and overlapping 15-mer HIV-1 rev peptides (D) for 6 h. The proportion of antigen-specific gated lymphocytes expressing IFN γ and either CD3 (solid bars) or both CD3 and CD8 (open bars) was assessed by flow cytometry. The mean \pm SD is shown for the three vaccine groups for each antigen.

level 8 weeks following inoculation and retained some CD4⁺ T cells (4.8% of total lymphocytes, compared to preinfection level of 16.4%) out to 12 weeks following challenge.

The group of five macaques receiving the DNA vaccine prime and HPV-SHIV VLP boost vaccine regimen had SHIV RNA levels that were modestly but significantly lower than that of the control macaques 2 weeks postchallenge (mean 7.8 vs 8.6 log₁₀ copies/ml in control macaques, $P = 0.03$, Student's t test). The SHIV RNA levels of the group was not significantly lower by 8 weeks postchallenge (mean 6.4 vs 6.7 log₁₀ copies/ml in controls). The three DNA vaccine prime and HPV-SHIV VLP boost vaccinated animals with the lower SHIV RNA levels at peak and set point (M28, M29, M30) had some retention of CD4 T cells out to 12 weeks following challenge. Macaques M28, M29, and M30 had a mean %CD4 T cell level of 11.5% 12 weeks following challenge, com-

pared to 29.7% in these three animals preinfection and 0.9% in the five control animals 12 weeks postchallenge. As a group, macaques receiving the DNA prime/HPV-SHIV VLP boost vaccine regimen demonstrated a borderline significant retention of CD4⁺ T cells in comparison to the control group (7.0% vs 0.9% at week 12, $P = 0.056$).

DISCUSSION

This study examined the immunogenicity and protective efficacy of chimeric HPV-SHIV VLP vaccines, expressing SIV p27 gag and the regulatory proteins HIV-1 tat and rev, delivered both mucosally and systemically in macaques. Very limited immunogenicity was detected following vaccination, even when the HPV-SHIV VLP vaccination was preceded by a DNA vaccine prime encoding the same SHIV antigens. Weak anti-HPV antibody

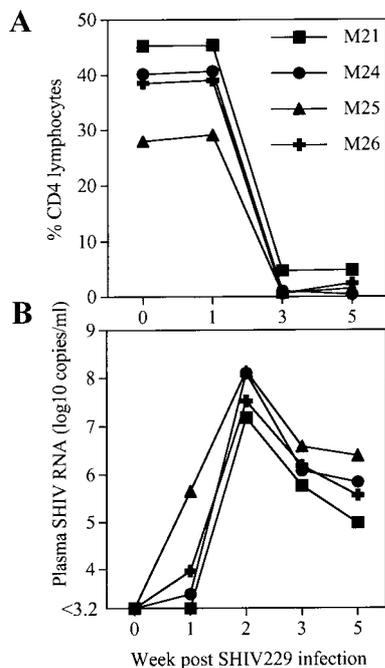


FIG. 6. Validation of intrarectal SHIV challenge stock. A macaque–PBMC grown SHIV_{229(macm)} stock was assessed for infectivity via intrarectal administration in pigtailed macaques at two doses (2×10^5 TCID₅₀ in two animals: M21 and M26, and 4×10^4 TCID₅₀ in two animals: M24 and M25) as assessed by serial analyses of (A) peripheral CD4+ T cells by flow cytometry and (B) plasma SHIV RNA levels by real-time PCR.

responses were detected by EIA, suggesting that delivery of the VLPs was achieved. However, only weak anti-SIV gag responses were detected by Western blot in a subset of DNA prime/HPV–SHIV VLP boost vaccinated animals, and no anti-SHIV responses were detected by EIA. T cell immunogenicity was also limited with no anti-SHIV T cell responses detected by lymphoproliferation or IFN γ ELISPOT and only weak anti-SIV gag and HIV-1 tat CD8+ T cell responses detected by intracellular IFN γ staining, again only in animals receiving the DNA prime/HPV–SHIV VLP boost vaccine regimen.

Previous experience has demonstrated the enhanced level of T cell immunogenicity of DNA prime/live vector boosting vaccination. It might have been expected that, if the HPV–SHIV VLP vaccines were capable of even weakly stimulating or boosting T cell responses, the DNA prime/HPV–SHIV VLP boost vaccine regimen would have produced detectable levels of T cell immunogenicity by assays such as lymphoproliferative assays and IFN γ ELISPOT. The weak anti-SHIV T cell responses detected by intracellular IFN γ staining following all vaccines could have been stimulated primarily by the DNA vaccines alone, although this was not directly assessed at that time point. Taken together, the immunogenicity results suggest that the HPV–SHIV VLP vaccines studied were not a highly effective delivery vector for anti-SHIV immune responses in outbred primates.

A model utilizing known MHC haplotypes and specific epitopes to detect immune responses (such as inbred mice or MamuA*01+ rhesus macaques) may have been a more sensitive model in which to detect anti-SHIV T cell responses. However, given previous experience with measuring anti-HIV-1 or SIV T cell responses in outbred primates, and recent or concurrent studies using alternate vaccine strategies which induced anti HIV-1 or SIV T cell responses, the HPV–SHIV VLP vaccine regimens do not appear highly T cell immunogenic (Kent *et al.*, 2001b, 1998). The significant differences in T cell immunogenicity observed between previous inbred murine studies of chimeric HPV–HIV VLP vaccines (Peng *et al.*, 1998; Liu *et al.*, 2000) and this outbred macaque study were remarkable. At least two reasons may explain the weak immune responses. First, only three of seven chimeric HPV constructs formed VLPs, while the rest were chimeric pentamers. Although pentamers and chimeric pentamers are immunogenic at inducing L1 specific antibodies (Yuan *et al.*, 2001; Liu *et al.*, manuscript in preparation), their ability to deliver the incorporated proteins might be compromised. The incorporated sequences did not predict protein production levels or the formation of VLPs (Liu *et al.*, manuscript in preparation). Second, the absolute amount of each of the capsids/capsomers used in this study may have been insufficient to deliver the incorporated proteins to the immune system of the primates studied and higher doses should be assessed in future studies. It is also possible that the administration of multiple HPV L1 VLPs and pentamers limited the ability of the animals to generate adequate immunity to the HIV-1 or SIV inserts, although this seems unlikely.

The efficacy of the vaccines was evaluated utilizing a highly pathogenic mucosal SHIV challenge. The challenge stock was grown in macaque PBMC and validated prior to challenge of the vaccinated animals. Animals receiving the HPV–SHIV VLP vaccine alone were not, as a group, significantly protected from high plasma SHIV RNA levels or CD4+ T cell loss, although one of the five animals had a slightly lower SHIV RNA level and stably retained some CD4+ T cells out to 12 weeks following challenge. Animals receiving the DNA prime/HPV–SHIV VLP boost vaccine regimen, as a group, had significantly lower SHIV RNA levels early following SHIV challenge. Three of the five animals were protected from the rapid and complete CD4+ T cell loss observed in controls out to 12 weeks following challenge. There was no correlation between the animals that demonstrated weak anti-SHIV antibody or T cell responses and protection from SHIV challenge in these groups of five animals (not shown).

It is of interest that vaccination with the DNA prime/HPV–SHIV VLP boost regimen was able to provide sufficiently blunted peak SHIV RNA responses to rescue some CD4+ T cells from the acutely pathogenic effects

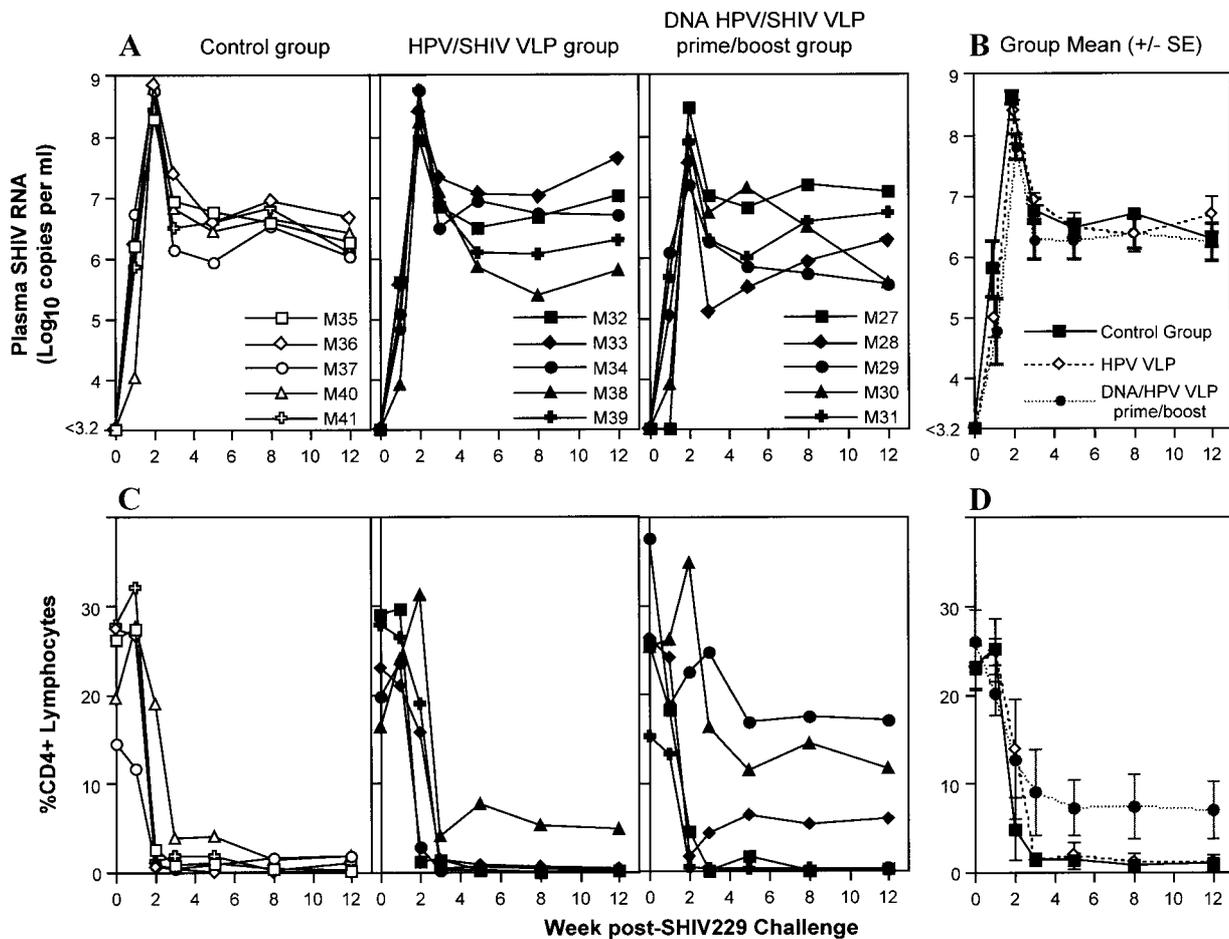


FIG. 7. Outcome of SHIV challenge. All vaccinated and control macaques were challenged intrarectally with SHIV_{229(mn)} and assessed for (A) CD4+ T cell loss by FACS and (C) plasma SHIV RNA by real-time PCR. Mean responses of CD4+ T cells and SHIV RNA of each vaccine group are shown in (B) and (D).

of the SHIV challenge. It is possible that additional mucosal immune responses were induced by the regimen that, together with modest systemic responses, made this a partially effective vaccine strategy. Alternatively, the partial protection observed in this group may have been derived from the DNA vaccines only, similar to previous observations of SIV and SHIV DNA vaccination studies in macaques (Barouch *et al.*, 2000; Lu *et al.*, 1996), although this does not explain the partial protection observed in one of the HPV-SHIV VLP only immunized animals.

The data also suggest that the SHIV-macaque challenge model, which although it is highly pathogenic over 2–3 weeks, requires only modest (mean 0.6 log₁₀ copies/ml difference between vaccinated and control macaques in this study) control of the early virologic profile to significantly affect the immunologic outcome. This is consistent with observations in HIV-1 infected humans where modest (0.5 log₁₀) differences in virologic set point 6–12 months after seroconversion profoundly affect the time to develop clinical AIDS or death, although the time frame is much longer (Mellors *et al.*, 1996).

There are substantial improvements that can, and must, be made to chimeric HPV-HIV VLP vaccines to improve their immunogenicity and efficacy in primates. Incorporating additional SHIV antigens into the vaccines may provide broader protective efficacy in outbred subjects. The incorporation of larger SHIV proteins into HPV-VLP vaccines will ultimately require novel technologies as the restriction in the size of the chimeric protein that can be incorporated into L1 means that it is impractical to construct and produce large numbers of VLP vaccines. The HPV L2 capsid protein may be able to incorporate larger chimeric proteins and more efficiently form true VLPs. Tethering proteins to the external portions of HPV L1 or L2 VLPs may also be an effective method to deliver larger proteins, as well as potentially direct the VLPs to specific cell types. VLPs utilized with mucosal adjuvants can increase the immunogenicity of VLPs in mice and this may be required in primates (Gerber *et al.*, 2001; Greer *et al.*, 2000). Future generations of chimeric HPV-HIV VLPs may be more effective and viable mucosal vaccine vectors and proceed to human trials.

MATERIALS AND METHODS

HPV–SHIV VLP vaccines

Recombinant HPV virus-like particles encoding HIV-1 tat, HIV-1 rev, and SIV p27 gag were produced by expression of a fusion protein between the L1 protein of HPV strain 6 and fragments of either SIV P27 gag (two fragments), HIV-1 tat (2 fragments), or HIV-1 rev (three fragments) as previously described (Liu *et al.*, 2000, 1998; Peng *et al.*, 1998). Briefly, a plasmid encoding a C-terminal deleted HPV6b L1 had approximately 150 nucleotide fragments spanning HIV-1_{AD8} tat, HIV-1_{NL4.3} rev, or SIV_{mac239} p27 gag inserted into the terminal *NcoI* site. The plasmids were transfected into baculovirus using a BaculoGold transfection kit (PharMingen, San Diego, CA) and VLPs were produced by infection of SF9 cells. The seven HPV–SHIV VLP vaccines expressing SIV_{mac239} gag p27 (part), HIV-1_{AD8} tat, and HIV-1_{NL4.3} rev were pooled and administered to two of the three groups of five macaques. HPV–SHIV VLPs were administered intramuscularly (20 μ g each of the seven constructs [140 μ g in total]) IM split between left and right quadriceps) as well as intrarectally (20 μ g each construct by atraumatic instillation 4 cm into the rectum) three times as noted in Fig. 1. Control macaques received HPV VLPs not containing SHIV antigens.

DNA vaccines

A mixture of three plasmids, encoding SIV_{mac239} gag (pSIVoptgag), HIV-1_{NL4.3} tat (pCMVNLtat), and HIV-1_{NL4.3} rev (pRSVrev), were pooled and administered to macaques twice as a priming vaccine to one of the three groups of five macaques. Five macaques received plasmid pCMV-empty, not encoding SIV or HIV-1 genes, as a negative control.

pSIVoptgag, encoding codon optimized SIV_{mac239} gag, was a kind gift from Dr. Gwen Heidecker (Merck Research Laboratories, PA) (Egan *et al.*, 2000; Shiver *et al.*, 2002). Plasmid pCMVNLtat, encoding the HIV-1_{NL4.3} tat, was constructed from plasmid vector pEGFP-N1 (Clontech BD Biosciences, CA) by replacing the EGFP coding sequence with the *SalI*–*BamHI* restricted tat fragment from the cDNA clone pCR2-tat1 as previously described (Purcell and Martin, 1993). Expression of tat was under the control of the human cytomegalovirus (CMV) immediate-early promoter. Plasmid pRSVrev was sourced from Dr. Brian Cullen (Hope *et al.*, 1990b). HIV-1_{NL4.3} rev expression was under the control of the rous sarcoma virus (RSV) promoter.

Functional expression of tat from the construct pCMVNLtat was confirmed *in vitro* by cotransfection of HeLa cells with pLTR-EGFP plasmid and FACS analysis of EGFP expression (Kent *et al.*, 2001a). *In vivo* functional expression of tat was confirmed by intradermal (gene gun) coinoculation with pLTR- β gal (expressing β -galac-

tosidase) in macaques (Kent *et al.*, 2001a). Rev expression was assessed *in vitro* by cotransfection of pRSVrev and the CAT-RRE reporter, pDM128 (Hope *et al.*, 1990a), into HeLa cells and subsequent measurement of chloramphenicol acetyltransferase activity (Sambrook *et al.*, 1989).

For each of the three DNA vaccines, plasmid DNA/*E. coli* (DH5 α) was amplified in 2.5 L Luria–Bertani medium by antibiotic selection. Endotoxin-free plasmid DNA was purified using the Qiagen Giga kit (Hilden, Germany).

Plasmids pSIVoptgag, pCMVNLtat, and pRSVrev were combined for IM and epidermal (gene gun) immunization. Intramuscular delivery used 500 μ g of each plasmid in normal saline, delivering half the vaccine to each of the right and left quadriceps muscle bundles. Gene gun immunization used 1 μ m gold beads coated with a mixture of the three plasmids (1 μ g each plasmid), delivering 15 shots (15 μ g/plasmid in total) to shaved lower abdomen skin at 350 psi as previously described at the times shown in Fig. 1 (Kent *et al.*, 2001b, 1998).

Macaques

Colony bred, juvenile (2–4 kg), SRV-D free pigtailed macaques (*M. nemestrina*, $n = 15$) were studied. Animals were sedated with ketamine prior to vaccinations and procedures, and the relevant animal experimentation and ethics committees approved the studies.

ELISA assay for HPV L1 specific Ig G antibodies

Measurement of HPV L1 specific IgG antibodies in serum was performed in flat-bottom polystyrene microtiter plates (Maxisorp) as described (Liu *et al.*, 1998). Briefly, plates were coated with 50 μ l of nonchimeric HPV6bL1 VLPs at 10 μ g/ml in PBS overnight at 4°C and then blocked with 100 μ l 5% milk in PBS at 37°C for 1 h. The plates were washed using 0.5% PBS Tween 20 (PBST) and sera were added. After incubation at 37°C for 1 h, the plates were washed three times with PBST, and HRP-conjugated goat anti-rhesus IgG (Southern Biotechnology, Birmingham, AL) was added at 1:1000 dilutions and incubated at 37°C for 1 h. A 5-mg OPD tablet (Sigma) was dissolved in 12.5 ml H₂O with 4 μ l H₂O₂. Substrate (50 μ l) was added and the reaction was stopped with 3 N HCl, and plates were read at 495 nm using a Bio-Rad 4500 reader.

ELISA for SIV gag, HIV-1 tat, and rev specific IgG antibodies

Specific antibodies against vaccine antigens were analyzed post DNA and HPV immunizations. Antigens SIV-gag, HIV-1tat, and HIV-1rev were obtained through the AIDS Research and Reference Reagent Program (NIAID, NIH). Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with antigen at 1 μ g/ml in 50 μ l PBS overnight at 4°C. Antigen was removed from the wells

and the wells were washed using three PBST wash cycles (400 μl /well) prior to blocking with 10% skim milk powder/PBS (200 μl /well). Plasma collected from the macaques prior to immunization, 4 weeks post DNA immunizations, and 1 week post HPV VLP immunizations was diluted 1 in 5, 1 in 50, and 1 in 500 (in PBS/1% skim milk powder) and 100 μl was added per well in duplicate. Plasma was incubated with the antigen-coated wells for 1 h at room temperature. Wells were washed with PBST as before and HRP-conjugated goat anti-rhesus IgG (Southern Biotechnology) was added at a dilution of 1:2000 and incubated at room temperature for 1 h. Plates were washed four times with PBST prior to detection using TMB peroxidase substrate (Kirkgaard, Gaithersburg, MD) and absorbance was read at 450 nm (Titertek). Polyclonal antibodies against SIV gag (monkey anti SIV (Kent *et al.*, 2001b)), HIV-1 tat (rabbit anti-HIV-1 tat antibody; Intracel Issaquah, WA), and HIV-1 rev (rabbit anti-HIV-1 rev antibody; Dr. Alan Cochrane, University of Toronto) were titrated and used as a positive control for each assay. Anti-SIV gag specific immune responses were also assessed by Western blot utilizing HIV-2 antigen (genetically similar to SIV) labeled strips, previously shown to efficiently detect anti-SIV gag immune responses (Kent *et al.*, 2001b).

Proliferation assays

Antigen-specific T cell proliferation assays were performed postimmunization as described previously (Kent *et al.*, 1997, 1998, 2000). Antigens used for stimulation were whole Aldrithiol-2 inactivated SIV_{mac239} and supernatant from the SUPT1-CCR5 CL.30 cell line in which the virus was grown as a negative control (10 $\mu\text{g}/\text{ml}$; kindly provided by the AIDS Vaccine Program, National Cancer Institute, MD (Arthur *et al.*, 1998)); SIV_{mac239} p55 gag protein 10 $\mu\text{g}/\text{ml}$ and VSV-N protein derived from similar baculovirus cultures as a negative control; 3 $\mu\text{g}/\text{ml}$ HIV-1 tat peptide pool of 15-mer peptides overlapping by 11 aa; and 3 $\mu\text{g}/\text{ml}$ HIV-1 rev peptide pool of 15-mer peptides overlapping by 11 aa (obtained through the AIDS Research and Reference Reagent Program, NIAID NIH). PBMC were also activated with pokeweed mitogen (Sigma) as a positive control for activation.

ELISPOT quantification of IFN γ production

ELISPOT assays were used to detect antigen specific macaque IFN γ production following immunization according to the manufacturer's instructions (U-CyTech by diagnostics, Utrecht, The Netherlands) as previously described (Dale *et al.*, 2000; Kent *et al.*, 2001b). Briefly, 96-well flat-bottom, transparent microtiter plates were coated with 5 μg (in 50 μl) of anti-IFN γ mAb MD-1 (U-Cytech) and incubated overnight at 4°C. Plates were washed five times with PBST (PBS containing 0.05% Tween 20) to remove capture antibody and blocked with

PBS containing 1% BSA for 1 h at 37°C. A total of 5×10^5 batch thawed PBMC in RPMI medium + 5% FCS were cultured overnight separately in 48-well plates with antigen (single SIV gag, HIV rev, or HIV tat 15-mer peptide pools, overlapping by 11 aa, from the AIDS Research and Reference Reagent Program, NIAID, NIH) or mitogen (SEB, staphylococcal enterotoxin B, Sigma, Australia). A total of 2×10^5 washed nonadherent cells (in 100 μl) was transferred to the ELISPOT plate in duplicate wells, incubated for 5 h for expression and capture of IFN γ , and then stored overnight at 4°C. The cells were then discarded, and remaining cells were lysed with 200 μl /well of ice-cold deionized water for 15 min and washed. Captured cytokine was detected by 1 h incubation at 37°C with 1 μg (in 100 μl) of rabbit polyclonal, biotinylated anti-IFN γ (pAb, U-Cytech) and washed. A total of 50 μl per well of an anti-biotin IgG gold conjugate (GABA, U-Cytech) was added for 1 h at 37°C and spots were developed. Spots were counted using an ELISPOT reader (AID, Strassberg, Germany).

Intracellular detection of IFN γ cytokine secretion

Induction of SHIV-specific intracellular IFN γ expression in CD3+8+ lymphocytes was assessed by flow cytometry as previously described (Maecker *et al.*, 2001). Briefly, 200 μl whole blood was incubated with 3 $\mu\text{g}/\text{ml}$ of whole inactivated SIV, overlapping 15-mer SHIV peptide sets (SIV-1 gag, HIV-1 tat, and HIV-1 rev, described above), or control antigens and the costimulatory antibodies CD28 and CD49d (BD Biosciences, Pharmingen, San Diego, CA) for 6 h. Brefeldin A (10 $\mu\text{g}/\text{ml}$; Sigma) was included during the last 4 h of the incubation. Anti-CD3-PE and anti-CD8-PerCP (BD) were added to each well and incubated for 30 min. Red blood cells were lysed (FACS lysing solution, BD) and washed with PBS and the remaining cells were permeabilized (Cytofix-Cytoperm, BD). Permeabilized cells were then incubated with anti-IFN γ -FITC antibody (Mabtech, Sweden) prior to paraformaldehyde fixation and acquisition (FACScan, BD). Acquisition data were analyzed using CellQuest (BD). The percentage of antigen-specific gated lymphocytes expressing IFN γ was assessed in both total CD3+ lymphocytes and the CD3+CD8+ subset.

SHIV challenge stock

SHIV_{229(mn)} was a kind gift from Dr. Michael Agy (Univ. of Washington). The SHIV_{229(mn)} strain is based on SHIV_{IIIB} encoding HIV-1_{HXBc2} tat, rev, and env on a SIV_{mac239} backbone and was passaged through *M. nemestrina* *in vivo* to become pathogenic (Thompson *et al.*, 2000). To generate the challenge stock, SHIV_{229(mn)} was expanded on PHA-activated *M. nemestrina* PBMC as follows: 1×10^7 PBMC purified from each of 3 macaques were pooled and activated with phytohemagglutinin (PHA 10 $\mu\text{g}/\text{ml}$; Murex Biotech Ltd.) and IL-2 (50 U/ml; Hoffmann-La Roche,

Nutley, NJ) for 3 days before being infected with SHIV_{229(mn)} at an m.o.i. of 0.1 TCID₅₀/cell. This culture was maintained by changing the culture medium every second day. On day 7, the cells were pelleted and used to infect a larger number of PHA/IL-2-activated *M. nemestrina* PBMC (3×10^6 PBMC pool from 14 macaques). The infection was maintained for a further 10 days and the supernatant was collected and titrated. The SHIV_{229(mn)} virus stock was 2×10^5 TCID₅₀/ml on the CEM×174 T cell line.

Assessment of the challenge stock in four macaques

To assess the *in vivo* infectivity of the amplified SHIV_{229(mn)} challenge stock, four pigtailed macaques were inoculated intrarectally as previously described (Kent *et al.*, 2001b). A similarly grown SHIV_{229(mn)} stock was previously titrated by vaginal mucosal infection of *M. nemestrina* and was highly infectious (Thompson *et al.*, 2000). Two macaques were infected with 0.2 ml (4×10^4 TCID₅₀) and two with 1.0 ml (2×10^5 TCID₅₀) using two equal doses over 2 consecutive days and the SHIV infection was followed over 5 weeks.

Immunized and control macaques were challenged with this macaque-PBMC amplified SHIV_{229(mn)} stock intrarectally using two 0.25 ml (10^5 TCID₅₀ total) on 2 consecutive days.

CD4 T cell and SHIV RNA analyses

CD3+ CD4+ T lymphocyte populations were determined using FACS. A total of 200 μ l whole blood was incubated with CD3-PE and CD4-FITC (anti-human CD3-PE, anti-human CD4-FITC; BD PharMingen) antibodies prior to lysis of red blood cells using FACS lysis buffer (BD) as previously described (Kent *et al.*, 2001b). Cells were acquired using a BD FACSort and analyzed using CellQuest (BD). RNA was extracted from plasma separated from EDTA-anticoagulated blood using the QIAamp viral RNA kit (Qiagen). SHIV viral RNA was quantified using real-time PCR as previously described (Jin *et al.*, 1999; Kent *et al.*, 2001b).

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